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Efecto de la inhibición de PARP-1 sobre angiogénesis, metástasis y mimetismo vasculogénico en melanoma

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Tesis Doctoral

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D. FCO. JAVIER OLIVER POZO, Investigador Científico del Departamento de Biología Celular e Inmunología del Instituto de Parasitología y Biomedicina "López-Neyra" del CSIC en Granada,

CERTIFICA que **Dª ANDREÍNA CECILIA PERALTA LEAL**, Licenciada en Biología, ha realizado bajo su dirección y en el Departamento de Biología Celular e Inmunología del Instituto de Parasitología y Biomedicina "López-Neyra" del CSIC en Granada, el trabajo titulado: **"Efecto de la inhibición de PARP-1 sobre angiogénesis, metástasis y mimetismo vasculogénico en melanoma"** reuniendo el mismo las condiciones necesarias para optar al grado de Doctor por la Universidad de Granada.

En Granada, a 12 de Marzo del 2012

Vº Bº Director
Fco. Javier Oliver Pozo

La interesada
Andreína Cecilia Peralta Leal

A mi mamá y mi abuela Carmen lo mejor de mi vida

A mi Tía Gladys y Guiomar Moctezuma mis mayores motivaciones para trabajar en cáncer

Y a todos los pacientes que sufren esta cruel enfermedad.

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ÍNDICE

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I. ABREVIATURAS

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ADP: adenosín difosfato [*adenosine diphosphate*]

AJs: uniones adherentes *[Adherens junctions]*

AKT: Proteina Kinasa *[Kinese Protein]*

APC: poliposis adenomatosa coli proteínas *[adenomatous polyposis coli protein]*

AR: receptor androgeno *[androgen receptor]*

ATP: adenosín 5'-trifosfato [*adenosine-5'-triphosphate*]

BER: reparación por escisión de bases [*base excision repair*]

bHLH-PAS: básico hélice-giro-hélice Per-ARNT-Sim [*basic helix-loop-helix Per-ARNT-Sim*]

BMP: proteína morfogenética ósea *[Bone Morphogenetic Protein]*

CAF: fibroblastos asociados con el cáncer *[Cancer-Associated Fibroblast]*

CBP: proteína de unión *[binding protein]*

CBS: sitio de unión a catenina *[catenin binding site]*

CDH1: E – Cadherina *[E-cadherin]*

CDH2: N Cadherina [*N-cadherin]*

CDH3: P Cadherina [*P-cadherin]*

CDH4: *R*- Cadherina *[R-cadherin]*

CDH13: T Cadherina *[T-cadherin]*

CTF: carboxi-terminal del fragmento *[carboxy-terminal fragment]*

CPE: carboxipeptidasa E [*Carboxypeptidase E]*

DBD: dominio de unión al DNA [*DNA binding domain*]

Del-1: Antiangiogénico antitrombina III *[Antiangiogenic antithrombin III]*

DNA: ácido desoxirribonucleico [*deoxyribonucleic acid*]

DNA-PK: proteína kinasa dependiente del DNA [*DNA dependent protein kinase*]

DNA-PKcs: subunidad catalítica de la proteína kinasa dependiente del DNA [*catalytic subunit of the DNA dependent protein kinase*]

DSB: rotura de cadena doble [*double-strand break*]

EC: células endoteliales *[endothelial cell]*

E-cadherin: cadherina epitelial *[epithelial-cadherin]*

ECM: matriz extracelular *[extracellular matrix]*

Ecs: endothelial cells *[células endoteliales]*

EGF: factor de crecimiento epidérmico *[epidermal growth factor]*

EMT: transición Epitelio mesénquima *[Epithelial-to-Mesenchymal Transition]*

EndMT: transición Endotelio mesénquima *[Endothelial to Mesenchymal* Transition]

EPCs: células progenitoras endoteliales *[endothelial progenitor cells]*

ERK: señal extracelular -quinasas reguladas *[extracellular-signal-regulated kinases]*

FAT4: miembro de la familia cadherina 14 (CDHF14) *[cadherin family member 14 (CDHF14)]*

FGF: Factor de crecimiento de fibroblasto [*Fibroblast growth factors]*

FGFR: Factor de crecimiento del receptor de fibroblastos *[Fibroblast growth factor receptor]*

FOXC2: proteína Forkhead box C2 *[Forkhead box protein C2]*

GSK3b: quinasa glucógeno sintasa 3 beta *[Glycogen synthase kinase 3 beta]*

HGF: Factor de crecimiento de hepatocitos *[Hepatocyte growth factor]*

HIF: factor inducible por hipoxia [*hypoxia-inducible factor*]

HR: recombinación homóloga [*homologous recombination*]

HRE: elemento de respuesta a hipoxia [*hypoxia response element*]

HUVEC: Células Endoteliales de cordón umbilical Humano *[Human Umbilical Vein Endothelial Cell`s]*

(IA): Intracellular Anchor Domain [Dominio de anclaje intracelular]

IGF-1: factor de crecimiento de insulina 1 *[Insulin-like Growth Factor 1]*

IGM: crecimiento vascular por intususcepción [Intussusceptive Microvascular Growth(

IR: radiación ionizante [ionizing radiation]

MEF: fibroblasto embrionario murino [mouse embryonic fibroblast]

MET: Transicion Epitelio Mesenquima [Mesenchymal-to-Epithelial Transition]

Mg2+: ion magnesio [magnesium ion]

MNU: N-metil-N-nitrosourea [N-methyl-N-nitrosourea]

MPCD: Dominio conservado citoplasmático proximal de membrana [Membrane proximal domain conserved]

mRNA: ácido ribonucleico mensajero [*messenger ribonucleic acid*]

NAD^+: dinucleótido de nicotinamida adenina [*nicotinamide adenine dinucleotide*]

N-cadherin: Cadherina Neural *[Neural-Cadherin]*

NF-kB: factor nuclear kappa-cadena ligera-potenciador de células B activadas *[nuclear factor kappa-light-chain-enhancer of activated B cells]*

NHEJ: unión de extremos no homólogos [*non-homologous end-joining*]

NLS: señal de localización nuclear [*nuclear localization signal*]

O_2: oxígeno molecular [*molecular oxygen*]

ODDD: dominio de degradación dependiente del oxígeno [*oxygen dependent degradation domain*]

PAR: poli(ADP)ribosa [*poly(ADP)ribose*]

PARG: poli(ADP-ribosa) glicohidrolasa [*poly(ADP-ribose) glycohydrolase*]

PARP: poli(ADP-ribosa) polimerasa [*poly(ADP-ribose) polymerase*]

PCDH8: Protocadherina 8 *[protocadherin-8]*

PHD: proteínas que contienen el dominio prolil hidroxilasa [*prolyl hydroxylase domain-containing proteins*]

PI3K: fosfoinosítido 3 kinasa [*phosphoinositide 3-kinase*]

PIKK: kinasa tipo fosfatidilinositol 3 kinasa [*phosphatidylinositol 3-kinase like kinase*]

(PL): Dominio rico en prolina *[Proline-rich domain]*

PLGF: Placental growth factor *[Factor de crecimiento placentario]*

PM: Membrana de Plasma *[plasma membrane]*

pVHL: proteína von Hippel-Lindau [*von Hippel-Lindau protein*]

RET: reordenado durante la transfección *[rearranged during transfection]*

ROS: especies reactivas de oxígeno [*reactive oxygen species*]

RUD: Dominio de unidad repetida *[Repeating Unit Domain]*

Smad: Fenotipo Pequeño y Madres [Small phenotype and Mothers]

TGF-β: Factor de crecimiento transformante Beta *[Transforming Growth Factor-β]*

TGF-β2: Factor de crecimiento transformante Beta 2 *[Transforming Growth Factor-β2]*

TJ: unión estrecha *[Tight Junction]*

(TM): Región transmembranal [transmembrane region]

TK: dominio de la cinasa *[tirosina tyrosine kinase domain]*

Twist: hélice-lazo-hélice básica *[basic helix-loop-helix]*

VE-Cadherin: Cadherina Endotelial Vascular *[Vascular-endothelial cadherin]*

VEGF: Factor de crecimiento endotelial vascular *[Vascular Endothelial Gowth Factor]*

VM: vasculogenic mimicry *[vasculogénica mímetica]*

ZEB1: Zinc finger E-box-binding homeobox 1 *[Dedo de zinc caja E de unión homeobox 1]*

ZEB2: Zinc finger E-box-binding homeobox 2 *[Dedo de zinc caja E de unión homeobox 2]*

β-cnt: β-catenina [β-catenin]

ΔN: grupo amino terminal [terminal amino group]

II. RESUMEN

RESUMEN

La inhibición de PARP produce efectos anti-tumorales cuando se usa como monoterapia o en combinación con la quimioterapia o la radioterapia en distintos tipos de tumores. Recientemente se ha encontrado por distintos laboratorios, incluido el nuestro, que la inactivación de PARP limita la angiogénesis tumoral. El presente trabajo se ha orientado a comprender las bases de la acción antiangiogénica de los inhibidores de PARP y su implicación en la metástasis, usando un modelo de melanoma murino metastásico. El análisis proteómico de las células endoteliales HUVECs, tras el tratamiento con inhibidores de PARP, revelaron una disminución en los niveles de vimentina (un filamento intermediario involucrado en la angiogénesis y marcador específico de la transición endotelio-mesénquima (EndMT)). Asimismo, hemos constatado que la VE-cadherina, un marcador endotelial de la normalización vascular, aumentó su expresión en HUVEC bajo tratamientos con inhibidores de PARP o el silenciamiento de PARP-1. En un modelo de melanoma hemos demostrado que la vimentina, por sí sola, es suficiente para inducir las transformaciones celulares que conducen a la transición epitelio mesénquima (EMT), afectando a la señalización a través de ILK/GSK 3–β y conduciendo a un aumento de la motilidad y migración celular. En un modelo murino de melanoma metastásico, la inhibición de PARP contrarrestó la capacidad de las células de melanoma para formar metástasis en el pulmón y exhibieron una disminución en la angiogénesis tumoral y un aumento de la supervivencia en comparación con el grupo control. Por otra parte el silenciamiento estable de PARP-1 en células de melanoma también dio lugar a un aumento significativo en la

supervivencia. Estos resultados sugieren que la inhibición de PARP interfiere con los procesos clave que promueven la metástasis, afectando al cambio en el fenotipo que permite a las células de melanoma adquirir propiedades invasivas.

III. INTRODUCCIÓN.

1. ANGIOGÉNESIS

1.1. Angiogénesis normal y patológica.

La “Angiogénesis” y “Arterogénesis” son tipos de neovascularización, (formación de nuevos vasos sanguíneos a partir de la proliferación y migración de células endoteliales maduras que proceden de un vaso preexistente (Mackiewicz et al., 2002)), mientras que otros tipos de neovascularización como la “Vasculogénesis” son la formación de vasos sanguíneos por células endoteliales progenitoras (Mackiewicz et al., 2002), (Nikitenko, 2009).

La vasculogénesis es un proceso importante durante el desarrollo embrionario. En el embrión los vasos sanguíneos ayudan al crecimiento de los órganos aportando oxígeno, nutrientes y señales de crecimiento para la órgano- morfogénesis.

La angiogénesis es un proceso fisiológico que ocurre en el organismo adulto durante la cicatrización y procesos relacionados con la reproducción en las mujeres durante el ciclo menstrual (en la formación del revestimiento del útero en el proceso de ovulación) (Fraser and Duncan, 2005) y durante el embarazo (en la formación de la placenta) (Kaufmann et al., 2004). En condiciones normales la angiogénesis se regula por inductores e inhibidores; a partir de este principio se plantea la hipótesis del balance del cambio angiogénico (Hanahan and Folkman, 1996). En condiciones donde se altera este balance, el resultado es el aumento o disminución de la angiogénesis y otros procesos de neovascularización, ocasionando el crecimiento anormal de vasos sanguíneos, bien sea en exceso o en defecto, dando lugar a condiciones patológicas en el organismo. El defecto de angiogénesis ocurre en enfermedades cardiovasculares, arterioesclerosis y complicaciones isquémicas cardiovasculares (Cao et al., 2005); mientras que el exceso de angiogénesis ocurre en cáncer, enfermedades de la piel, diabetes (Martin et al., 2003), artritis reumatoide (Koch, 2003), (Carmeliet, 2005; Hillen and Griffioen, 2007) y mas de 70 patologías (Carmeliet, 2005). Durante el crecimiento tumoral, la vasculogénesis y la arteriogénesis también son importantes en la progresión del tumor. El microambiente tumoral aporta

señales para el reclutamiento de células endoteliales progenitoras de médula ósea (Mackiewicz et al., 2002) y las condiciones óptimas para su diferenciación en el endotelio maduro (De Palma et al., 2003; Nikitenko, 2009; Peters et al., 2005; Spring et al., 2005).

Las células tumorales requieren un aporte sanguíneo para su crecimiento y para metástasis. Los tumores pueden aumentar su tamaño aproximadamente 1-2 milímetros sin que se restrinjan sus demandas metabólicas. Por el contrario, para crecer por encima de ese tamaño, el tumor debe cambiar a un fenotipo angiogénico (Carmeliet and Jain, 2000). En algunas patologías como el desarrollo neoplásico y otros desórdenes malignos, el crecimiento de vasos está desregulado dando lugar a una angiogénesis o linfogénesis aberrante. La angiogénesis o linfogénesis excesiva ocurre no sólo en cáncer sino también en aterosclerosis, psoriasis, obesidad y ceguera (Tabla 1) (Nikitenko, 2009). La células tumorales y estromales secretan factores angiogénicos y linfangiogénicos, como los pertenecientes a la familia de VEGF, que actúan directamente sobre sus respectivos receptores en las células endoteliales quiescentes de los vasos preexistentes activando los programas angiogénicos y respuestas biológicas de migración, invasión, división celular, proteolisis, expresión de proteínas antiapoptóticas y finalmente el desarrollo de nuevos capilares. (Ferrara and Kerbel, 2005; Nikitenko, 2009) (Kerbel and Folkman, 2002) (Carmeliet, 2005).

1.2. Formación de vasos sanguíneos en cáncer: tipos.

Inicialmente se pensaba que la angiogénesis en tumores malignos se producía exclusivamente a partir de vasos preexistentes. No obstante, estudios recientes han identificado otros procesos de angiogénesis y remodelación vascular (Dome et al., 2007).

Los mecanismos de neovascularización en tumores **(Figura 1),** pueden dividirse en los siguientes tipos: angiogénesis por brotes vasculares (“sprouting”) **(Figura 1a)**, vasculogénesis a partir de células precursoras de células endoteliales **(Figura 1b)**, angiogénesis por invaginación

(intususcepción) **(Figura 1c),** co-opción vascular **(Figura 1d),** mimetismo vascular **(Figura 1e).** Asimismo, en el tumor se observa la diferenciación de células endoteliales a partir de células tumorales **(Figura 1f)** y el proceso de linfoangiogénesis **(Figura 1g)**. (Hillen and Griffioen, 2007)

Tabla 1. Angiogénesis y linfoangiogénesis en neoplasia "endotelial" y "no endotelial"
Adaptado de (Nikitenko, 2009)

Sistema/órgano	**Ejemplos de procesos caracterizados por una angiogénesis/linfangiogénesis * anormal**	**Papel de las células endoteliales maduras o progenitoras**
Sistema reproductivo (útero, ovario, mama)	Neoplasias[a,b]	Neovascularización[e]
Huesos, articulaciones	Neoplasias[a,b]	Neovascularización[e]
Hígado, riñón, pulmón y otros epitelios	Neoplasias[a,b]	Neovascularización[e]
Cerebro, nervios, ojos	Neoplasias[a,b]	Neovascularización[e]
Piel	Neoplasias[a,b]	Neovascularización[e]
	Kaposi's sarcoma[a,d]	Células tumorales progenitoras[e,f]
Vasos sanguíneos	Hemangioma[c]	Células tumorales[f]
	Hemangioendotelioma[c]	Células tumorales[f]
Vasos linfáticos	Metástasis tumoral[b]	Vía para la diseminación de las células tumorales[e]
	Desórdenes limfoproliferativos[b,c] (ej. Linfangioma, linfangiosarcoma)	Células tumorales[f]
		Neovascularización[e]
Hematopoyesis	Sarcoma de Kaposi [a,d]	Células tumorales progenitoras[e,f]
	Neoplasias hematológicas[d]	Neovascularización[e]

[a] Aumento de la vascularización atribuible a una angiogénesis normal,
[b] Aumento de la linfoangiogénesis o creciemiento alterado del endotelio linfático,
[c] Hiperplasia de células endoteliales,
[d] Diferenciación anormal de las células progenitoras o reprogramación de las células endoteliales
[e] La línea de células endoteliales es el tipo celular no neoplásico de apoyo del tumor
(Endothelial lineage cells are the non-neoplastic tumour-supporting cell type)
[f] La línea de células endoteliales es el tipo celular principal (incluyendo el neoplásico)

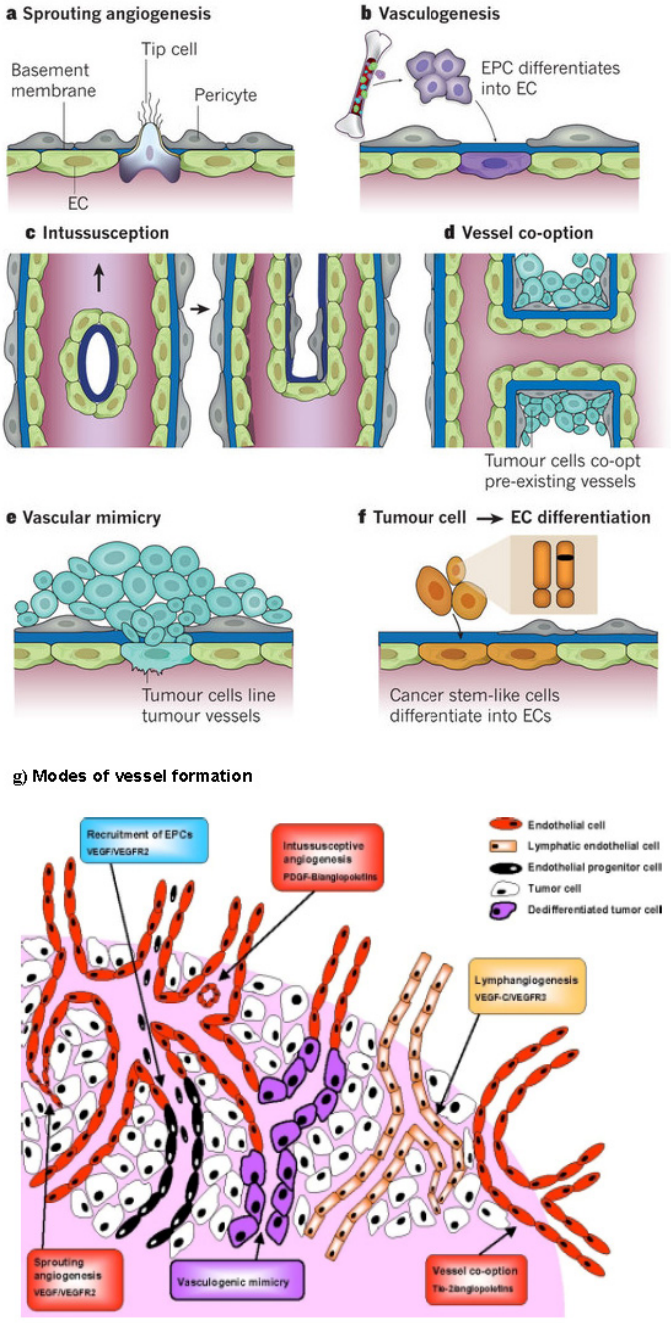

Figura 1. Modelos de formación de vasos. Se conocen diferentes mecanismos de vascularización en tejidos normales y tumorales. **a-c**: la formación de vasos puede ocurrir por angiogénesis por brotes **(a)**; por reclutamiento de células derivadas de la medula ósea y/o de células progenitoras endoteliales residentes en la pared vascular, (EPCs) "endothelial progenitor cells", que se diferencian a células endoteliales (ECs) **(b)**; por el proceso de separación de vasos conocido como intususcepción o invaginación **(c). d-f**: co–opción vascular de vasos sanguíneos preexistes en células tumorales **(d);** vasos tumorales formados a partir de células tumorales (mimetismo vascular o mimetismo vasculogénico **e);** o por células endoteliales con anormalidades citogenéticas en sus cromosomas, derivadas de células cancerosas progenitoras **(f);** tejidos normales con aparición de angiogénesis, vasculogénesis e invaginación **(a-c)** tumores que pueden presentar cualquiera de las seis formas de formación de vasos. Diferentes mecanismos de vascularización que pueden observarse en un mismo tumor incluyendo linfoangiogénesis **(g)** *Adaptado de* (Carmeliet and Jain, 2011a) (Hillen and Griffioen, 2007)

1.2.1. Angiogénesis a partir de vasos preexistentes.

1.2.1.a. Angiogénesis por brotes vasculares ("sprouting").

Es el mecanismo más conocido por el cual el tumor promueve su vascularización formando nuevos capilares a partir de los preexistentes. Este proceso fue descrito por Folkman en los años 70. Las etapas en que se divide este proceso son: 1) Alteración de la membrana basal, en la que hay una activación de las células endoteliales por factores de crecimiento específicos que se unen a sus receptores. A continuación, la matriz extracelular y la membrana basal que rodea a las células endoteliales se degradan por proteasas permitiendo que las células endoteliales invadan la matriz, proliferen y migren a través de ella (Dome et al., 2007). 2) Formación de un cordón sólido por células endoteliales sucesivas. 3) Formación de un lumen por la curvatura de cuerpos celulares endoteliales simples o en paralelo. Así estas células polarizadas crearán la luz de un vaso inmaduro. Posteriormente, se sintetiza membrana basal y se reclutan pericitos/células murales y se genera matriz extracelular, estabilizando estos vasos inmaduros. (Dome et al., 2007) (Ferrara et al., 2003) (Hillen and Griffioen, 2007) **(Figura 2a)**.

1.2.1.b. Angiogénesis por invaginación o intususcepción .

Este mecanismo es una variante de la angiogénesis, también llamada crecimiento vascular por intususcepción, "Intussusceptive Microvascular Growth" (IGM), siendo un proceso rápido (tiene lugar en horas o incluso en minutos), ya que no es necesaria la proliferación de células endoteliales, la degradación de membrana basal ni la invasión de tejido conectivo (Burri et al., 2004). Durante este proceso, las células endoteliales del pulmón aumentan su tamaño más de 20 veces.

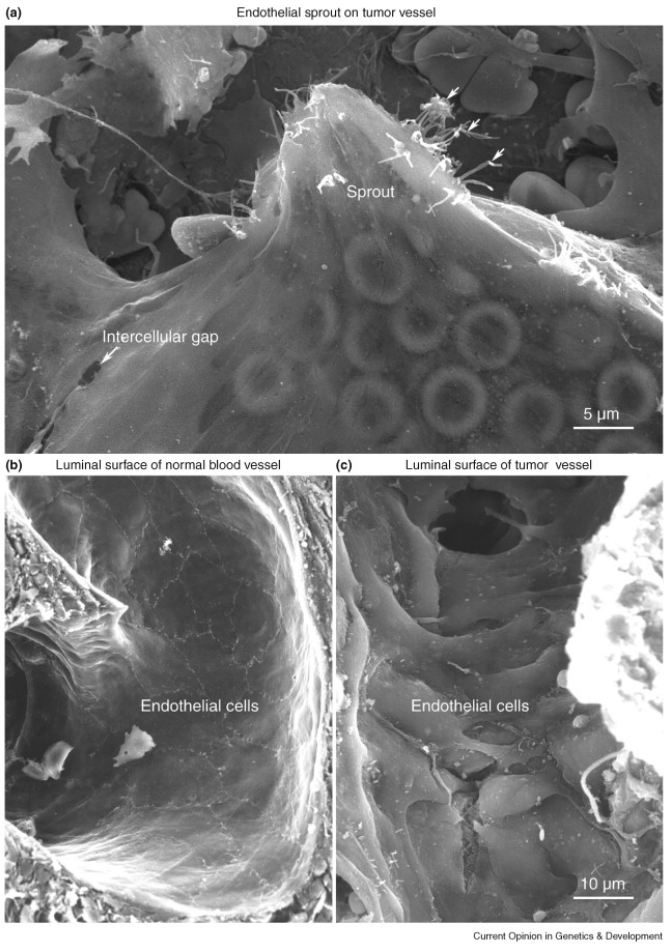

Figura 2. Angiogénesis por formación de brotes. La micrografía electrónica muestra **(a)** Superficie externa (abluminal) de un brote endotelial en un tumor de islote pancreático de un RIP- Tag2 de ratón transgénico. Múltiples filopodios (flechas cortas) extendidos desde la superficie de la célula endotelial cerca de la punta del brote. Se observa un espacio como una apertura en la unión celular endotelial (flecha larga). Se observan eritrocitos intravasculares a través del endotelio delgado y casi transparente. La micrografía electrónica comparada **(b)** Monocapa de células endoteliales lisa, que se estrecha cubriendo la superficie luminar de los vasos sanguíneos normales **(c)** Desorganización de vasos en el endotelio un tumor RIP – Tag2. La sangre fue retirada por perfusión vascular. *Adaptado de (Baluk et al., 2005)*

La intususcepción consiste en la división de los vasos preexistentes en dos nuevos vasos, mediante la formación de un tronco transvascular en el interior de la luz del vaso. Después de la angiogénesis y vasculogénesis se expanden redes capilares, en corto tiempo y consumiendo poca energía. El proceso consta de las siguientes etapas: 1) “kissing contact”: las paredes de células endoteliales de posición opuesta tienen un contacto como de “beso” por el cual forman un puente transluminal. 2) Reorganización de uniones interendoteliales y perforaciones de bicapas endoteliales. 3) Formación de un tronco intersitial con pericitos y miofibroblastos que invaden y forman la pared intersticial con nuevos vasos. 4) Crecimiento del diámetro

del tronco, retracción de células endoteliales y formación de dos vasos separados (Carmeliet and Jain 2011a, ; Hillen and Griffioen, 2007). **(Figura 2c)**

1.2.2. Vasculogénesis a partir de células precursoras de células endoteliales o vasculogénesis postnatal.

La formación de nuevos vasos puede producirse a partir de células precursoras de endotelio (CPE) (angioblastos), almacenadas en la médula ósea (Reyes et al., 2002) y reclutadas hacia los tejidos (Kumar *et al*., 2005), por lo que no es un proceso exclusivo que necesite de vasos preexistentes. Las CPE pueden ser reclutadas por tejidos tumorales y citoquinas de la médula ósea (Asahara and Kawamoto, 2004); la citoquina más característica es VEGF. Estas CPE expresan marcadores de células madre hematopoyéticas (Conway *et al*., 2001) así como marcadores endoteliales específicos como CD34, CD31, receptor del factor de crecimiento del endotelio vascular-2 (VEGFR-2) y Tie-2. (Asahara et al., 1997; Hillen and Griffioen, 2007; Kalka et al., 2000). El reclutamiento de CPEs se induce por varios factores de crecimiento, quimoquinas y citoquinas, las cuales se producen durante procesos como el estrés fisiológico (isquemia en tejido), ejercicio físico y crecimiento tumoral. La movilización de las células progenitoras comienza con la activación de la metaloproteasa 9, promoviendo las uniones de la membrana a los ligandos en forma soluble. El reclutamiento e integración de CEPs incluye múltiples pasos, como la quimioatracción, la activación del "homing" dentro de la vasculatura angiogénica, la transmigración al espacio intersticial, la incorporación dentro de la microvasculatura y la diferenciación dentro de las células endoteliales maduras (Hillen and Griffioen, 2007). Después de la diferenciación a célula endotelial madura, las CEPs pierden sus propiedades progenitoras y comienzan a expresar marcadores endoteliales como VE-cadherina, factor Von Willebrand y la óxido nítrico-sintetasa endotelial (Hanahan and Weinberg, 2000).

Existe controversia sobre la contribución de las CPE al crecimiento de los vasos en procesos tumorales (Dome et al., 2007). Algunos autores

afirman que las CPE representan un papel principal en la angiogénesis tumoral (Rajantie *et al*., 2004; Peters *et al*., 2005), sin embargo, otros afirman que su contribución en la vascularización de las neoplasias es mínima (Machein *et al*., 2003; Gothert *et al*., 2004).

1.2.3. Coopción vascular "Vessel cooption".

Es un estado en el que el crecimiento de los tumores y la metástasis comienzan como una masa avascular y luego se induce el desarrollo de nuevos vasos para crecer más allá de unos pocos milímetros en tamaño. Sin embargo, se ha sugerido que muchos tumores pueden crecer en un estado avascular, principalmente en tejidos bien vascularizados como el cerebro o el pulmón (Hillen and Griffioen, 2007; Holmgren et al., 1995; Pezzella et al., 1997; Wesseling et al., 1994). De esta manera, las células tumorales podrían crecer asociadas a microvasos preexistentes sin necesidad de una respuesta angiogénica (Hillen and Griffioen, 2007).

Durante este proceso se desarrollan vasos largos y sinuosos sin membrana basal dentro nódulos tumorales. Holash encontró en sus estudios iniciales que en procesos de coopción había una alta expresión de angiopoyetina 2 en tumores de dos semanas y tumores con un estadío tardío con un centro necrótico (Holash et al., 1999). Sin embargo, la expresión de VEGF es un poco más baja en tumores de estadíos tempranos e incrementa en estadíos tardíos.(Hillen and Griffioen, 2007)

1.2.4. Mimetismo vasculogénico ("*vasculogenic mimicry*" (VM)).

Con este término se describe la formación de canales por parte de células no endoteliales, que tienen la capacidad de conducir fluidos como plasma y células rojas, y hace referencia a dos procesos: 1) mimetismo, porque son canales que no son vasos sanguíneos y simplemente imitan su función, y 2) vasculogénico porque no se forman a partir de un vaso preexistente a pesar del hecho de que ellos distribuyen plasma y pueden contener células sanguíneas (Folberg and Maniotis, 2004).

En 1999 Maniotis describe la formación de canales constituidos por células de melanoma altamente agresivas y genéticamente desreguladas, obteniéndose células con características parecidas a células endoteliales. Se forman estructuras pseudovasculares producto de un mecanismo especializado donde se presenta plasticidad en células tumorales para adquirir características parecidas a células endoteliales (Hendrix et al., 2003). El mimetismo vasculogénico aporta a los tumores una vía de perfusión independiente de la angiogénesis sin la participación de las células endoteliales (Folberg et al., 2000) (Folberg and Maniotis, 2004). En este proceso, se forman redes de estructuras parecidas a los vasos, rodeadas de matriz extracelular y ricas en laminina (Paulis et al.). En cánceres humanos VM depende de la vía de activación de VE-cadherina (CD144), también involucrada en la diferenciación endotelial (Hendrix et al., 2001). El VM ha sido observado en osteosarcoma, cáncer de mama, cáncer de colon, carcinoma hepatocelular, glioma, cáncer de ovario, cáncer de próstata, y melanoma uveal (Chang et al., 2000; Kobayashi et al., 2002; Zhang et al., 2007). La presencia de VM en los tumores da una característica de agresividad y esto es clínicamente significativo y corresponde con un aumento del 50% de riesgo de muerte por metástasis

La detección morfológica de los canales vasculares asociados a la matriz se hace por tinciones histológicas, siendo la más usada la PAS-positiva con ácido periódico de Schiff (Pezzella et al.1997), (Folberg et al., 2000). En el melanoma uveal, el VM en la forma de PAS y CD144 positivo, CD31 negativo la formación de canales está asociado con incremento de mortalidad, actividad mitótica y tipo de célula (Maniotis et al., 1999).

1.3. Regulación de la Angiogénesis

1.3.1. Balance Angiogénico “Angiogenic switch”

Normalmente, el crecimiento de nuevos vasos sanguíneos es controlado por un delicado equilibrio entre los activadores angiogénicos, como el factor de crecimiento endotelial vascular (VEGF), factores de crecimiento de fibroblastos (FGF), factor de crecimiento derivado de las plaquetas (PDGF) y factor de crecimiento epidérmico (FEAG) y los inhibidores angiogénicos (factores antiangiogénicos) trombospondina 1,

angiostatina, endostatina y tumstatin entre otros (**Tabla 2)** (Bergers and Benjamin, 2003; Kalluri, 2003). Durante el "cambio angiogénico", en el tumor se activa la vascularización y crece más allá de su límite de difusión, la balanza se inclina hacia el lado pro-angiogénico (Hanahan and Folkman, 1996). Esto se logra a través de los factores intrínsecos de las células tumorales, pero las células del estroma también (especialmente las células mieloides) contribuyen a este proceso (Murdoch et al., 2008). Asímismo las proteasas desempeñan un papel crucial, por ejemplo, en la liberación de factores pro-angiogénicos de la ECM y en la activación de los inhibidores de la angiogénesis (Bergers and Benjamin, 2003; Kalluri, 2003; Overall and Kleifeld, 2006). El cambio angiogénico puede ocurrir ya en las etapas premalignas de la tumorigénesis.

Tabla 2. Factores angiogénicos y antiangiogénicos endógenos (Makrilia et al., 2009).

Factores angiogénicos	**Factores antiangiogénicos**
Factor de crecimiento del endotelio vascular (VEGF)	Angiostatina
Angiopoyetinas	Endostatina
Factores de crecimiento fibroblastoides ácidos y básicos	Trombospondina 1/2
Factor de crecimiento endotelial derivado de plaquetas (PDEGF)	Vasostatina
Factor de crecimiento transformante α/β	Factor asociado a plaquetas 4
Factor de necrosis tumoral α	Osteopontina
Factor de crecimiento epidermal	Metaloproteinasas inhibidoras de tejido (TIMP)
Ciclooxigenasa 2	Interleucina 12
Interleucina 6/8	

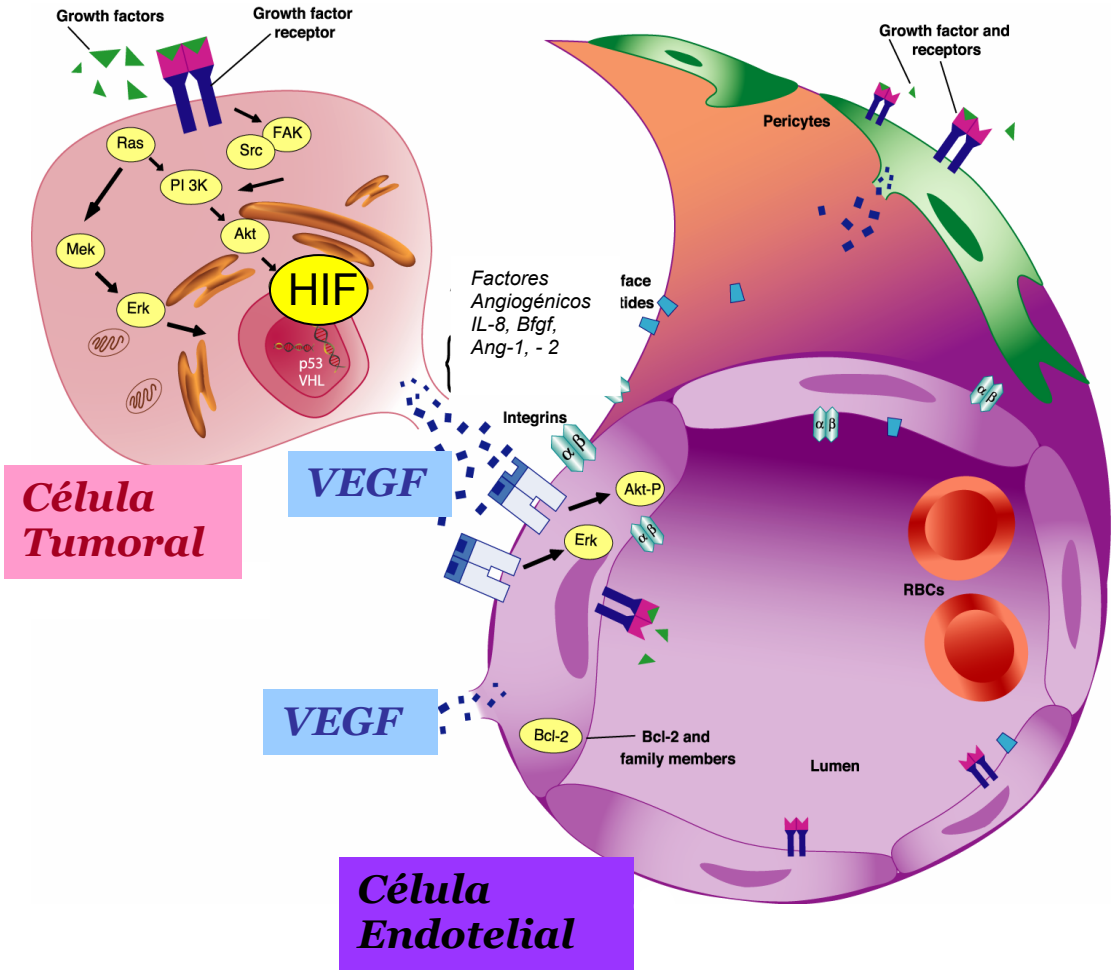

Figura 3. Proceso de activación de la angiogénesis en el crecimiento tumoral. Las células tumorales estimulan factores de crecimiento activando cascadas de señalización celular donde intervienen PI3K y Akt, que activan al factor de transcripción HIF. La activación de factores angiogénicos como IL-8, Bfgf, Ang-1 y 2, y VEGF estimula la liberación de proteínas de la matriz (MMPs) por parte de las células endoteliales permitiendo su migración, proliferación y desarrollo neovascular en vasos adyacentes.

1.3.2. Hipoxia

La iniciación de la angiogénesis durante el desarrollo tumoral, en condiciones de hipoxia, se promueve por la activación de factores como HIF y diversos factores de crecimiento, siendo uno de los más importantes VEGF **(Figura 3)**. El crecimiento tumoral genera un microambiente de hipoxia que induce la formación de una nueva estructura vascular activando el proceso de angiogénesis para obtener oxígeno y nutrientes. En tumores sólidos la presión parcial de oxígeno (PpO_2) es menor a 1-10 mmHg. Esta disminución de los niveles de oxígeno estabiliza y activa a HIF, que induce la transcripción de genes como *VEGF* **(Figura 4)** (Brahimi-Horn and Pouyssegur, 2006).

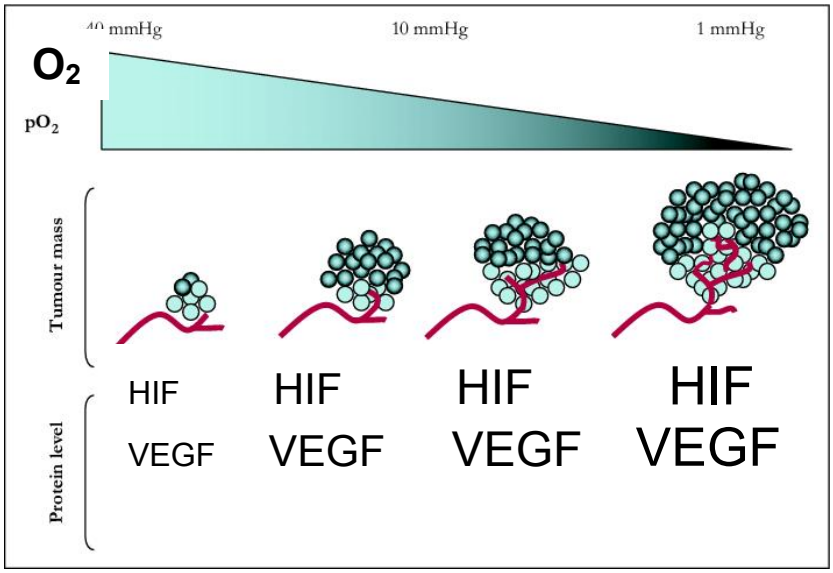

Figura 4. Estrés hipóxico en células tumorales. Durante la baja presión parcial de oxígeno (PpO_2) se estabiliza y activa HIF, que a su vez activa al factor de crecimiento endotelial vascular A (VEGF-A). Esto tiene lugar en colaboración con otros factores conduciendo al desarrollo de nuevos vasos sanguíneos que penetran la masa del tumor reestableciendo el suplemento de oxígeno y nutrientes en las células tumorales. *Adaptado de* (Brahimi-Horn and Pouyssegur, 2006).

HIF-1 funciona como un regulador de la respuesta celular a las variaciones de la concentración de oxígeno y sufre cambios conformacionales inducidos por dichas variaciones. Estructuralmente, HIF-1 está formado por dos subunidades α/β, perteneciente a la familia bHLH-PAS (Basic helix-loop-helix/Per-ARNT-Sim) característico de muchos factores de transcripción **(Figura 5)**. La subunidad β se expresa constitutivamente y su actividad es independiente de oxígeno. La hipoxia celular induce la expresión de la subunidad α, mientras que en tensiones normales de oxígeno se mantiene a niveles bajos en la mayoría de las células. Los niveles de HIF-1α determinan la capacidad de unión al ADN y la actividad transcripcional. En la inducción de HIF-1 en la respuesta a la hipoxia hay un aumento en la expresión de mRNA, estabilización de la proteína y localización nuclear (Mabjeesh and Amir, 2007).

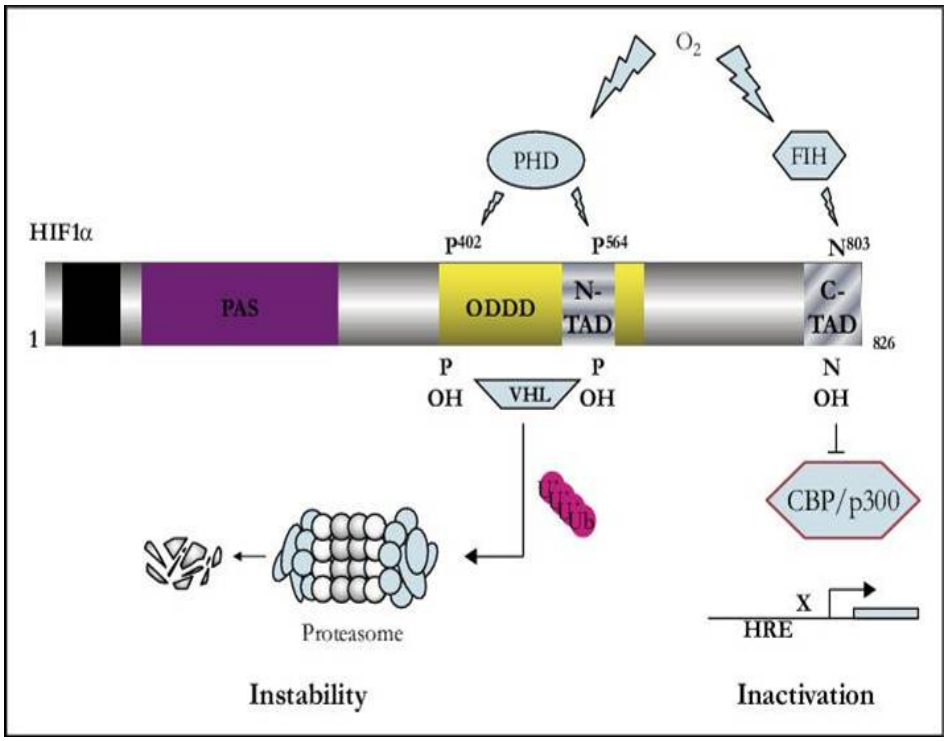

Figura 5. La sub-unidad alfa de HIF 1. En humanos contiene un N-terminal básico-helix-loop-helix (bHLH) y un dominio Per-Arnt-Sim (Pezzella et al. 1997) que es requerido para la heterodimerización e interacción con ADN. Esta posee un dominio dependiente de la degradación de oxigeno (ODDD) que contiene dos residuos de prolina (402 and 564) que son hidroxilados en presencia de oxigeno por proteínas con dominio prolil hidroxilasa (PDH) generandose una unión de von Hippel-Lindau (VHL) E3 ubiquitina ligasa componente que marca a HIFα con cadenas de poliubiquitina que son reconocidas por el proteasoma 26S generando la degradación de HIFα. Adicionalmente en presencia de oxigeno, otras hidrolasas que inhibe el factor HIF-1 (HIF) hidroxilatos como residuos de asparagina (803) en el dominio C-terminal de activación transcriptional (C-TAD) de interacción con un co-activator necesario CBP/p300 produciendose la inactivacion de la actividad transcripcional de HIF a través de la unión de elementos específicos de respuesta a hipoxia (HRE) de genes blanco. *Adapatado de* (Brahimi-Horn and Pouyssegur, 2006)

Un amplio rango de estímulos fisiológicos y patológicos activan al sistema HIF, incluyendo factores de crecimiento como insulina, IGF-1, EGF y oncogenes como ras y Myc. Las mutaciones de genes supresores de tumores como p53 y pVHL también conlleva a la inducción y amplificación de HIF-1. Más de 200 genes son activados por HIF-1 durante la hipoxia para generar una adaptaión a ese microambiente tumoral, incluyendo VEGF, eritropoietina y muchas enzimas implicadas en el metabolismo de glucosa, hierro y nucleótidos. VEGF es el mitógeno endotelio-específico más potente conocido y se ha demostrado que tiene un papel central en la angiogénesis. VEGF es el gen más importante en la activación de la angiogénesis durante hipoxia y es el principal regulador en la formación de nuevos vasos

sanguíneos (Hickey and Simon, 2006). Así mismo, la actividad de VEGF es modulada a nivel transcripcional, postranscripcional y postraduccional en varios tipos celulares. La modificación postraduccional más importante de VEGF es la poli ADP ribosilación, ya que afecta su actividad angiogénica. Estudios recientes han demostrado que el lactato afecta la modificación de VEGF por PAR, observándose un aumento de VEGF modificado por PAR tanto en extractos de células endoteliales como en el VEGF secretado al medio en condiciones de crecimiento sin lactato (Kumar et al., 2007).

La angiogénesis es activada durante el desarrollo tumoral en condiciones de hipoxia y por la activación de factores como HIF y diversos factores de crecimiento, siendo uno de los más importantes VEGF. La sobreexpresión de HIF-1 en tumores se correlaciona con un incremento en la mortalidad (Maxwell et al., 1999). En modelos experimentales la reducción de HIF-1 impide el crecimiento tumoral y la angiogénesis (Ryan et al., 1998). El efecto de la expresión de HIF-1 en los distintos tumores parece depender del tipo de cáncer, además de la presencia o ausencia de alteraciones genéticas que afectan al equilibrio entre efectos pro o antiapoptóticos. La hipoxia generada en los tumores afecta negativamente, complicando el tratamiento de la radio y quimioterapia. Las células cancerosas en hipoxia son más resistentes a los tratamientos de radio y quimioterapia (Unruh et al., 2003). HIF-1 por sí mismo media la resistencia a la quimio y radioterapia. Por lo tanto, la inhibición de HIF-1 podría reducir la angiogénesis, la diseminación y el crecimiento tumoral, además de aumentar la eficacia de estos tratameintos. Hoy en día existe un gran interés en el desarrollo de inhibidores de la activación de HIF-1 para mejorar los actuales tratamientos antineoplásicos.

1.4. Normalización vascular

La normalización vascular, o el restablecimiento de la estructura y función en los vasos sanguíneos, surge como estrategia para tratar el cáncer y otros trastornos vasculares (Mazzone et al., 2009). Se ha propuesto que "normalizar" los vasos sanguíneos desregula las vías de señalización que contribuyen al crecimiento del cáncer (Jain, 2009). Estudios preclínicos y

clínicos iniciales revelan que la normalización de las anomalías vasculares se está convirtiendo en un paradigma terapéutico complementario para el cáncer y otras enfermedades vasculares (Carmeliet and Jain 2011b). Los tumores suelen tener un abundante número de vasos, sin embargo, estos tienen un mal funcionamiento generando condiciones de hipoxia y privación de nutrientes. Existen grandes diferencias entre los vasos de los tejidos sanos y los vasos de los tumores (**Figura 6).** La presencia de vasos anormales en el tumor generan puertas de entrada para la difusión de las células tumorales, facilitando la diapédesis de células tumorales, ya que no proporcionan una barrera física que restringa la migración de células tumorales transendoteliales (Mazzone et al., 2009) y también porque las células endoteliales tumorales producen niveles elevados de proteasas, moléculas de adhesión y otros factores que facilitan diapédesis (Sullivan and Graham, 2007). Las características de este ambiente alterado, puede estimular la progresión de esta enfermedad y la resistencia al tratamiento. Las terapias antiangiogénicas tradicionales intentan reducir el suministro vascular al tumor, pero su éxito está limitado por la falta de eficacia o el desarrollo de resistencias. Los vasos alterados del tumor también pueden impedir el funcionamiento de las células inmunitarias, así como el transporte y/o distribución de quimioterapia y oxígeno. De esta manera los vasos sanguíneos alterados también pueden conducir a una resistencia de las células tumorales a la radioterapia y la quimioterapia.

Existen diversos factores que intervienen en la formación de vasos anormales en el tumor **(Figura 7).** Sin embargo, es importante señalar el aumento de activadores angiogénicos entre los que destaca VEGF, factor clave que estimula el crecimiento angiogénico de las células endoteliales, la migración, la permeabilidad, la formación del lumen y la superviviencia (Nagy et al., 2007). Varios estudios preclínicos han demostrado que los altos niveles de VEGF observados en los tumores inducen a la formación de vasos anormales (Jain, 2005). Otra molécula importante a destacar es la proteína de dominio prolil hidroxilasa 2 (PHD2 también conocida como EGLN1), un sensor del balance de los niveles de oxígeno, que interviene en la regulación de la desorganización de vasos en el tumor (Aragones et al., 2009) (Majmundar et al.). PHD2 es una enzima sensora de oxígeno que

hidroxila al factor inducible por hipoxia (HIF), en presencia de oxígeno, para su degradación vía proteasoma (Aragones et al., 2009; De Bock et al., 2009). HIF1α y HIF2α difieren entre sí, no sólo por su especificidad en cuanto a sus dianas, sino también por su regulación dependiente de oxígeno. HIF1α sólo se activa cuando los niveles de oxígeno se reducen sustancialmente, mientras que HIF2α puede ser activado por una hipoxia menos severa. Bajo condiciones de hipoxia, la PHD2 está inactiva y HIF inicia una serie de respuestas para aumentar el suministro de oxígeno (en parte a través de la angiogénesis) (Rey and Semenza 2010). Un estudio reciente demuestra que células endoteliales con niveles reducidos de PHD2 pueden estabilizar los vasos tumorales mediante la sobreregulación de la producción de los receptores de VEGF soluble 1 (VEGFR-1) y VE-cadherina. Estas observaciones sugieren que los agentes antiPHD2, son un blanco terapéutico de gran interés y podrían utilizarse para sensibilizar a las células tumorales en la terapia de radiación y quimioterapéuticos (Mazzone et al., 2009).

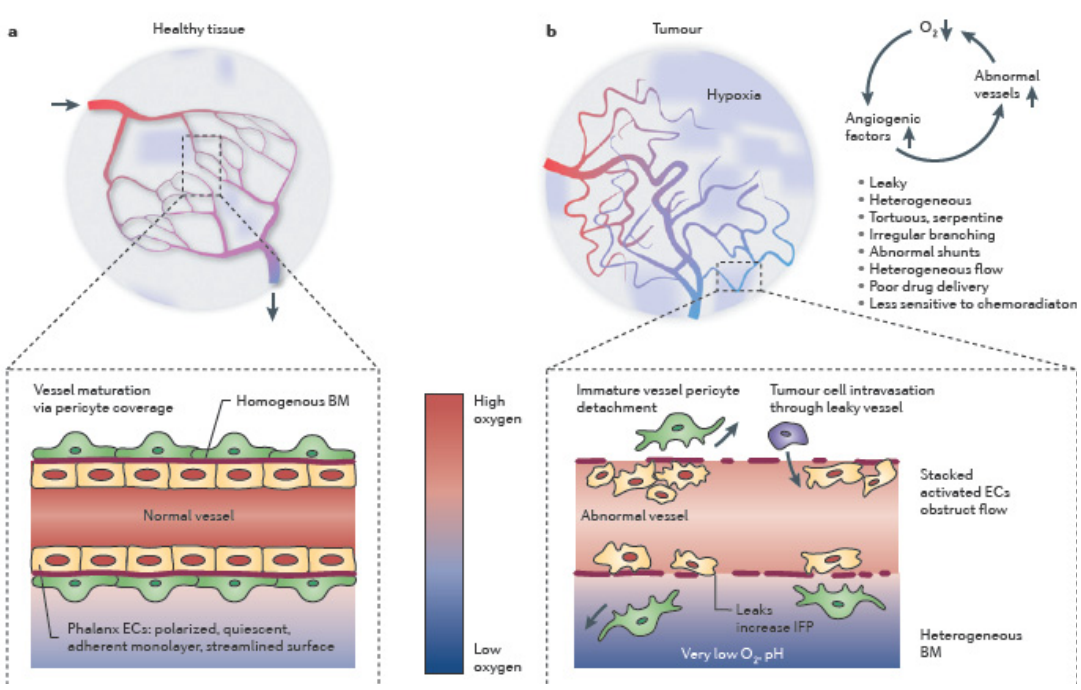

Figura 6. Los vasos tumorales son estructuralmente y funcionalmente anormales. a) En el tejido sano, un patrón regular y la estructura vascular está formada (panel superior), por vasos con pared y endotelio normal (panel inferior). B) En los tumores, la estructura vascular (panel superior), así como el endotelio y la pared del vaso (panel inferior) muestran anomalías estructurales y funcionales, dando lugar a regiones de hipoxia severa (representado por el sombreado azul) BM, membrana basal. EC células endoteliales. IFP la presión del líquido intersticial. *Adaptado de* (Carmeliet and Jain 2011b).

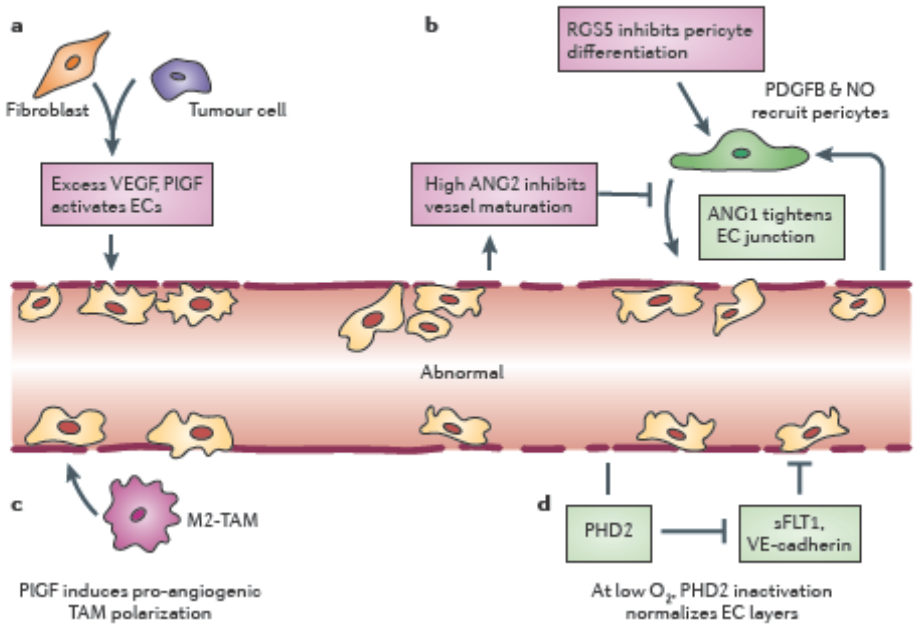

Figura 7. Cambios moleculares que conducen a los vasos anormales. El exceso de producción de factores pro-angiogénicos que actúan sobre las células endoteliales (ECS) (a) o de defectos en el reclutamiento de pericitos o maduración conlleva a la formación anormal de los vasos del tumor (b). Datos recientes muestran que la polarización de los macrófagos asociados a tumores (TAM) a un fenotipo parecido a M2 puede promover la formación anormal de los vasos (c). La inactivación parcial de la enzima sensora de oxígeno prolil hidroxilasa 2 (PHD2), a baja tensión de oxígeno normaliza los vasos mediante retroalimentación negativa (d).ANG, angiopoyetina; sFlt1, soluble similar al FMS de la tirosina quinasa 1, NO óxido nítrico, PDGFB, β-tipo de derivado de plaquetas receptor del factor de crecimiento; PlGF factor de crecimiento placentario, RGS5, regulador de la señalización de la proteína G-5, VE-cadherina, cadherina endotelial vascular (una molécula de unión) VEGF, factor de crecimiento endotelial vascular *Adaptado de* (Carmeliet and Jain 2011b).

1.5. Terápia antiangiogénica

La angiogénesis ha sido la diana terapéutica por excelencia durante décadas. Sin embargo, también existe la terapia proangiogénica en los casos de enfermedades donde el desequilibrio entre de factores de crecimiento da lugar a una angiogénesis insuficiente. En nuestro caso, nos centraremos en las terapias antiangiogénicas relacionadas con la progresión tumoral. Aunque existen puntos comunes, dependiendo del tipo de cáncer, la diana terapéutica puede variar. Las dianas terapéuticas más comunes están indicadas en la **Figura 8.** Asimismo una de las diana terapeuticas de gran interes durante muchos años ha sido VEGF. Numerosas publicaciones de

estudios precínico y clínicos se han realizado para distintos tipos de cáncer y diferentes estados de progresión de la enfermedad, bien sea a nivel del tumor primario o en la micrometástasis o macro metástasis, como se indica en la **Figura 9.**

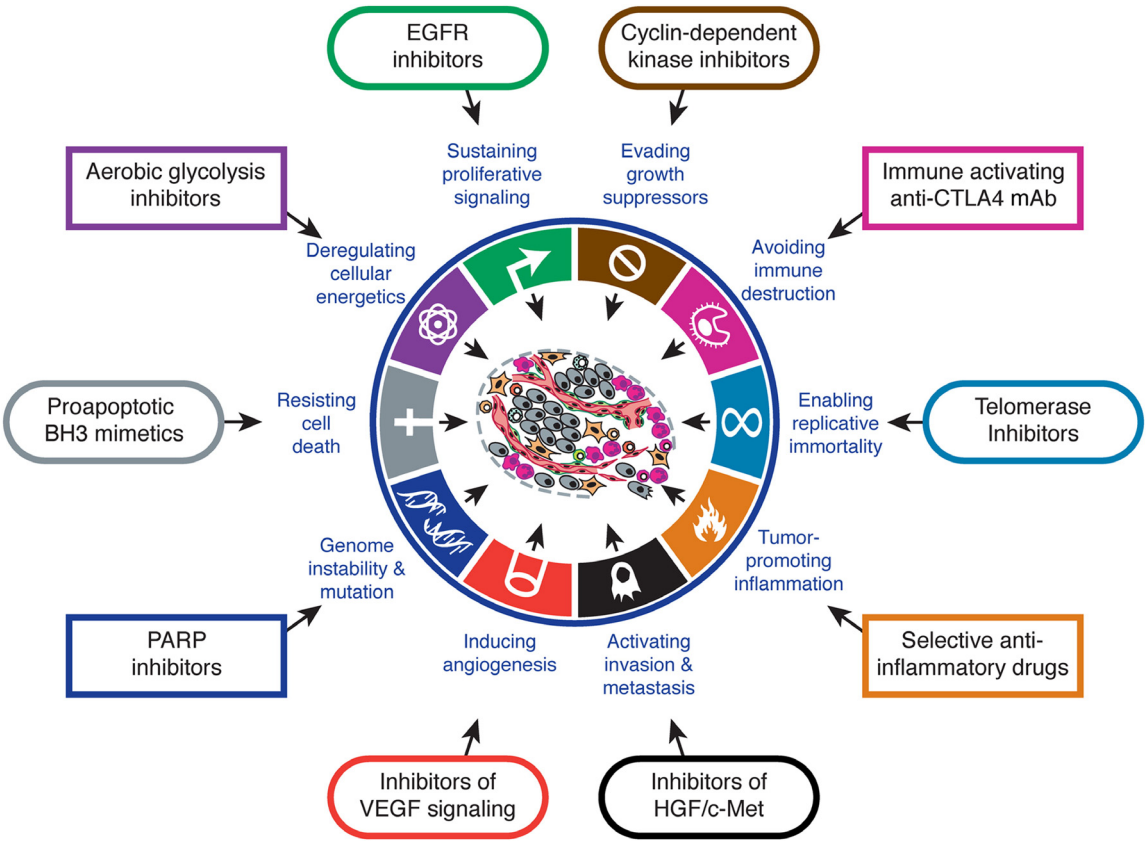

Figura 8. Dianas Terapéuticas en el tratamiento del cáncer. Fármacos aprobados en uso clínico de forma segura como tratamiento de cáncer en humanos o en ensayo clínico interfieren con las alteraciones adquiridas durante el crecimiento y progresión tumoral. Adicionalmente, las investigaciones sobre fármacos han sido desarrolladas para cada diana teniendo como objetivos las diferentes características posibles (inestabilidad genómica y mutaciones, inflamación promovida por el tumor) y características emergentes distintivas (desregulación celular energética y evasión de la destrucción de la respuesta inmunitaria) las cuales son también una promesa terapéutica en cáncer. Los fármacos señalados están mostrados en los ejemplos de la figura. Hay diferentes proyectos de producción de fármacos candidatos con diversas dianas moleculares y modos de acción. *Adaptado de* (Hanahan and Weinberg 2011).

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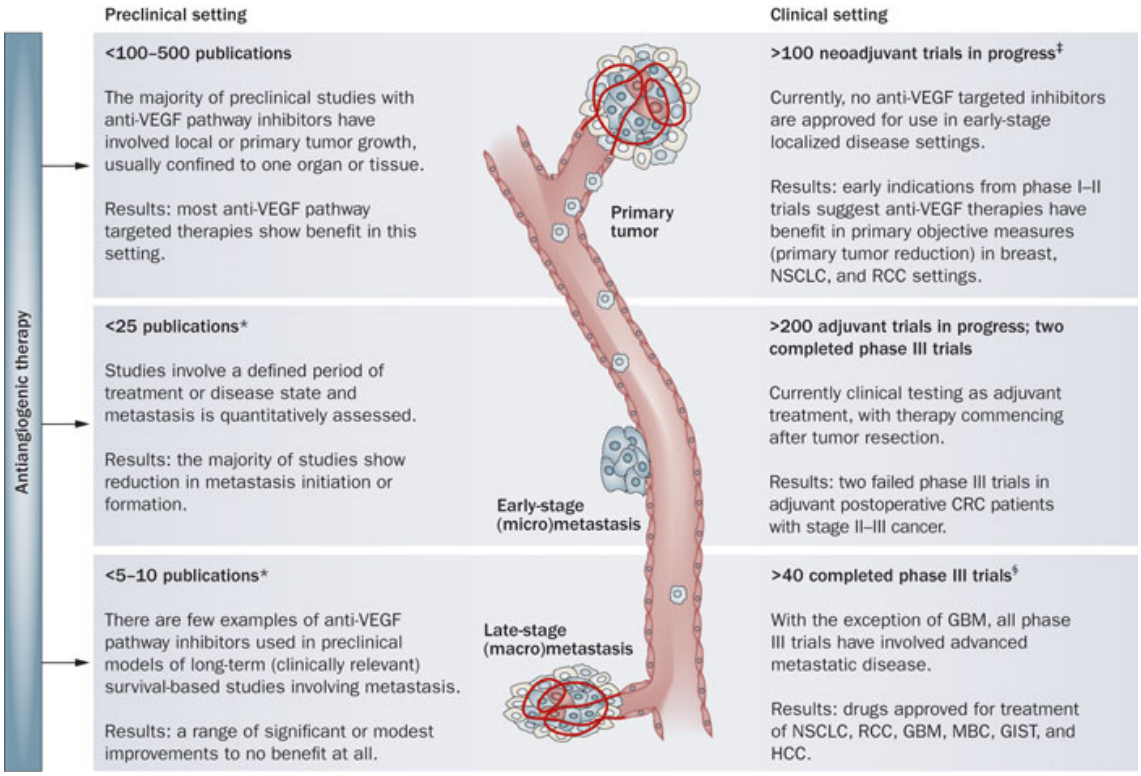

Figura 9. Eficacia variable del VEGF en el seguimiento de terapias. En la figura se muestra la diferencia entre pruebas preclínicas y clínicas. El número de estudios que se ha completado en clínica está inversamente correlacionado con el número de publicaciones preclínicas. Abreviaciones: CRC, cáncer colorectal; GBM, glioblastoma muliforme; GIST, tumor gastrointestional estromal HCC, carcinoma hepatocelular; MBC, cáncer de mama metastático; NSCLC, cáncer de pulmón de célula no pequeña; RCC, carcinoma de célula renal. *Adaptado de (Ebos and Kerbel 2011)*

2. TRANSICIÓN EPITELIO MESÉNQUIMA

2.1. Transición Epitelio Mesénquima

La Transición Epitelio Mesénquima (*Epithelial–Mesenchymal Transition*) (EMT) es un proceso biológico donde células epiteliales polarizadas, que normalmente interactúan con la membrana basal a través de su superficie basal, presentan múltiples cambios bioquímicos y morfológicos, los cuales le permiten adquirir un fenotipo de células

mesenquimales, aumentando su capacidad migratoria e invasiva, confiriéndoles resistencia a la apoptosis, y aumentando la producción de componentes secretores (Kalluri and Neilson, 2003). En el proceso de la EMT se activa la degradación de la membrana basal subyacente así como los cambios que conducen a la formación de una célula mesenquimal, capaz de migrar lejos de la capa epitelial en la que se originó. En este proceso están involucrados diferentes mecanismos moleculares con el fin de iniciar la EMT y asegurar su finalización. Entre estos mecanismos están incluidos la activación de factores de transcripción, la expresión de determinadas proteínas de la superficie celular, la reorganización y expresión de proteínas del citoesqueleto, la producción de enzimas que degradan la ECM y los cambios en la expresión de microARN específicos. En muchos casos, existen una serie de factores utilizados como biomarcadores **(Tabla 3)** que indican Transición Epitelio Mesénquima **(Figura 10).**

El proceso de EMT se presenta en diferentes situaciones biológicas, incluyendo la conversión de células epiteliales a mesenquimales durante el desarrollo y la edad adulta, la reparación de tejidos y en situaciones patológicas, por ejemplo durante inflamación y cáncer; donde se ha visto que la EMT es responsable del inicio de la conducta invasiva y metastática de tumores epiteliales, como son los carcinomas de alto grado. La EMT se puede clasificar en tres subtipos diferentes basados en el contexto biológico en que se produce. **(Figura 11)** (Kalluri and Weinberg, 2009)**.** La EMT Tipo 1 se asocia con la implantación y la gastrulación embrionaria, dando lugar al mesodermo, endodermo y células móviles de la cresta neural. El epitelio primitivo, en concreto el epiblasto, da lugar a un mesénquima primario a través del proceso de EMT. La EMT Tipo 2 se desarrolla en largos períodos de tiempo y puede llegar a destruir el órgano afectado si el daño inflamatorio primario no se elimina o atenúa. En la EMT Tipo 3, los epitelios secundarios asociados a muchos órganos pueden transformarse en células tumorales que permitirán la invasión y metástasis. En este caso, se producen células neoplásicas que previamente han sufrido cambios genéticos y epigenéticos en los genes que favorecen la expansión clonal y el desarrollo de tumores localizados. Estos cambios, que afectan sobre todo a oncogenes y genes

supresores de tumores, se asocian con los circuitos de regulación de EMT para producir resultados muy diferentes de los observados en los otros dos tipos de EMT (Kalluri and Weinberg, 2009).

Tabla 3. Marcadores asociados a la Transición Epitelio Mesénquima (EMT) en muestras clínicas para el pronóstico de pacientes (Iwatsuki et al.)

EMT-associated gene	Characteristics	Cancer types	Reference (author)
Epithelial marker			
E-cadherin	Type I cell–cell adhesion glycoprotein	Breast cancer	Gould Rothberg and Bracken[25]
		Gastric cancer	Chan *et al.*[24]
		Colorectal cancer	Doridi *et al.*[84]
Claudin-1	Tight junctions restrict lateral diffusion of lipids and membrane proteins	Lung cancer	Chao *et al.*[85]
		Renal cell carcinoma	Fritzsche *et al.*[86]
		Ovarian carcinoma	Kleinberg *et al.*[87]
Mesenchymal marker			
Vimentin	Intermediate filaments represent a third class of cytoskeletal elements	Breast cancer	Thomas *et al.*[88]
		Lung cancer	Al-Saad *et al.*[89]
		Gastric cancer	Utsunomiya *et al.*[90]
N-cadherin	Type I cell–cell adhesion glycoprotein	Esophageal cancer	Yoshinaga *et al.*[91]
		Lung cancer	Nakashima *et al.*[92]
		Urothelial tumor	Lascombe *et al.*[93]
Fibronectin	High-molecular weight extracellular matrix glycoprotein	Bladder tumor	Mutlu *et al.*[94]
		Colorectal cancer	Inufusa *et al.*[95]
		Ovarian carcinoma	Franke *et al.*[96]
Transcription factor			
Snail	Zinc finger transcriptional repressor	Adenocortical carcinoma	Waldmann *et al.*[97]
		Esophageal cancer	Natsugoe *et al.*[98]
		Hepatocellular carcinoma	Miyoshi *et al.*[99]
Slug	Zinc finger transcriptional repressor	Lung cancer	Shih *et al.*[100]
		Colorectal cancer	Shioiri *et al.*[101]
		Esophageal cancer	Uchikado *et al.*[102]
Twist	Basic helix-loop-helix transcription factors	Cervical cancer	Shibata *et al.*[103]
		Ovarian carcinoma	Hosono *et al.*[104]
		Breast cancer	Martin *et al.*[105]

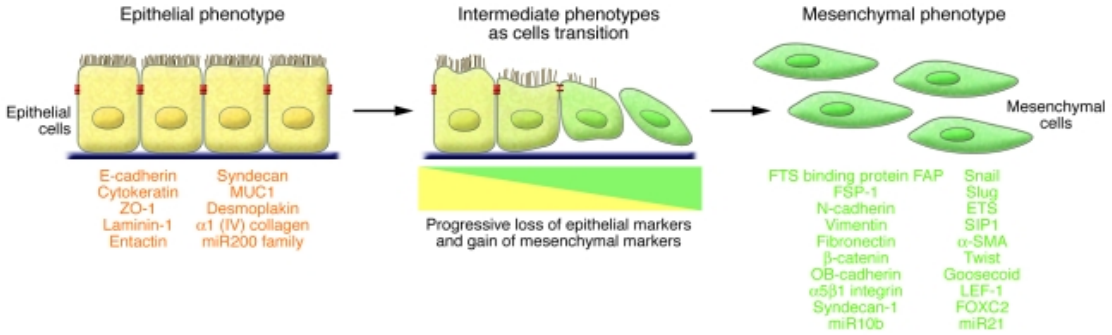

Figura 10. La Transición Epitelio Mesenquima (EMT) (*Epithelial–Mesenchymal Transition*). Este proceso implica una transformación funcional y morfológica de células epiteliales polarizadas en movimiento, y de células mesenquimales que secretan componentes de la matriz extracelular. Los marcadores epiteliales y mesenquimales de EMT están enumerados en esta figura. La colocalización de estos dos conjuntos de marcadores define un fenotipo intermedio de EMT, indicando que las células han pasado sólo parcialmente a través de este proceso. La detección de células que expresan ambos conjuntos de marcadores, hace que sea imposible identificar a todas las células mesenquimales que se originan en el epitelio a través de EMT, ya que muchas células mesenquimales expresan todos los marcadores epiteliales una vez que la transición se ha completado. Por esta razón, la mayoría de los estudios en ratones usan líneas celulares epiteliales marcadas para abordar la gama completa de los cambios inducidos por la EMT. ZO-1 (zona occludens 1); MUC1 (mucina 1 asociada a superficie celular); miR200 (microRNA 200); SIP1, (proteínas de la superficie de neuronas motoras asociadas a la proteina 1); FOXC2 (*Forkhead Box C2)* (Kalluri and Weinberg, 2009).

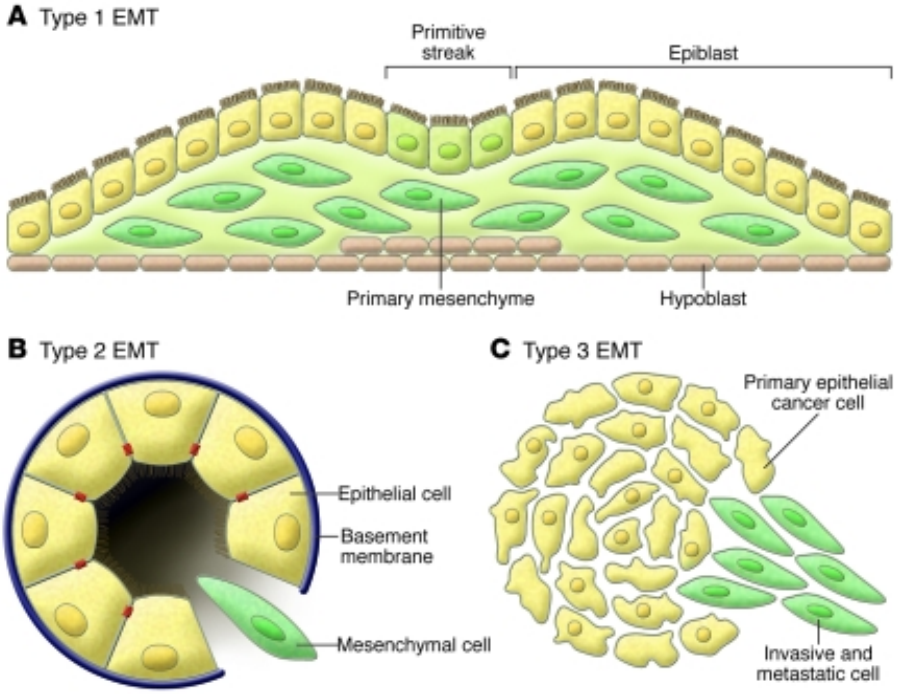

Figura 11. Diferentes tipos de EMT. (A) La EMT Tipo 1 se asocia con la implantación y gastrulación embrionaria, dando lugar al mesodermo, endodermo y células móviles de la cresta neural. El epitelio primitivo, en concreto el epiblasto, da lugar a un mesénquima primario. Éste puede formar epitelios secundarios a través de un nuevo proceso de EMT. Se sugiere que los epitelios secundarios pueden diferenciarse para formar otros tipos de tejidos epiteliales y que la EMT puede generar las células del tejido conectivo, incluyendo los astrocitos, adipocitos, condrocitos, osteoblastos y células musculares. (B) La EMT de Tipo 2 tiene lugar en el contexto de la inflamación y la fibrosis. A diferencia de la EMT de Tipo 1, el Tipo 2 se expresa en largos períodos de tiempo y puede llegar a destruir un órgano afectado si la respuesta inflamatoria primaria no se elimina o atenúa. (C) Finalmente, la EMT de Tipo 3 que ocurre en epitelios secundarios asociados a muchos órganos, que puede transformar células normales en tumorales que más tarde permiten la invasión y la metástasis (Kalluri and Weinberg, 2009).

Durante la EMT las uniones adherentes se modifican considerablemente, debido a que hay un reemplazamiento de E-cadherina por N-cadherina, un proceso llamado “cambio de cadherina” *(cadherin switching),* que se encuentra asociado al desarrollo tumoral. (Cavallaro et al., 2002; Voulgari and Pintzas, 2009). El desarrollo de la EMT está asociado con la agresividad tumoral y un mal pronóstico en carcinoma (De Wever et al., 2008, Hugo et al., 2007) (Sigurdsson et al. 2011). Esta conversión de células tumorales hacia un fenotipo mesenquimal implica la pérdida y disminución de la expresión de marcadores epiteliales como E-.cadherina y queratina, y por otro lado se acompaña de un incremento de marcadores mesenquimales como N-cadherina, vimentina y fibronectina, aumentando de

esta manera la movilidad y dando lugar a un fenotipo invasivo. (Moustakas et al., 2007, (Peinado et al., 2007), (Zeisberg and Neilson, 2009).

En los últimos veinte años se han estudiado en detalle las numerosas vías y factores involucrados en el proceso de activación de EMT. Algunos de los factores implicados en la inducción de este proceso son 1) los factores de crecimiento/diferenciación TGF-β, VEGF, HGF, EGF, FGF y la vía Wnt; 2) los factores de transcripción Snail, Slug, Twist, ZEB1, ZEB2 FOXC2 y E47; 3) determinados componentes de la matriz extracelular **(Figura 12).** (Moustakas et al., 2007, Peinado et al., 2007, (de Herreros et al. 2010).

La EMT Tipo 3 está asociada con progresión tumoral y metástasis. La excesiva proliferación de células epiteliales y la angiogénesis son el sello distintivo de la iniciación y el crecimiento inicial de los principales tumores epiteliales (Hanahan and Weinberg 2000). En muchos de estos estudios, se ha propuesto la activación de un programa de EMT como el mecanismo fundamental para la adquisición de fenotipos malignos por las células de tumor epitelial (Thiery 2002). Muchos estudios *in vitro* y en modelos murinos han demostrado que las células de carcinoma pueden adquirir un fenotipo mesenquimal, expresando marcadores como α-SMA, FSP1, vimentina y desmina (Yang and Weinberg 2008). Estas células normalmente se localizan en el frente invasivo del tumor primario, y son las que finalmente participan en los procesos de invasión y metástasis (intravasación, transporte a través de la circulación, extravasación, formación de micrometástasis, y finalmente colonización) (Thiery 2002, Fidler 2008, Brabletz 2001). **(Figura 13).**

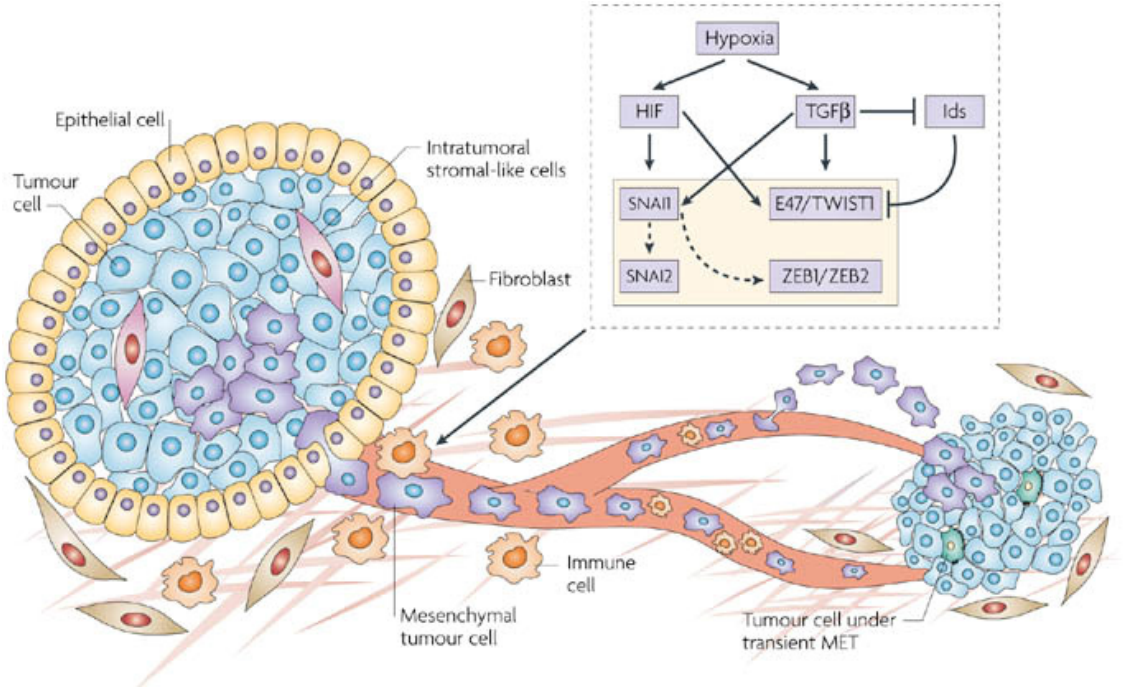

Figura 12. Figura esquemática de la interacción potencial de represores de CDH1 y el microambiente durante la progresión tumoral. Las señales derivadas de la hipoxia y el TGF-β podrían promover la interacción de Snail, Zeb y bHLH que regulan la represión CDH1 y la transición epitelio-mesénquima (EMT) durante la progresión tumoral. Snail1 podría estar implicado en el fenotipo migratorio inicial y considerado como un marcador temprano de EMT, que a veces contribuye a la inducción de otros factores. Por el contrario, Snail 2, ZEB1, ZEB2 y/o TWIST podrían ser responsables del comportamiento migratorio de las células, así como de su malignidad (Peinado et al., 2007)

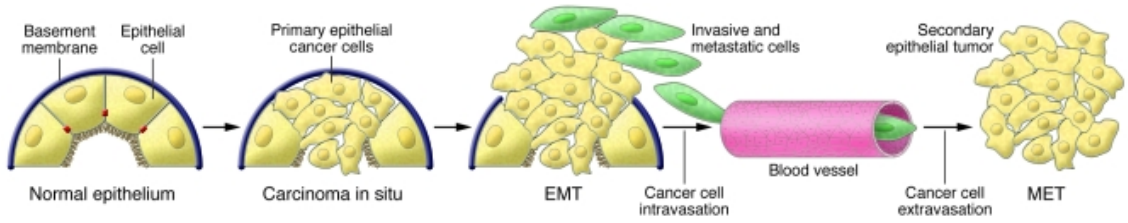

Figura 13. La progresión desde el epitelio normal al carcinoma invasivo pasa por varias etapas. La etapa de carcinoma invasivo consiste en la pérdida de polaridad de las células, lo que conduce a un desprendimiento de las mismas de la membrana basal. La composición de la membrana basal también cambia, alterando las interacciones célula-ECM y las redes de señalización. El siguiente paso consiste en la EMT y un cambio angiogénico, que facilita la fase maligna del crecimiento tumoral. La progresión de esta etapa a cáncer metastásico también incluye EMT, permitiendo que las células del cáncer entren en la circulación y salgan del torrente sanguíneo formando micro y macro-metástasis, lo que puede implicar el proceso inverso (MET), y así una reversión a un fenotipo epitelial. (Kalluri and Weinberg, 2009)

2.2. Transición endotelio-mesénquima (EndMT)

Dada la importancia de la interacción entre el endotelio vascular y las células cancerosas, especialmente en el proceso de invasión, es importante estudiar su relación con la EMT. En este contexto, se ha descrito el proceso de Transición endotelio-mesénquima (EndMT). En la EndMT las células endoteliales se despegan a partir de una capa de células organizadas e invaden el tejido subyacente **(Figura 14).** Este fenotipo denominado mesenquimal puede ser caracterizado por la pérdida de las uniones célula-célula, la adquisición de propiedades invasivas y migratorias, la pérdida de marcadores endoteliales, tales como CD31 (también conocido como molécula de adhesión de las plaquetas de las células endoteliales molécula-1 (PECAM-1)), y el aumento de los marcadores mesenquimales como los fibroblastos específicos de la proteína 1 (FSP1, también conocida como S100A4) o α-actina de músculo liso (αSMA) (Potts y Runyan, 1989; Nakajima 2000; Armstrong y Bischoff, 2004; Arciniegas et al, 2007; Zeisberg et al, 2007a, 2007b). Los primeros estudios donde se definió EndMT se realizaron inicialmente en el desarrollo embrionario del corazón. Se han publicado evidencias que muestran que la EndMT puede ocurrir después del nacimiento, así como en una variedad de procesos patológicos, incluyendo el cáncer y la fibrosis cardiaca (Zeisberg et al, 2007a, 2007b).

En el caso del cáncer, durante la EndMT, el 40% de las células afectadas son los fibroblastos asociados al cáncer (CAFs), células que juegan un papel importante en la progresión tumoral y pueden alterar el microambiente de varias maneras. Los CAFs pueden afectar a las moléculas de la matriz extracelular y secretar factores paracrinos alterando el comportamiento de los diferentes tipos celulares dentro del tumor. Además, los CAFs liberan señales potencialmente oncogénicas, como el factor de crecimiento transformante TGF-β, y son una fuente importante de VEGF, que promueve la angiogénesis (Kalluri y Zeisberg 2006).

La EndMT es catalogada muchas veces como una forma especializada de la EMT. Tanto EMT y EndMT dan lugar a células que tienen un fenotipo mesenquimal similar, y las evidencias sugieren que ambos procesos utilizan vías de señalización común. No obstante, dadas las

enormes diferencias entre las células endoteliales y epiteliales se deben profundizar en el estudio de ambos procesos (Potenta et al., 2008). Específicamente, las células endoteliales expresan diferentes proteínas implicadas en la unión célula-célula, como son las cadherinas ya descritas. La EndMT tiene gran importancia en cáncer y angiogénesis. Estudios recientes han demostrado que EndMT puede ocurrir en una variedad de procesos patológicos como el cáncer (Zeisberg et al, 2007a) y la fibrosis cardiaca (Zeisberg et al, 2007b). Con respecto al cáncer, EndMT es reconocida como una fuente única de CAFs (Zeisberg et al, 2007a). Se ha demostrado que los CAFs pueden facilitar la progresión tumoral de varias maneras (Kalluri y Zeisberg, 2006). Estudios realizados en tumores murinos han mostrado que una cantidad significativa de CAFs surgen a través de EndMT. Estos CAFs fueron identificados como una población única de células que coexpresan el marcador CD31 endotelial, junto con uno de los marcadores mesenquimales, FSP1 o αSMA. Estos datos sugieren que EndMT es un mecanismo importante para el reclutamiento de CAFs al estroma tumoral y que estos CAFs pueden tener un papel único en la progresión tumoral. Se ha demostrado que los vasos angiogénicos pueden someterse a EndMT. Los autores especulan que EndMT pueda jugar un papel en los brotes de la angiogénesis, donde las células apicales de los mismos, llevan un plexo vascular emergente y migran hacia los tejidos adyacentes. Mientras que las células migratorias sin luz (Gerhardt et al, 2003), las células de la punta tienen un fenotipo que parece presentar una EndMT. En el frente angiogénico, las células endoteliales migran y están expuestas a factores de crecimiento y moléculas de la matriz intersticial, como el colágeno de tipo I, que difieren de los componentes normales de la membrana basal vascular (Davis y Senger, 2005). Quizás en respuesta a estos factores, algunas células endoteliales pueden sufrir EndMT y mantener su fenotipo mesenquimal indefinidamente. Por otra parte, resultados previos han sugerido que las células de soporte vascular, tales como pericitos y/o células del músculo liso, pueden surgir del propio endotelio, y por lo tanto EndMT puede ser un mecanismo importante en el reclutamiento de estas células durante la angiogénesis (Armulik et al, 2005). Además, estas células de soporte vascular son un componente importante de los vasos maduros

(Armulik et al, 2005), y por lo tanto, EndMT puede jugar un papel importante en la estabilización de la estructura vascular, en la vasculogénesis y la angiogénesis.

Por otro lado, se ha demostrado la inducción de EndMT *in vitro* en células endoteliales. Diversos estudios han demostrado que la EndMT puede ser modulada en respuesta a la alteración en las vías de TGF-β. Se ha observado en estudios recientes que TGF-β2 (transforming growth factor-β2) estimula la EndMT a través de Smad (Medici et al.2011)(van Meeteren and ten Dijke 2012). También es probable que otras vías de señalización interactúen con TGF-β y Notch para mediar EndMT, por ejemplo, VEGF, NFAT, BMP, Wnt/β-catenina, ErbB y NF1/Ras durante el desarrollo cardíaco (Armstrong and Bischoff, 2004). Se piensa que Snail, que regula negativamente la expresión de VE-cadherina, interrumpe las uniones adherentes y permite que las células endoteliales se despeguen y entre en el proceso de EndMT. (Thiery and Sleeman, 2006)

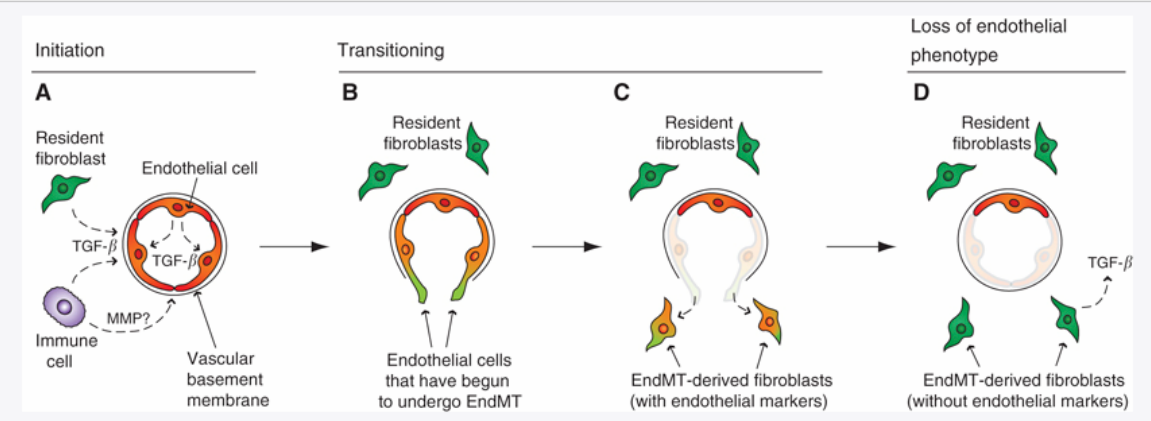

Figura 14. Etapas de EndMT. (A) La Transición Endotelio Mesénquima se inicia por las señales inflamatorias autocrinas y/o paracrinas originadas dentro de los tejidos circundantes, como el TGF-β. Las posibles fuentes incluyen los fibroblastos locales (verde) o las células inmunitarias (púrpura). Por otra parte, el endotelio (rojo) puede sufrir EndMT en respuesta directa a la lesión vascular. Es probable que la membrana basal vascular sea degradada por metaloproteinasas de la matriz (MMP), que se derivan de las células inmunitarias locales. (B-C) Durante la transición de las células endoteliales (rojo/verde) éstas adquieren un fenotipo migratorio, atraviesan la membrana basal vascular y comienzan a expresar marcadores mesenquimales, como FSP1, al mismo tiempo que expresan marcadores endoteliales. (D) Las células que han sufrido EndMT (verde) pierden su fenotipo endotelial. Es probable que estos fibroblastos derivados de EndMT produzcan diversos factores de crecimiento, como el TGF-β. Se desconoce si los vasos afectados son repoblados, y si siguen siendo funcionales después de la salida de las células endoteliales residentes (Potenta et al., 2008).

Tabla 4. Comparación de las células epiteliales, endoteliales y mesenquimales (Potenta et al., 2008).

Figure and tables index

	Epithelial cell	Endothelial cell	Mesenchymal cell
Cell–cell junctions:	adherens junctions w/E-cadherin tight junctions desmosomes	adherens junctions w/VE-cadherin *limited* tight junctions	None (or focal)
Organised cell layer:	✔	✔	No
Apico-basolateral polarity:	✔	✔	No
Basement membrane:	✔	✔	No, but makes interstitial matrix
Migratory:	No	No	✔
Intermediate filament:	Cytokeratin	Vimentin	Vimentin
Markers:	E-cadherin, claudins, occludins, desmoplakin, cytokeratin, mucin-1	CD31, VE-cadherin, Tie1, Tie2, VEGFR	FSP1, ∝SMA, vimentin, fibronectin, vitronectin, collagen types I and III

2.3 Cadherinas

Las cadherinas son un grupo de proteínas que participan en la adhesión célula-célula y la diferenciación celular, así como en el establecimiento y mantenimiento de la homeostasis de los tejidos. En el proceso de oncogénesis esta adhesión organizada es afectada por cambios genéticos y epigenéticos, resultando en un cambio en la señalización, pérdida de inhibición por contacto, alteración de la migración celular e interacción estromal. Una molécula de cadherina está formada por tres regiones: un dominio extracelular (EC); una región transmembranal (TM) y un dominio citoplasmático proximal de membrana conservado (MPCD). Asimismo dependiendo de la subfamilia puede tener otras regiones como un sitio de unión a catenina (CBS); un dominio extracelular proximal de membrana conservado (MPED); un dominio de anclaje intracelular (IA); un dominio rico en prolina (PL); un dominio de unidad repetida (RUD) y un dominio terminal (TD) **(Figura 15)** (Sánchez-Sanchez et al., 2005). El dominio EC (N-terminal), contiene los sitios que determinan la especificidad de la molécula para unirse a otras moléculas y los sitios encargados de la unión de calcio. Las cadherinas poseen una estructura altamente conservada entre los diferentes miembros de la superfamilia, especialmente

en el dominio extracelular. Esta estructura está formada por diferentes repeticiones extracelulares de dominio de cadherina (CD o EC) (Takeichi, 1995) (Peinado et al., 2004). Las cadherinas clásicas presentan 5 dominios (EC1 a EC5). El dominio EC1 es el más alejado de la membrana o también llamado distal, hasta EC5 que es el más cercano a la membrana o proximal. Cada uno de éstos (EC1 hasta EC5) está formado por aproximadamente 100 aminoácidos, incluyendo 10 aminoácidos que participan entre la unión de dominios. En esta unión interdominio se encuentran tres sitios responsables de la unión de calcio y contiene un dominio conservado His-Ala-Val (HAV) (Trejo Códova, 2008). Estas estructuras son responsables de la interacción homofílica de estas moléculas, especialmente el dominio N-terminal EC1. Sin embargo otras secuencias también son requeridas para la interacción entre cadherinas de diferentes subfamilias. La asociación de iones calcio con una unión a la región que conecta dos de los dominios EC induce el cambio conformacional necesario para que el dominio extracelular de las cadherinas formen sus interacciones celulares (Peinado et al., 2004). La región transmembrana es una glicoproteína caracterizada por la presencia de dos o más motivos repetidos de 110 residuos de aminoácidos, que atraviesa la membrana celular (Hulpiau and van Roy, 2009). En el citoplasma de la célula se ubica la región intracelular o carboxi-terminal (C-terminal); esta región conecta a las cadherinas con los filamentos de actina del citoesqueleto, unión que es necesaria para estabilizar la adhesión intercelular dependiente de cadherinas. El dominio citoplasmático de las cadherinas está también conservado dentro de las subfamilias, y en el caso de las cadherinas clásicas, este es el dominio que interactúa con cateninas, y que une las cadherinas al citoesqueleto de actina. Los dominios citoplasmáticos de las cadherinas son cruciales en la fuerza de las interacciones y en la señalización celular entre célula y célula (Peinado et al., 2004) (Hulpiau and van Roy, 2009). Las cadherinas son proteínas que pertenecen a una superfamilia de moléculas de adhesión celular estudiadas desde los años 90s. Las cadherinas se pueden clasificar en 5 subfamilias:

1) Cadherinas clásicas tipo I, localizadas en las uniones adherentes, incluidas E-cadherina, N-cadherina, P-cadherina, y R-cadherina.

2) Cadherina atípicas o altamente relacionadas tipo II, que incluyen las VE-cadherina, K-cadherina y H-.

3) Cadherinas desmosomales (desmocolinas y desmogleínas) que forman las uniones desmosomales, incluyen desmocolinas-1, -2, -3.

4) Protocadherinas implicadas en el desarrollo neuronal, incluyen Protocadherinas-α3, β1, -1, -8;

5) Cadherinas parecidas a Fat y cadherinas Flamingo (Nollet et al., 2000; Peinado et al., 2004). Las cadherinas están expresadas en varios tipos de tejidos con alguna especificidad: la E-Cadherina es el mayor representante de la células epiteliales, la N-cadherina en el sistema nervioso, células de músculo liso, fibroblastos y células endoteliales, la VE-cadherina es específica de endotelio (Cavallaro et al., 2006; Dejana et al., 2008; Francavilla et al., 2009; Gumbiner, 2005). En diferentes tipos de cáncer se han identificado diversas. En Melanoma la N-cadherina, P-cadherina y VE-cadherina (Berx and van Roy, 2009) (Harmon et al., 2009).

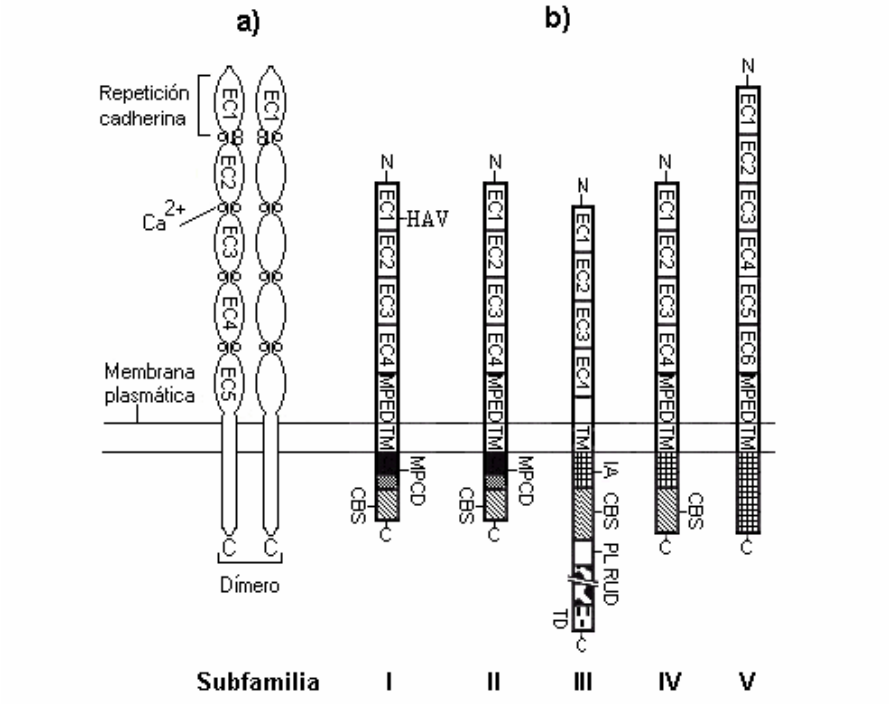

Figura 15. Estructura de la subfamilia (superfamilia) de las cadherinas. EC: Dominio extracelular cadherina, TM: Región transmembranal, MPCD: Dominio citoplasmático proximal de membrana conservado, CBS: Sitio de unión a catenina, MPED: Dominio extracelular proximal de membrana conservado, IA: Dominio de anclaje intracelular, PL: Unidor rico en prolina, RUD: Dominio de unidad repetida, TD: Dominio terminal. *Adaptados de* (Sánchez-Sánchez et al., 2005).

En el desarrollo tumoral las moléculas de adhesión celular “cell adhesion molecules” (CAMs) juegan un papel importante. Se pueden destacar tres tipos celulares donde las cadherinas juegan un papel importante: las células tumorales, las células endoteliales y otro tipo de célula estromal **(Figura 16)**.

Las células endoteliales forman la vasculatura y constituyen la mayor barrera entre la sangre y el resto del cuerpo. La disfunción endotelial es el resultado frecuente de la alteración de la permeabilidad de la monocapa de células endoteliales y esta es la característica principal de muchas patologías y enfermedades como la aterosclerosis, la diabetes, la hipertensión, la inflamación, los tumores y la metástasis (Harris and Nelson).

Las células endoteliales utilizan dos tipos de complejos de adhesión para mediar la interacción célula-célula: uniones adherentes y rígidas **(Figura 17)**. (Wallez and Huber, 2008). Las uniones adherentes participan en múltiples funciones, incluyendo el establecimiento y el mantenimiento de la adhesión célula-célula, remodelado del citoesqueleto de actina, señalización intracelular y regulación transcripcional. Las uniones rígidas regulan la permeabilidad de la monocapa y juegan un papel importante en el rol de las células endoteliales como barrera. El componente transmembrana de las uniones adherentes de las células endoteliales es la VE-cadherina (cadherina-5, CD144), cadherina clásica tipo II endotelial (Lampugnani et al., 2006) la cual se une con su región citoplasmática a los miembros de la familia de proteínas que contienen repeticiones “armadillo”, incluyendo p120-catenina, β-catenina y placoglobina (Wallez and Huber, 2008). Los complejos de VE-cadherina se asocian con el citoesqueleto de vimentina en algunas localizaciones vasculares (Kowalczyk et al., 1998). Estas asociaciones están mediadas por placoglobina y que interactúa con desmoplaquina la cual está asociada a vimentina. VE-cadherina se encuentra específicamente en todos los subtipos de endotelios (Lampugnani et al., 2006).

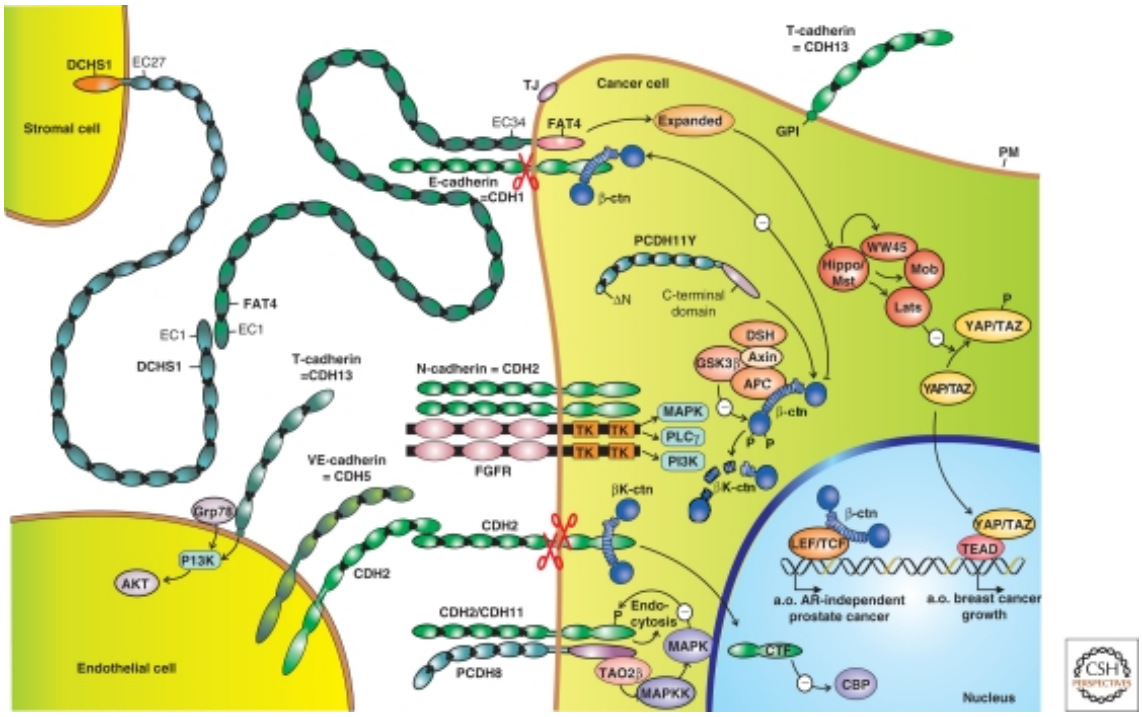

Figura 16. Selección de los patrones de expresión y las actividades de los miembros de la superfamilia de cadherina en cáncer. Tres tipos de células se representan parcialmente en la figura: una célula de cáncer, una células endotelial, y otro tipo de célula estromal. Los dominios de la proteína de color verde son totalmente homólogas para estos en el prototipico E-cadherina/CDH1. Los dominios en otros colores se desvían sustancialmente en la estructura y función. Los puntos negros representan iones Ca^{2+}. Las flechas con símbolos de signo menos se refieren a la influencia inhibitoria directa o indirecta. Se muestran las cadherinas en la superficie de las células del cáncer donde se expresan en la zona apical de la membrana (T-cadherina/CDH13 por encima de la unión fuertes, TJ), o en las membranas laterales (E-cadherin/CDH1, N-cadherin/CDH2, cadherin-11/CDH11). Los tres últimos probablemente se producen como cis-homodímeros. La CDH1 es frecuentemente inactivada en las células cancerosas, mientras que las cadherinas "mesenquimales" CDH2 y CDH11 son a menudo aumentadas. La CDH2 pueden interactuar con el receptor del factor de crecimiento de fibroblastos (FGFR), potenciando su señalización a través de las enzimas MAPK, PLCγ, y PI3K. Las cadherinas son propensas al procesamiento proteolítico (símbolos de tijeras), que libera tanto el ectodominio o un fragmento carboxi-terminal (CTF). En el caso de CDH2, este CTF se ha demostrado que entra en el núcleo e inhibe la proteína de unión CREB (CBP). En la activación neural, CDH2 y CDH11 están asociadas con protocadherina-8 (PCDH8 o Arcadlin), lo cual resulta en la activación de la TAO2β MAPKKK, llevando eventualmente a la endocitosis de las cadherinas. PCDH8 es a menudo silenciada en las células cancerosas, pero no está claro si se trata de una relación causal con la regulación de las cadherinas mesenquimales. La actividad transcripcional de β-catenina (β-cnt) en un complejo nuclear con LEF/TCF, lleva, entre otros efectos, al crecimiento del cáncer de próstata independiente del receptor de andrógeno (AR) a ser este fenómeno es inhibido por el secuestro de β-ctn por la E-cadherina o por la degradación de β-ctn después de la fosforilación (-P) por GSK3β. La degradación de β-ctn se produce en un complejo de la degradación citoplasmática con APC (poliposis adenomatosa), Axin y Disheveled (DSH). Sin embargo, la actividad nuclear β-ctn-LEF/TCF es estimulada por un mecanismo desconocido en una variante del citoplasma protocadherina-11Y (PCDH11Y) que carecen de un péptido señal debido a un grupo amino terminal truncado (ΔN). Las células endoteliales asociados al tumor expresan VE-cadherina/CDH5, CDH2 y T-cadherina/CDH13. La segunda está vinculada a la PM a través de anclaje de glicosilfosfatidilinositol (GPI) y las señales secretadas a través de Grp78/BiP a un vía anti-apoptótica PI3K-AKT. Dachsous-1 (DCHS1) y FAT4 son enormes proteínas relacionadas con cadherina, que interactúan entre sí de forma heterofílica (diferentes tipos de proteínas) y de forma heterotípica (diferentes tipos de células). El silenciamiento de FAT4 humanos se ha visto en cáncer de mama y su activación está relacionada con una vía Hipo-YAP, no resuelta, la cual controla el tamaño de los órganos en *Drosophila* y está afectada en varios cánceres humanos (Mackiewicz et al.) repeticiones de cadherina extracelular; (PM) membrana plasmática; (TK); dominio de la tirosina quinasa. *Adaptado de (Berx and van Roy, 2009)*.

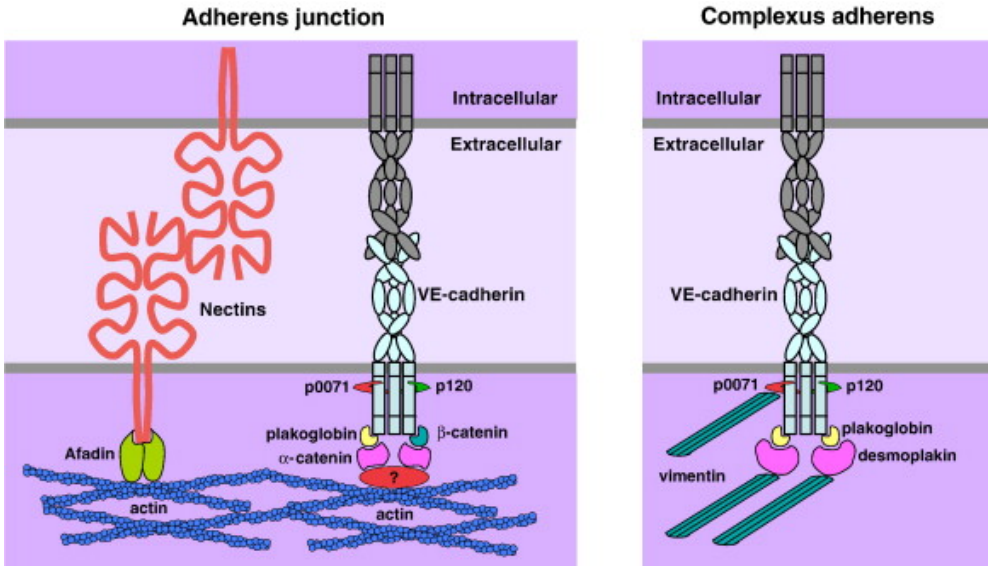

Figura 17. Uniones adherentes y complejos adherentes en células endoteliales. En las uniones adherentes AJs "Adherens junctions" la VE–cadherina interactúa directamente en una zona distante de la membrana con α y β catenina y en la zona de la juxtramembrana con p120 y p0071. El enlace molecular exacto entre el complejo VE-cadherina-catenina y los filamentos de actina son poco conocidos. Las nectinas están unidas al citoesqueleto de actina vía afadina. En los complejos adherentes, la VE–cadherina está asociada con filamentos de vimentina vía plakoglobina/desmoplaquina o p0071. *Adaptado de (Wallez and Huber, 2008).*

La VE-cadherina participa en múltiples aspectos de la biología de las células endoteliales, y es indispensable en la maduración, extensión y remodelación de los vasos sanguíneos que caracterizan la angiogénesis. (Morita et al., 1999). La VE-cadherina es esencial para controlar la permeabilidad de la monocapa endotelial y la angiogénesis. Están descritos varios mecanismos que regulan la VE-cadherina, incluyendo la modulación de la actividad de la VE-Cadherina a través de la fosforilación y el control de los niveles de VE-cadherina disponibles para las uniones adherentes tanto de la proteína como del mRNA. La fosforilación de VE-cadherina conduce a la desestabilización de los complejos de las uniones adherentes y al incremento de la permeabilidad de la monocapa. Diversos factores solubles como el factor de crecimiento endotelial vascular (VEGF) "*Vascular Endothelial Growth Factor*", factor de necrosis tumoral alfa (TNFα) "*tumor necrosis factor"*, factor de activación de plaquetas (PAF) "*Platelet-activating factor"*, trombina e histamina fosforilan a VE-cadherina. Asimismo cuando p120-catenina, β-catenina y placoglobina están fosfoforiladas hay una desestabilización de las uniones celulares (Harris and Nelson). La interacción entre VE-cadherina y p120-catenina es importante en la función

de la barrera endotelial (Iyer et al., 2004). Los ratones deficientes en VE–cadherina mueren durante el desarrollo embrionario por el daño en la formación de lumen vascular por apoptosis de las células endoteliales (Carmeliet et al., 1999).

Es importante destacar otras uniones: las uniones celulares rígidas, compuestas por proteínas transmembranas que incluyen las claudinas, ocludinas y moléculas de unión por adhesión "junctional adhesion molecules" (JAMs) **(Figura 18)**. En las proteínas de membrana asociadas con el citoplasma están la "zonula occludens" (ZO), "AF-6/afadin", y PAR-3. De los componentes transmembrana, la claudina-5 es específicamente expresada en células endoteliales. Existen otras moléculas de adhesión características de células endoteliales, la denominada "platelet and EC adhesion molecule "PECAM-1 o también llamada CD31(Woodfin et al., 2007).

En las células tumorales un miembro prototípico, E-cadherina, ha sido caracterizado como un potente supresor de la invasión y la metástasis. La región promotora de E-cadherina se representa a través de elementos de control en regiones proximales, ejerciendo un efecto positivo o negativo sobre la expresión de la E-cadherina **(Figura 20).**

Los cambios en la expresión de las cadherinas juegan un papel crítico durante la progresión del tumor; tales cambios pueden ser concomitantes o el resultado de la conversión de células tumorales nacientes de un fenotipo epitelial a un fenotipo mesenquimal. La pérdida de expresión o función de la E-cadherina en carcinomas epiteliales ha sido considerada como la razón principal para la ruptura del contacto estrecho célula-célula del tejido epitelial, conduciendo a la progresión tumoral y a un estado invasivo metastático (Hajra and Fearon, 2002). Se ha observado que la función de la E-cadherina está ausente en muchos cánceres epiteliales debido a: 1) inactivación mutacional de E-cadherina o de genes de cateninas, 2) represión transcripcional o 3) proteolisis del dominio extracelular, lo que indica que estas moléculas juegan un importante papel supresivo en la tumorigénesis epitelial.

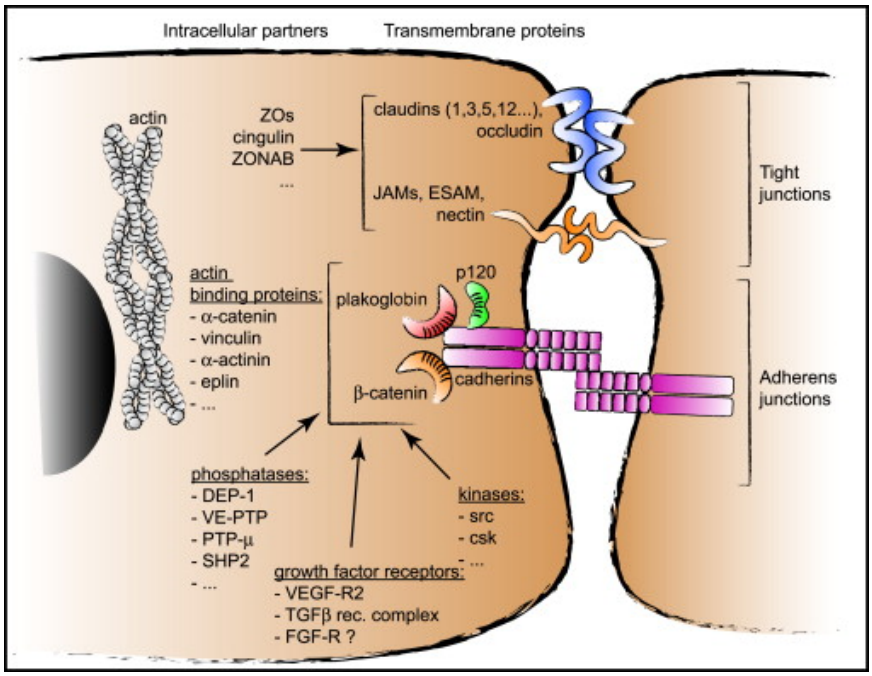

Figura 18. Representación esquemática de las uniones fuertes y las uniones por adherencias en células endoteliales. En las células endoteliales, la adhesión por uniones fuertes es mediada por claudinas, ocludinas, miembros de la familia JAM y ESAM. Los componentes citoplasmáticos de las uniones fuertes son ZO proteínas, cingulinas, ZONAB y otros. En las uniones adherentes, la adhesión es promovida por cadherinas (VE-cadherina y N-cadherina) las cuales se unen a p120, β-catenina y placoglobina. Las nectinas y sus compañeros intracelulares afadina/AF-6 participan en la organización de ambas uniones fuertes y adherentes. Un largo grupo de proteínas unidas a actinas se encuentran asociadas en las uniones adherentes tales como cateninas, vinculinas, α-actina, eplina y otras. Además, fosfatasas (DEP-1, VE-PTP, PTPμ, SHP2, etc.) y quinasas (src, csk, y otras) están directa e indirectamente asociadas a los componentes de las uniones adherentes. Receptores de factores de crecimiento de: VEGF, receptores 2 (también llamados flk–1 o KDR) y complejo de receptor TGFβ pueden unirse al complejo de VE-cadherina. Esta interacción modula sus propiedades de señalización. *Adaptado de* (Dejana et al., 2009).

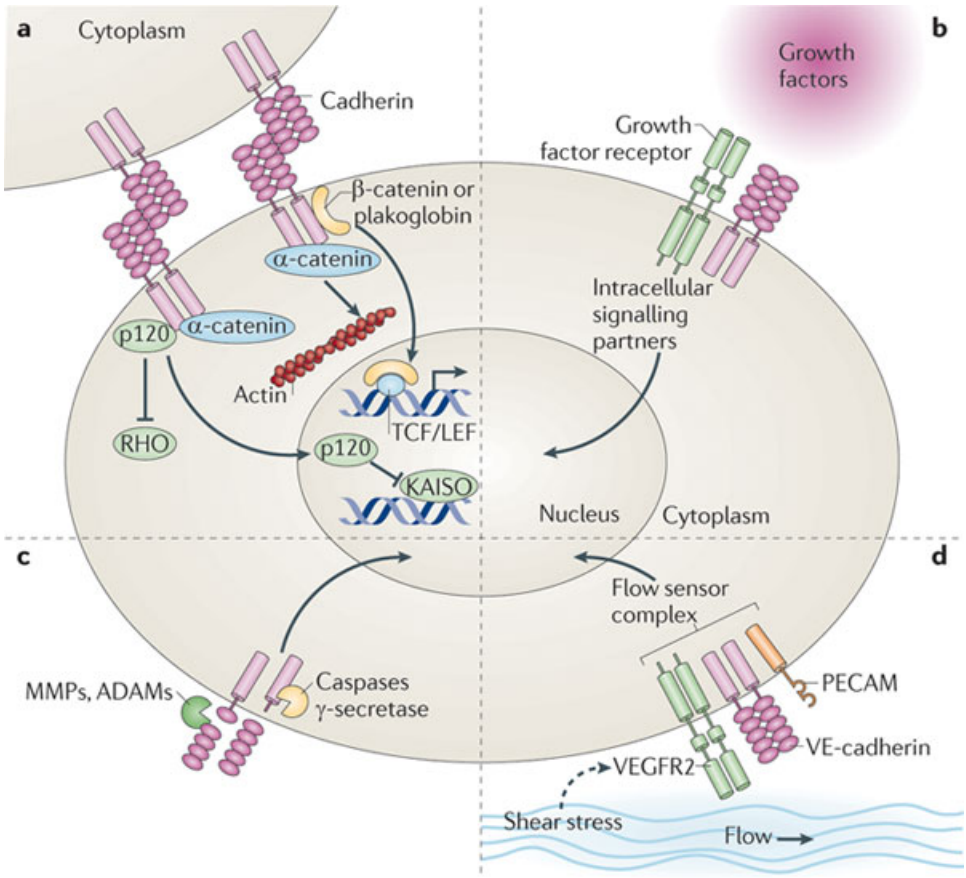

Figura 19. Vista esquemática de las vías de señalización reguladas por cadherinas. Señalización a través de cadherinas. Esquema de las vías de señalización reguladas por cadherinas. A) cadherinas pueden señalar indirectamente, mediante el reclutamiento de proteínas de señalización, incluyendo β-catenina, la p120-catenina (P120) y la unión plakoglobina (plakoglobina) a la membrana, la cual, una vez que se liberan de la cola de cadherina, se puede trasladar al núcleo y regulan la transcripción. Sin consolidar, el P120 también puede regular la motilidad celular al inhibir la actividad de Rho GTPasas. Cuando se libera en el citosol, α-catenina puede regular la agrupación de actina. La consolidacion de α-catenina en el complejo cadherina está mediada por β-catenina. B) las cadherinas pueden formar unidades de señalización mediante la interacción con los receptores del factor de crecimiento como el factor de crecimiento endotelial vascular 2 (VEGFR2), el receptor de factor de crecimiento epidérmico (EGFR), el receptor del factor de crecimiento de fibroblastos (FGFR), derivado de las plaquetas del receptor del factor de crecimiento (PDGFR) y el receptor de crecimiento transformante beta (TGF-Factor) Además, pueden formar unidades de señalización intracelulares asociadas con señalización tales como quinasas (por ejemplo, quinasas SRC familiares y la Tyr-proteína quinasa CSK (CSK)), así como con fosfatasas tales como la densidad mejorada-fosfatasa (DEP1, también conocida como RPTPη), Tyr-proteína fosfatasa no receptora tipo 11 (también conocido como SHP2) y endotelial vascular proteína fosfatasa Tyr (VE-PTP, también conocida como RPTPβ). Por último, las proteínas del adaptador, como miembros de la familia SHC también pueden formar unidades de señalización con cadherinas. C) Después de la familia de MMP (metaloproteasas de la matriz) o ADAM (miembro de la desintegrina y metaloproteasa) mediada por la proteasa del derramamiento de las ectodominias, proteasas intracelulares como las caspasas y la γ-secretasa que rompen la cola citoplasmática de cadherina, que luego pueden trasladar al núcleo y regular la transcripción. D) La cadherina vascular endotelial (VE-cadherina) en las células endoteliales puede formar un complejo sensor del flujo con plaquetas de moléculas de adhesión celular endotelial (PECAM) y VEGFR2. Este complejo de transferencias de señales intracelulares ayudan a las células a adaptarse a las condiciones de esfuerzo constante. LEF, factor potenciador linfoide, TCF, el factor de células T. *Adaptado de* (Cavallaro and Dejana 2011).

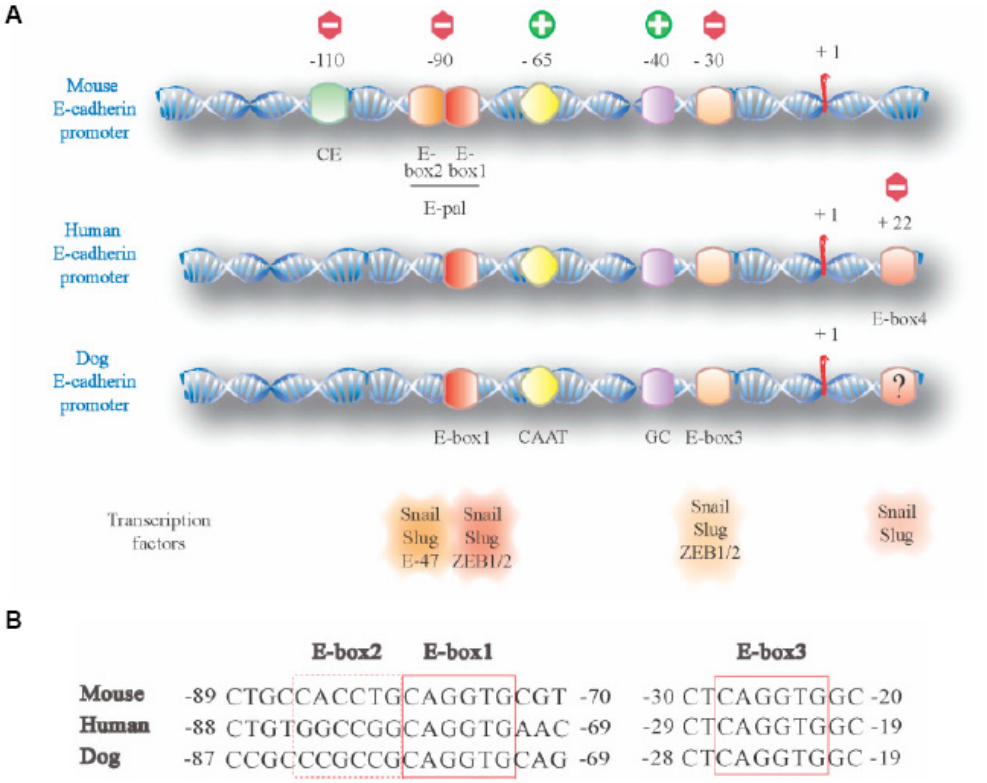

Figura 20. Representación esquemática de la región promotora y la secuencia E-caja de la E-cadherina en ratón, humano y perro. (a) La región promotora de E-cadherina se representa a través de elementos de control en regiones proximales (ejerciendo un efecto positivo o negativo sobre la expresión de la E-cadherina). La caja CAAT, GC y E-caja1/E-caja3 están conservadas en los tres promotores, mientras que E-caja2 solo está presente en la región promotora de ratón. Una E-caja adicional (E-caja4) aguas abajo del sitio de inicio de la transcripción está presente en el promotor humano, pero no está conservada en la región comprendida en el promotor de ratón. Los factores de transcripción que reconocen las distintas secuencias consenso de las E-cajas se muestran en la parte inferior de la figura **(b)**. Secuencias de las E-cajas presentes en la región proximal de las secuencias de E-cadherina de ratón, humano y perro. Se observa el alto grado de conservación de la E-caja1 y E-caja3. *Adaptado de* (Peinado et al., 2004)

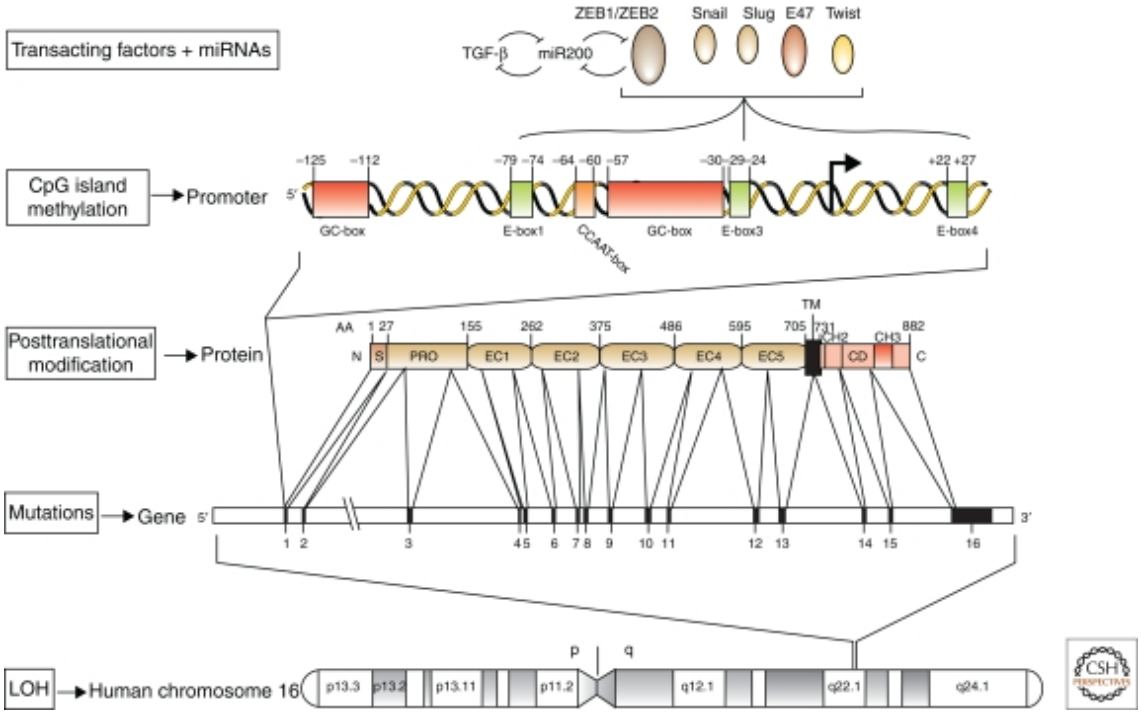

Figura 21. Distintos niveles de regulación de la expresión de E-cadherina en tumores humanos. La E-cadherina CDH1 está en el cromosoma 16q22.1 (que se muestra en la parte inferior). Esta región muestra con frecuencia pérdida de heterocigosidad (LOH) en diferentes tipos de carcinoma humano. Las mutaciones específicas de inactivación se encuentran dispersas en toda la región de codificación y son particularmente abundantes en el cáncer esporádico lobular de mama y el cáncer gástrico difuso. Las mutaciones en línea germinal pueden producir el síndrome hereditario de cáncer gástrico difuso. Además, modificaciones post-traduccionales, tales como la fosforilación, glicosilación o procesamiento proteolítico pueden afectar a la funcionalidad de E-cadherina. El silenciamiento epigenético se ha asociado con la metilación de CpG en la región promotora CDH1 o con unión directa de represores transcripcionales específicos a las secuencias de *E-box* en esta región. Los represores transcripcionales ZEB1/δEF1 ZEB2/SIP1 son reprimidos por miRNAs de la familia miR-200. A su vez, los factores de transcripción ZEB reprimen la transcripción de los genes miR-200. Así, un sistema regulador bifásico controla el equilibrio entre el estado epitelial y mesenquimal en respuesta a las señales entrantes. El TGF-β en el microambiente del tumor puede inducir la expresión de proteínas ZEB, al menos aguas abajo de la regulación por los microRNA de la familia miR-200. Esto resulta en un bucle de auto-mejora que conduce a desdiferenciación epitelial y la invasión. Ver texto para más detalles y referencias. AA, posición del aminoácido, C, carboxi-terminal, CD, dominio citoplásmico, CE, cadherina extracelular, N amino-terminal, PRO, propéptido, S, péptido señal TM, en la región transmembrana. Las flechas señalan la iniciación de la transcripción *Adaptado de* (Berx and van Roy, 2009).

2.4. Vimentina.

La vimentina es una proteína de 54 KDa expresada y altamente conservada perteneciente a las proteínas del tipo III, de la familia de proteínas de filamentos intermedios (FI) (Hillen and Griffioen). (Tang, 2008). La vimentina está constituida por 466 aminoácidos con un dominio α-hélice altamente conservado denominado dominio “rod”, que está limitado por un dominio no α-helice N-terminal "head" con 77 residuos, y un dominio C-

terminal "tail" con 61 residuos (Goldie et al., 2007) formando estructuras básicas de dímeros y tetrámeros de proteínas de FI **(Figura 22)** (Fuchs and Hanukoglu, 1983). Dentro de la estructura de la vimentina se pueden encontrar estructuras de homopolímero y heteropolímero (asociado con otras proteínas de FI tipo III y tipo IV) estructuras comunes dentro de los miembros de la familia de proteínas de FI. La presencia de dominios α-helice contribuye a la formación de polímeros de gran estabilidad y determina el estado de fosforilación de proteínas integrales (Ku et al., 1996). La vimentina es una de las proteínas más abundantes en las células mesenquimales y se usa frecuentemente como marcador de desarrollo de células y tejidos. Estudios recientes muestran la vimentina como una proteína crítica en procesos de adhesión, migración y señalización celular (Ivaska et al., 2007). La fosforilación es clave en la regulación de la dinámica de los FI, modulando la organización de redes de FI y su distribución subcelular (Omary et al., 2006). La vimentina tiene patrones de fosforilación complejos (Eriksson et al., 1992; Eriksson et al., 2004; Kochin et al., 2006) con sitios de fosforilación para diferentes quinasas que actúan de manera específica en diferentes procesos celulares, como mitosis (Takai et al., 1996), diferenciación (Gard and Lazarides, 1982) y estrés (Stefanovic et al., 2005). La vimentina es importante en la adhesión celular, regulando la función de integrinas (Ivaska et al., 2007). Asimismo, la vimentina juega un papel funcional importante en la regulación del contacto célula-célula, especialmente en células endoteliales (Vincent et al., 2004) **(Figura 23).**

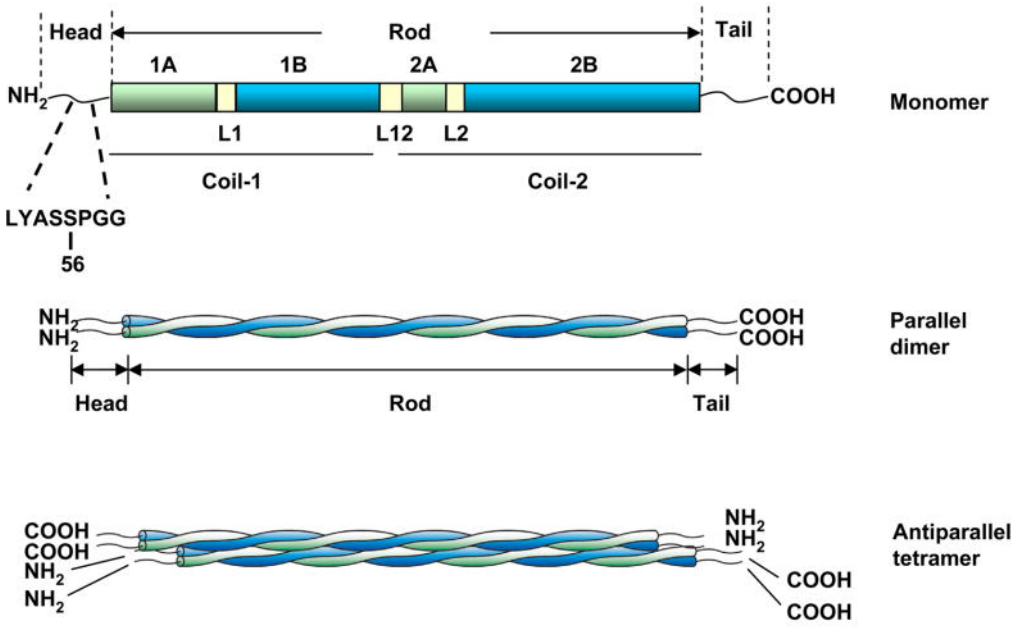

Figura 22. Estructura molecular de la vimentina en el músculo liso. La estructura de la vimentina se compone de un dominio central, un dominio N-terminal "head", y un dominio C-terminal "tail". Estos dominios están separados por 3 conectores conocidos como L1, L12 y L2. En su conjunto, dos monómeros enrollados entre sí constituyen un dímero paralelo. Un par de dímeros asociados lateralmente se asocian formando un tetrámero antiparalelo, que constituye una subunidad proteica soluble para el ensamblaje de FI en las células. La Ser-56 es el sitio de mayor fosforilación de la vimentina en el músculo liso en respuesta a la estimulación contráctil. La Desmina comparte una estructura molecular similar y el mismo proceso de ensamblaje (Tang, 2008).

La expresión de vimentina se ha encontrado en una amplia gama de tipos celulares incluyendo las células precursoras del páncreas, las células de Sertoli, las células precursoras neuronales, células trofoblásticas gigantes, fibroblastos y células endoteliales que revisten los vasos sanguíneos, las células tubulares renales, macrófagos, neutrófilos, células mesangiales, los leucocitos, y células del estroma renal (Satelli and Li 2011). La vimentina es considerada como un marcador del proceso de “EMT”. En ratones *knockout* para vimentina se ha visto un fenotipo normal sin defectos aparentes (Colucci-Guyon et al., 1994). Esta observación sugiere que la vimentina no es fundamental para la supervivencia del ratón bajo condiciones fisiológicas normales. Sin embargo, estudios más recientes muestran que los ratones *knockout* para vimentina tienen una deficiencia en el proceso de cicatrización de heridas tanto en embriones como en adultos porque sus

fibroblastos están afectados en su capacidad de migración (Eckes et al., 2000).

Diferentes resultados han sugerido que la vimentina juega un papel importante en el desarrollo del cáncer (Ivaska et al., 2007). Sin embargo, existe una discrepancia entre resultados, siendo esto atribuido al hecho de que la vimentina está expresada de forma diferencial en distintos tipos de células y puede tener funciones tejido-específicas, así como afectar la relación con otras proteínas de FI. En diferentes tipos de líneas celulares y tejidos tumorales, incluyendo cáncer de próstata, cáncer de mama, cáncer endometrial, tumores del sistema nervioso central, melanoma maligno y tumores del tracto gastrointestinal que incluyen páncreas, colorrectal y cáncer hepático, se ha observado un aumento de la expresión de vimentina. La información relacionada con los patrones de expresión y función de estas proteínas en diferentes tejidos cancerosos es de gran importancia en el diagnóstico, pronóstico y desarrollo terapéutico de estas enfermedades (Satelli and Li).

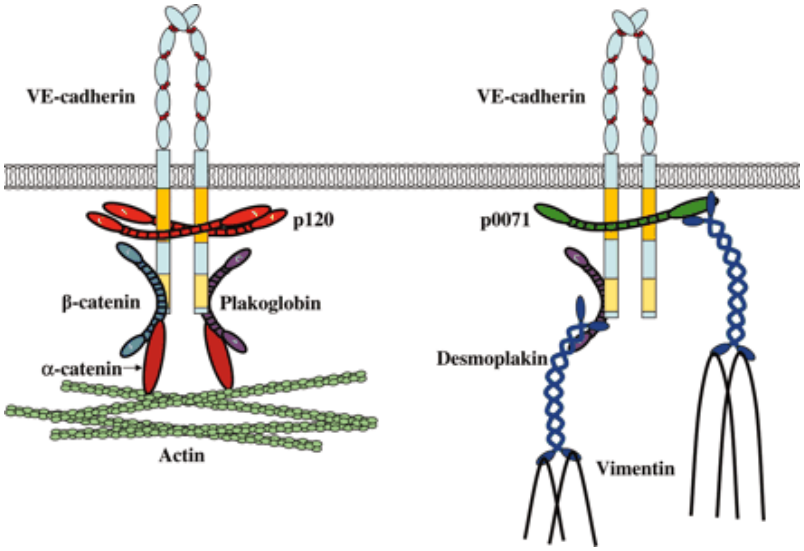

Figura 23. Representaciones esquemáticas de las interacciones entre proteínas dentro de las uniones intercelulares endoteliales. Se ha mostrado que la VE-cadherina participa en las interacciones proteicas que conducen a dos asociaciones de actina y vimentina P120 y p0071, que se asocian tanto con el dominio de la yuxtamembrana de la VE-cadherina, mientras que la β-catenina y la placoglobina asociada con el dominio de unión distal catenina y P120 se cree que median en el ensamblaje de estructuras adhesivas basadas en actina, mientras tanto la placoglobina y p0071 están asociadas con la desmoplaquina, que son proteínas de unión de filamentos intermedios. Es necesario tener en cuenta que el esquema presenta interacciones conocidas, pero no se pretende excluir a otras combinaciones de eventos de enlace que pueden ocurrir con las uniones intercelulares endoteliales (Vincent et al., 2004)

La evaluación del patrón de expresión de vimentina en los tejidos normales y cancerosos puede ser de gran utilidad en el diagnóstico y el pronóstico del tumor. La vimentina se utiliza principalmente como un marcador para la transición epitelio-mesénquima en asociación con otros marcadores conocidos. La mayoría de tumores sobreexpresan vimentina, sirviendo como un indicador de mal pronóstico **(Figura 24).** Se ha estudiado la expresión de vimentina en diferentes tipos de cánceres, como son cáncer de próstata (Zhao et al., 2008, Lang et al., 2002), tracto gastrointestinal (Fuyuhiro et al., 2010), mama (Guilles et al., 2003, Kokkinos et al., 2007), tumores del sistema nervioso (Yamada 1992, Fortin 2010). Asimismo, la vimentina se sobreexpresa en otros tipos de tumores, incluyendo cáncer de cuello uterino (Guilles et al., 1996, Gustmann et al., 1991), carcinoma de células renales (Williams et al., 2009), ciertos tipos de linfomas (Gustmann 1991), el carcinoma papilar de tiroides (Yamamoto 1992), y los carcinomas de endometrio (Coppola et al., 1998). Es importante resaltar los estudios de vimentina en melanoma. Recientes análisis proteómicos en una amplia gama de muestras de melanoma, demostraron que la sobrexpresión de vimentina en tumores primarios no sólo sirve como un marcador diagnóstico, sino que también tiene valor en la predicción de la metástasis hematógena (Li et al., 2010). Los estudios de expresión de vimentina pueden ser de gran utilidad en la predicción de la evolución clínica, proporcionando estrategias individuales de tratamiento para pacientes con melanoma. Asimismo, otros estudios han abordado la relación entre la sobreexpresión de vimentina y su asociación con metástasis y mayor potencial invasor de las células de melanoma (Chu 1996 et al., , Hendrix et al., 1992, Ben-Ze'ev et al 1985., Caselitz et al., 1983, Li et al., 2010). Por otro lado, es importante destacar los estudios de vimentina en cáncer de pulmón, en adenocarcinomas moderadamente y bien diferenciados, y en carcinomas de células gigantes (Upton et al., 1986). En cáncer de pulmón de células pequeñas no resecados, la sobreexpresión de vimentina puede predecir la supervivencia en pacientes (Al-Saad et al., 2008). En otro estudio, se observó que la vimentina glicosilada era regulada negativamente en adenocarcinomas de pulmón; por tanto, se propueso como un nuevo biomarcador para el diagnóstico funcional y la respuesta al tratamiento de cáncer de pulmón (Rho

et al., 2009). Además, la vimentina se expresa diferencialmente en líneas celulares de cáncer de pulmón, y la poli (ADP-ribosa) polimerasa 1 es un modulador positivo de la región promotora de vimentina al inducir su expresión en las células de cáncer de pulmón (Chu et al., 2007).

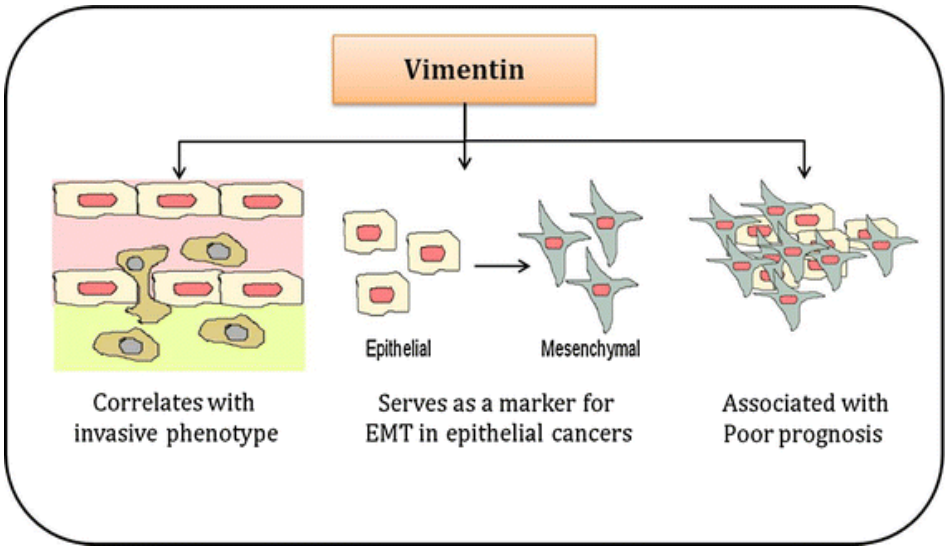

Figura 24. El papel de la vimentina en cáncer. La sobrexpresión de vimentina se asocia frecuentemente con una mayor capacidad migratoria/invasiva de células cancerosas. La vimentina se utiliza principalmente como un marcador para la transición epitelio-mesénquima en asociación con otros marcadores conocidos. La mayoría de tumores sobreexpresan vimentina, sirviendo como un indicador de mal pronóstico (Satelli and Li 2011).

La expresión de vimentina es transactivada por β-catenin/TCF que se une al sitio putativo 468 pb aguas arriba del sitio de iniciación de la transcripción del promotor de vimentina, aumentando así el potencial invasor de las células tumorales (Guilles et al., 2003). Se ha demostrado que NF-kB juega un papel importante en la regulación del proceso de EMT (Min et al., 2008) y que su inhibición en las células mesenquimales puede revertir el proceso de EMT (Huber et al., 2004) lo que sugiere que NF-kB es importante tanto en la activación como en el mantenimiento de la EMT. El elemento de respuesta a TGFβ1 se encuentra en la región promotora de vimentina sensible a AP1 y está involucrado en la regulación de la expresión de vimentina en mioblastos y miotubos (Wu et al., 2007). El gen de la vimentina puede sufrir modificaciones epigenéticas, en carcinomas colorrectales avanzados se ha observado con frecuencia esta modificación,

por lo que se ha propuesto como un marcador diagnóstico para la detección y seguimiento de pacientes (Shirahata et al., 2009).

El citoplasma de las células mesenquimales está constituido por FI de vimentina que participan en el mantenimiento e integridad de la citoarquitectura [5]. La vimentina se sabe que interactúa con una gran cantidad de proteínas y participa en diversas funciones celulares **(Tabla 5)**, también está involucrada en otros procesos que implican la formación de complejos con las moléculas de señalización celular y otras proteínas adaptadoras **(Figura 25)** y además, se une a Erk fosforilada, una proteína quinasa activada por mitógenos, para protegerlo de la desfosforilación (Person 2006). AKT1 quinasa se une a la vimentina fosforilada y de esta manera puede protegerla de la proteólisis inducida por caspasa, lo que conduce a un aumento de la motilidad y la capacidad invasiva de las células del sarcoma de tejidos blandos (Zhu et al., 2011). Se ha descrito que la vimentina fosforilada interactúa con proteínas 14-3-3, que participan en una multitud de procesos de señalización celular y regulación del ciclo celular. Esta interacción impide la formación del complejo Raf-14-3-3 y complejos similares, lo que sugiere que la vimentina regula la señalización intracelular y las rutas de control del ciclo celular mediante la modificación de la disponibilidad de 14-3-3 (Tzivion et al., 2000). Asimismo, la proteína Scrib, implicada en la migración celular, está protegida de la degradación proteasomal por la interacción con vimentina durante la EMT debido a la estabilización de Scrib derivada de esta interacción, promoviendo la migración de células y el aumento de su capacidad invasiva (Phua et al., 2009). Se ha demostrado, además, que la vimentina funciona como un regulador de los receptores de tirosina quinasa Axl, observándose un aumento en la migración de las células mediante la inducción de la expresión Axl (Vuoriluoto et al., 2011).

La vimentina está definida como una proteína estructural del citoesqueleto, sin embargo, se sabe que también puede ser una proteína extracelular y nuclear (Traub et al., 1994). Adicionalmente a los múltiples trabajos de Traub y colaboradores se han revelado diferentes funciones de la vimentina en relación a su interacción con estructuras de ADN, como ADN

satélite, (Tolstonog etal., 2000), ADN telomérico, ADN mitocondrial y retrotrasposones (Tolstonog et al., 2001, Ivaska et al., 2007).

Tabla 5. Resumen de las funciones de vimentina en diferentes procesos fisiológicos y moleculares con dianas implicadas en los efectos observados (Ivaska et al., 2007).

Cellular function	Target of regulation	Effect	Reference
Structural integrity of cells and tissues	Endothelial cell junctions and/or ECM interactions	Integrity of cell layers and tissues	[8]
Adhesion and migration	Integrins, cell adhesion molecules, cytoskeletal crosslinking proteins	Formation and turnover of adhesive structures	[8,36–39, 57,70]
Signal transduction	Protein kinases	Operation and organization of kinases	[57,83,84,88]
	14-3-3	Modulation of 14-3-3 interactions	[92,93]
	Receptors and receptor-associated proteins	Membrane localization of receptors	[75]
		Formation of a functional cell surface complex	[78,79]
	Transcription factors	Modulation of activity through sequestration	[105,110]
Apoptotic and immune defence	Cell death-regulating factors	Modulation of activity through sequestration	[104,105]
	Viral components	Regulation of subcellular localization	[21,106]
	Extracellular domains	Activation of innate immune system cells	
Regulation of genomic DNA	Genomic DNA	DNA recombination and repair	[111–114]

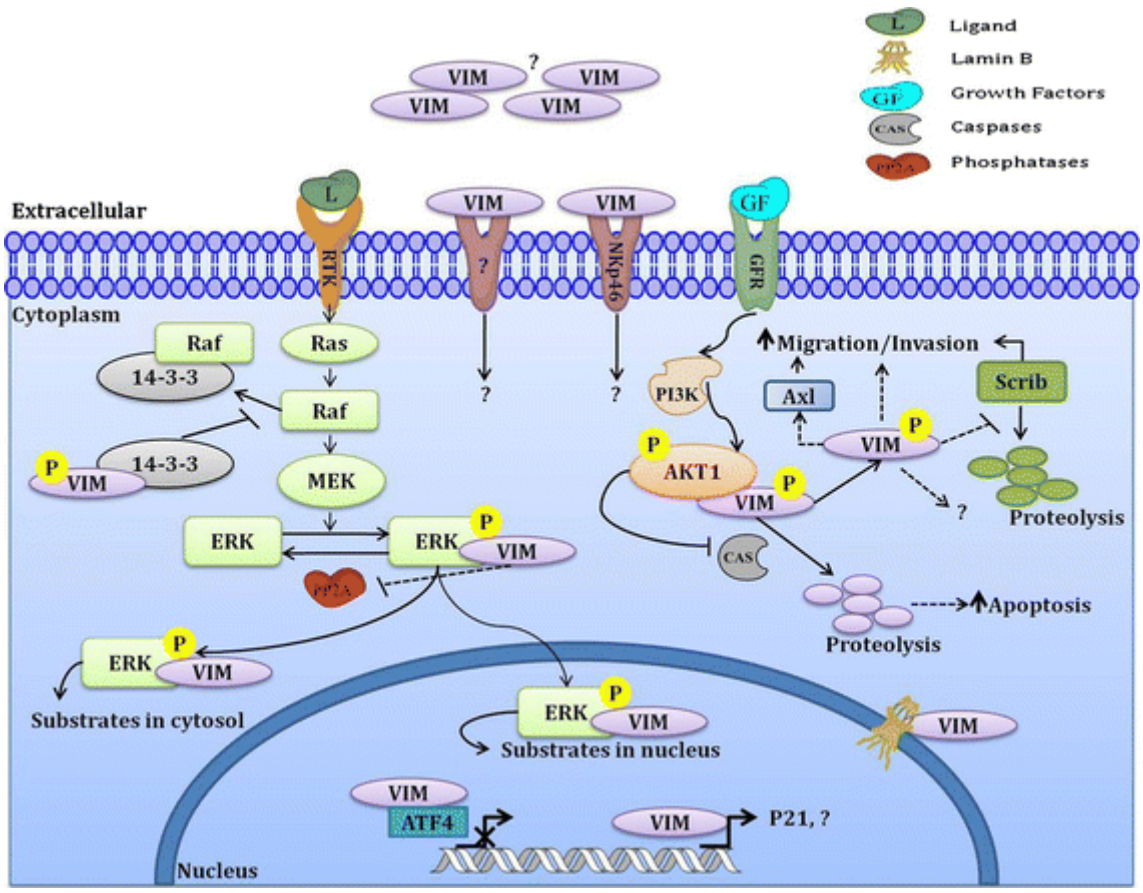

Figura 25. El papel de la vimentina en la señalización celular. En el citosol, se muestra que la vimentina interactúa con Erk fosforilado, protegiendo así su fosforilación por la inhibición de fosfatasas, lo que le permite recorrer largas distancias dentro de la célula. Además, la interacción de vimentina con las proteínas 14-3-3, impide la formación del complejo 14-3-3-Raf, regulando así varios procesos celulares por agotamiento de la disponibilidad de 14-3-3 libre. Además, se muestra que la quinasa AKT1 fosforila a vimentina, protegiéndola de la proteólisis inducida por caspasa. Por lo tanto, la vimentina participa en los procesos que conducen a una mayor capacidad migratoria e invasiva de las células. En el núcleo, la vimentina regula la expresión de p21. Además se conoce que forma un complejo con ATF4, impidiendo así la transcripción del mismo. En el medio extracelular, los receptores específicos de vimentina no han sido identificados; sin embargo, se ha demostrado que la vimentina actúa como un ligando específico para un posible receptor, NKp46, en células NK *(Natural Killer)*. Aunque los resultados indican que la vimentina también se secreta, ni la función de la proteína secretada ni el mecanismo de secreción han sido clarificados (Satelli and Li 2011).

2.5. Snail.

Snail es una proteína reguladora esencial en la EMT. Es un represor transcripcional de CDH1 (E-cadherina) e inductor de la invasión tumoral. La caracterización del factor Snail SNAI1 (Barrallo-Gimeno and Nieto 2005) como un represor transcripcional de CDH1 y un inductor de la EMT, (Batlle 2000, Cano et al., 2000) ha sido un avance importante, que ha proporcionado nuevos conocimientos sobre los mecanismos moleculares de la invasión tumoral. La superfamilia de Snail está constituida por Snail y Scratch. Existen tres miembros de esta familia descritos hasta la fecha en vertebrados: SNAI1, SNAI2 **(Figura 26)** y SNAI3 (Barrallo-Gimeno and Nieto 2005). Los miembros de la familia Snail son factores de transcripción que comparten una organización estructural común: 1) una región C-terminal muy conservada, que contiene de cuatro a seis dedos de zinc (tipo C_2H_2) y 2) una región N-terminal divergente. Estos dedos de zinc permiten a la proteína unirse al ADN de forma específica, a una secuencia consenso E2 de elementos de tipo C / A (CAGGTG) (Nieto 2002). Actualmente, se piensa que los factores de la familia Snail son represores de transcripción (Hemavathy et al., 2000). Su capacidad represora recae en el dominio SNAG (Peinado et al., 2004), formado por 7-9 aminoácidos, localizados en la parte

N-terminal de la proteína, los cuales están conservados entre Snail y las proteínas del factor de crecimiento de independencia (Gfi). En la región central de Snail, existe una región rica en serina-prolina, muy divergente entre los diferentes miembros de la familia. Las proteínas SNAI2 contienen en esta región un dominio llamado Slug, cuya función aun no ha sido definida. Por el contrario, existen dos dominios funcionales en la región central de las proteínas SNAI1: un dominio regulador que contiene una señal de exportación nuclear (NES) (Domínguez et al., 2003) y una caja de dominio de destrucción (Zhou et al., 2004). La fosforilación de los residuos de prolina/serina en ambas regiones y la posible modificación de los residuos de lisina, está implicada en la localización subcelular de SNAI1, la estabilidad de la proteína y su actividad represora (Domínguez et al., 2003). **(Figura 26).**

Snail impide la expresión de genes epitelio-específicos, como PTEN, MUC1, Claudin, y Occludin, así como algunos receptores del factor nuclear (receptor de la vitamina D, HNF-1α) (Peinado et al., 2007), De Herreros et al., 2010). Snail no sólo reprime genes epiteliales, sino que también estimula la transcripción de genes mesenquimales. Se ha propuesto que los efectos estimulantes de Snail son dependientes de la represión de E-cadherina y de la liberación de factores de transcripción retenidos por esta proteína. Así pues, la E-cadherina evita la sobreexpresión de genes mesenquimales inducidos por Snail (Solanas et al., 2008). Sin embargo, la estimulación de la transcripción de genes no puede explicarse exclusivamente por la inhibición de E-cadherina, ya que el silenciamiento génico del factor de transcripción CDH1, no promueve la activación de los genes mesenquimales en la misma medida que la expresión de Snail. Por otra parte, los efectos de Snail en los genes mesenquimales se detectan incluso en las células defectuosas para la expresión de E-cadherina (Solanas et al ., 2008). También se sabe que Snail interactúa con β-catenina en el núcleo (Stemmer et al., 2008), promoviendo la activación transcripcional de los genes diana de Wnt, lo que sugiere que Snail, al menos en ciertas condiciones, podría funcionar como un activador directo.

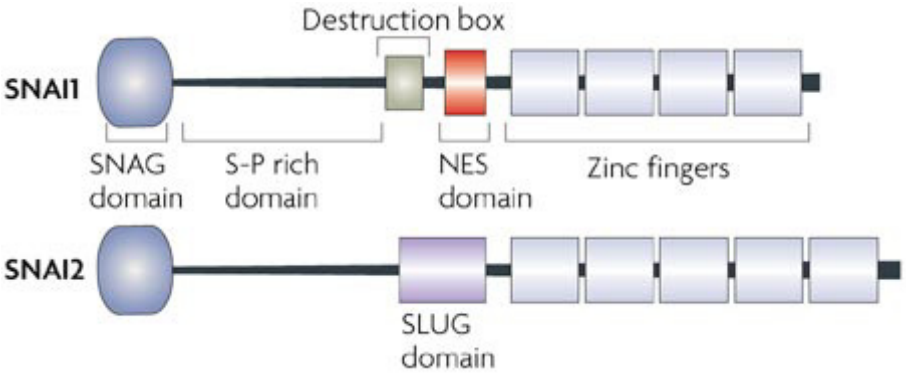

Figura 26: Esquema comparativo de los principales dominios estructurales SNAI1 y SNAI2 encontrados en mamíferos. *Adaptado de (Peinado et al., 2007)*

3. METASTASIS

La metástasis es la diseminación de células malignas de un tumor primario a lugares distantes y es la causa de muerte de la mayoría de casos de cáncer. Numerosas investigaciones son constantemente publicadas acerca de los diversos elementos involucrados en este proceso tan importante, sin embargo, sigue siendo uno de los aspectos más enigmáticos de la enfermedad. La metástasis se puede describir como un proceso de dos fases: La primera fase consiste en el desplazamiento físico de una célula tumoral a un órgano distante, mientras que la segunda abarca la capacidad de las células cancerosas para convertirse en una lesión metastásica en ese lugar lejano. Aunque aún queda mucho por aprender acerca de la segunda fase, se piensa que la comprensión de la primera fase ya está mucho más avanzada (Chaffer and Weinberg 2011)

La primera fase se presenta en una serie de pasos discretos, que han sido descritos en una "cascada metastásica". La visión clásica de la cascada metastásica, incluye: 1) Cambio fenotípico hacia célula mesenquimal (EMT) y capacidad para atravesar la membrana basal, 2) la disociación de las células tumorales del tumor primario; 3) invasión de los tejidos vecinos; 4) intravasación en sangre y vasos linfáticos; 5) transporte a través de los vasos; 6) extravasación de los vasos; 7) establecimiento de las células en tejidos distantes (que puede permanecer latentes durante un período prolongado de tiempo), y 8) formación de micrometástasis y macrometástasis/tumores secundarios (Chambers et al., 2002, Eccles and Welch 2007, Fidler 2003, Gupta and Massagué 2006, Geiger and Peeper 2009) **(Figura 27).** Observaciones recientes han sugerido un paso más, que se añade al principio de la cascada (y por lo tanto, designado como paso "0") en la creación de un "nicho premetastatico" en el sitio de destino, antes de que las células del tumor primero llegar a este lugar lejano. **(Figura 28)**

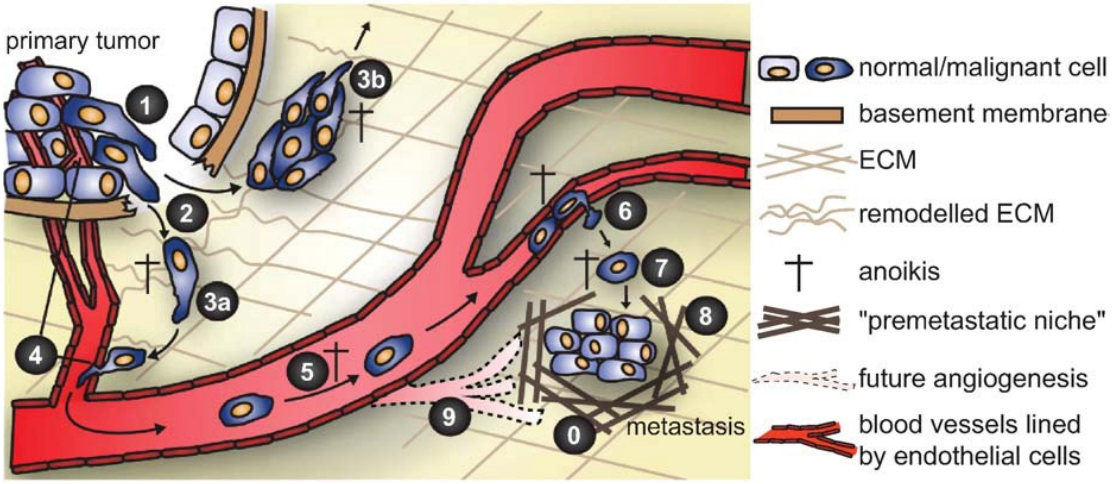

Figura 27. La cascada metastásica. 0) Es inducida por un tumor a distancia y mediada por células derivadas de la médula ósea, un "nicho premetastático" formas que se hacen evidente antes de la metástasis. **1)** Las células del tumor primario se someten a la transición epitelio-mesenquima (EMT) y a la adquisición de propiedades invasivas. **2)** La degradación de la membrana basal y la remodelación de la matriz extracelular (MEC) por proteinasas facilitando la invasión de células tumorales. **3)** Las células tumorales invaden los tejidos circundantes como células individuales **(3a)** o colectivamente **(3b). 4)** intravasación de las células tumorales en los vasos recién formados dentro o cerca del tumor. **5)** Las células tumorales son transportadas a través de la vasculatura y arrestadas en un lecho capilar donde son extravasadas **(6)**. 7) extravasación de las células tumorales puede permanecer latente durante años. 8) Eventualmente, algunas células diseminadas crecen fuera a un tumor secundario/macrometástasis, que requieren remodelación de ECM en desarrollo y angiogénesis (9). Las células fuera de su microambiente normal experimentan anoikis ("apoptosis inducida por desprendimiento"). Anoikis podría dificultar la metástasis en varios pasos de la cascada, como se indica en el esquema. No todos los pasos de la cascada metastásica ocurre necesariamente en forma lineal. Por ejemplo, los tumores premalignos ya puede ser vascularizado, mientras que el momento de la inducción del nicho premetastatic sigue siendo esquiva. *Adaptado de* (Geiger and Peeper, 2009).

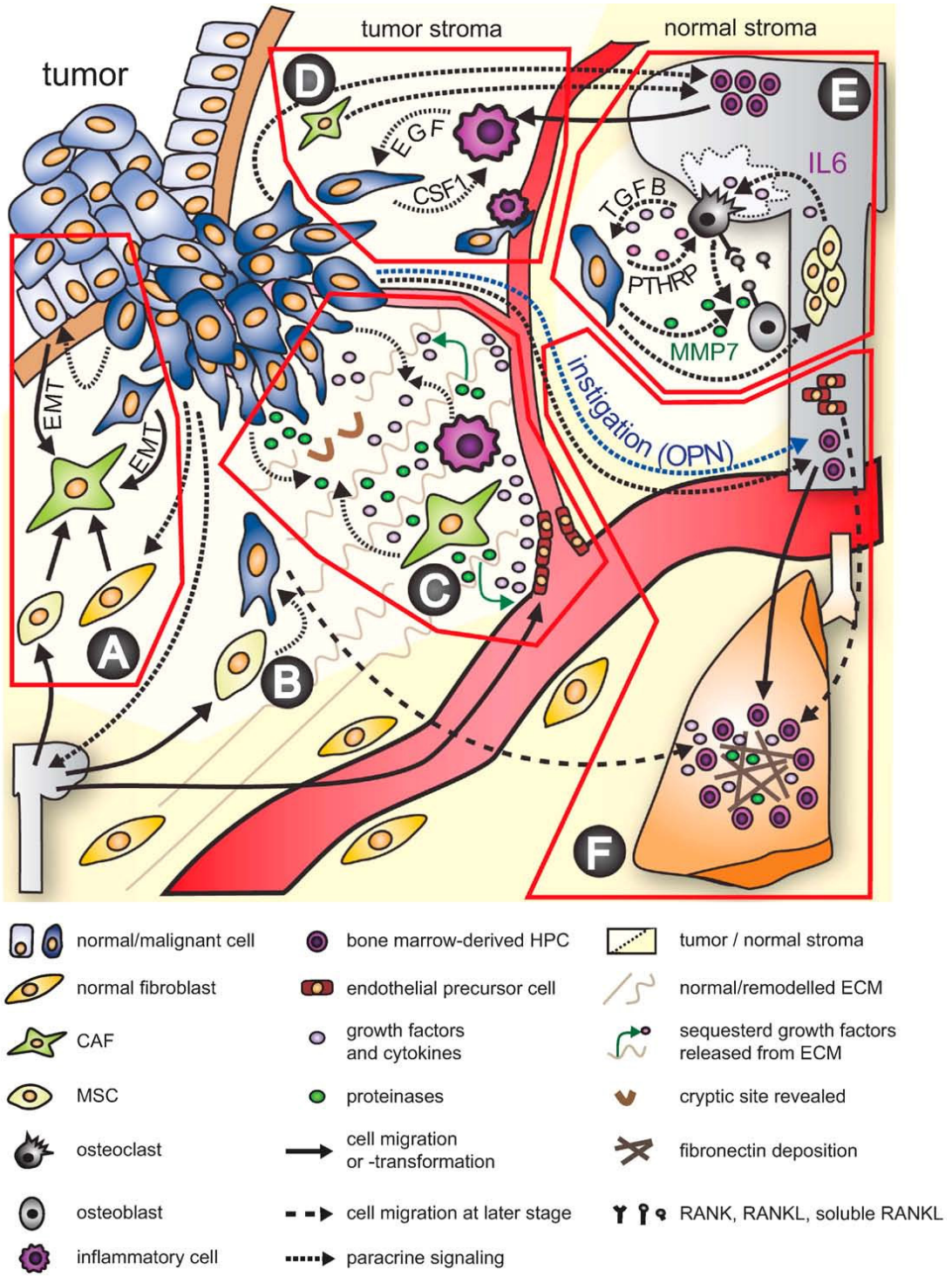

Figura 28. Ejemplos de las funciones del microambiente tumoral que promueven metástasis. promoción de A) Origen de los fibroblastos asociados al cáncer (CAF). Las células del tumor liberando factores de crecimiento y citocinas activa los fibroblastos normales o atraer derivadas de la médula ósea células madre mesenquimales (MSC) que se diferencian. CAF también se pueden derivar de las células tumorales o células epiteliales normales que han sido sometidos a EMT. B) Las células madre mesenquimales aumentar el potencial invasor de las células tumorales. C) Las células tumorales, CAF y células inflamatorias segregan factores que estimulan el crecimiento tumoral, la invasión y la angiogénesis. Proteinasas secretadas remodelación de la matriz extracelular (MEC) y revelan pro-migratorias sitios crípticos o el crecimiento liberar secuestrados y factores

angiogénicos. D) las células progenitoras hematopoyéticas (HPC) son activados por un tumor distante y asociados al tumor macrófagos (TAM) son reclutados a través de Csf1 señalización. TAM atraer a las células tumorales a los buques por EGFR estimular y facilitar intravasation. E) El "círculo vicioso de las metástasis óseas osteolíticas" implica PTHRP secretada por las células tumorales y factores de crecimiento, incluyendo TGFB, liberado de la degradación ósea por osteoclastos activados. Las células tumorales también pueden estimular el MSC en la médula ósea para que activan a los osteoclastos a través de IL-6. Liberación de MMP7 solubiliza RANKL, más aumento de la actividad de osteoclastos. F) La formación de un "nicho premetastatico" es provocada por la movilización de la médula ósea procedentes de células de un tumor a distancia. La médula ósea procedentes de células infiltrarse en el órgano diana, inducir la deposición de fibronectina y enriquecer el microambiente de los factores de crecimiento y proteasas. Sólo después de que el nicho se ha establecido, las células tumorales infiltrarse y colonizar estos sitios. El resultado de micrometástasis establecido es promovido por la activación de la médula ósea procedentes de células debido a la instigación sistémica mediada por el tumor segrega OPN y otros factores. *Adaptado de* (Geiger and Peeper, 2009).

Melanoma metastásico

El melanoma es un tumor generalmente cutáneo, pero también del intestino y el ojo (melanoma uveal) y altamente invasivo por su capacidad de generar metástasis (Chin et al., 2006). Además, estos tumores son marcadamente resistente a las terapias convencionales (Helmbach et al., 2003) El melanoma primario es altamente curable (>95%) a través de cirugía cuando el diagnóstico es temprano (Breslow, 1978). Mientras el pronóstico es excelente para pacientes diagnosticados en estadios tempranos, el resultado de la enfermedad avanzada es muy desfavorable. La terapia mas apropiada para el melanoma metastático aun no está definida. Monoterapia con compuestos metilantes como la dacarbazina ha sido una referencia en el tratamiento en melanoma pero en general los paciente no obtienen mejoras (Markovic et al., 2007b) El melanoma maligno tiene una incidencia y tasa de mortalidad que está incrementando (Markovic et al., 2007a). En nuestro país el melanoma representa el 1.3% y el 2.5% de todos los tumores malignos en varones y mujeres respectivamente, mientras que los valores mundiales son del 2.4% y del 4.9%, y es una de las neoplasias que ha experimentado un incremento más espectacular, ya que casi ha triplicado su incidencia en los últimos cuarenta años a un ritmo de un 4 % anual en Occidente.

En cuanto a los factores de riesgo cabe destacar:

1) Predisposición genética: Un 5-10% de los pacientes que sufren melanomas tienen antecedentes familiares de esta enfermedad. Parece que existen al menos 4 genes implicados en la aparición de melanomas familiares 10. Los aspectos moleculares de dos de ellos (el CDKN2A, un gen supresor tumoral que codifica la proteína p16, y el CDK4, un oncogen dominante) localizados en los cromosomas 9p21 y 12q14 respectivamente, han sido definidos.
2) Fototipo: Se acepta la existencia de un fototipo cutáneo especial asociado a una mayor predisposición de padecer melanoma; correspondería a personas que toleran mal el sol, se queman con facilidad y se broncean con dificultad. La incidencia de melanoma guarda relación con los fototipos bajos (fototipos I y II en la clasificación de los tipos de piel según su reactividad a la exposición solar) que presentan una piel muy sensible, se queman prácticamente siempre que se exponen a la radiación solar y nunca o mínimamente se pigmentan. Fenotípicamente se trata de pacientes caucásicos de piel blanca, ojos claros y pelo rubio o pelirrojo, que tienen un riesgo 12 veces superior de padecer un melanoma, respecto a individuos de piel morena, ojos marrones y pelo castaño o negro 20. Esta sensibilidad frente al sol se considera uno de los factores del huésped más estrechamente relacionados con el riesgo de padecer melanoma.
3) Factores ambientales, la radiación ultravioleta (RUV), es considerada hasta la fecha como el principal factor desencadenante conocido para el desarrollo de melanomas en una población genéticamente predispuesta. La exposición solar durante la infancia y adolescencia parece tener una importante influencia en el riesgo de padecer un melanoma. En la carcinogénesis cutánea por RUV parecen influir tres niveles: en primer lugar, daño celular que clínicamente se traduce en melanogénesis,

luego fallos en la reparación del ADN, y por último, depresión inmunológica que puede originar una pérdida de la inmunovigilancia . El espectro de la radiación ultravioleta A (320-340 nm) y radiación ultravioleta B (290-320 nm) además de producir mutaciones en el ADN sobre las células epidérmicas, impide su reparación por cambios oxidativos y favorece la inmunosupresión, por lo que se cumplen muchos de los requisitos necesarios para el desarrollo tumoral.

En general, además de una predisposición genética, parece existir una susceptibilidad especial a la RUV, que hace que las exposiciones solares puntuales e intensas de forma repetitiva, junto con el antecedente de quemaduras en la infancia, constituyan los principales factores de riesgo en la aparición de este tumor. Sin duda hoy en día el mejor tratamiento del melanoma es la cirugía en los estadios iniciales; para ello es necesario conocer muy bien las presentaciones clínicas y los grupos de riesgo, en base a los antecedentes hereditarios y al fenotipo cutáneo. Las campañas de difusión para localización de lesiones pigmentadas en la población general, y la educación en la edad escolar para fomentar el uso de fotoprotectores como lo que realmente son, no como productos con los que sin riesgo se puede tomar más el sol, constituyen la mejor prevención. En la progresión de la enfermedad, en el paso de una fase de crecimiento horizontal a una fase invasiva de crecimiento vertical, factor determinante del pronóstico, intervienen factores sólo parcialmente conocidos. La angiogénesis o desarrollo de nuevos vasos, es necesaria para mantener el crecimiento, invasión y capacidad metastásica de un tumor. Por tanto, es urgente un mejor conocimiento acerca de la biología -comportamiento y homeostasis- de las células de melanoma, que no se limite sólo al diagnóstico precoz o a la prevención tumoral, sino que también pueda aportar soluciones terapéuticas a corto plazo, para lo que se considera una de las mayores plagas oncológicas del nuevo Todos estos datos nos indican la urgencia de nuevas estrategias de tratamiento para este tipo de cáncer tan agresivo.

4. PARP-1

La proteína nuclear Poli(ADP-Ribosa) Polimerasa-1 (Alvarez-Gonzalez, Watkins et al.; Aarts and Tymianski) es una enzima presente en células eucariotas, descrita hace más de 40 años por Chambon y colaboradores (Chambon, Weill et al. 1963). PARP-1 (113 kDa) es una proteína nuclear que cataliza la transferencia de monómeros de ADP-ribosa, obtenidos del sustrato NAD+, sobre los grupos carboxílicos de los aminoácidos glutámico, aspártico y lisina de una serie de proteínas nucleares. Es una proteína altamente conservada en la escala filogenética; ha sido descrita en muchos eucariotas (excepto en levaduras), y está formada por seis dominios estructurales **(Figura 29)**, de los cuales sólo cuatro están perfectamente estudiados funcionalmente. La modificación transitoria de proteínas nucleares por poli-ADP-ribosilación constituye un tipo de modificación post-transduccional necesaria para la activación así como la desactivación de una serie de procesos celulares (Kim, Zhang et al. 2005)

4.1. Estructura

PARP-1 ha sido implicada en muchas funciones biológicas especialmente en procesos de reparación de daños al ADN de cadena simple y doble, inducidos por diferentes agentes genotóxicos como la radiación ionizante, los radicales libres, los inhibidores de la topoisomerasa I y II y los agentes alquilantes **(Figura 30)**. Una vez detectado el daño en el ADN la PARP-1 une al mismo a través de las estructuras en dedos de zinc presentes en el dominio de unión al ADN, y se activa. Esta activación requiere la formación de homodímeros principalmente a través de la estructura FII (segundo dedo de zinc) y cataliza la hidrólisis de β-nicotinamida adenina dinucleótido (NAD+), en nicotinamida y monómeros de ADP-ribosa, que posteriormente utilizará como sustrato para formar polímeros largos y ramificados de poli (ADP-ribosa) sobre residuos γ-carboxilo de ácido glutámico y ácido aspártico y lisinas de determinadas proteínas nucleares mediante enlace tipo éster (Schreiber, Dantzer et al. 2006) **(Figura 30)**.

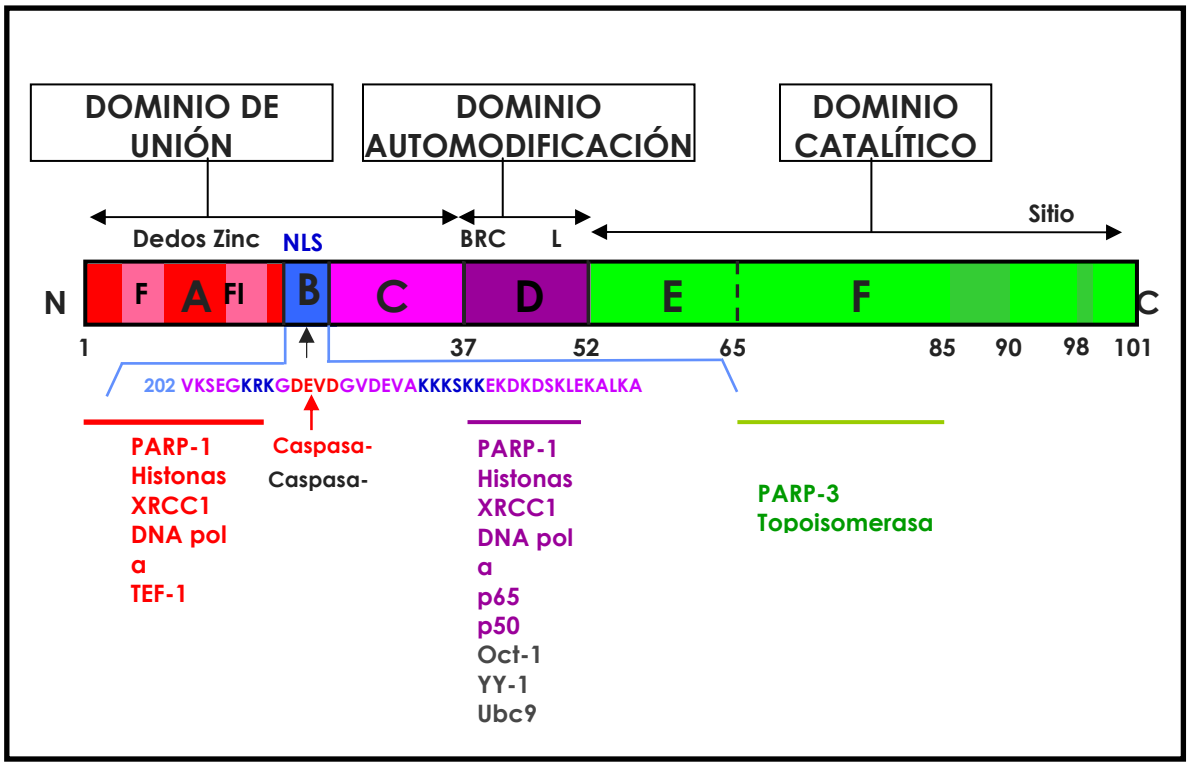

Figura 29: Dominios estructurales de PARP-1. Dominio de unión al ADN (DBD); FI y FII, estructura en dedos de zinc; Secuencia de localización nuclear (NLS); dominio de automodificación y dominio catalí tico. Motivo BRCT (*BRCA1 like C-terminus)*; lugar de corte de las caspasas-3 y -7; motivo cremallera de leucina (LZ, *Leucine zipper*). Además se indican los posibles sitios de interacción con otras proteínas. *Adaptados de* (Aguilar-Quesada, Munoz-Gamez et al. 2007)

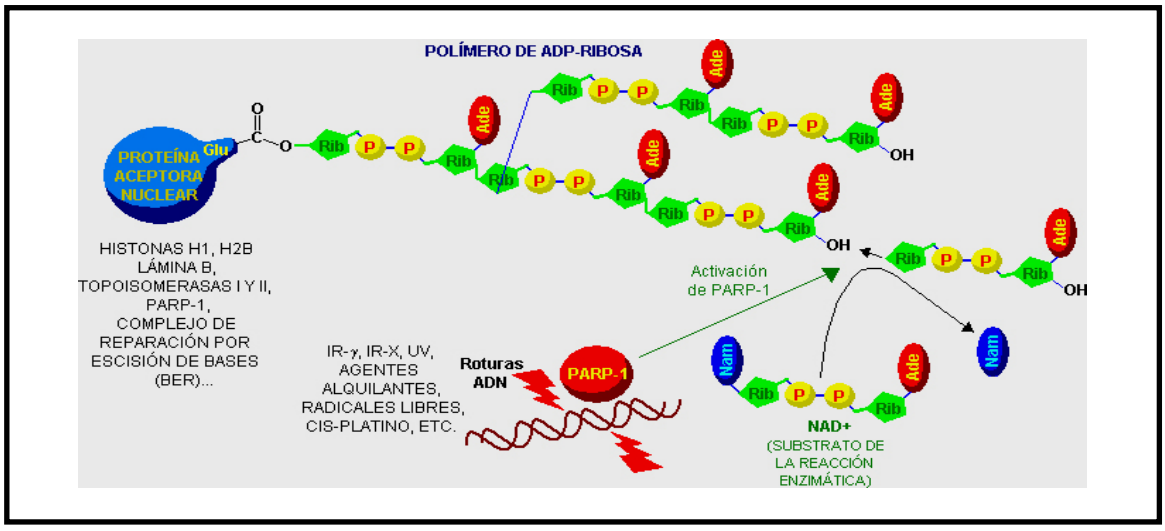

Figura 30. Mecanismo de activación de PARP-1 tras el daño en el ADN. Determinados agentes genotóxicos activan PARP-1. Se muestra el procedimiento de activación, síntesis de polímero a partir de NAD+ y la consiguiente modificación de determinadas proteínas nucleares. *Adaptados de* (Aguilar-Quesada, Munoz-Gamez et al. 2007)

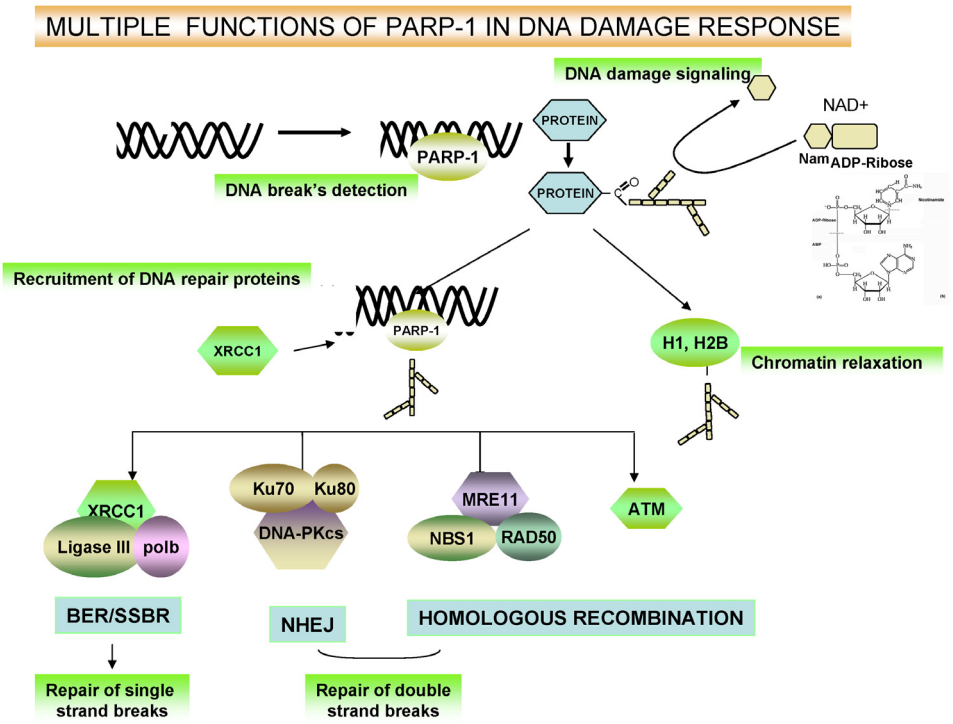

Figura 31. Papel de la poli (ADP-ribosila)ción en la respuesta al daño en el ADN. PARP-1 detecta roturas en el ADN e inicia la síntesis de un polímero ramificado de ADP-ribosa (Pezzella, Pastorino et al.) sobre los residuos de glutamina, ácido aspártico y lisina de las proteínas aceptoras. La poli(ADP-ribosila)ción de PARP-1 (automodificacion) permite el reclutamiento de proteínas de reparación que contienen un dominio de unión a poli (ADP-ribosa) en el sitio del daño. La poli (ADP-ribosila)ción de las proteínas que El poli (ADP-ribosila)ción de las histonas permite la apertura de la estructura de la cromatina. *Adaptado por* (Dantzer, Noel et al.).

PARP-1 tiene la capacidad de formar polímeros que varían desde unas pocas unidades hasta más de 200 monómeros, mediante tres actividades enzimáticas diferentes (Alvarez-Gonzalez, Watkins et al. 1999): (1) la actividad de ***mono-ADP-ribosilación del sustrato (iniciación)***, donde se produce el anclaje del primer monómero de ADP-ribosa al grupo carboxílico del sustrato mediante enlace tipo éster; (2) la actividad de ***elongación*** del polímero mediante la incorporación de las unidades de ADP-ribosa mediante enlace □-glicosídico 1''→2'; (3) finalmente la actividad de ***ramificación***, que se produce de forma irregular con una pauta de una ramificación por cada 20-50 unidades de ADP-ribosa de la cadena principal, mediante enlace glicosídico 2''→1''(Schreiber, Dantzer et al. 2006).

Se han identificado 18 proteínas que presentan homología de secuencia con el dominio catalítico de PARP-1, sin embargo presentan diferencia en función, estructura y localización funcional **(Figura 32).** La proteína PARP-1 es el principal miembro de esta familia y se le han atribuido múltiples funciones (Ame, Spenlehauer et al. 2004). (Miwa and Masutani, 2007; Schreiber et al., 2006),

4.2. Función.

Varias son las funciones que se le pueden atribuir a PARP-1 (Schreiber, Dantzer et al. 2006), pero las funciones principales son: 1. Reparación del ADN y mantenimiento de la integridad genómica, 2. Muerte celular, 3 Modificación de la estructura de la cromatina y regulación transcripcional.

4.2.1. Reparación del ADN y mantenimiento de la integridad genómica

PARP-1 es un sensor molecular de daños en el ADN, de modo que su actividad catalítica se estimula más de 500 veces por unión a roturas del ADN, y tiene una función clave en la organización espacial y temporal de su reparación. A través de la asociación física con proteínas nucleares, o mediante la poli(ADP-ribosil)ación de las mismas permite la supervivencia celular y el mantenimiento de la integridad genómica (Schreiber, Dantzer et al. 2006).

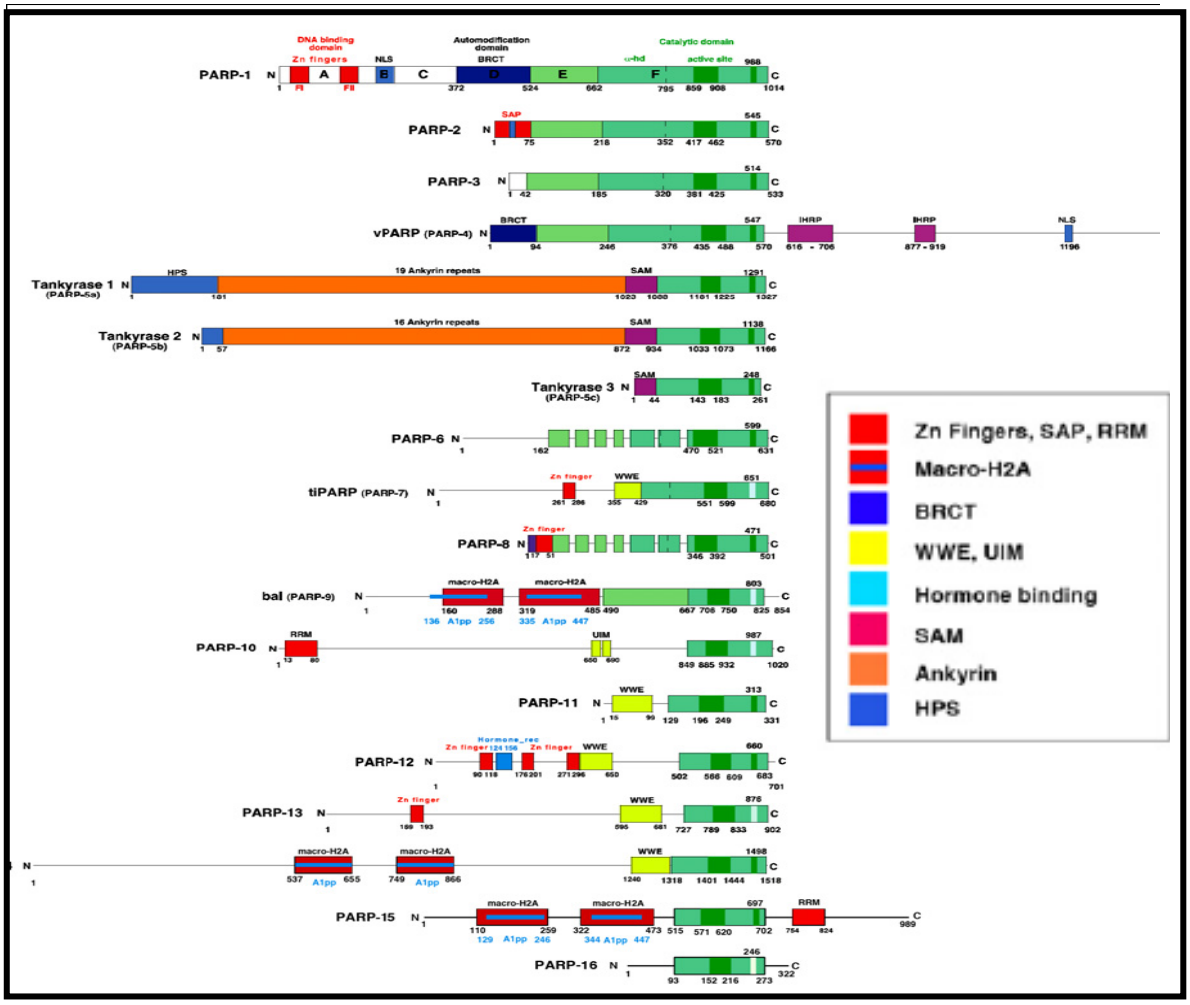

Figura 32. Familia de PARP. **Estructura en dominios de los miembros de la superfamilia PARP.** La caracterización de la familia de proteínas PARP se ha realizado en base a datos del NCBI usando el dominio catalítico de la PARP-1 como motor de búsqueda. Se representan con recuadros coloreados los dominios más característicos localizados en los diferentes miembros de la familia. La gran diversidad de dominios presentes en las proteínas de la famila PARP sugiere la implicación de estas proteínas en una gran variedad de procesos celulares. Figura *Adaptada de*(Ame, Spenlehauer et al. 2004).

En respuesta a daños en el ADN, bien sea la ruptura directa del esqueleto azúcar-fosfato o daños en las bases, se produce una respuesta bioquímica inmediata por parte de PARP-1. PARP-1 se une al ADN dañado a través de su doble dedo de zinc del dominio de unión al ADN (Kim, Zhang et al. 2005) para la reparación a través de las rutas de SSBR (reparación de roturas de cadena sencilla) o BER (reparación por excisión de bases). Esta unión induce un cambio conformacional en la proteína PARP-1, que activa el centro catalítico y dispara su actividad enzimática (Meyer-Ficca, Meyer et al. 2005). PARP-1 interacciona físicamente y funcionalmente con varias

proteínas implicadas en rutas de reparación del ADN, modificándolas por poli(ADP-ribosil)ación y/o reclutándolas al ADN dañado (Lan et al., 2004; Okano et al., 2003). Así, la síntesis de polímero permite: la modificación de las histonas, o la interacción específica no covalente de estas histonas con el polímero libre o unido a PARP-1, que contribuye a la relajación y apertura de la fibra de cromatina de 30 nm, incrementando el acceso al daño; la señalización del lugar y del alcance del daño en el ADN, de manera que la célula establece una respuesta adaptativa de acuerdo a la extensión del daño; y por último, el reclutamiento rápido de factores SSBR/BER al lugar de la lesión (Schreiber, Dantzer et al. 2006). La propia PARP-1, gracias a su automodificación, favorecerá el reclutamiento de proteínas de reparación, y regulará su propia actividad **(Figura 31).** La auto-poli(ADP-ribosil)ación modifica las propiedades de unión de PARP-1 con proteínas y la presencia de cargas negativas promueve la liberación de PARP-1 del ADN, inactivándose.

Por ejemplo, XRCC1, la proteína central de SSBR/BER, interacciona principalmente con PARP-1 poli(ADP-ribosil)ada, siendo indispensable el polímero para el reclutamiento de XRCC1 al ADN dañado y por tanto, para el proceso de SSBR/BER (Caldecott, 2003; Masson et al., 1998; Okano et al., 2003).

Las roturas de cadena sencilla pueden originarse en la célula de forma directa por fuentes endógenas (productos del metabolismo) o exógenas (por ejemplo, radiación ionizante); como resultado de la inestabilidad inherente al ADN o indirectamente tras la iniciación de la reparación por excisión de bases (BER), que se encarga de reparar los daños producidos en las bases del ADN. Además, si estas roturas persisten, pueden convertirse en roturas de cadena doble (Horton, Watson et al. 2008).

PARP-1 también es activada por DSBs (roturas de cadena doble) (Miwa and Masutani 2007). Las DSBs pueden ser reparadas por dos rutas principales dependiendo del contexto del daño en el ADN, HR (recombinación homóloga), mecanismo libre de errores, o NHEJ (unión de extremos no homólogos), mecanismo propenso a errores (Kanaar, Wyman et al. 2008). Ambos mecanismos están bien definidos, y más del 90% de las DSBs en las células de mamífero se reparan por NHEJ (Hakem 2008).

Aunque PARP-1 parece que no es necesaria para los propios mecanismos de reparación de DSBs (Yang, Cortes et al. 2004) varios estudios la han implicado en el equilibrio entre ambos, HR y NHEJ. Ku70/80 (componente de la maquinaria NHEJ) tiene una alta afinidad por DSBs, que es reducida por poli(ADP-ribosil)ación (von Kobbe et al., 2003). Se ha publicado que la HR es considerablemente inhibida por la proteína Ku, indicando que PARP-1 suprime el bloqueo de la reparación por HR (Hochegger et al., 2006). Además, la proteína WRN es reclutada por interacción con Ku70/80 a DSBs, y es necesaria para la completa activación de PARP-1 (von Kobbe et al., 2003). También, PARP-1 está implicado, junto con Ligasa III, en una ruta alternativa de NHEJ de más baja fidelidad (Wang et al., 2006). Sin embargo, se ha descrito que PARP-1 promueve la HR, antagonizando con NHEJ (Saberi et al., 2007). Así, aunque los motivos de estas discrepancias no están claros, todos estos trabajos implican a PARP-1 en la regulación de la elección de la ruta de reparación de DSBs (Shrivastav, De Haro et al. 2008). La reparación de SSBs es dependiente de PARP-1 y la reparación de DSBs, dependiente de HR (Bryant et al., 2005; Farmer et al., 2005).

El ADN dañado que no se repara apropiadamente puede dar lugar a inestabilidad génomica, predisponiendo al organismo a cáncer (Hakem 2008) **(Figura 33)**. Ya se ha comentado la contribución de los cambios genéticos en el desarrollo tumoral. Así, PARP-1, como factor de reparación del ADN, está estrechamente ligado al proceso de carcinogénesis.

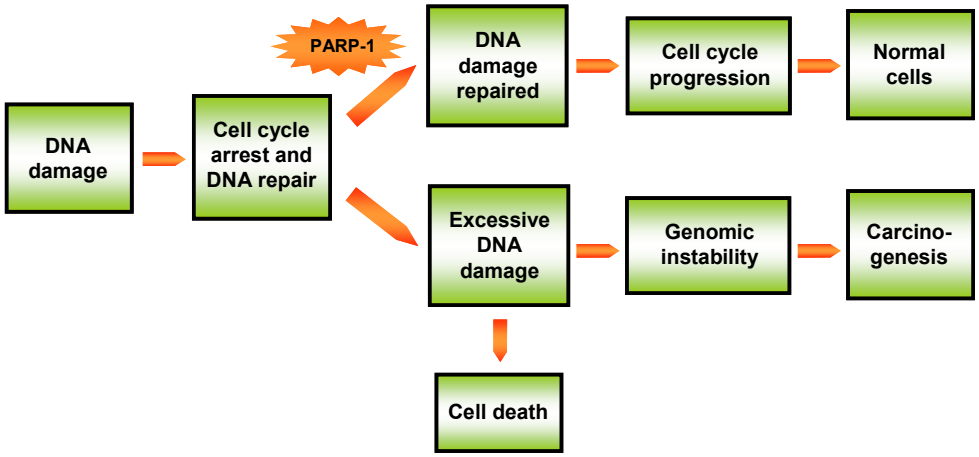

Figura 33. Correlación entre daños en el ADN y carcinogénesis. *(Adaptado de* (Bernstein, Bernstein et al. 2002)

4.3.2. Muerte celular

Con niveles leves o moderados de daño en el ADN, PARP-1 actúa como un factor de supervivencia implicado en la detección del daño del ADN y en su reparación, sin el riesgo de arrastrar genes mutados. Por el contrario, con grandes niveles de daño en el ADN, PARP-1 promueve mecanismos de muerte celular (Burkle 2001). Así, la intensidad del estímulo genotóxico, determinará el destino celular (Jagtap and Szabo 2005) **(Figura 34).**

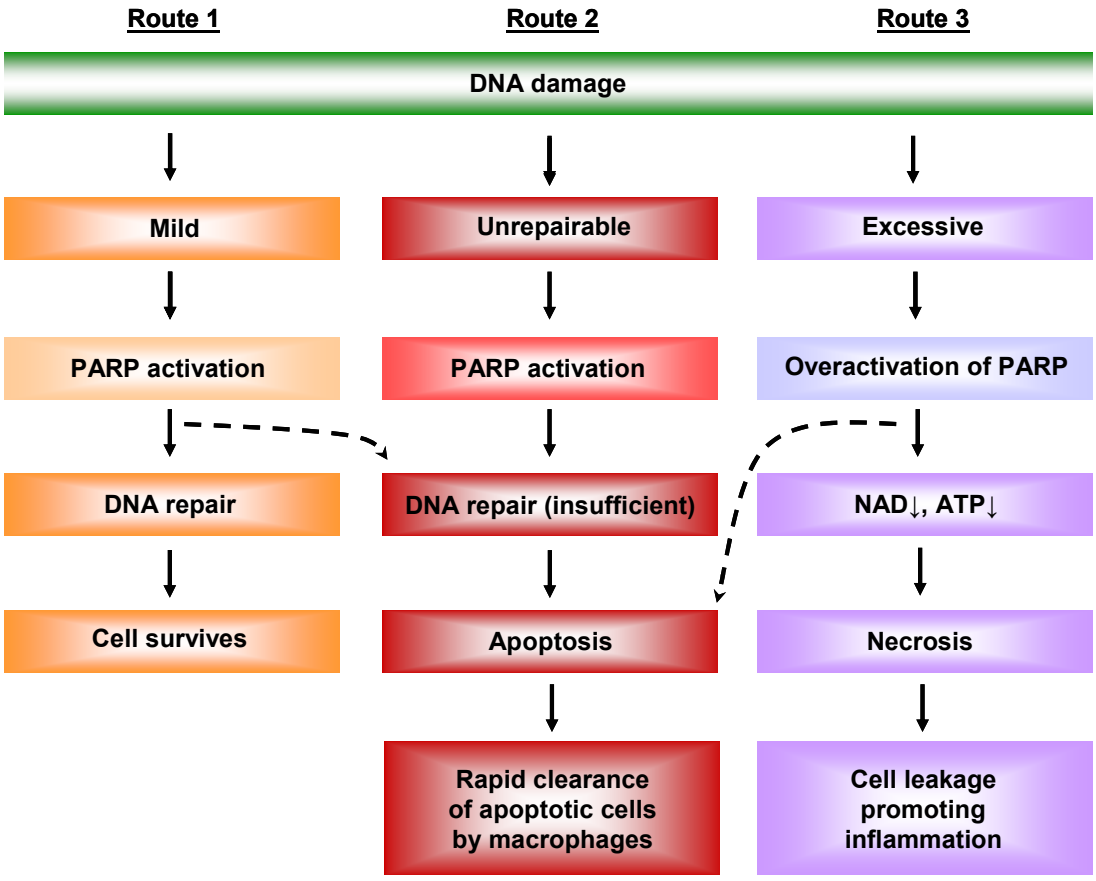

Figura 34. La intensidad del estímulo de daño del ADN determina el destino celular: supervivencia, apoptosis o necrosis. Dependiendo de la intensidad del estímulo, PARP-1 regula tres rutas diferentes. En el caso de daño leve en el ADN, la poli(ADP-ribosil)ación facilita la reparación del ADN y por tanto, la supervivencia (ruta 1). Estímulos genotóxicos más intensos activan la apoptosis para eliminar células con ADN dañado (ruta 2). Un daño en el ADN muy grave puede causar sobreactivación de PARP-1, lo que agota los niveles celulares de NAD^+ y ATP, bloqueando la apoptosis y favoreciendo la necrosis (ruta 3). La inhibición de PARP-1 en células que entran en la ruta 1 impide la reparación y, por tanto, las desvía a la ruta 2 (flecha discontinua de la izquierda). La inhibición de PARP-1 en células que entran en la ruta 3 conserva los niveles de energía celular y permite el proceso de apoptosis (flecha discontinua de la derecha). *(Adaptado de (Jagtap and Szabo 2005).*

PARP-1 está implicado tanto en apoptosis como en necrosis. La apoptosis es un proceso de muerte celular programada en el que la célula es desmantelada de manera organizada y sistemática dentro de vesículas recubiertas por membranas que son fagocitadas, de manera que se evita la liberación de los componentes intracelulares al tejido adyacente. Sin embargo, la necrosis es un proceso de muerte celular en el que la célula se hincha y se rompe, liberando todos los componentes intracelulares al tejido adyacente, lo que promueve una respuesta inflamatoria (Edinger and Thompson 2004).

Los estudios iniciales que relacionaban a PARP-1 con muerte celular se centraban en la inactivación de PARP-1 y en su papel potencial en apoptosis (Edinger and Thompson 2004). Durante la apoptosis, las caspasas 7 y 3 (proteasas proapoptóticas) rompen a PARP-1 en dos fragmentos, separando su DBD de su dominio catalítico, lo que hace que la enzima conserve su actividad enzimática basal, pero no la actividad inducida por el daño en el ADN (Kaufmann, Desnoyers et al. 1993). Este procesamiento es independiente de la actividad catalítica de PARP y se considera un marcador de apoptosis, y no un ejecutor del proceso (Virag and Szabo 2002). La ruptura proteolítica de PARP-1 impide su sobreactivación en respuesta a la fragmentación del ADN que se produce en la apoptosis y por tanto, mantiene la energía celular para ciertos pasos dependientes de ATP de este tipo de muerte celular (Aikin et al., 2004; Herceg and Wang, 1999). Además, previniendo intentos inútiles de reparación del ADN, la ruptura de PARP-1 quizá ayude a las células a entrar en apoptosis (Aikin et al., 2004; Soldani and Scovassi, 2002)

Independientemente, la activación de PARP-1 promueve un tipo de muerte celular apoptótica independiente de caspasas a través de AIF (Yu et al., 2006; Yu et al., 2002) AIF (apoptosis-inducing factor) es una oxidorreductasa mitocondrial que se libera al citoplasma para inducir muerte celular en respuesta a la activación de PARP-1. En este caso, se ha identificado al polímero como la señal de muerte que determina la liberación mitocondrial de AIF y su traslocación al núcleo (Yu, Andrabi et al. 2006; David, Andrabi et al. 2009). Ya en el núcleo, AIF induce condensación de la cromatina y fragmentación del ADN (Susin et al., 1999). Como las caspasas

no están implicadas en este proceso, PARP-1 está intacto para ser activado por el ADN fragmentado, lo que origina una masiva síntesis de polímero, caída de los niveles de NAD^+ y ATP, y por tanto, muerte celular.

El destino de la célula tras el daño en el ADN, como ya se ha comentado, va a depender de la magnitud de la lesión. Pero además, dicha magnitud también será clave para el tipo de muerte celular dependiente de PARP-1. Una lesión grave del ADN induce apoptosis, con las caspasas 3 y 7 inactivando a PARP-1, de modo que células con este daño son eliminadas. Pero si la magnitud del daño supera un determinado umbral, la sobreactivación de PARP-1 reduce los niveles de su sustrato NAD^+ y, como consecuencia, de ATP. Este estado energético celular comprometido no permite que la apoptosis tenga lugar, por lo que las células mueren por necrosis (Jagtap and Szabo 2005).

Con este planteamiento, tras la inhibición de PARP-1, se presentaría la siguiente situación: células con un daño en el ADN leve reparable, así como aquellas fuertemente dañadas, serían eliminadas por apoptosis **(Figura 34).** La inhibición de PARP en estas últimas, que normalmente morirían por necrosis, conservaría los niveles de ATP necesarios para la apoptosis (Hassa, Buerki et al. 2003; Jagtap and Szabo 2005). Dentro del contexto de la carcinogénesis, se trata de una idea interesante, ya que la inhibición de PARP-1 favorecería la muerte por apoptosis de células tumorales, inducida por agentes radio- y quimioterapéuticos, evitando la necrosis, y por tanto, la respuesta inflamatoria derivada, relacionada funcionalmente a su vez, con el desarrollo tumoral.

4.3.3. Modificación de la estructura de la cromatina y regulación transcripcional

PARP-1 puede actuar como un estimulador o como un represor de la transcripción. NF-κB es un factor de transcripción clave en la regulación de procesos celulares esenciales relacionados con la respuesta inflamatoria, la apoptosis, y la migración celular, entre otros. PARP-1 se ha sido descrito que actúa como coactivador transcripcional de NF-κB en estos procesos (Oliver, Menissier-de Murcia et al. 1999; Hassa, Buerki et al. 2003; Carrillo,

Monreal et al. 2004). PARP-1 tiene un importante papel en la regulación epigenética de la estructura de la cromatina y en transcripción génica en condiciones fisiológicas en la que la integridad del ADN se mantiene (Aguilar-Quesada et al., 2007; Schreiber et al., 2006)

PARP-1 fue identificado en los años 80 como TFIIC, un factor que suprimía la transcripción aleatoria inducida por roturas, de la RNA polimerasa II, pero que no era necesario para la transcripción basal específica de promotor (Slattery et al., 1983). Cada vez más, numerosos trabajos ponen en evidencia que PARP-1 regula la transcripción que tiene lugar en respuesta a estímulos biológicos, químicos o físicos, a través de al menos dos mecanismos no excluyentes entre sí **(Figura 35):** modulación de la estructura de la cromatina y/o interacción directa con factores de transcripción y/o sitios de unión a factores de transcripción (Schreiber, Dantzer et al. 2006).

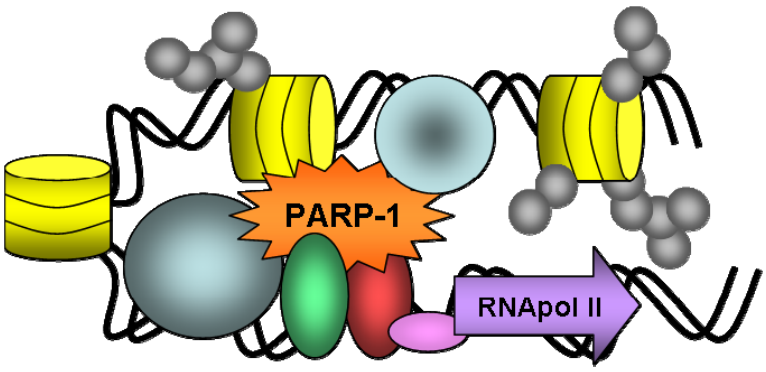

Figura 35. Mecanismos de regulación de la transcripción por PARP-1. Se han propuesto dos modos de regulación de la transcripción por PARP-1. Primero, por modificación de las histonas (en amarillo) y otros factores asociados a cromatina, modulando la estructura de la misma; y segundo, como cofactor transcripcional, de forma dependiente o independiente de su actividad enzimática. *(Adaptado de(Kraus and Lis 2003)*

Modificación de la estructura de la cromatina

PARP-1 afecta a la estructura de la cromatina al modificar con poli(ADP-ribosa) al núcleo de histonas y proteínas asociadas a cromatina, de manera que favorece la disociación de los nucleosomas y la descondesación de la cromatina (D'Amours et al., 1999; Kraus and Lis, 2003; Mathis and Althaus, 1987; Oei et al., 1998; Rouleau et al., 2004).

PARP-1, por tanto, tiene efectos contrarios sobre la estructura de la cromatina (compactación frente a descondesación) dependiendo del tipo de cromatina, pero también en función de señales fisiológicas. De hecho, PARP-1 modula reversiblemente la estructura de la cromatina de forma dependiente de NAD^+ (Kim, Mauro et al. 2004). PARP-1 se incorpora a la cromatina gracias a sus propiedades específicas de unión a nucleosomas y promueve la formación de estructuras compactas y transcripcionalmente reprimidas de la cromatina. En presencia de NAD^+, PARP-1 se automodifica y se disocia de la cromatina, formándose estructuras descondensadas y transcripcionalmente activas de la cromatina(Kim, Zhang et al. 2005).

Por otro lado, se ha sugerido que la poli(ADP-ribosil)ación determina y mantiene los patrones de metilación del ADN genómico (Zardo et al., 2003). Dada la importante contribución de la metilación del ADN en la organización de la estructura de la cromatina (Robertson 2002) la alteración dependiente de poli(ADP-ribosil)ación, de la extensión y de los patrones de metilación del ADN, podría suponer otra manera por la que PARP-1 modulara la estructura de la cromatina (Kim, Zhang et al. 2005).

Control por PARP-1 de la maquinaria transcripcional

PARP-1 puede modular la transcripción actuando directamente sobre factores de transcripción o cofactores como parte de complejos de unión a activadores/promotores **(Figura 36)** (Kraus and Lis 2003).

PARP-1 afecta a la expresión génica mediada por numerosos factores de transcripción, entre otros: AP-1, AP-2, p53, NF-κB, B-Myb, TEF-1, Sp1, YY1, STATs (Anderson et al., 2000; Butler and Ordahl, 1999; Cervellera and Sala, 2000; Ha et al., 2002; Ha et al., 2001; Kannan et al., 1999; Kiefmann et al., 2004; Le Page et al., 1998; Martin-Oliva et al., 2006; Oei et al., 1997; Oliver et al., 1999; Whitacre et al., 1995; Zaniolo et al., 2007; Zingarelli et al., 2004) los cuales, no necesariamente requieren la actividad enzimática de PARP-1. Esto es gracias a que PARP-1 facilita, junto con otros cofactores estructurales, las interacciones cooperativas entre activadores específicos de secuencia y diferentes complejos coactivadores, proporcionando un

armazón para estabilizar el complejo de preiniciación (Hassa and Hottiger 2002). Además, PARP-1 se ha identificado entre los componentes del complejo cofactor-1 (Guermah et al., 1998), esencial para la actividad de factores de transcripción como NF-κB, Sp1 y Oct-1...

PARP-1 tiene diferentes efectos sobre los factores de transcripción en función de la presencia de otros cofactores, el contexto del promotor, el tipo celular, el estado proliferativo de la célula, la concentración de NAD^+ y la presencia de roturas denle el ADN, siendo el mecanismo de acción también distinto (Aguilar-Quesada et al., 2007; D'Amours et al., 1999; Hassa and Hottiger, 2002; Kraus and Lis, 2003). En presencia de NAD^+, la represión por parte de PARP1 de la transcripción implica la poli(ADP-ribosil)ación de factores de transcripción específicos como p53 y fos (Amstad et al., 1992; Wesierska-Gadek and Schmid, 2001), lo que impide su unión a las secuencias consenso de ADN respectivas y la formación de complejos de transcripción activos, teniendo PARP-1 en este caso un efecto negativo sobre la transcripción (Butler and Ordahl, 1999; Oei et al., 1998; Rawling and Alvarez-Gonzalez, 1997; Simbulan-Rosenthal et al., 2001). Sin embargo, en respuesta al daño en el ADN, la actividad catalítica de PARP-1 se dispara y la automodificación de PARP-1 impide su interacción con el promotor, permitiendo la transcripción, por ejemplo, de genes dependientes de p53 que favorezcan la parada del ciclo celular. En ausencia de NAD^+, PARP-1 promueve la transcripción dependiente de activador, interaccionando con factores asociados a la RNA polimerasa II (Meisterernst et al., 1997) y numerosos factores de transcripción como TEF-1, AP-2, p53, NF-κB, B-Myb, Oct 1 y YY1 (Butler and Ordahl, 1999; Cervellera and Sala, 2000; Hassa et al., 2003; Kannan et al., 1999; Nie et al., 1998; Oei et al., 1997; Oliver et al., 1999; Wesierska-Gadek et al., 2003)

No puede obviarse el hecho de que PARP-1 puede ejercer una doble regulación con efectos contrarios sobre algunos factores de transcripción (Li et al., 2004) y es posible que algunos estudios de PARP-1 hayan caracterizado y descrito sólo uno de los dos efectos.

Así, para el caso de la transcripción mediada por NF-κB, PARP-1 puede actuar como inhibidor o como activador. La interacción directa de PARP-1 con NF-κB inhibe la unión de NF-κB al ADN, pero esta inhibición se

reduce por autopoli(ADP-ribosil)ación de PARP-1 (Chang and Alvarez-Gonzalez, 2001) Por otro lado, varios estudios demuestran que la actividad coactivadora de PARP-1 para la expresión génica dependiente de NF-κB parece depender del estímulo y del tipo celular (Hassa and Hottiger 2002). De modo que PARP-1 interacciona directamente con NF-κB sin que la unión al ADN ni la actividad enzimática de PARP-1 sean necesarias para la completa activación de NF-κB en respuesta a varios estímulos (Hassa et al., 2001). Además, la transactivación dependiente de NF-κB, de promotores en los que PARP-1 participa, no sólo requiere la actividad enzimática del coactivador de NF-κB, p300/CBP, sino que además la propia PARP-1 es acetilada en respuesta a estímulos inflamatorios (Hassa et al., 2003).

Se ha descrito un tercer modo de regulación de la transcripción por parte de PARP-1 que consiste en la organización de dominios en la cromatina (Schreiber, Dantzer et al. 2006). Los aisladores son elementos que organizan el genoma en dominios reguladores discretos, limitando la acción de potenciadores y silenciadores a través de un mecanismo de bloqueo posicional (Bell, West et al. 2001). PARP-1, por su parte, regula por poli(ADP-ribosil)ación a la proteína aisladora de la cromatina unida a ADN, CTCF (CCCTC-binding factor) (Yu et al., 2004).

4.3.4. Otras funciones

PARP-1 también interviene en otros procesos celulares tales como:

Replicación del ADN:. PARP-1 interacciona con un complejo multiproteico que contiene importantes proteínas de replicación del ADN, muchas de las cuales son poli(ADP-ribosil)adas (Simbulan-Rosenthal et al., 1996). Además, se ha propuesto que PARP-1 participa en un mecanismo de supervivencia que controla la progresión de la horquilla de replicación en presencia de daños en el ADN (Dantzer et al., 1998). Por otra parte, se ha sugerido el papel de PARP-1 en la respuesta celular a la parada de la horquilla de replicación (Yang, Cortes et al. 2004).

Función del aparato mitótico: PARP-1 se asocia con varios componentes del aparato mitótico, necesario para la segregación correcta de los cromosomas durante la división celular (Smith 2001). Por ejemplo, PARP-1 se localiza en los centrómeros (Saxena et al., 2002) y en los centrosomas (Augustin et al., 2003), hecho que une los mecanismos de supervivencia al daño en el ADN con el control de la fidelidad mitótica (Schreiber, Dantzer et al. 2006)

Control del ciclo celular: PARP-1 está implicado en la regulación de los puntos de control del ciclo celular tras el daño en el ADN a través de p53. Además, la entrada en la fase S desde la fase G_0 disminuye en varios tipos celulares en ausencia de PARP-1 (Miwa and Masutani 2007).

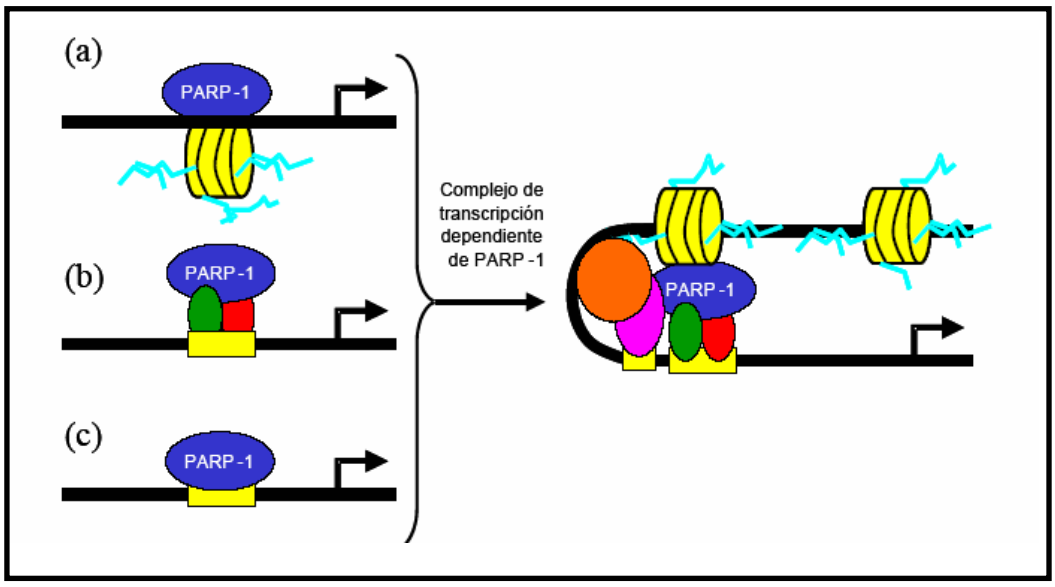

Figura 36: Mecanismos de activación de la transcripción génica regulados por PARP-1. Figura adaptada de (Kraus and Lis 2003)

4.4. Inhibidores de PARP

El uso de inhibidores de PARP-1 cada vez más específicos y potentes, así como el desarrollo de ratones deficientes en PARP-1 y el establecimiento de líneas celulares deficientes en PARP-1, han sido de gran importancia para establecer su papel biológico y contribuir a entender el

proceso de la poli (ADP-ribosilación). PARP-1 está relacionada con procesos patológicos que cursan con inflamación, siendo de gran importancia en enfermedades como la artritis y la diabetes así como otras condiciones fisiopatológicas (Virag and Szabo 2002). Por tal razón el estudio de los inhibidores de PARP-1 ha sido un área de gran interés por su gran aplicación terapéutica (Jagtap and Szabo 2005; Fong, Boss et al. 2009).

4.4.1. Estructura de Inhibidores de PARP

Inicialmente la síntesis de inhibidores de PARP-1 se basó en su acción en la unión al dominio catalítico de PARP-1, ejerciendo una inhibición competitiva mediante la ocupación transitoria del centro de unión para NAD^+. Los resultados obtenidos mediante el uso de los inhibidores clásicos en el campo de la quimio- y radiosensibilización atrajo el interés hacia el desarrollo de nuevos fármacos cada vez más potentes y más selectivos frente a PARP. Gracias al mayor conocimiento adquirido sobre la estructura del centro catalítico de PARP-1, una **segunda generación de inhibidores** más potentes y específicos fue desarrollada durante la década de los 90. La mayor contribución a la evolución de los inhibidores de PARP fue realizada por Banasik (Banasik et al., 1992), través del estudio de 170 fármacos como potenciales drogas inhibitorias de PARP-1 y permitió identificar una gran variedad de compuestos con potente actividad inhibitoria. Estos estudios han permitido dar a conocer las características moleculares necesarias que caracterizan a un potente inhibidor de PARP, capaz de bloquear eficazmente su actividad enzimática **(Figura 37).** En la **Figure 40** se muestran los inhibidores de PARP clásicos y en la **Figure 41** los inhibidores de PARP utilizados en este trabajo.

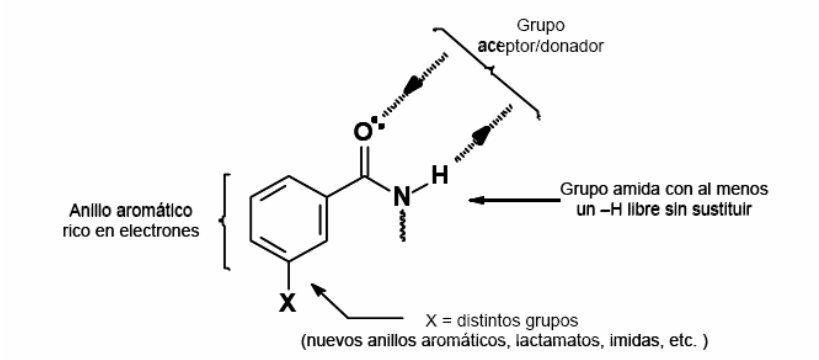

Figura 37. Características estructurales de inhibidores de PARP-1

4.4.2. Inhibidores de PARP en cáncer

El uso de inhibidores de PARP como monoterapia se ha planteado en células tumorales deficientes en recombinación homóloga, como las de cáncer de mama familiar, deficientes en BRCA1 o BRCA2 (Bryant, Schultz et al. 2005; Farmer, McCabe et al. 2005; Fong, Boss et al. 2009). Estas células acumulan SSBs por inhibición de PARP, que durante la replicación del ADN se convierten en DSBs al paso de la horquilla de replicación. Debido al defecto en HR, las DSBs no pueden repararse, desencadenando muerte celular por letalidad sintética **(Figura 38).** (Yap, Sandhu et al.). Este tratamiento, por tanto, tiene aspectos que lo diferencian claramente de terapias previas antitumorales, entre los que cabe destacar que no son necesarios agentes genotóxicos adicionales para causar muerte celular, por lo que se espera que haya pocos efectos sobre las células sana "no mutadas" (en las que la recombinación homóloga no está afectada), que de hecho muestran muy baja toxicidad a la inhibición de PARP (Bryant and Helleday 2004; Yelamos, Buendia et al. 2004). En la figura 40 y Tabla 6 se muestras inhibidores de PARP usados en estudios clínicos en cáncer y otras enfermedades (Peralta-Leal et., 2009).

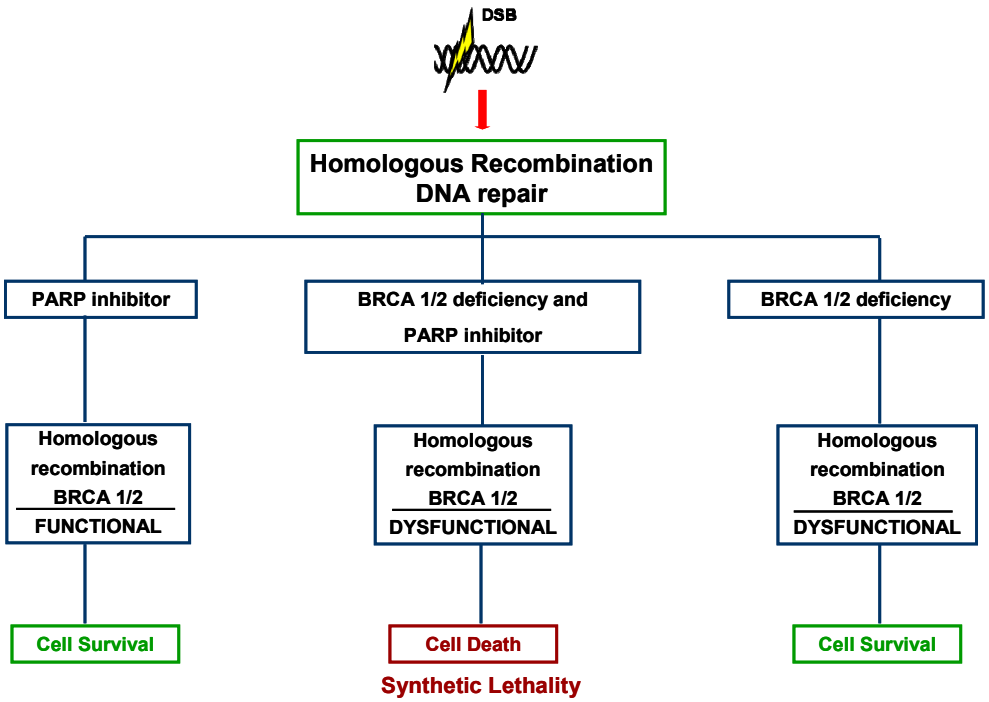

Figura 38. Letalidad Sintética: Mientras que la eliminación de una sóla ruta de reparación del ADN (mediante la inhibición de PARP o la mutación en BRCA1/2) no afecta a la viabilidad celular, la doble deficiencia resulta letal al no poder la célula superar la presencia de daños de cadena doble (DSBs). De esta manera la inhibición de PARP sólo afecta a células deficientes en la reparación por recombinación homóloga. Pero no a las células sanas.

4.4.3. Inhibidores de PARP en Angiogénesis

La inhibición farmacológica de PARP-1 o los ratones deficientes de esta enzima (*parp*-1-/-), han mostrado un fuerte retraso y una reducción en el desarrollo de lesiones premalignas cutáneas (Martin-Oliva et al., 2004). Estudios de expresión génica masiva han puesto de manifiesto que PARP-1 coopera con la activación de NF-kB y AP-1 y regula la expresión de multitud de genes implicados en la promoción tumoral (Martin-Oliva et al., 2006). Uno de los genes que se ha encontrado que disminuye fuertemente en ausencia/inhibición de PARP es el del factor de transcripción HIF-1 alfa (factor inducible por hipoxia-1). Actualmente existe un gran interés en estudiar el efecto de los inhibidores de PARP sobre la angiogénesis. Estudios en HUVEC han sugerido que los inhibidores de PARP pueden disminuir la angiogénesis afectando a la migración y formación de tubos endoteliales inducida por VEGF o FGF (Rajesh et al., 2006a; Rajesh et al., 2006b).

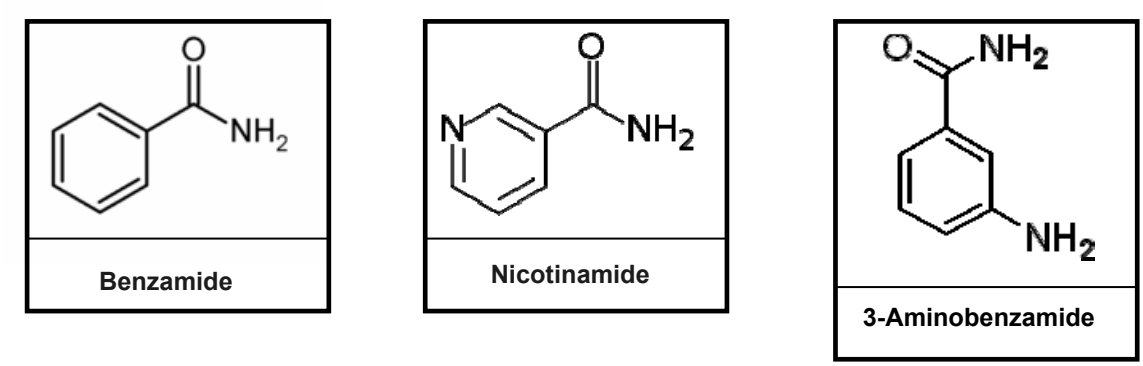

Figure 39. Inhibidores de PARP. Los Inhibidores clásicos son nicotinamida, benzamida, y sustituidos de benzamida, en particular 3-aminobenzamide. **.** *Adaptado de (Peralta-Leal et al., 2009)*

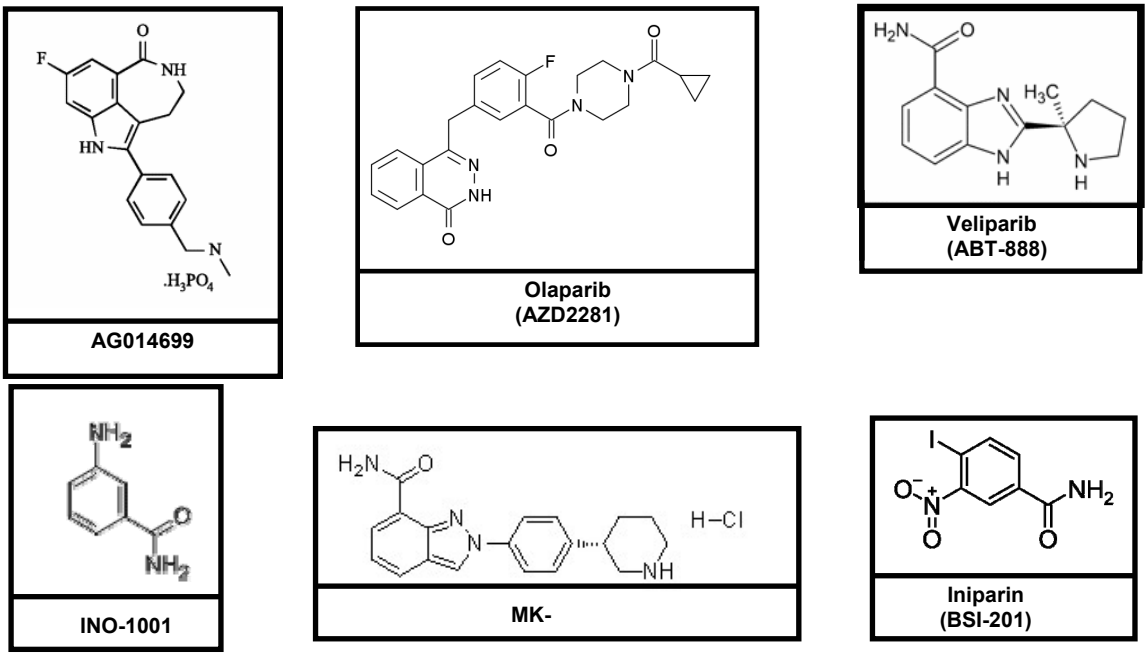

Figure 40. Estructura de Ihnibidores de PARP usados en estudios clínicos. Los Ihhibidores son: **AG014699** (PF01367338) Pfizer (8-fluoro-2-(4-methylaminomethylphenyl)-1,3,4,5-tetrahydroazepino[5,4,3-*cd*] indol-6-1). **Olaparib** (AZD2281, KU-0059436) AstraZeneca. 4-[3-(4 Cyclopropanecarbonylpiperazine-1-carbonyl)-4-fluorobenzyl]-2*H*-phthalazin-1-1 . C24H23FN4O3. **Veliparib** (ABT888) Abbott Laboratories. 2-[(*R*)-2-Methylpyrrolidine-2-yl]-1*H*-benzimidazole-4-carboximide. 13H16N4O. d/da/Veliparib_skeletal.svg, **Iniparib** (BSI 201, NSC-746045; IND-71677) Sanofi-aventis.http://en.wikipedia.org/wiki/File: Iniparib. svg, **(Weil 2010)MK-4827** (S)-2-(4-(piperidin-3-yl)phenyl)-2H-indazole-7-carboxamide Hydrochloride **NO-1001.** *Adaptado de* (Peralta-Leal et al., 2009)

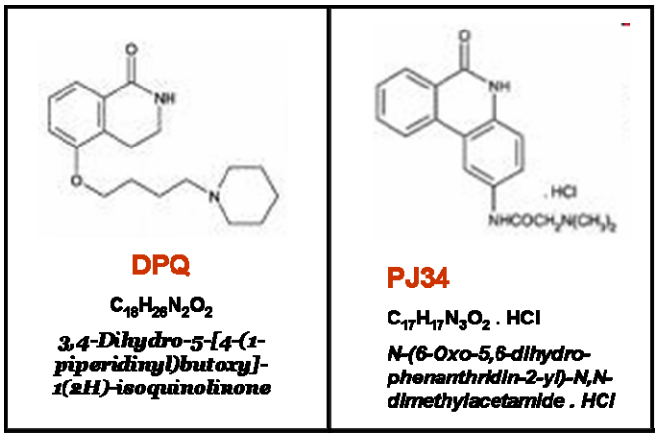

Figura. 41. Estructura de Inhibidores de PARP DPQ y PJ34

Además, se ha demostrado que los los Inhibidores PARP impiden la aparición de brotes angiogénicos utilizando anillos aórticos de rata en un ensayo ex vivo (Rajesh et al., 2006a; Rajesh et al., 2006b).. La inhibición de PARP también ha demostrado ser eficaz en limitar la angiogénesis en un modelo de membrana corio-alantoidea (Rajesh et al., 2006a; Rajesh et al., 2006b). Más recientemente, un estudio sobre la función in vivo de PARP-1 en la agresividad del melanoma, se ha demostrado una notable disminución en la vascularización del tumor tras la eliminación de PARP-1 (Tentori et al., 2007). Se han encontrado resultados similares en un modelo de cáncer de pulmón, mostrando que el tratamiento de rayos X en combinación con el inhibidor de PARP ABT-888 se traducía en una disminución del 50% en el factor de Von Willenbrand del tumor en comparación con el tratamiento con rayos X únicamente (Albert et al., 2007).

Los mecanismos moleculares que subyacen a los efectos de la PARP-1 en la angiogénesis aún no están claros. En nuestro laboratorio hemos demostrado en un modelo de cáncer de piel, que el tratamiento de ratones con el inhibidor de PARP reduce en un 80% el número de vasos sanguíneos en el tumor (Martín-Oliva et al, 2006), Además, en el mismo estudio se mostró que la actividad de PARP tiene la capacidad de modular la expresión de genes implicados en la angiogénesis, sobre todo el factor inducible por hipoxia (HIF), cuya actividad disminuye notablemente en presencia del inhibidor de PARP DPQ o tras la eliminación de PARP-1. HIF-α ha sido ampliamente relacionado en la progresión del tumor al mediar una

respuesta global a la hipoxia, incluyendo la formación de vasos nuevos. Hay resultados que sugieren que la ausencia de PARP–1 y limita la activación de HIF-1α mediante la reducción de los niveles de óxido nítrico y el estrés oxidativo (Martinez-Romero, Canuelo et al.), y que la modificación de HIF-1α por PAR podría estabilizar la proteína frente a degredación por pVHL/proteasoma (Gonzalez-Flores, Oliver FJ, manuscrito en preparación).

La principal conclusión derivada de estas publicaciones que tratan de PARP y la angiogénesis es el requisito de la integridad de la vía de señalización mediada por PARP-1 para tener un desarrollo adecuado de la red angiogénica, por lo que la PARP-1 es un blanco atractivo para la reducción de la angiogénesis en el cáncer y otras enfermedades causadas por una disfunción angiogénica.

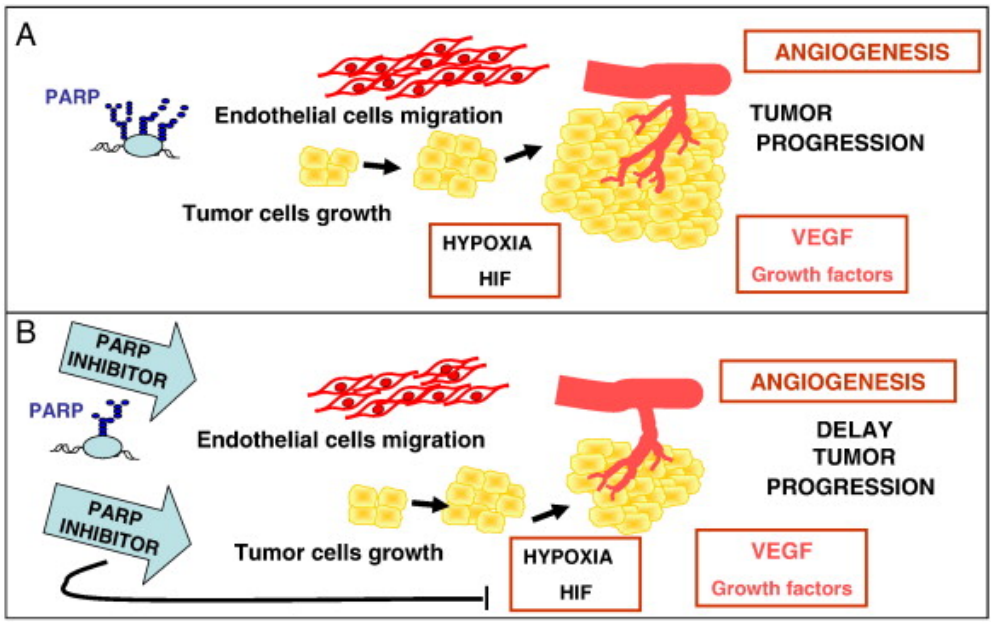

Figura 43. Inhibidores de PARP en Angiogénessis. (A) Progresión tumoral. La hypoxia estimula la expansion y el remodelaje de la vasculature pre-existente para aumentar el flujo sanguíneo hacia tefidossin oxíhgeno. Este proceso se lleva a cabo a través de la activación de genes diana de HIF tales como VEGF y otros factores de crecimiento. (B) Los inhibidores de PARP producen un retraso en el desarrollo del tumor en parte debido a su efecto anti.angiogénico (Peralta-Leal et al., 2009)

Tabla 6. Estudios clínicos con inhibidores de PARP en cáncer. *Adaptado de* (Yelamos et al., 2011)

Inhibitor	Company	Indications	In combination with	Clinical phase	Route
AZD2281 (olaparib)	AstraZeneca	Breast neoplasms in BRCA1/2 mutation carriers	-	II	Oral
		Ovarian neoplasms in BRCA1/2 mutation carriers	-	II	
		Avanced or metastatic solid tumours	-	I	
		Avanced serous ovarian cancer	Paclitaxel and Carboplatin	II	
		Solid tumours	Cisplatin and Gemcitabine	I	
		Ovarian cancer, triple-negative breast cancer	-	I	
		Malignant solid tumours	Topotecan	I	
		Melanoma neoplasm	Dacarbazine	I	
		Avanced solid tumours	Bevacizumab	I	
ABT-888 (Veliparib)	Abbott	Metastatic breast cancer in BRCA1/2 mutation carriers	Temozolomide	II	Oral
		Non-hematologic malignancies and metastatic melanoma	Temozolomide	I	
		Adult refractory solid tumours and lymphomas	-	I	
		Adult refractory solid tumours and lymphomas	Metronomic Cyclophosphamide	I	
BSI-201 (Iniparib)	Sanofi-Aventis	Non-small cell lung cancer stage IV	Gemcitabine and Cisplatin	II	Intravenous
		BRCA1/2 associated advanced epithelial ovarian, Fallopian tube or primary peritoneal cancer	-	II	
		Triple-negative metastatic breast cancer	Gemcitabine and Cisplatin	III	
		Platinum-sensitive recurrent ovarian cancer	Gemcitabine and Carboplatin	II	
AG014699	Pfizer	Advanced solid tumours	Temozolomide	I	Intravenous
		Ovarian cancer, breast cancer in BRCA1/2 mutation carriers	-	II	
CEP-8983/9722	Cephalon	Advanced solid tumours	Temozolomide	I	Subcutaneous
MK-4827	Merck	Advanced solid tumours, BRCA1/2 mutant tumours	-	I	Oral
INO-1001	Inotek	Stage III or IV melanoma	Temozolomide	Ib	Intravenous

IV. JUSTIFICACIÓN Y OBJETIVOS

JUSTIFICACIÓN

La angiogénesis es la formación de nuevos vasos sanguíneos a partir de los ya existentes mediante un proceso que implica migración de células endoteliales. Este proceso es de gran importancia en el desarrollo tumoral así como en el proceso de metástasis. La inhibición de la angiogénesis ha sido blanco terapéutico por excelencia desde hace décadas. Multiples blancos han sido estudiados y hoy en día ha aumentado considerablemente la esperanza de vida en los pacientes con cáncer. Los inhibidores de PARP se están convirtiendo en la terapia prometedora anticáncer y actualmente están siendo objeto de ensayo clínico. Por otro lado mecanismos alternativos de formación de vasculatura crean resistencia en la terapia antitumoral cuando está basada únicamente en la utilización de la angiogénesis como blanco terapéutico. Uno de estos mecanismos es la formación de una "pseudovasculatura" en paralelo formada por células tumorales desreguladas genéticamente (mimetismo vasculogénico).

Las líneas de investigación actuales de nuestro grupo se centran en el estudio del papel de PARP-1 en desarrollo tumoral. En estudios previos en el laboratorio se ha demostrado que la inhibición farmacológica de PARP-1 o ratones deficientes de esta enzima (parp-1-/-), presentan un fuerte retraso y una reducción en el desarrollo de lesiones premalignas epidérmicas. En estos tumores tanto la vascularización como los niveles de activación de factores de transcripción que intervienen en los procesos proangiogénicos se encuentran disminuidos (Martín-Oliva et al., 2004 y Martín-Oliva et al., 2006).

Durante el desarrollo de este trabajo otros grupos de investigación han demostrado que los inhibidores de PARP diminuyen la angiogénesis; sin embargo aun no están bien definidos los mecanismos que regulan este proceso de gran importancia en el desarrollo tumoral. Nuestro estudio lo hemos centrado en caracterizar las propiedades antiangiogénicas y antimetastásicas de los inhibidores de PARP en el contexto del melanoma metastásico, así como el efector de estos inhibidores sobre la vasculatura formada por mimetismo vasculogénico.

Dados estos resultados previos del laboratorio y en base a lo descrito en la literatura, iniciamos una serie de experimentos encaminados a dar respuesta a la siguiente hipótesis

La vasculatura es un factor determinante en el desarrollo de la agresividad tumoral y de la respuesta al tratamiento antineoplásico. PARP-1 puede jugar un papel modulador en la progresión del tumor hacia metástasis, y su ausencia podría mejorar el control de la progresión tumoral.

Para comprobar esta hipótesis diseñamos los siguientes OBJETIVOS:

OBJETIVO GENERAL

Estudio del efecto de los inhibidores de PARP-1 sobre los procesos de angiogénesis, metástasis y mimetismo vasculogénico.

OBJETIVOS ESPECÍFICOS

1. Caracterizar el efecto de los inhibidores de PARP sobre la angiogénesis en modelos *in vitro* e *in vivo* en células endoteliales HUVEC.

2. Identificar mediante proteómica (2D-DIGE) proteínas cuya expresión resulte modificada por la inhibición de PARP en HUVEC

3. Estudiar la expresión génica global (mediante microarray y PCR cuantitativa) en células endoteliales HUVEC en función de la de inhibición de PARP.

4. Estudio del efecto de los inhibidores de PARP sobre el desarrollo tumoral en células de melanoma B16F10.

4.1. Estudio de la formación de la formación de estructuras de tipo endotelial (mimetismo vasculogénico) en matrigel *in vitro*

4.2. Estudio de la migración celular por formación de herida *in vitro*

5. Estudio del efecto de los inhibidores de PARP o silenciamiento de PARP-1 sobre el crecimiento tumoral y metástasis producido por la inoculación intravenosa de la línea B16-F10 en ratones.

6. Caracterización de la alteración inducida por la inhibición de PARP en la señalización del cambio fenotípico de células endoteliales y epiteliales a un mesenquimales (EndMT y EMT, respectivamente).

V. RESULTADOS

A. PARP-1 PARTICIPA EN EL MELANOMA METASTÁSICO A TRAVES DE LA REGULACIÓN DE LA PLASTICIDAD VASCULAR Y LA INTERACCIÓN CON VIMENTINA QUE INDUCE LA TRANSFORMACIÓN MALIGNA

RESUMEN

La inhibición de PARP pueden inducir efectos anti tumorales cuando se usa como monoterapia o en combinación con la quimioterapia o la radioterapia en tumores diversos ajustes, sin embargo, la base para las actividades contra la metástasis como resultado de la inhibición de PARP sigue siendo una incógnita. El análisis proteómico de las células endoteliales reveló que la vimentina, un filamento de intermediarios involucrados en la angiogénesis y el marcador específico del EndMT (transición endotelio mesénquimal), disminuye bajo la pérdida de la función de PARP-1 en las células endoteliales. Asimismo, en este trabajo se observó que la VE-cadherina, un marcador endotelial vascular de la normalización, se reguló en HUVEC bajo tratamientos con inhibidores de PARP o el silenciamiento de PARP-1. En las células del melanoma, la inhibición de PARP fué capaz de reducir marcadores pro-angiogénicos, tanto en cultivos celulares como en modelos in vivo Hemos demostrado que la vimetina por sí sola es suficiente para inducir el aumento de los efectos fenotípicos mesenquimales / prometastasicos en las células del melanoma, incluyendo OLK/GSK 3–b dependiente E-cadherina bajo regulada, Snail 1 junto a la activación y la motilidad celular y el aumentó de la migración. En un modelo murino de melanoma metastásico, la inhibición de PARP puede contrarrestar la capacidad de las células del melanoma para formar metástasis en el pulmón. Los ratones implantados con células de melanoma metastático, que fueron tratados también con el inhibidor de PARP DPQ, exhibieron una drástica disminución tanto en la metástasis de pulmón como en la angiogénesis tumoral en comparación con el grupo de ratones control. El grupo de ratones tratados también experimentó un aumento de la supervivencia en comparación con el grupo control. Un silenciamiento estable de PARP-1 en células de melanoma también dio lugar a un aumento significativo en la supervivencia. Estos resultados sugieren que la inhibición de PARP interfiere con los procesos clave que promueven la metástasis, como el cambio en el fenotipo que permite a las células de melanoma adquirir propiedades invasivas.

PARP-1 participates in metastatic melanoma through regulation of vascular plasticity and interaction with vimentin-induced malignant transformation (EN REVISIÓN)

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Key words: *Metastasis, angiogenesis, vimentin, Snail-1, E-cadherin, EMT, PARP-1, transcriptional regulation, melanoma.*

PARP inhibition can induce anti-neoplastic effects when used as monotherapy or in combination with chemo- or radiotherapy in various tumor settings; however, the basis for the anti-metastasic activities resulting from PARP inhibition remains unknown. Proteomic analysis of endothelial cells revealed that vimentin, an intermediary filament involved in angiogenesis and a specific hallmark of EndoMT (endothelial to mesenchymal transition) transformation, was down-regulated following the loss of PARP-1 function in endothelial cells. VE-cadherin, an endothelial marker of vascular normalization, was up-regulated in HUVEC that were treated with PARP inhibitors or following PARP-1 silencing. In melanoma cells, PARP inhibition was able to reduce pro-angiogenic markers both in cultured cells and in vivo. We demonstrated that vimentin alone is sufficient to induce increased mesenchymal/prometastasic phenotypic effects in melanoma cells, including ILK/GSK3-b-dependent E-cadherin down-regulation, Snail1 activation and increased cell motility and migration. In a murine model of metastatic melanoma, PARP inhibition can counteract the ability of melanoma cells to metastasize to the lung. Mice implanted with metastatic melanoma cells that were also treated with the PARP inhibitor DPQ exhibited a drastic decrease in both lung metastasis area and tumor angiogenesis compared to control mice. The mice also experienced an increased length of disease-free survival compared to controls. Stable knockdown of PARP-1 in melanoma cells also resulted in a significant increase in disease-free survival. These results suggest that inhibition of PARP interferes with key metastasis-

promoting processes, ultimately suppressing the invasion and colonization of distal organs by aggressive metastatic cells.

Introduction

Metastatic melanoma is a fatal malignancy that is remarkably resistant to treatment; however, the mechanisms regulating the transition from the primary local tumor growth to distant metastasis remain poorly understood. Metastasis, defined as the spread of malignant tumor cells from the primary tumor mass to distant sites, involves a complex series of interconnected events. Understanding the biochemical, molecular, and cellular processes that regulate tumor metastasis is of vital importance. The metastatic cascade is thought to be initiated by a series of genetic alterations, leading to changes in cell-cell interactions that allow the dissociation of cells from the primary tumor mass. These events are followed by local invasion and migration through proteolitically modified extracellular matrix (ECM). To establish secondary metastatic deposits, the malignant cells evade host immune surveillance, arrest in the microvasculature, and extravasate from the circulation. Finally, tumor cells can invade the local ECM, proliferate, recruit new blood vessels by induction of angiogenesis, and then expand to form secondary metastatic foci (1). Several key steps in metastatic progression involve tumor-associated endothelial cells (EC) (2). Both angioinvasion and angiogenesis require disruption of endothelial integrity for tumor cell transmigration across the endothelium, EC migration and EC access for mitogenic stimulation. An essential step in angioinvasion and angiogenesis is the disruption of the adherens junctions between EC. Vascular endothelial cadherin (VE-cadherin; also known as cadherin 5) is the most

important adhesive component of endothelial adherens junctions. The interactions between VE-cadherin and the cytoskeleton are mediated by catenins that interact with the C-terminal region of the VE-cadherin cytoplasmic tail. When an endothelial monolayer is subjected to chemical or mechanical wounding, the monolayer is disrupted, and the catenin proteins dissociate from VE-cadherin (3). The dissociation of the VE-cadherin/catenin complex results in EC retraction, exposure of the basement membrane matrix, and loss of endothelial integrity.

Epithelial-mesenchymal transition (EMT) is a trans-differentiation characterized by decreased epithelial markers such as E-cadherin (4). EMT is a dynamic process resulting in the acquisition of cell motility with decreased adhesive ability for body organization that includes embryonic development and wound healing. Currently, EMT is thought to be a key step in the process of cancer metastasis (5). Molecular markers of EMT include E-cadherin down-regulation responsible for the loss of cell-cell adhesion, up-regulation of matrix-degrading proteases and mesenchymal-related proteins such as vimentin and N-cadherin, actin cytoskeleton reorganization, and up-regulation and/or nuclear translocation of transcription factors underlying the specific gene program of EMT, such as β-catenin and members of the Snail1 family (6).

The nuclear protein PARP-1, known to function as a DNA damage sensor and to play a role in various DNA repair pathways, has recently been implicated in a broad variety of cellular functions, including transcriptional regulation (7). PARP

inhibitors exhibit antitumor activity in part due to their ability to induce synthetic cell lethality in cells deficient for homologous recombination repair (8-11). PARP inhibitors also possess anti-angiogenic properties (12-15), and recently, our group reported that PARP inhibition results in the down-regulation of Snail1 by accelerating the degradation of this protein. Our present study indicates that PARP inhibition, as monotherapy, displays an anti-metastatic effect in a model of murine melanoma by modulating endothelial cell plasticity, EMT and vasculogenic mimicry. We have identified vimentin as an upstream modulator of EMT sufficient to induce tumor cell transformation through the ILK/GSK3-b signalling axis. The ability of PARP inhibition to modulate vimentin levels (and hence EMT), the interference with vasculogenic mimicry, and the modulation of endothelial plasticity allow PARP inhibitors to exert a multifaceted antimetastatic effect to counteract the progression of malignant melanoma. Additionally, PARP-1 expression was found to be elevated in human melanoma samples and to correlate with vimentin and other EMT markers.

RESULTS

The effect of PARP inhibition on endothelial cell integrity involves vimentin down-regulation and increased expression of VE-cadherin

A number of reports from various laboratories, including ours, have identified a novel and unexpected effect of PARP inhibitors that reveals a relationship between PARP and angiogenesis, raising the possibility that PARP inhibitors may be useful as anti-angiogenic agents (15). In our present study, we disrupted PARP activation in HUVECs in an attempt to elucidate the mechanisms by which PARP-1 influences endothelial cell dynamics. We have

previously shown that PARP inhibitors are able to reduce angiogenesis both in vitro and in vivo as measured by in vitro (in matrigel) endothelial tube formation (14, 15) or after subcutaneous matrigel injection (**Fig.** S1) in the presence and absence of DPQ. To further explore the molecular mechanisms involved in the effect of PARP on endothelial cell plasticity, we performed a proteomic analysis using primary HUVEC in the presence or absence of the PARP inhibitor DPQ (Fig. 1a, Table 1, (Supplementary Fig. 1). The expression levels of a number of proteins were altered following PARP inhibition, as detected by 2D DIGE electrophoresis (figure S2) and mass spectrometry analysis (Fig. 1A,Table 1). A statistically significant down-regulation of vimentin (a class III intermediary filament), tropomyosin alpha-4 chain (involved in stabilizing actin filaments), endoplasmin (a molecular chaperone involved in processing and transport of secreted proteins), mitochondrial ATP synthase ATPB5, protein disulfide isomerase PDIA6, heat-shock 70kD protein-5 (glucose-regulated protein, 78kD), heat shock protein 90 kDa alpha (cytosolic), class B member 1, HSP90AB1 and HSPD1 occurred following PARP inhibition

Due to its important role in the biology of endothelial cells, we focused our studies on vimentin, the main structural protein of intermediary filaments. It has been reported that vimentin can be targeted for tumor inhibition due to its specific up-regulation in tumor vasculatures (16, 17). To confirm the results of our proteomic analysis, we performed western blot analysis in HUVEC either treated with DPQ or left untreated. In **Fig.**s 1B and C, western blot and immunofluorescence analysis indicates that vimentin expression is down-regulated in HUVEC cells treated with DPQ. PARP-1 knockdown also resulted

in a strong down-regulation of vimentin (**Fig.** S3). **Fig.** 1D shows that PARP inhibition affected not only vimentin levels but also Snail1, VE-cadherin protein and mRNA levels. Endothelial to mesenchymal transition (EnMT) is a process by which endothelial cells disaggregate, change shape, and migrate into the surrounding tissue. The process of endo-MT is characterized by the loss of endothelial cell markers, such as vascular endothelial (VE)-cadherin, and the expression of mesenchymal cell markers, such as vimentin and Snail1 (18, 19). Endothelial cell migration was strongly inhibited by PARP inhibition (**Fig.** 1E). These results suggest that PARP inhibition inhibits the acquisition of a mesenchymal phenotype by endothelial cells.

Interplay between vimentin and PARP-1 modulates the expression and activity of proteins involved in EMT of melanoma cells

Vimentin is a well-known marker of epithelial to mesenchymal transition (EMT), which is a hallmark of primary tumor progression to a metastatic phenotype. We tested the impact of vimentin down-regulation (induced by PARP inhibition or vimentin silencing) on EMT differentiation in various melanoma cell lines. One major event in the process of EMT is the down-regulation of E-cadherin expression through the activation of the transcription factor Snail-1. Snail1 and vimentin levels were both down-regulated following DPQ treatment, indicating a disruption of the EMT pathway in the absence of PARP activation (Fig. 2A and B). Vimentin and Snail-1 mRNA levels were decreased after PARP inhibition. Using two different luciferase reporter plasmids under the control of a Snail responsive sequence and the E-cadherin promoter, we found that PARP inhibition negatively influenced the activation of Snail1 and activated the expression of the E-cadherin promoter (Fig. 3G, 3H, and Fig. 3C). Wound

healing experiments also identified decreased wound closing following treatment with a PARP inhibitor (**Fig.** 2D). Similar results were found using the murine melanoma cell line B16F10 (**Fig.** S4).

We next sought to determine if alterations in vimentin levels were sufficient to alter or reverse EMT progression. Vimentin is known to positively influence tumor cell migration. We have previously evaluated the effect of both PARP-1 and vimentin silencing on the expression of AXL, a key determinant of cell migration and EMT promotion (20). Following PARP-1 silencing in melanoma cells, the EMT marker Snail1 decreases while E-cadherin becomes upregulated (**Fig.** 3A). Interestingly, AXL expression is also down-regulated in parallel with decreased levels of vimentin. Vimentin knockdown also caused a global alteration in the expression of EMT markers. Under these conditions, AXL levels were decreased (**Fig.** 3B), suggesting that vimentin down-regulation is sufficient to drive tumor cells toward a more mesenchymal state. To further characterize the implications of vimentin disruption in the context of EMT, we expressed GFP-vimentin in a human breast tumor cell line possessing an epithelial phenotype (MCF7) and lacking endogenous vimentin expression. GFP-vimetin over-expression alone was able to induce a mesenchymal phenotype characterized by SNAL1 up-regulation, loss of E-cadherin, increased VE-cadherin expression and increased cell motility (**Fig.** 3B and C). Mechanistically, this action of vimentin was related to its ability to modulate GSk3-b activation. Specifically, while inhibition of GSk3-b with Li activates EMT and results in e-cadherin down-regulation (**Fig.** 3B), concomitant vimentin over-expression results in an additional increase in Snail1-1. VE-cadherin, an

established marker of malignant transformation in tumor cells, is increased after vimentin expression and following Li treatment. Alternately, E-cadherin was down-regulated following Gsk3-b inhibition by Li or exogenous expression of vimentin. Concomitant Li-treatment of cells expressing vimentin resulted in a further reduction in e-cadherin levels (**Fig.** 3B).

The trajectories of cell migration were determined using video-microscopy and analyzed using imageJ. Global trajectories of MCF7 cells following expression of GFP-vimentin in the presence or absence of PARP inhibitor and Hepatocyte growth factor (HGF) were determined. Treatment with the PARP inhibitor PJ34 resulted in decreased cell motility under non-stimulated conditions and after HGF treatment (**Fig.** 3D). Vimentin expression alone was able to increase cell motility (**Fig.** 3D, right), and PARP inhibition was able to inhibit this increased cell motility under all experimental conditions.

GSK3-b activation could be achieved through the silencing of its upstream inhibitor integrin-linked kinase (ILK). ILK knockdown resulted in Snail1 down-regulation and increased e-cadherin expression (fig 3C). Interestingly, exogenous vimentin expression completely prevented siILK-induced e-cadherin up-regulation and only partially prevented the reduction of Snail1 expression. These results suggest that vimentin, when over-expressed, is sufficient to induce the phenotypic changes associated with a mesenchymal phenotype, regardless of the activation status of GSK3-b. The ability of vimentin to induce EMT, however, is strongly affected by ILK activation, as siILK-induced e-cadherin accumulation is completely prevented by vimentin over-expression.

PARP inhibition suppresses vasculogenic mimicry in malignant melanoma cells

The formation of patterned networks of matrix-rich tubular structures in three-dimensional culture is a defining characteristic of highly aggressive melanoma cells. Melanoma cell plasticity contributes to the lack of efficient therapeutic strategies targeting metastatic tumors. This vascular mimicry is clinically significant and increases the risk of metastatic disease (21). It has been demonstrated that aggressive melanoma cells in which VE-cadherin was repressed could not form vasculogenic-like networks (22), suggesting that tumor-associated misexpression of VE-cadherin (observed in melanoma cells) is instrumental in allowing endothelial cells to form vasculogenic networks. We measured VE-cadherin levels in B16F10 cells after treatment with the PARP inhibitor DPQ. VE-cadherin was expressed in B16F10 cells (but not in poorly aggressive melanoma cells), and its expression was down-regulated following PARP inhibition (**Fig.** S5). Vasculogenic mimicry has been measured in vitro using B16F10 and B16F1 cells cultured in matrigel coated plates (**Fig.** 4A). All markers of VM structure formation (covered area, tube length, branching points and loops) were significantly decreased after treatment with the PARP inhibitor PJ34 (**Fig.** 4B). In vivo, VM was evaluated in melanoma subcutaneous xenografts (counts by PAS-positive andCD31-negative pattern), and PARP inhibition again significantly decreased VM (**Fig.** 4C).

PARP inhibition protects against lung-metastasis of murine melanoma cells.

We next aimed to examine the effect of PARP inhibition on melanoma tumor growth of cells subcutaneously implanted in C57/BL6 mice. Mice were treated

every two days with 15 mg/kg (i.p.) of the PARP inhibitor DPQ or vehicle. A significant difference in tumor growth was found after 14 days of tumor implantation in the DPQ-treated group compared to the control (**Fig.** S6).

To evaluate the direct effects of the PARP inhibitor DPQ on tumor metastasis, we used a well-characterized model of experimental lung metastasis in which we i.v. injected B16F10 melanoma cells into our experimental mouse model(23). B16F10 cells were tail vein injected into mice, and the mice were then treated with 15 mg/kg of the PARP inhibitor DPQ or vehicle three times per week over a three-week period. Photon emission was acquired every two days. Seven days after B16F10 cell injection, a photon signal was already detected in the lungs (Fig. 5A and B), and DPQ treatment significantly suppressed B16F10 cell lung metastasis compared to the control throughout the duration of the experiment (21 days). Metastatic foci were also detected in other organs upon mice autopsy. These organs included the liver, kidney, spleen, gut, stomach and heart (**Fig.** S7). In all cases, the incidence of metastatic foci was reduced compared to lung metastasis. DPQ-treated mice exhibited a decreased incidence of extra-pulmonary metastasis compared to the control. Pathologic analysis of the lungs showed a decrease in size and number of metastatic foci (more than 80%) after DPQ treatment (Fig. 5C and D) that was accompanied by a reduced number of tumor vessels in both primary subcutaneous tumors and lung metastasis (Fig. 5E), suggesting that the anti-angiogenic effect of the PARP inhibitor may be involved in the observed reduction in metastatic progression. Apoptotic and mitotic indices were not significantly different in tumors derived from DPQ-treated or untreated mice (**Fig.** S8). We also performed a Kaplan Meyer curve to compare the mortality of both groups of

mice, and we observed a statistically significant difference in the survival rate from <4 weeks in the untreated group to >8 weeks in the DPQ-treated mice (Fig. 5E). We also injected B16F10 cells stably expressing shRNA targeting PARP-1 (**Fig.** 5F), and we observed a significant increase in survival in mice injected with the PARP-1 deficient cells.

To investigate in vivo the effect of PARP inhibition on the expression of Snail1 and E-cadherin, we performed immunohistochemistry for these EMT markers in metastatic lung tumors (Fig. 5G). We observed that Snail1 was highly expressed in the vessels of tumors derived from the untreated group. This expression exhibited both nuclear and cytoplasmic distribution as previously reported (24). Metastatic lung tumors derived from DPQ-treated mice displayed reduced expression of Snail1 as well as an increase in E-cadherin expression, similar to the results obtained in cultured melanoma cells. These data indicate that the in vivo expression of EMT markers within tumors is also reduced following treatment with PARP inhibitor.

Human melanoma tissue array

To determine the correlation between PARP-1 expression and disease progression in human melanoma, we used IHC to analyze the levels of vimentin, PARP-1, Snail1, E-cadherin and MITF in nodular and metastatic melanoma frozen biopsies. Vimentin was expressed in all biopsies derived from both nodular and metastatic melanoma; however, the level of expression was elevated in nodular melanoma, which is the initial stage of the disease. PARP-1 expression was positively correlated with vimentin expression, suggesting an

association between the in vivo expression of both proteins (Fi. S9, Table S1). Expression of the Snail1 and Microphthalmia-associated transcription factor (MITF), which is a melanocyte marker, is also increased in metastatic melanoma. Interestingly, nodular melanoma did not express Snail1 while 40% of metastatic melanoma samples displayed Snail1 expression. Loss or reduction of E-cadherin and increased expression of EMT markers is frequently associated with the development of an invasive phenotype in cancer. Expression of E-cadherin in normal melanocytes is significantly reduced during the initial steps of melanoma progression (25); however, elevated levels of E-cadherin are found at advanced stages of the disease (26). E-cadherin expression was similar in both nodular and metastatic melanoma (Table 2), which is in agreement with previous publications. These findings suggest that in human melanoma, there is a complex interconnection between the expression levels of various disease markers and the expression of PARP-1, although we have detected a strong correlation between vimentin and PARP-1 expression (**Fig.** S8).

Discusion

PARP inhibitors are a novel and important class of anticancer drugs, and there are now more than 40 clinical trials that are ongoing or in development to study the effectiveness of PARP inhibitors in the treatment of various cancers. Given the enormous interest in this target, it is important to understand the underlying mechanisms by which PARP-1 and other PARPs function in tumor cell biology. Until recently, the development of PARP-1 inhibitors has focused almost exclusively on the function of this enzyme in DNA repair. Emerging literature,

however, indicates other activities of PARP-1 that may explain the in vivo potency of some PARP-1 inhibitors that cannot be entirely attributed to their apparent in vitro activity and that could provide additional targets for anti-cancer therapies. In addition to its direct role in DNA-damage recognition and repair, PARP-1 can regulate the function of several transcription factors, including p53 and NFκB. In the context of certain cancers, PARP-1 interacts with the transcription factors HIF1 (14) and Snail1 (27). The mechanisms underlying the effects of PARP inhibition on vascular plasticity and metastasis remain relatively unknown. Our current study identifies PARP-1 as a pivotal modulator of the molecular and functional changes characteristic of EndoMT (involved in the loss of function of tumor-associated vessels) and of the phenotypic switch that facilitates the acquisition of pro-metastatic capacities by tumor cells. Proteomic analysis of endothelial cells that have been treated with a PARP inhibitor identifies the intermediary filament protein vimentin as a target of PARP inhibition. Intermediary filaments such as vimentin and keratins are known to play non-mechanical roles in protein trafficking and signaling (reviewed in (28)), which in turn influence cellular processes such as cell adhesion and polarization. Vimentin is abundantly expressed by mesenchymal cells and plays a critical role in wound healing, angiogenesis, and cancer growth. Vimentin has also been described as a tumor-specific angiogenesis marker, and targeting endothelial vimentin in a mouse tumor model significantly inhibited tumor growth and reduced microvessel density (29).

Vimentin is both an EMT and ENMT marker and is also over-expressed in tumor samples compared to normal tissues. This protein also contributes to tumor phenotype and invasiveness (16). Our findings indicate that PARP

inhibitors reduce the metastatic potential of melanoma cells, at least in part, through their ability to down-regulate vimentin expression.

A recent publication identified a mechanism by which PARP-1 can regulate vimentin expression in lung cancer cells at the transcriptional level. They demonstrated that PARP-1 binds to and activates the vimentin promoter independent of its catalytic domain (30). Our results indicate that either loss of PARP-1 or the inhibition of this protein results in the down-regulation of vimentin expression. Given the effect of PARP inhibition on vimentin expression, it is likely that an additional regulatory mechanism not involving transcriptional regulation by PARP-1 exists that does not require PARP-1 activation. The final link between PARP inhibition and vimentin expression may involve the ability of PARP-1 and poly (ADP-ribose) to modulate Snail1 and HIF-1 dependent transcription (31-33). Our results also reveal that vimentin levels are not merely a hallmark of EMT. While silencing of vimentin in melanoma cells can reverse the EMT phenotype, in part by promoting down-regulation of the protein kinase AXL that is involved in cell motility, forced expression of vimentin in tumor cells lacking this protein (MCF7, human breast tumor cells) is sufficient to cause the switch from epithelial to mesenchymal phenotype. GSK3-b is an upstream regulator of key factors involved in EMT such as Snail1 and β–catenin. We hypothesized that vimentin may be involved in the modulation of this upstream regulator of EMT. Indeed, vimentin expression potentiated Li-(a GSK beta inhibitor) induced EMT (**Fig.** 3B) and counteracted the inhibitory action of ILK-silencing (leading to GSK3 beta activation) in the context of EMT (**Fig. 3D**). Mechanical signals can inactivate GSK3β resulting in stabilization of β-catenin.

Intermediate filaments are important in allowing individual cells, tissues and organs to cope with various types of stress, and they play a significant role in the mechanical behavior of cells (34). It is possible that the signaling pathway that integrates PARP activation with altered vimentin expression and fluctuations in GSK3-β activity could be related to the capability of PARP inhibitors to inactivate AKT signaling (35), which would result in GSK3-b activation and the modulation of its downstream signaling, ultimately resulting in the reversal of EMT. Within tumor cells, this mechanism could be supported by the ability of PARP-1 to modulate metastasis-related transcription factors such as Snail1 and HIF-1.

Vasculogenic mimicry, as a de novo tumor microcirculation pattern, differs from classically described endothelium-dependent angiogenesis. This is a unique process characteristic of highly aggressive melanoma cells found to express genes previously thought to be exclusively associated with endothelial cells and is characteristic of aggressive melanoma tumor cells. HIF-1α and HIF-2α, transcription factors that are stabilized during conditions of oxygen depletion (hypoxia), are the master regulators of VE-cadherin. HIF-mediated transcriptional regulation during hypoxia is critical as this process induces genes that are essential for tumor cell adaptation to the stress of oxygen depletion. As a result, the expression of HIF target genes is associated with increased malignancy. Although the expression of VE-cadherin is not hypoxia-regulated, HIF-2α, but not HIF-1α, activates the VE-cadherin promoter by binding to the HRE during normoxic conditions [41]. HIF-2α expression is associated with developing endothelium [42], proper vascular development [43]

and increased tumor malignancy [44] [45], raising the possibility that it may be an important protein that functions in the induction of tumor cell plasticity. It is likely that HIF-2α expression in aggressive tumor cells may be directly associated with dedifferentiation towards the endothelial lineage through the transcriptional induction of VE-cadherin. In agreement with the theory that HIF-2α can act as an inducer of dedifferentiation, HIF-2α specifically regulates Oct-4 gene transcription [46] [47]. Oct-4 is a transcription factor essential for maintaining stem cell pluripotency. Its expression is induced with the purpose of stimulating a dedifferentiation response. In HIF-2α knock-in tumors, an increased fraction of undifferentiated cells was observed, and this phenotype could be reversed by knock-down of Oct-4. These findings identify a role for HIF-2α in mediating endothelial differentiation, and given the increased expression of this protein in aggressive tumours, HIF-2α is emerging as an interesting candidate gene in the study of VM.

Using a mouse model of melanoma lung metastasis, we also present in vivo evidence indicating that targeting PARP strongly reduces metastatic dissemination of melanoma cells, at least in part through inducing a reduction in tumor microvessel density along with changes in the expression pattern of EMT markers (Snail, vimentin and E-cadherin) within the tumor.

Snail is a master regulator of EMT, and the activation of this protein can mediate tumor invasiveness through the transcriptional repression of E-cadherin expression. Regulating the activity of E-cadherin repressors represents a potentially beneficial strategy to fight cancer progression, and PARP inhibitors

accomplish this function by interfering with Snail1 activation. The underlying mechanisms by which PARP-1 activity influences Snail are currently under intensive investigation in our laboratory.

Results from human tissue arrays of melanoma suggest a complex interaction between PARP-1 expression and melanoma progression. It is difficult to verify EMT experimentally in vivo due to the reversible and dynamic nature of the process. Although melanoma cells are not epithelial in nature, the EMT for this tumor is well characterized and the relevance of the cadherin switch has been previously described using several experimental approaches, demonstrating that melanoma cell lines transfected with N-cadherin are morphologically transformed from an epithelial-like shape to a fibroblast-like shape(36). Adenoviral re-expression of E-cadherin in melanoma cells down-regulates endogenous N-cadherin and reduces the malignant potential of these cells (36).

Over the past few years, PARP has emerged as a strong and effective target for first line anticancer therapy. Due to its ability to regulate a number of cellular functions (from DNA repair to cell death and transcription), inhibition of PARP may affect multiple facets of tumor metabolism. These findings strongly indicate that several novel activities of PARP-1 may contribute to the effects of anti-cancer therapy targeting this protein by interfering with tumor physiology and the tumor microenvironment. Given these findings, it is of vital importance that we elucidate mechanisms regulating novel functions of PARP-1 and poly (ADPribose) in tumor biology so that PARP inhibitors can ultimately make the transition to routine clinical use.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVEC) were cultured in EGM®-2 Endothelial Cell Growth Medium-2 (LONZA). Cells were subjected to experimental procedures within passages 3–6. B16-F10-luc-G5 cells stably expressing plasmids pGL3 control (SV40-luc) (Promega) and pSV40/Zeo (Invitrogen) and B16-F10 cells were cultured in DMEM containing 10% fetal bovine serum, 0.5% gentamicin (Sigma, St. Louis, MO), and 4.5% glucose. All cells were cultured at 37ºC (5% CO2). Cells were treated with the PARP inhibitors 3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone (DPQ) or [N-(6-Oxo-5,6-dihydro-phenanthridin-2-yl)-N,N-dimethylacetamide] (PJ-34) (Alexis Biochemicals, San Diego, CA) for 22 hours. For capillary-like formation assays, 25 µL of Matrigel (BD Biosciences) were spread onto eight-chamber BD Falcon glass culture slides (BD Biosciences) or onto 96-well plates. Cells were seeded at 2.5 × 10^4 cells per well (high density) in eight-chamber slides and at 5 × 103 cells per well (low density) in 96-well plates and maintained in RPMI supplemented with 1% FBS (37).

Western blot and qPCR were performed according to previously published methods (15).

In vitro angiogenesis assay

The effect of PARP inhibitors on the formation of tube-like structures in Matrigel (BD Biosciences) was determined according to manufacturer instructions. Briefly, 24-well plates were coated with 100 μl of BD Matrigel™ Basement Membrane Matrix and allowed to solidify at 37 ºC in 5% CO_2. Cells were treated with DPQ or PJ34. After 22 to 24 h of incubation at 37 ºC in 5% CO_2, the cells were fixed with 3.7% formaldehyde, and images were acquired using an Olympus CKX41 microscope. The formation of tube-like structures was then quantified. Each treatment was performed in triplicate, and the experiment was independently repeated at least three times.

Matrigel angiogenesis assay in vivo

C57BL/6 mice background (8 weeks old) were subcutaneously (s.c.) flank-injected with 600 µl of matrigel (BD Biosciences) supplemented with VEGF (100 ng/ml) (Peprotech) and heparin (Sigma, 19 U). The negative controls contained heparin alone. Each group consisted of four animals. After seven days, mice were sacrificed and matrigel plugs were extracted. The angiogenic response was evaluated by macroscopic analysis of the plug at autopsy and by measurement of the hemoglobin (Hb) content within the pellet of matrigel. Hb was mechanically extracted from pellets reconstituted in water and measured using the Drabkin (Sigma-Aldrich) method by spectrophotometric analysis at 540 nm. The values were expressed as optical density (OD)/100 mg of matrigel.

Migration/invasion assays

HUVEC and B16F10 cells were cultured on coverslips in six-well cell culture dishes. Monolayer cultures were stained with 10 μM CellTracker Green CMFDA

(5-chloromethylfluorescein diacetate) (Invitrogen) according to manufacturer recommendations or with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) (post-fixation). A wound was induced in the confluent monolayer cultures, and the cultures were then treated with the indicated inhibitor. The cells were fixed with 3.7% buffered formaldehyde and then prepared for immunofluorescence. Images were captured using a confocal microscope (LEICA TCS SP5 Argon Laser 488 nm, HeNe Laser 543 nm when the cells were stained with CellTracker Green CMFDA Abs [522 nm] and Em [529 nm] and Zeiss Axio Imager A1 microscopy for cells stained with DAPI).

In vivo bioluminescence assay

Eight-week-old male C57/Bl6 albino mice *(The Jackson Laboratories*, Bar Harbor, MN, USA) were injected subcutaneously with B16-F10-luc-G5 cells ($1x10^5$) and intravenously with B16-F10-luc-G5 cells ($1x10^5$ or $5x10^5$). Three times per week the mice were injected intraperitoneally with DPQ dissolved in phosphate-buffered saline/10% DMSO at a dose of 15 mg/kg body weight. Mice were injected intraperitoneally with D-luciferin solution dissolved in phosphate-buffered saline at a dose of 150 mg/kg body weight. After 5 to 8 minutes, the animals were anesthetized in the dark chamber using 3% isoflurane in air at 1.5 L/min and O_2 at 0.2 L/min/mouse, and animals were imaged in a chamber connected to a camera (IVIS, Xenogen, Alameda, CA). Exposure time was 3 min in *large binning*, and the quantification of light emission was performed in photons/second using Living Image software (Xenogen). Tumor growth was monitored at 0, 2, 7, 14 and 21 days by in vivo imaging and calliper

measurement. After 21 days, mice were sacrificed, and their organs were removed and stored in buffered formalin (3.7%) until histological staining.

Indirect immunofluorescence

Immunostaining for vimentin and E-Cadherin was performed on cells plated onto coverslips and grown for 24 h prior to experimental treatments. The culture medium was removed, and the cells were fixed (Paraformaldehyde 3%, Sucrose 2% in PBS) for 10 minutes at room temperature. Permeabilization was performed using 0.2% Triton X-100 in PBS. The coverslips were rinsed three times in PBS prior to incubation with primary antibody for 1 h at 37°C in the dark and then rinsed three times in PBS before incubation with the secondary antibody. The primary antibodies used in these experiments were vimentin and VE-cadherin from Santa Cruz Biotechnology (Santa Cruz, CA), and the secondary antibody was FITC-conjugated anti-mouse IgG (Sigma, St. Louis, MO) at a concentration of 1:200. Antibodies were diluted in PBS containing 2% bovine serum albumin. Nuclear counterstaining with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) was performed after removal of excess secondary antibody. Slides were prepared using Vectashield mounting medium (Vector Lab., Inc., Burlingame, CA 94010), coverslipped and stored in the dark at 4°C. Immunofluorescence images were obtained in the linear range of detection to avoid signal saturation using a fluorescent microscope (Zeiss Axio Imager A1).

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AUTHOR CONTRIBUTIONS

A.P.L. and M.R performed most of the experiments. A.G-F., J.M.R-V., J.M-M. and L.L performed the western blots and transient and permanenet silencing, and helped with the mice experiments. FO performed the immunohistochemistry for the human and mouse experiments and clinicopathologic patient information. R. F. performed the vasculogenic mimicry assays. J.C.R.M., A.G.H. andd J.M.R.A. designed the experiments. F.J.O. directed the study.

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Table 1. Proteins differentially expressed and identified by mass spectrometry analysis in HUVEC. The level of expression of various proteins in HUVEC was altered following PARP inhibition as determined by 2D-DIGE, and the proteins were positively identified using mass spectrometry analysis. Of particular interest for this study was vimentin, the major structural protein of intermediary filaments (spot 1). Expression of this protein was decreased in HUVEC following PARP inhibition. The proteins were identified by MALDI-TOF. Sequence coverage (%) and number of peptides were identified with ≤1% FDR (false discovery rate cut-off against decoy-concatenated randomized database). Coverage and score was determined using the MASCOT algorithm. The average ratio of protein expression between the control and cells treated with the PARP inhibitor DPQ was determined in HUVEC.

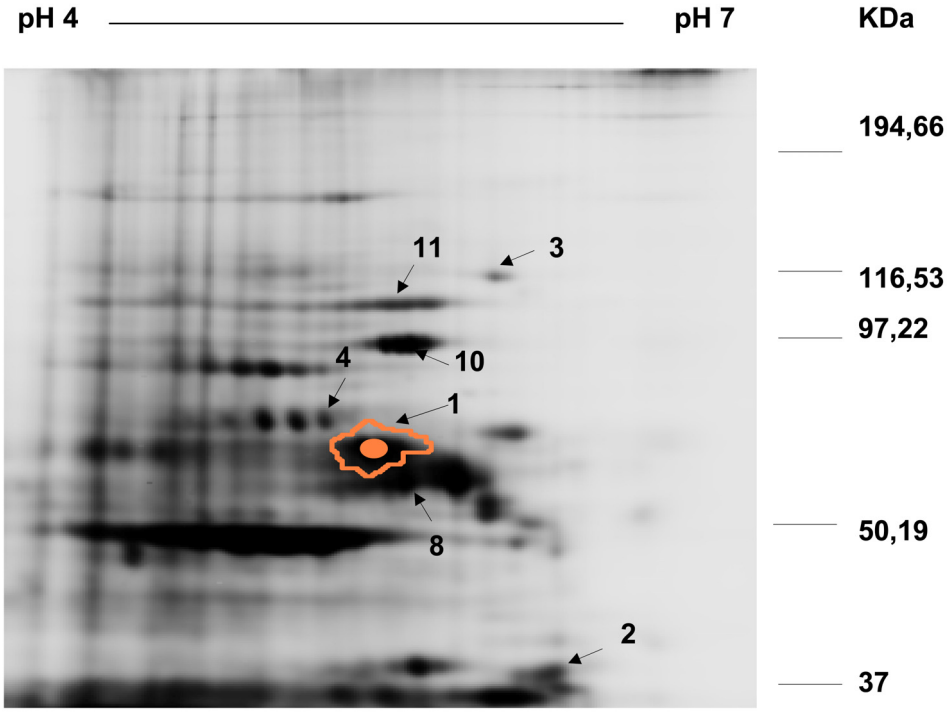
DOWN-REGULATED PROTEINS

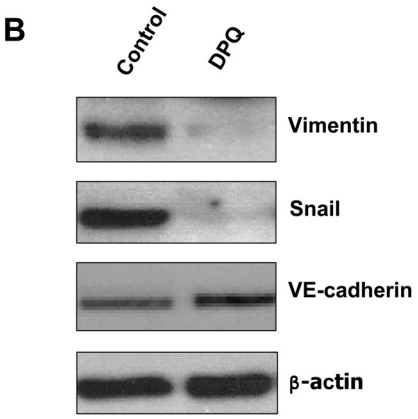
Spot No.	SwissProt Access No.	Gene Symbol	Protein name	MW (Da)	p/	SCORE	Seq Cov %	Av ratio	No. Pep	Function
1	P08670	VIM	Vimentin	53676	5.06	319	58%	-1.21	26	class-iii intermediate filaments
2	P67936	TPM4	Tropomyosin alpha-4 chain	28619	4.67	52	16%		40	stabilizing cytoskeleton actin filaments
3	P14625	HSP90B1	Endoplasmin	92696	4.76	157	28%	-1.32	37	molecular chaperone that functions in the processing and transport of secreted proteins.
4	P10809	HSPD1	60 kda heat shock protein, mitochondrial	61187	5.70	61	22%	-1.38	7	implicated in mitochondrial protein import and macromolecular assembly. folding of imported proteins. polypeptides generated under stress conditions in the mitochondrial matrix
8	P06576	ATP5B	ATP synthase subunit beta, mitochondrial	56525	5.26	251	63%	-1.25	21	molecular chaperone that functions in the processing and transport of secreted proteins.
	Q15084	PDIA6	Protein disulfide-isomerase A6	48490	4.95	61	22%	-1.25	7	catalytic activity. catalyzes the rearrangement of -s-s- bonds in proteins.
10	P11021	HSPA5	78 kda Glucose-regulated protein	72402	5.07	159	32%	-2,14	17	subunit structure. interacts with mica on the surface of tumor cells, leading to mica disulfide bond reduction which is required for its release from tumor cells.
11	P08238	HSP90AB1	Heat shock protein hsp 90-beta	83554	4.97	69	18%	-1.31	13	molecular chaperone. has atpase activity.

Figures

Figure 1. **PARP inhibition down-regulates vimentin expression and inhibits endothelial-to-mesenchymal transition in HUVECs.**

Cell extracts from HUVEC either treated with vehicle or 20 μM DPQ were subjected to 2D electrophoresis as described in Materials and Methods. Image analysis software (DeCyder TM) indicated that seven proteins exhibited decreased expression in HUVEC treated with DPQ compared to untreated cells. Proteins were identified using MALDI TOF. Spots labeled with arrows indicate proteins that were identified by mass spectrometry (see Table 1). The spot with the arrow is vimentin **(A)** PARP inhibition reduced the expression of both vimentin and Snail-1 and up-regulated VE-cadherin in human endothelial cells (HUVEC) as determined by immunoblotting **(B)**, indirect immunofluorescence **(C),** and mRNA levels **(D)**. PARP inhibition decreased HUVEC cell migration (E). Cells were compared in the presence or absence of serum (*P < 0.05, ***P < 0.001 control *versus* PJ-34).

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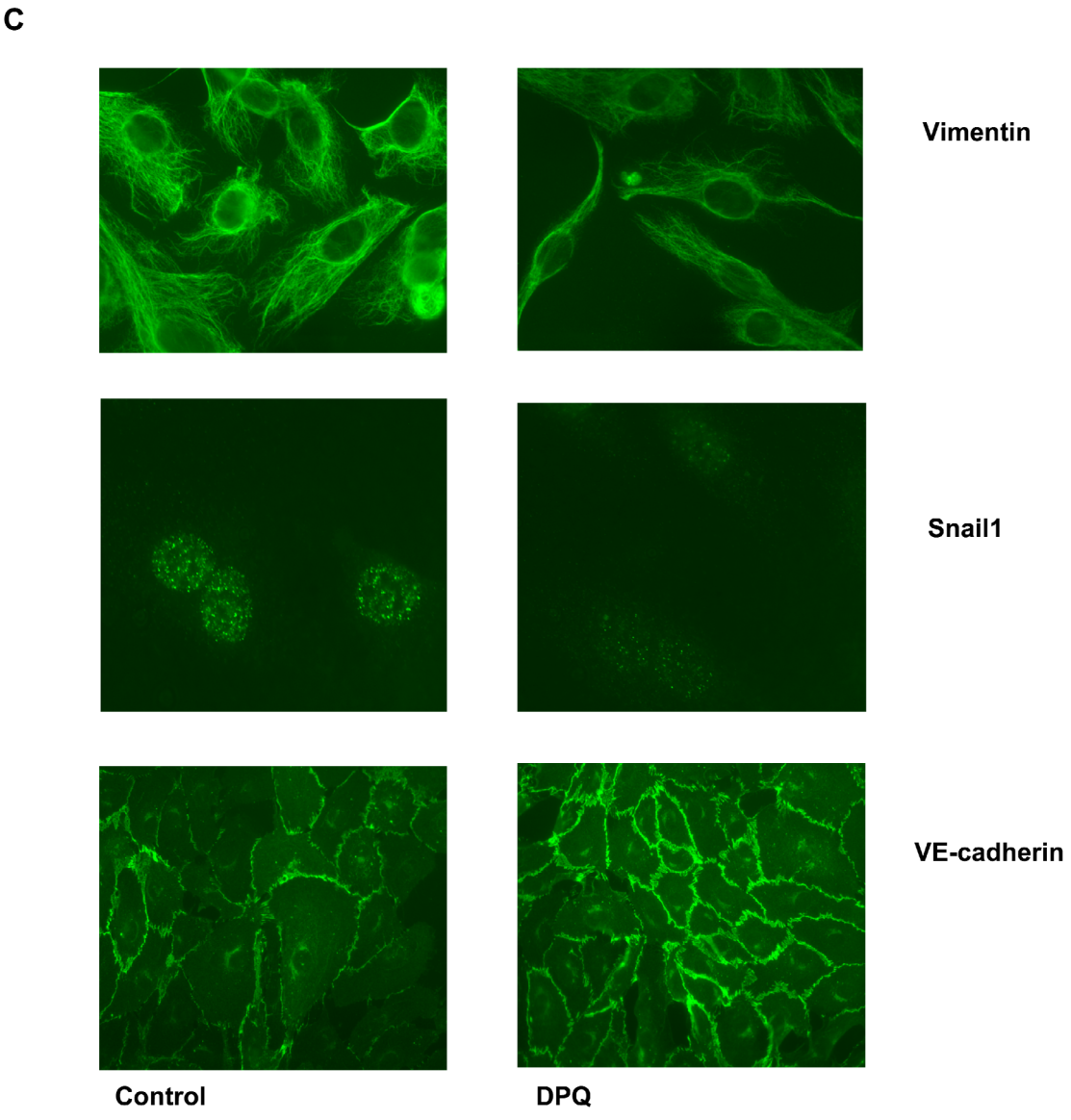
β-actin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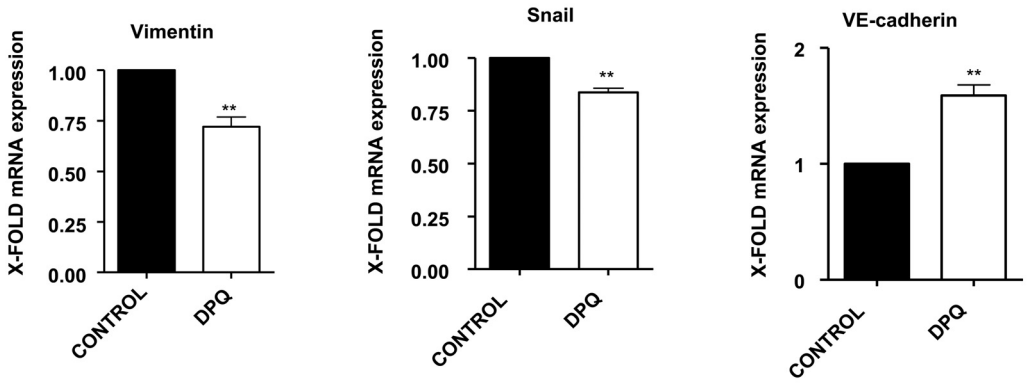
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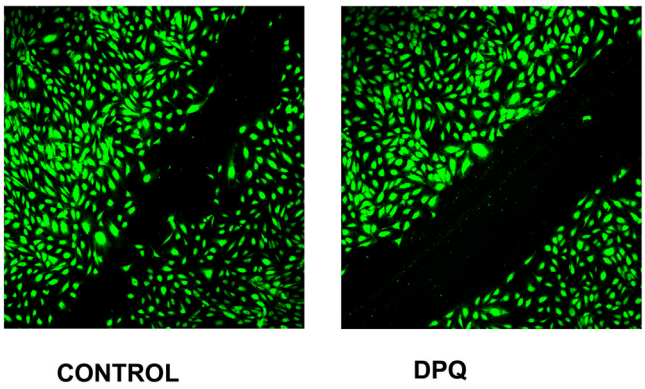
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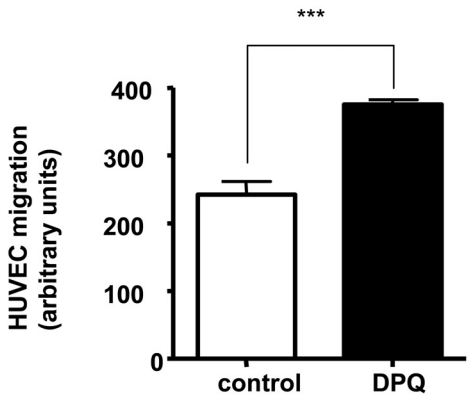
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HUVEC migration
(arbitrary units)
400
300
200
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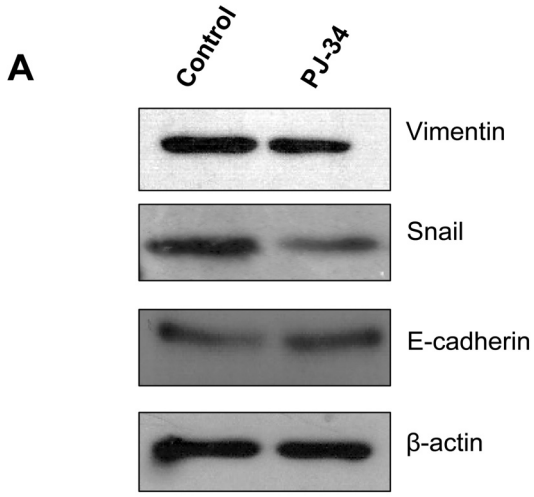
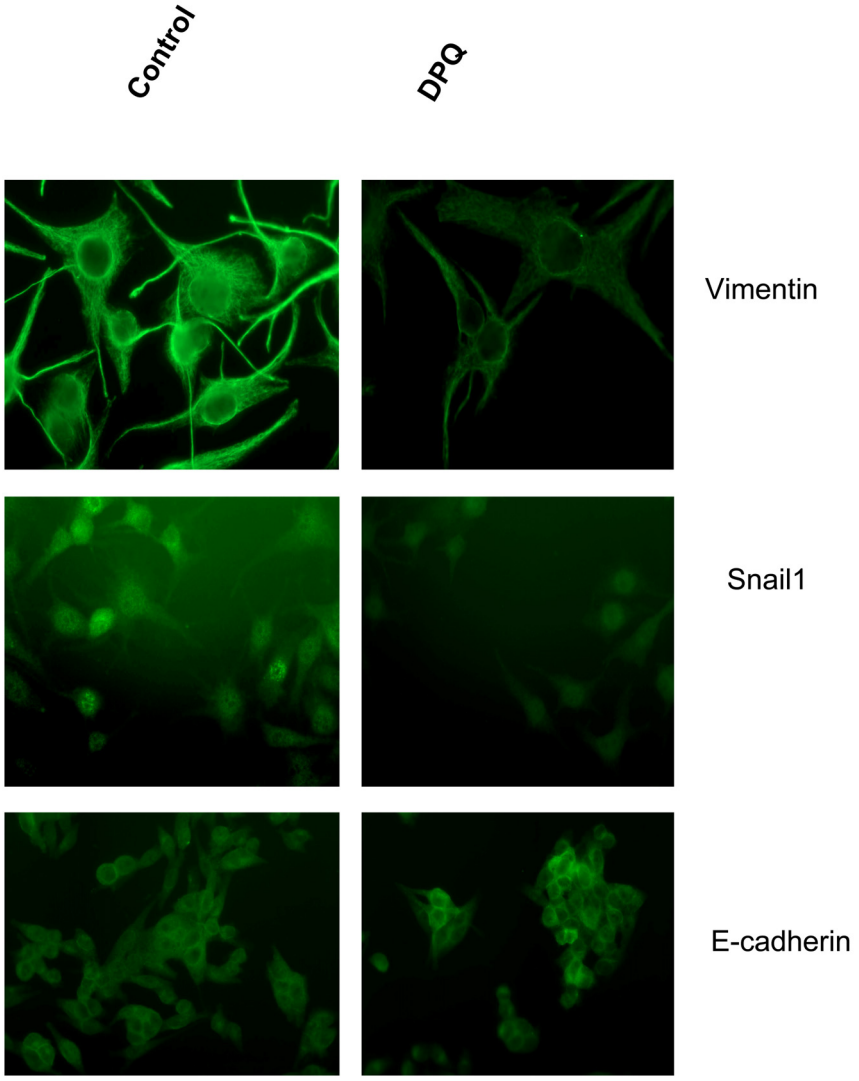
control
DPQ

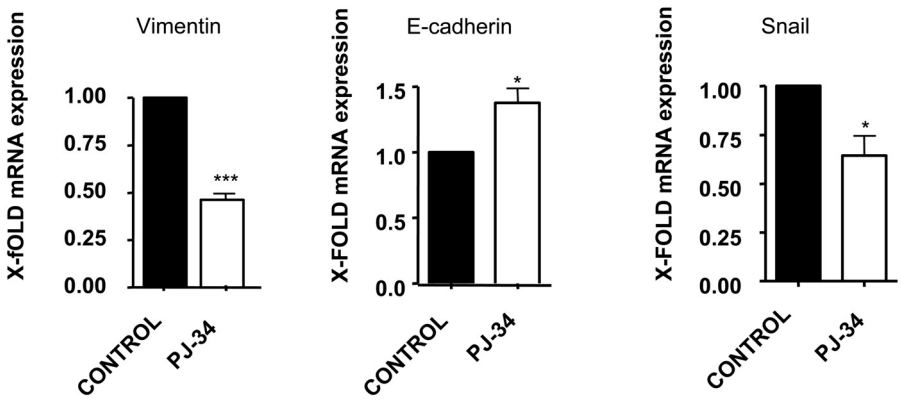
Figure 2: PARP inhibition inhibits the acquisition of an EMT phenotype in malignant melanoma cells. Human melanoma G361 cells and murine B16F10 melanoma cells (Suplemmentary Fig. S2) were used for these experiments. Cells were treated with either DPQ or PJ34 (40 μM) for 18 hours. IF, western blot or qPCR assays were performed to evaluate the effects of PARP inhibition on EMT markers. PARP inhibition reduced the expression of vimentin and Snail-1 and up-regulated E-cadherin in human melanoma cells as determined by immunoblotting (**A**), indirect immunofluorescence (**B**), and mRNA levels **(C).** Cells were compared in the presence or absence of serum (*P < 0.05, ***P < 0.001, PARP Inhibitor groups *versus* the control). β-actin was used as an internal control for protein loading. **(D)** Snail-1 and E-cadherin promoter activity are regulated by PARP inhibitors. Luciferase activity was determined after transfecting the constructions into G361 cells. Firefly Luciferase was standardized to the levels of Renilla Luciferase. Cells were cotransfected with 0.5 μg renilla as a transfection control and 0.5 μg of Snail1 or E-cadherin using jetPEI cationic polymer transfection reagent according to the manufacturer's instructions. Cells were compared in the presence or absence of serum (*P < 0.05, ***P < 0.001 control *versus* PJ-34). The expression of both Firefly and Renilla luciferase was analyzed 48 h after transfection. Cloning of the human Snail1 promoter (-869/+59) into pGL3 basic (Promega) was described previously (38). The E-Cadherin promoter was cloned into pGL3-basic (Promega) to generate pGL3-E-cadherin (-178/+92). **(E)** Inhibitory effect of PARP on B16F10 motility. Treatment with the PARP inhibitor PJ34 (40 μM) or DPQ (40 μM) decreased cell migration in vitro. Migration was quantified as distance between Wound Healing limits (*** p <0.001 *versus* control).



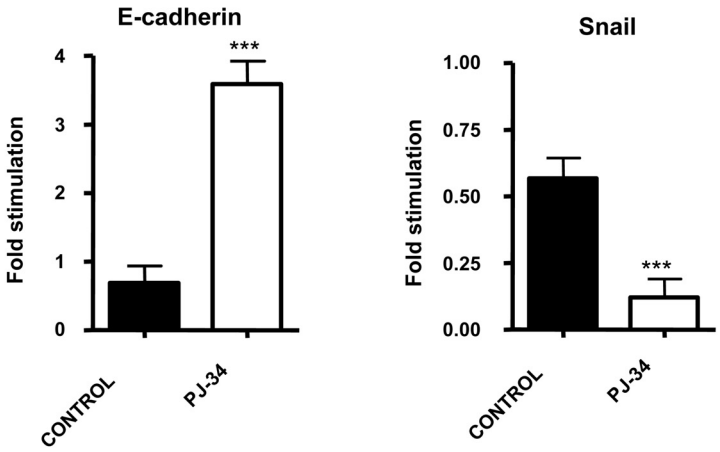
B


Control
DPQ
Vimentin
Snail1
E-cadherin

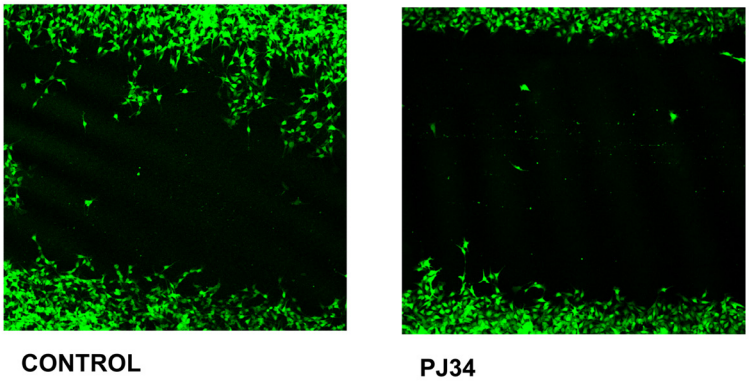
C


Vimentin
X-fOLD mRNA expression
1.00
0.75
0.50
0.25
0.00
***
CONTROL
PJ-34
E-cadherin
X-FOLD mRNA expression
1.5
1.0
0.5
0.0
*
CONTROL
PJ-34
Snail
X-FOLD mRNA expression
1.00
0.75
0.50
0.25
0.00
*
CONTROL
PJ-34

D


E-cadherin
***
Fold stimulation
4
3
2
1
0
CONTROL
PJ-34
Snail
1.00
0.75
0.50
0.25
0.00
***
Fold stimulation
CONTROL
PJ-34

E


CONTROL
PJ34

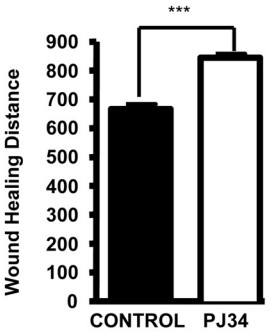
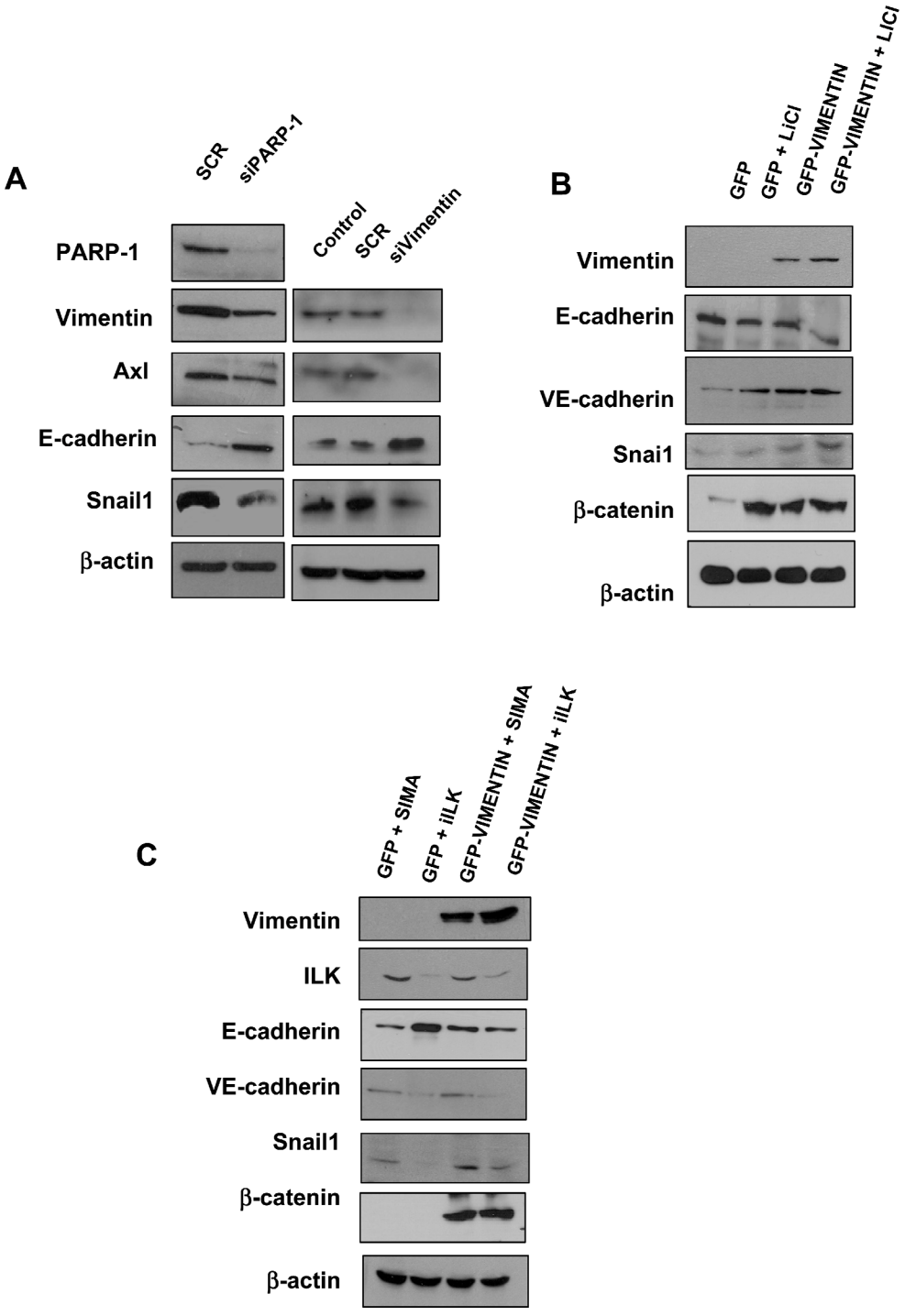
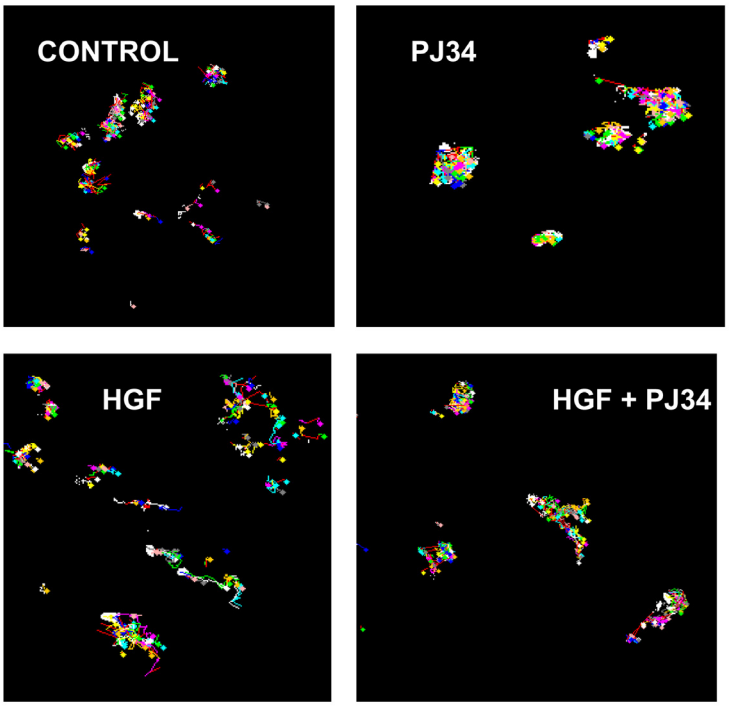

***
900
800
700
600
500
400
300
200
100
0
Wound Healing Distance
CONTROL
PJ34

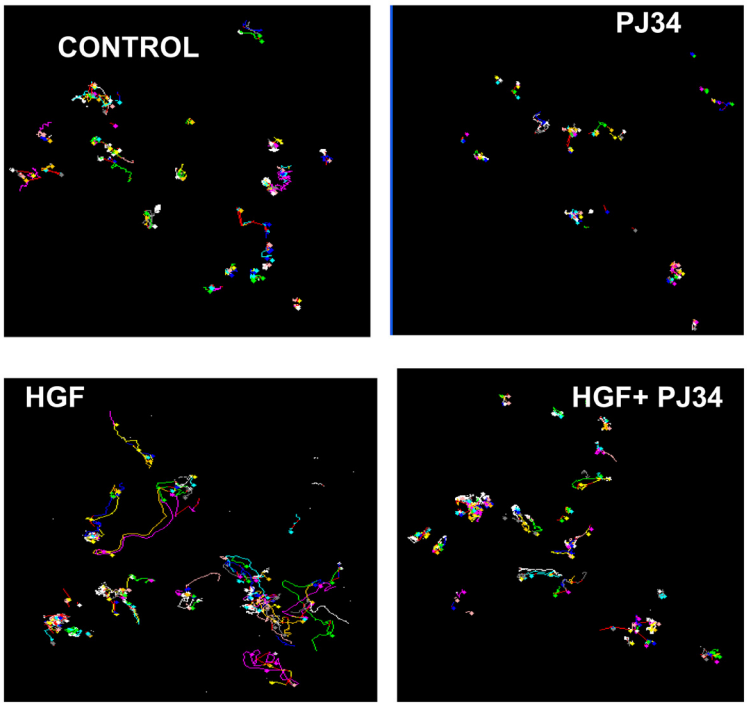
Figure 3.- PARP-1 or vimentin is sufficient to reverse EMT and confer increased cell motility. (A) Human melanoma cells (G361) were silenced for PARP-1 or vimentin and the expression levels of E-cadherin, Snail1, AXL, PARP-1, and vimentin were determined by immunoblot (B, C). Forced expression of vimentin drives human breast tumor epithelial cells (MCF7) to a mesenchymal phenotype through the integrin-linked-kinase/GSK□-b axis. 3 mM LiCl was used to inhibit GSK□-b, as detected by the accumulation of beta-catenin. (C) ILK was knocked down to analyze the significance of the interaction between vimentin and ILK in promoting the transition to a mesenchymal phenotype. (D) Cell migration was analyzed in MCF7 cells transfected with either GFP or GFP-vimentin using video-microscopy and imageJ free software. While vimentin was able to increase the length of the trajectories in the absence or presence of hepatocyte growth factor (HGF), treatment with PARP inhibitor resulted in a sustained reduction in cell motility.


A
SCR
siPARP-1
PARP-1
Vimentin
Axl
E-cadherin
Snail1
β-actin
Control
SCR
siVimentin
B
GFP
GFP + LiCl
GFP-VIMENTIN
GFP-VIMENTIN + LiCl
Vimentin
E-cadherin
VE-cadherin
Snai1
β-catenin
β-actin
C
GFP + SIMA
GFP + iILK
GFP-VIMENTIN + SIMA
GFP-VIMENTIN + iILK
Vimentin
ILK
E-cadherin
VE-cadherin
Snail1
β-catenin
β-actin

D



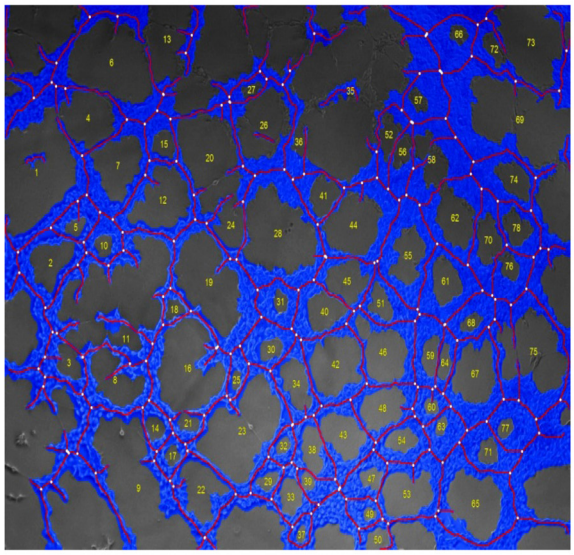
Mock transfected



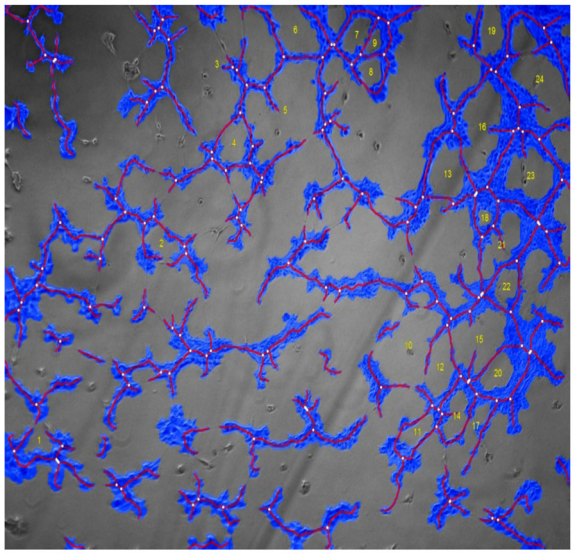
Vimentin transfection

Figure 4.- Vasculogenic mimicry is reduced by PARP inhibition in cell cultures and in xenogafts of malignant melanoma. (A,B) B16F10 cells were cultured on polystyrene-treated culture slides and treated with the PARP inhibitor PJ34 at 20 □M or left untreated. Following treatment, pictures were taken and analyzed using Wimasis image analysis software. Four independent experiments were performed (* p<0.05; **p<0.005). (C) In vivo vasculogenic mimicry was evaluated in xenografts by counting PAS positive CD31 negative structures from 6 mice.

A

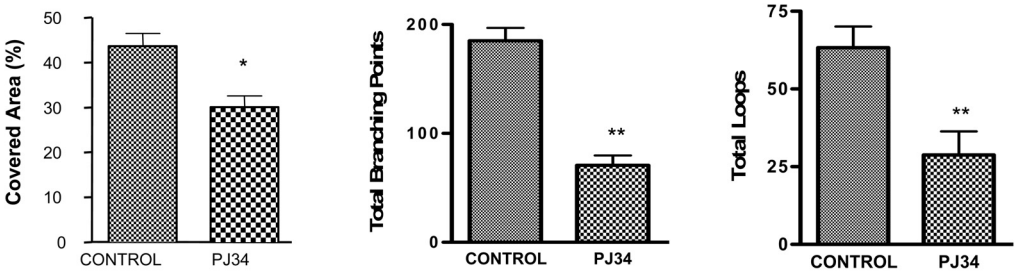


CONTROL



PJ34

B


Covered Area (%)
50
40
30
20
10
0
*
CONTROL
PJ34
Total Branching Points
200
100
0
**
CONTROL
PJ34
Total Loops
75
50
25
0
**
CONTROL
PJ34

C

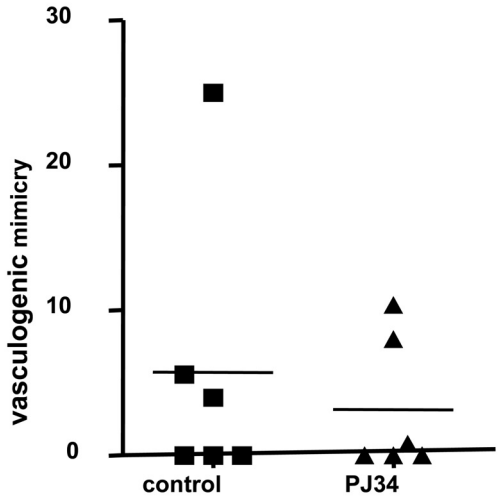
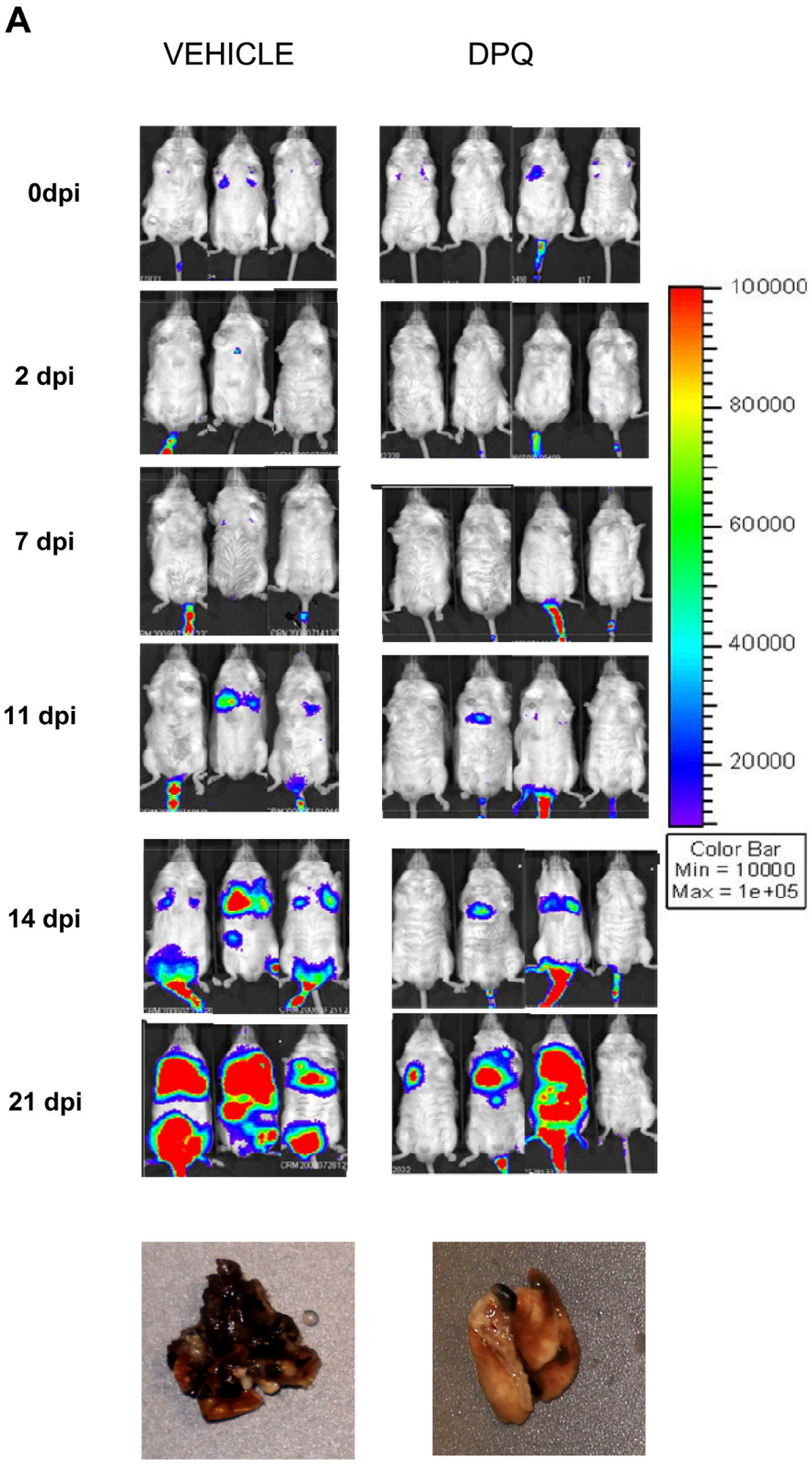
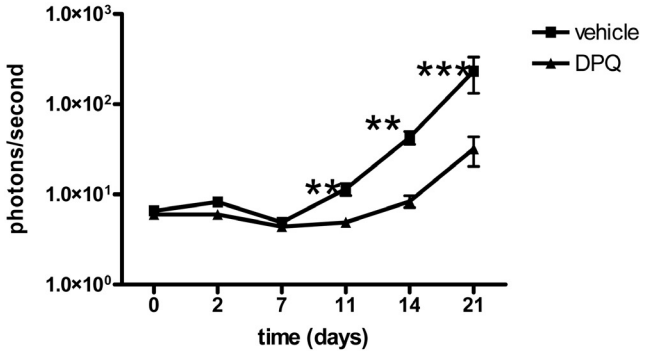

vasculogenic mimicry
30
20
10
0
control
PJ34

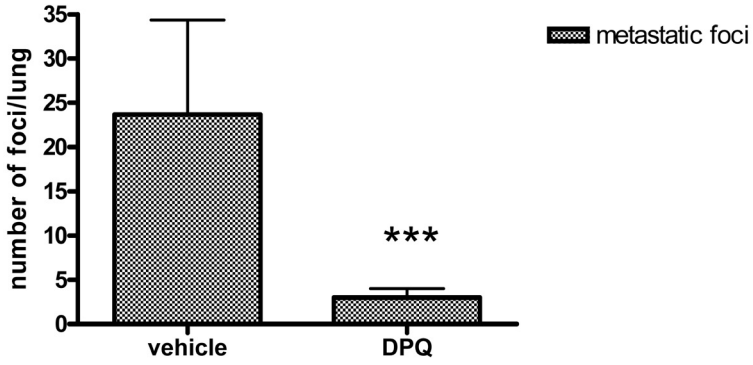
Figure 5.- Decreased melanoma-induced lung metastasis following PARP inhibition. Mice were inoculated with the murine melanoma cell line B16F10-luc. Localization, and the intensity of luciferase expression was monitored by in vivo bioluminescence imaging (dpi, days post cells injection) (A). Quantification of luciferase activity over time shows the average light emission in photons/s (B) (**P<0.01, ***P<0.001). The observed results are from a representative experiment (vehicle n=3, DPQ n=4). (C, D) The number of metastatic foci/lung were counted macroscopically (***P<0.001). (E) Angiogenesis was measured using a specific endothelial cell marker (tomato lectin) and measured as blood vessels per mm^2 in tumor sections of lung metastasis (Columns, mean ± SE. *P < 0.05, with respect to vehicle control and DPQ–treated mice. (F) Immunohistochemistry staining of Snail and E-Cadherin in lung metastasis. (G) Kaplan-Meyer survival curve shows the survival advantage of DPQ-treated mice following intravenous tail injection of melanoma cells as previously described (** P < 0.01).


A
VEHICLE
DPQ
0dpi
2 dpi
7 dpi
11 dpi
14 dpi
21 dpi
100000
80000
60000
40000
20000
Color Bar
Min = 10000
Max = 1e+05

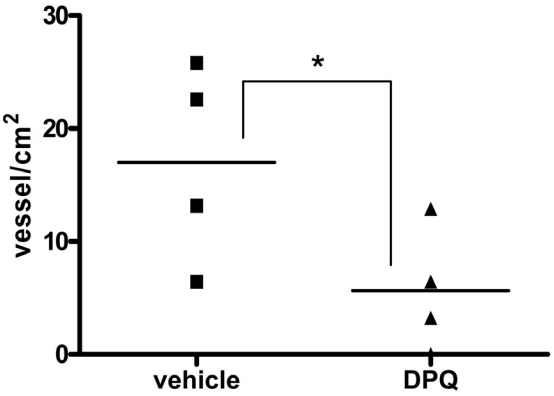
B


1.0×10³
1.0×10²
1.0×10¹
1.0×10⁰
photons/second
0
2
7
11
14
21
time (days)
**
**
***
vehicle
DPQ

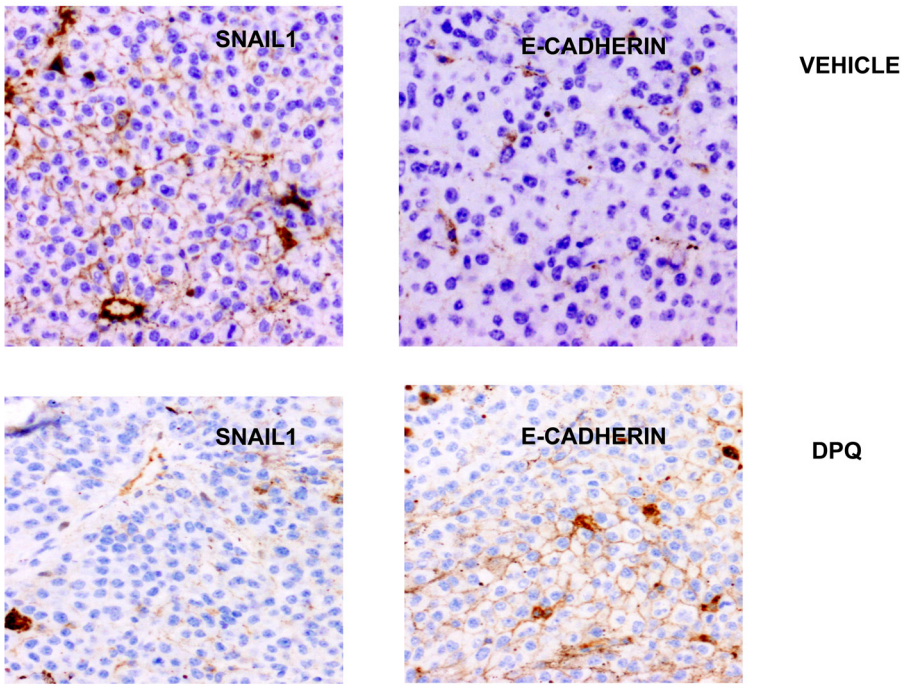
C


35
30
25
20
15
10
5
0
number of foci/lung
***
vehicle
DPQ
metastatic foci

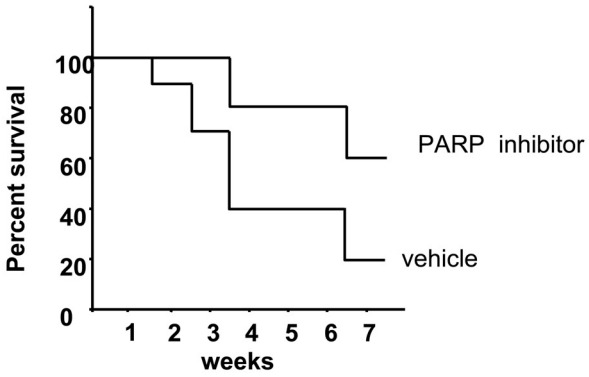
D


30
20
10
0
vessel/cm²
*
vehicle
DPQ

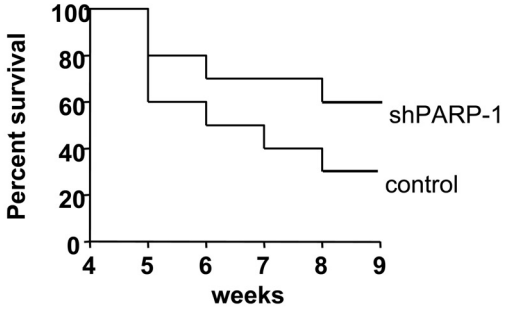
E


SNAIL1
E-CADHERIN
VEHICLE
SNAIL1
E-CADHERIN
DPQ

F


Percent survival
100
80
60
40
20
0
1 2 3 4 5 6 7
weeks
PARP inhibitor
vehicle

G

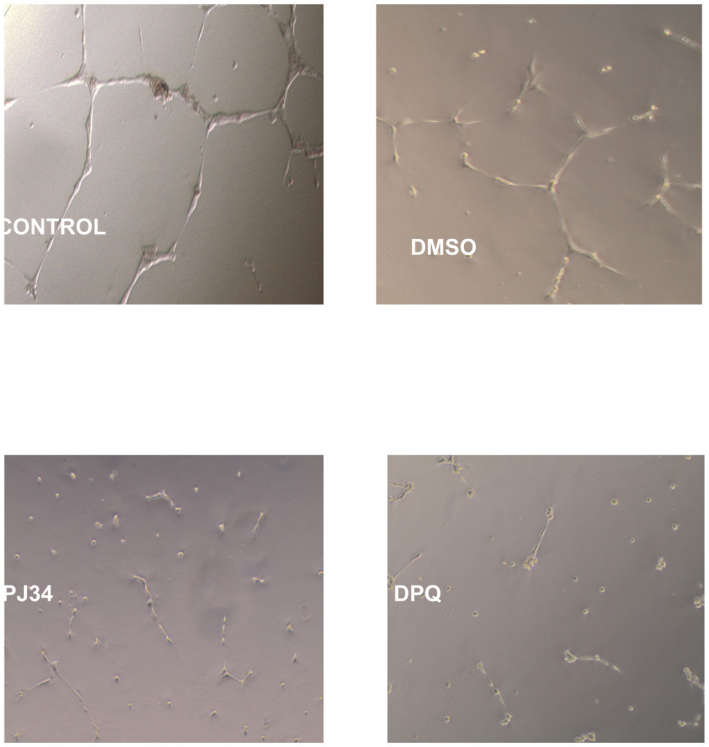

Percent survival
100
80
60
40
20
0
4 5 6 7 8 9
weeks
shPARP-1
control

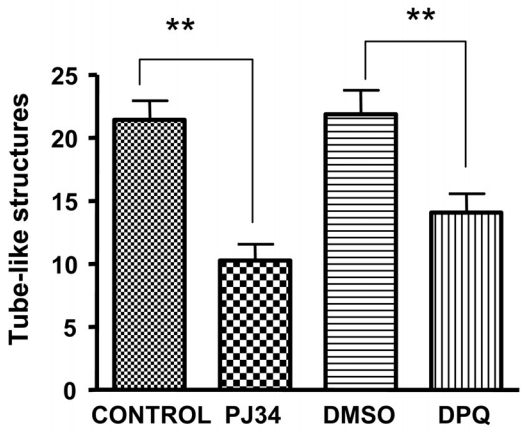
SUPPORTING INFORMATION

Figure legends and Methods

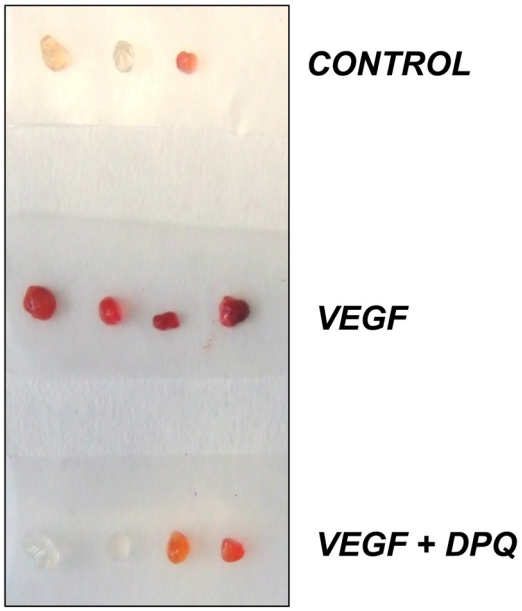
S1. The PARP inhibitors DPQ or PJ34 inhibits VEGF-induced tube formation of HUVECs in vitro. Cells were collected and seeded in Matrigelcoated 48-well plates and then incubated in the absence (Control) or presence of VEGF and DPQ or PJ34 (40 μM). After 48 h, the morphological changes of the cells and any tubes formed were observed and recorded under a microscope. Five random fields were observed for each well. Micrographs were taken × 40. Results are representative of 4 experiments in triplicate. The number of tube was counted (figure 1A right) (n = 4), and mean is shown. Bars ± SEM (**P < 0.01 versus control). After subcutaneous matrigel injection in the presence and absence of DPQ PARP inhibitor DPQ decrease VEGF-induced in vivo angiogenesis in HUVEC. The formation of vessel in vivo was assessed after injection of HUVEC with matrigel plug contains VEGF and heparin. The neovascularizaton was evaluated by measurement of HB content of matrigel plug. The histogram represents the mean (n=4) of the content, expressed as absorbance (DO)/100 mg of matrigel plug (B).

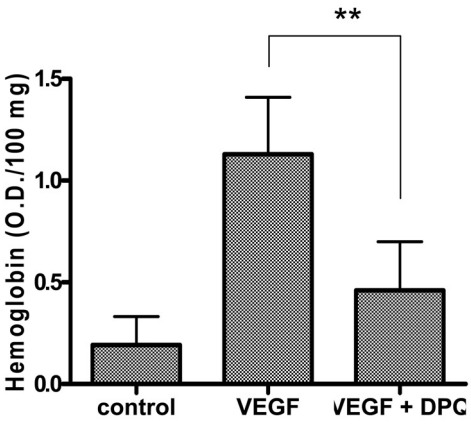
A


CONTROL
DMSO
PJ34
DPQ


**
**
Tube-like structures
25
20
15
10
5
0
CONTROL
PJ34
DMSO
DPQ

B


CONTROL
VEGF
VEGF + DPQ

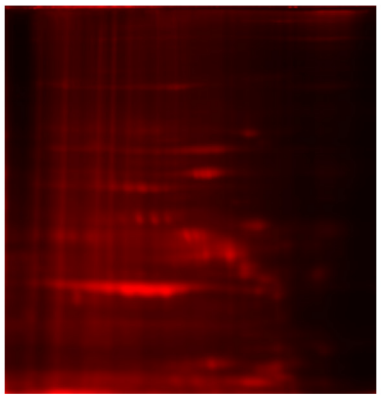

**
Hemoglobin (O.D./100 mg)
1.5
1.0
0.5
0.0
control
VEGF
VEGF + DPC

S2. **2D-DIGE (Differential In-Gel Electrophoresis)**

HUVEC were solubilized in 2-D DIGE sample buffer (40 mM Tris, 7 M Urea, 2 M Thiourea, 1% ASB-14), sonicated and then the concentration was determined using the RC DC Protein Assay (Bio- Rad). Fifty µg of protein was then labeled with 400 pmol of CyDye DIGE Fluor minimal dyes (GE Healthcare) and incubated on ice in the dark for at least 30 min according to manufacturer instructions (Cy3, Cy5 for samples and Cy2 for internal control consisting of equal parts of all samples). The reaction was halted by the addition of 10 mM lysine and incubated on ice for 10 min. Samples were loaded onto IPG strips (7 cm, pH 4–7) (Bio-Rad) by passive rehydration for 15 h and subjected to isoelectrofocusing using the PROTEAN IEF Cell System (Bio-Rad) according to the manufacturer's protocols (250 V, 20 min, Linear; 4000V, 2 hr Linear; 4000 V, 10.000 v-hr, Rapid). Upon IEF, strips were incubated in equilibration buffer (6 M urea, 2% SDS, 0.375 M Tris-HCl pH 8.8, and 20% glycerol and 2% w/v DTT) for 10 min and then in the same buffer without DTT and containing 4.5% iodoacetamide for 10 min. For the second dimension, strips were loaded on top of 7.5% polyacrylamide gels and electrophoresed at 150 V for 1 h. The 2D gels were then scanned using a Typhoon Imager (GE Healthcare) at 100 µm resolution with λex/λem of 488/520, 532/580, and 633/670 nm for Cy2, Cy3, and Cy5, respectively. The photomultiplier tube was set to ensure that the maximum pixel intensity was between 90,000 and 99,000 pixels. Image analysis was performed using DeCyder 6.5 software (GE Healthcare) as described in the user manual. Six independent experiments were performed for each experimental setup. Briefly, the differential in-gel analysis (DIA) module was used for spot detection, spot volume quantification and volume ratio normalization of different samples in the same gel (Supplementary Fig. 1). Differentially expressed spots were considered for identification based upon the fold change (>1.1) and the t-test ($p<0.05$). Preparative gels were run with 200 µg protein following the same procedure described above. Proteins were visualized by staining with SYPRO Ruby Protein Gels Stain (Bio-Rad), and images were acquired by a Typhoon Imager using λex/λem of 532/560 nm. Differentially represented spots were excised manually or with EX Quest Spot Cutler Biorad, and gel specimens were processed with a digestor (DigestPro MS, Intavis). The protein was reduced with DTT (10 mM) for 45 min to 56 ºC, alkylated with iodoacetamide (55 mM) for 30 min at room temperature and then digestion was performed using 10 ng/µl trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega) for 4 h at 37 ºC. The resulting peptides were extracted with 0.2% trifluoroacetic acid (TFA). The sample was concentrated for centrifugation under a vacuum and then identified using MALDI-TOF MS. The Mass Spectrometry analysis was performed using a Voyager DE-PRO (Applied Biosystems, Barcelona, Spain) instrument in reflectron mode. Spectra were externally mass calibrated using peptides derived from trypsin autodigestion. For the analysis, 1 _l of the peptide extract and 1 _l of matrix (CHCA, 2.5 mg/mL) were loaded in the MALDI plate and allowed to dry. Identification of peptide mass was performed using the NCBI or Swiss-Prot databases (European Bioinformatics Institute, Heidelberg, Germany) using MASCOT software (Matrix Science, London, UK). Differential protein expression analysis of HUVEC with DPQ 22 h by s. Total protein extracts were was labelled with Cy3 (A) (Control), Cy5 (B) (DPQ) dyes and an

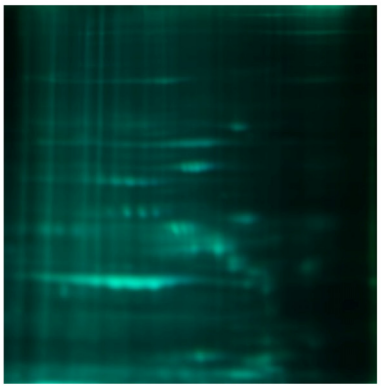
internal standard Cy2 (C). Typhoon Imager (GE Healthcare) at 100 µm resolution with λex/λem of 488/520, 532/580, and 633/670 nm for Cy2, Cy3, and Cy5 respectively. . 2-D-DIGE were performed on IPG strips 7 cm, pH 4–7 (first dimension) and 7.5% polyacrylamide gels (second dimension). The Image analysis DeCyder TM Sofware indicate those differential spots detected in HUVEC treated with DPQ cells that were subsequently identified. Difference between spot 1 HUVEC Control and DPQ decrease its expression with DPQ. Vimentin is identificated in the spot 1 by MALDI-TOF.

A



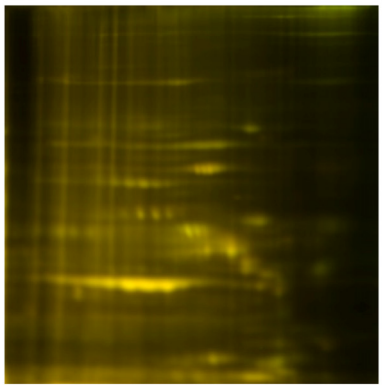
Cy3 Control

B



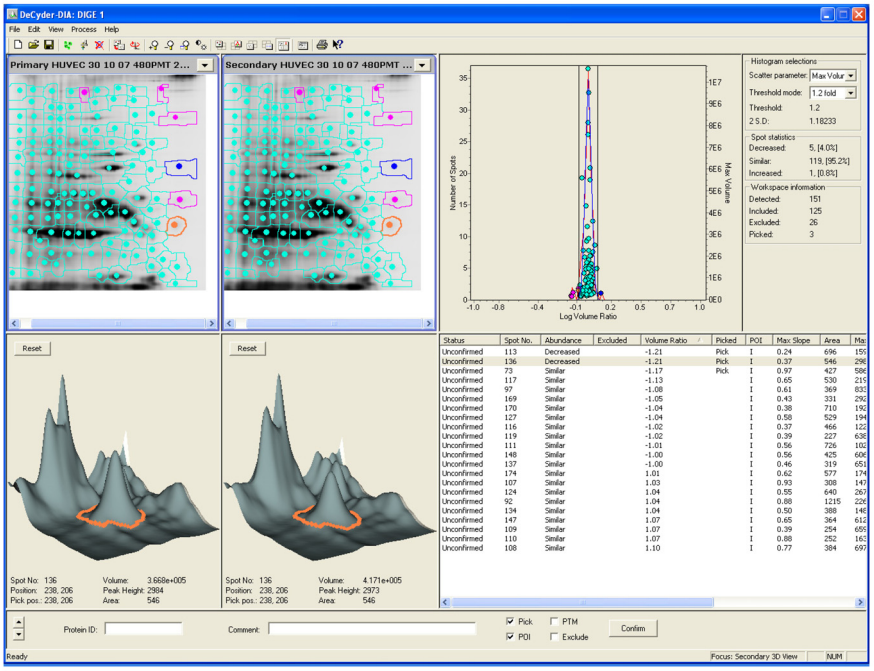
Cy5 DPQ

C



Cy2 Standard

D


DeCyder-DIA: DIGE 1
File Edit View Process Help
Primary HUVEC 30 10 07 480PMT 2...
Secondary HUVEC 30 10 07 480PMT ...
Number of Spots
Max Volume
Log Volume Ratio
Histogram selections
Scatter parameter: Max Volur
Threshold mode: 1.2 fold
Threshold: 1.2
2 S.D.: 1.18233
Spot statistics
Decreased: 5, [4.0%]
Similar: 119, [95.2%]
Increased: 1, [0.8%]
Workspace information
Detected: 151
Included: 125
Excluded: 26
Picked: 3
Reset
Reset
Spot No: 136
Position: 238, 206
Pick pos.: 238, 206
Volume: 3.668e+005
Peak Height: 2984
Area: 546
Spot No: 136
Position: 238, 206
Pick pos.: 238, 206
Volume: 4.171e+005
Peak Height: 2973
Area: 546
Status
Spot No.
Abundance
Excluded
Volume Ratio
Picked
POI
Max Slope
Area
Protein ID:
Comment:
Pick
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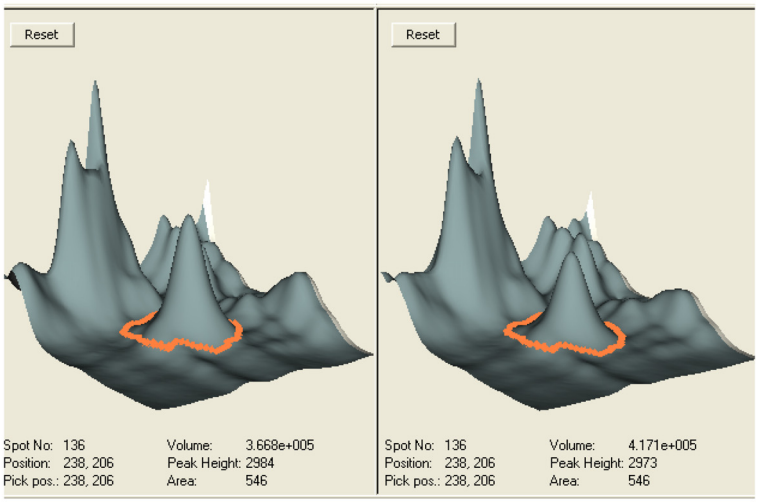
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Volume: 3.668e+005
Peak Height: 2984
Area: 546
Spot No: 136
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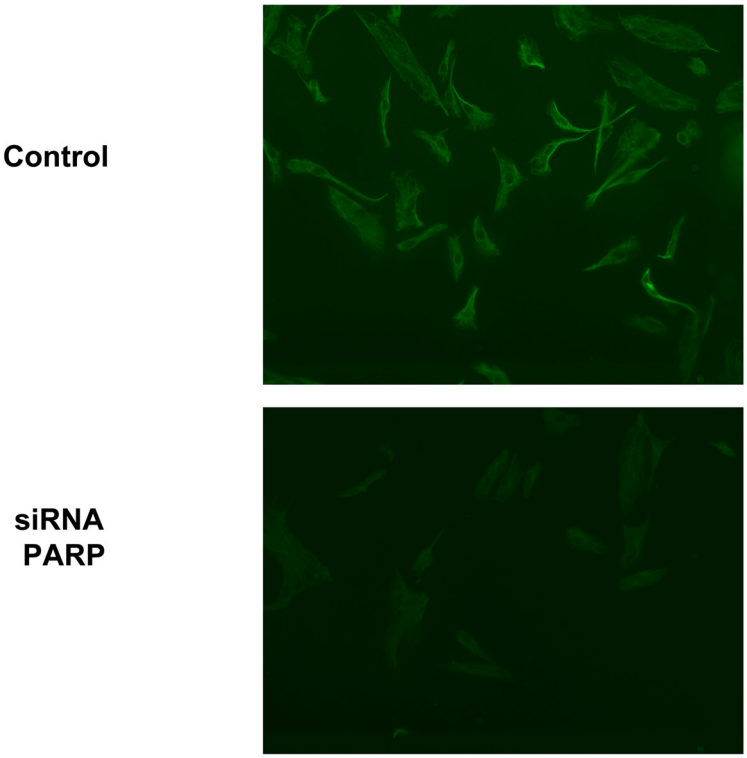
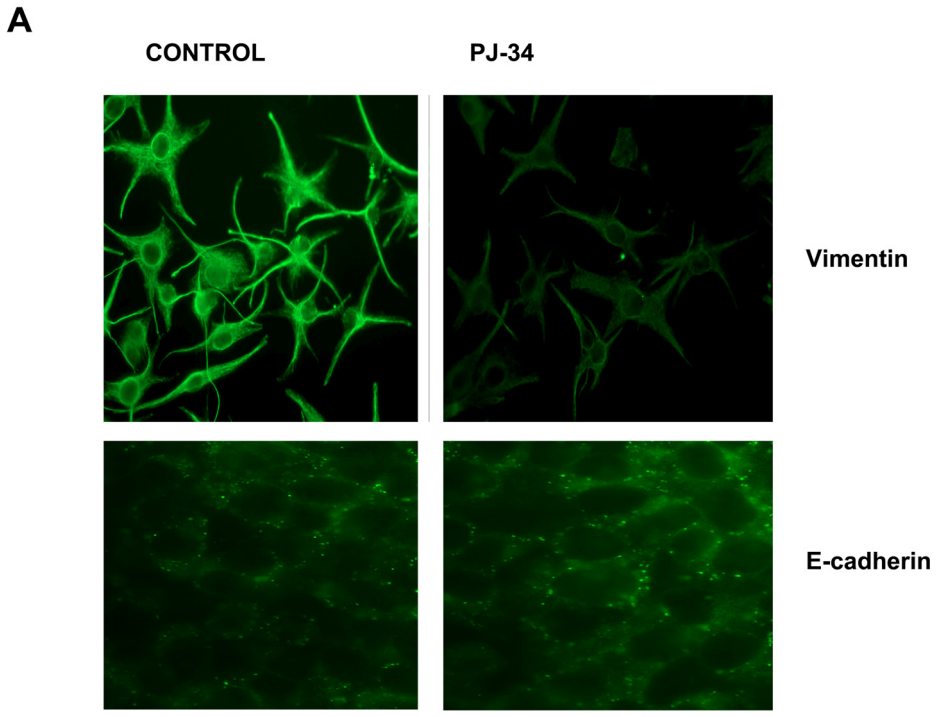
Pick pos.: 238, 206
Volume: 4.171e+005
Peak Height: 2973
Area: 546

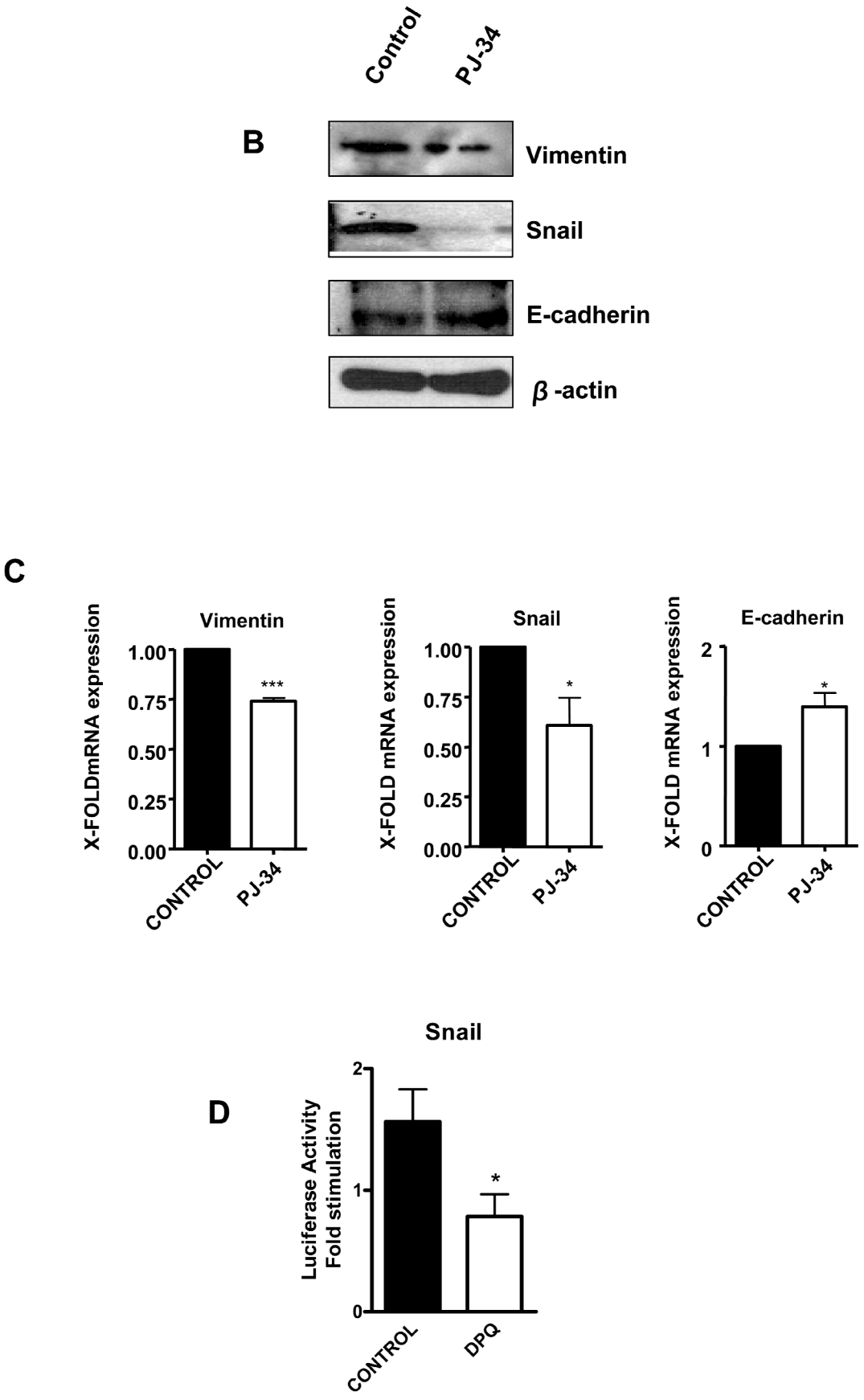
S3. PARP-1 knockdown in HUVECs leads to vimentin down regulation.



S4. **PARP inhibition reduced the expression of Vimentin and SNAIL-1 and up-regulates E-cadherin murine B16F10 melanoma cells.**

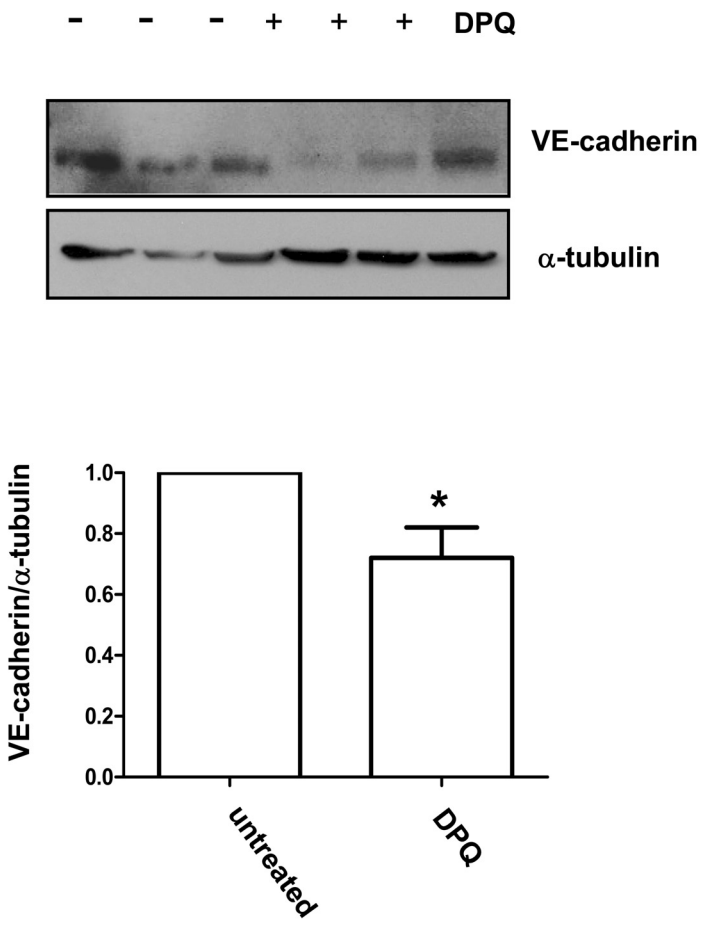
Cells were treated with either of the PARP inhibitors DPQ or PJ34 (not shown) at 40 μM during 18 hours. IF **(A),** western blot **(B)** or qPCR **(C)** were performed to evaluate the impact of PARP inhibition on on EMT markers. *P < 0.05, ***P < 0.001 PARP Inhibitor groups versus the control. β-actin was used as internal controls for protein loading. SNAIL-1 and E-cadherin promoter activity are regulated by PARP inhibitors. Luciferase activity **(D)** was determined after transfecting the constructions into the B16F10 cells. Firefly Luciferase was standardized to the value of Renilla Luciferase. Cells were co-transfected with 0.5 μg renilla as control of transfection together with 0.5 μg of the Snail or E-cadherin plasmid using jetPEI cationic polymer transfection reagent according to the manufacture´s instructions. Fold: cells with serum vs. cells without serum. *P < 0.05, ***P < 0.001 control versus PJ-34. The expression of Firefly and Renilla luciferases was analyzed 48 h after transfection, according of the manufacturer´s instructions. Cloning of the human SNAIL1 promoter (-869/+59) in pGL3 basic (Promega), was described previously (Barbera et al., 2004). Ecadherin promoter were cloned into pGL3-basic (Promega) to generate pGL3- E-cadherin (-178/+92).S5.- VE-cadherin levels are **

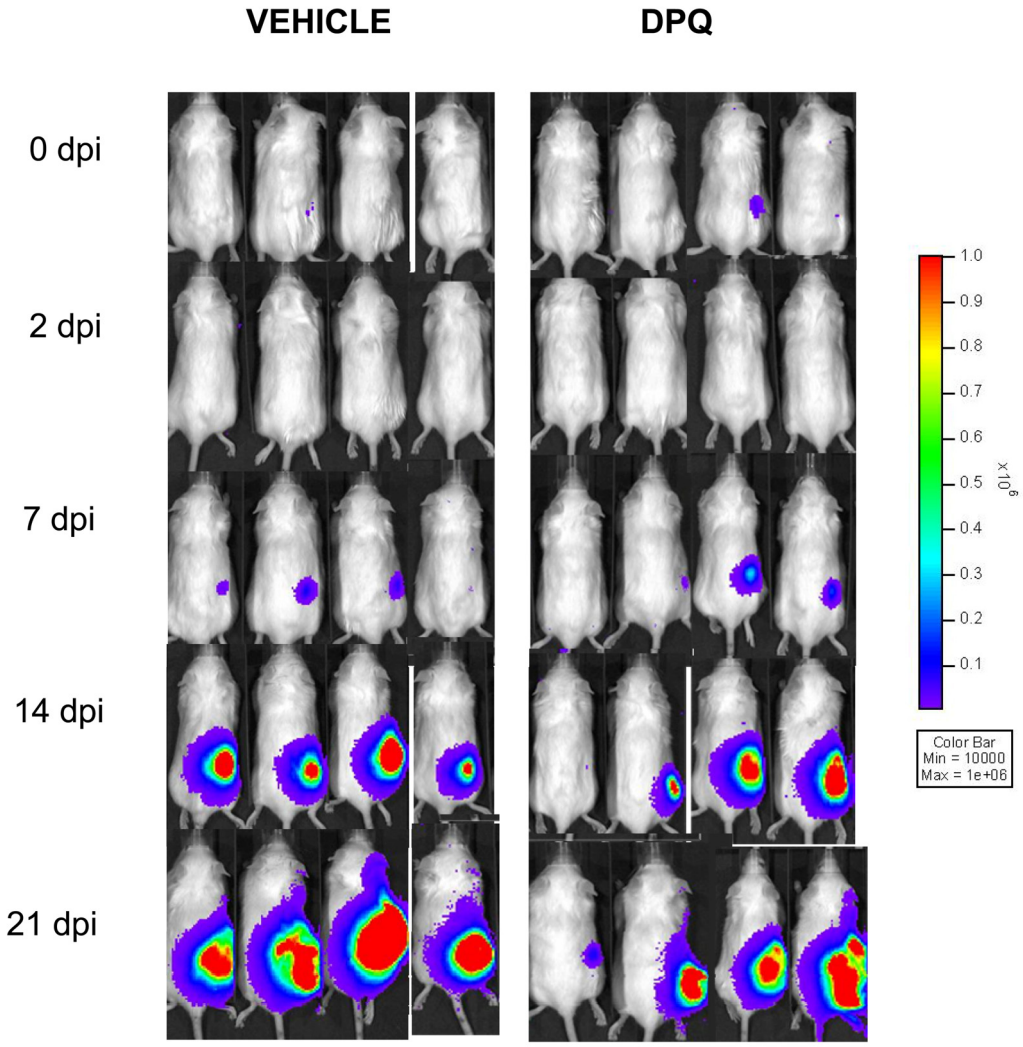


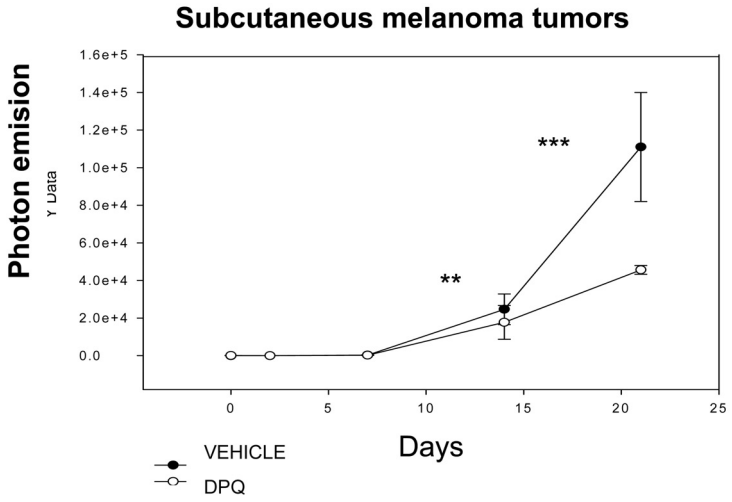

B
Control
PJ-34
Vimentin
Snail
E-cadherin
β -actin
C
Vimentin
X-FOLDmRNA expression
1.00
0.75
0.50
0.25
0.00
***
CONTROL
PJ-34
Snail
X-FOLD mRNA expression
1.00
0.75
0.50
0.25
0.00
*
CONTROL
PJ-34
E-cadherin
X-FOLD mRNA expression
2
1
0
*
CONTROL
PJ-34
D
Snail
Luciferase Activity
Fold stimulation
2
1
0
*
CONTROL
DPQ

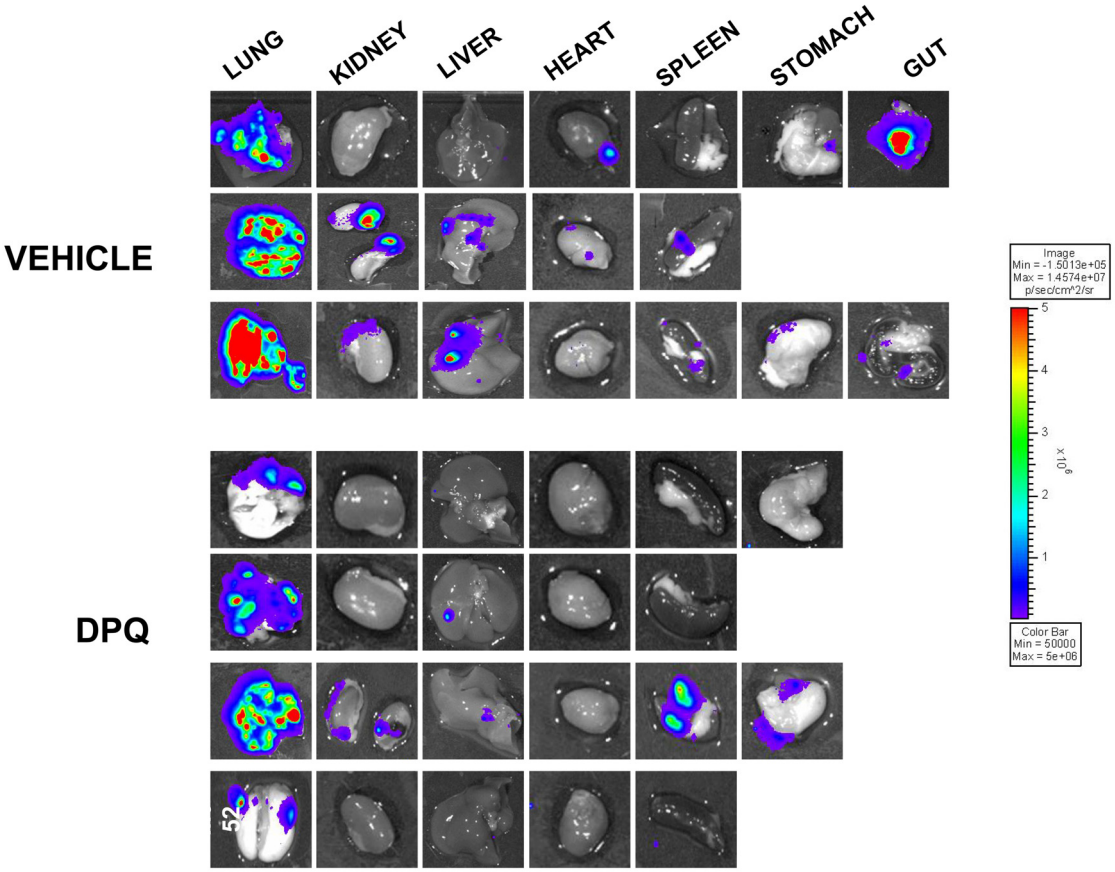
S5. **Development of subcutaneous xenografts of melanoma is reduced by treatment with the PARP inhibitor DPQ.**

C57Bl6 albino mice (Jackson Laboratories, Philadelphia, USA), were inoculated with B16F10-luc cells as explained in Methods. Localization and intensity of luciferase expression was monitored by in vivo bioluminescence imaging. Quantitation of luciferase activity over time in photons/s, is represented in the color bar. Vehicle (n=4), DPQ (n=4). **P < 0.01; ***P < 0.001.

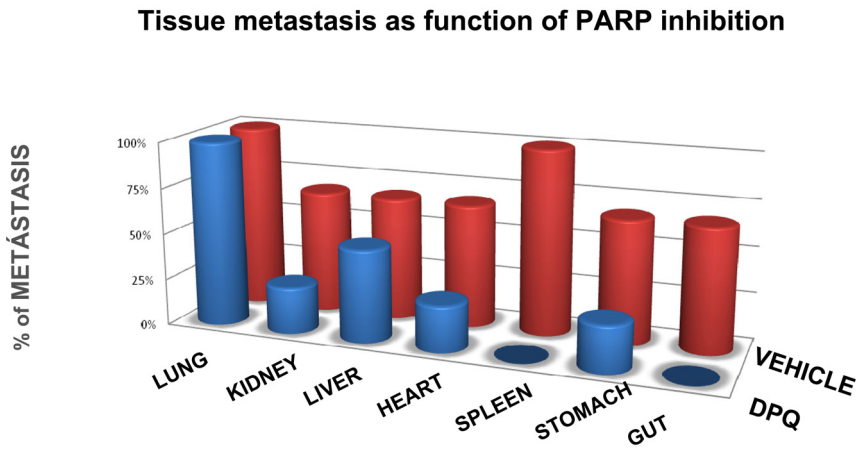



VEHICLE
DPQ
0 dpi
2 dpi
7 dpi
14 dpi
21 dpi
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
x10⁶
Color Bar
Min = 10000
Max = 1e+06


Subcutaneous melanoma tumors
Photon emision
Y Data
1.6e+5
1.4e+5
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
0.0
***
**
0
5
10
15
20
25
Days
VEHICLE
DPQ


LUNG
KIDNEY
LIVER
HEART
SPLEEN
STOMACH
GUT
VEHICLE
DPQ
52
Image
Min = -1.5013e+05
Max = 1.4574e+07
p/sec/cm^2/sr
5
4
3
2
1
x 10^6
Color Bar
Min = 50000
Max = 5e+06

S6. Ex-vivo photon emission: Treatment with the PARP inhibitor DPQ reduced lung and extra-pulmonary melanoma-induced metastasis.



S7. **Cell proliferation and apoptosis are not affected by PARP inhibition in metastasis.** Mitosis and apoptosis were evaluated in histological metastasis slides and evaluated in a blind observation by two different pathologists.

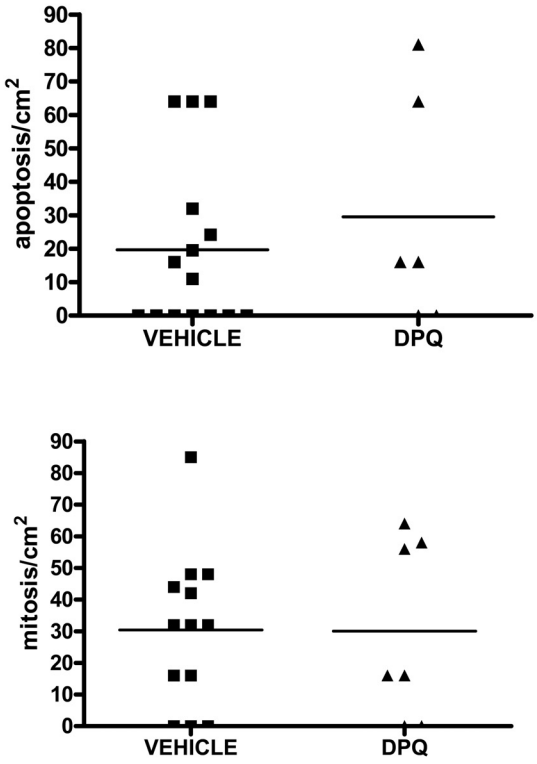
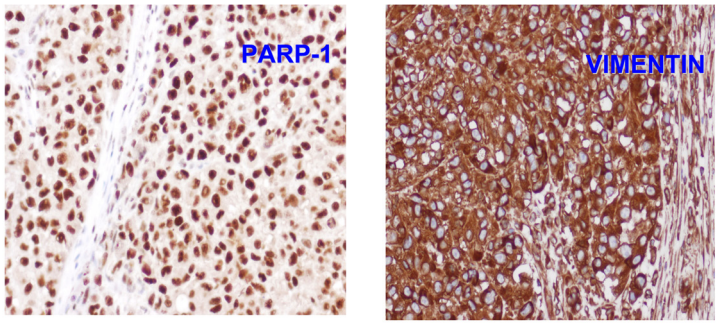


Table S1

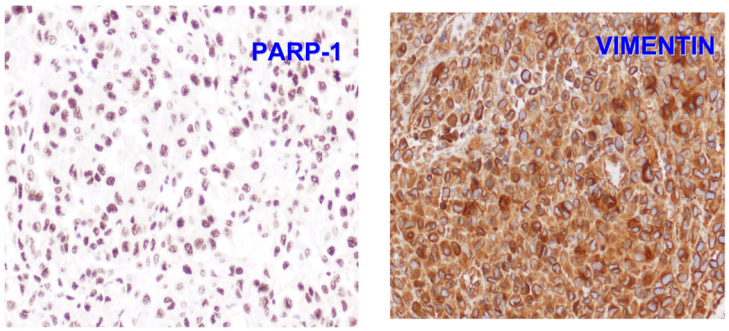
	Nodular melanome	Metastasis
Vimentin	10/10	20/20
PARP-1	9/10	18/20
Snail-1	0/10	8/20
E.cadherin	9/10	16/20
MITF	3/10	8/20

S8. Human melanoma tissue array.

Expression of PARP-1 and EMT marker in nodular and metastatic human melanoma. PARP-1 expression correlates with Vimentin in nodular and metastatic melanoma. SNAIL-1 and E-cadherin expression do not correlate with PARP-1 positivity. Results for all the different markers are presented in **



NODULAR MELANOMA



METASTATIC MELANOMA

Supplementary references

Barbera, M. J., Puig, I., Dominguez, D., Julien-Grille, S., Guaita-Esteruelas, S., Peiro, S., Baulida, J., Franci, C., Dedhar, S., Larue, L., and Garcia de Herreros, A. (2004). Regulation of Snail transcription during epithelial to mesenchymal transition of tumor cells. Oncogene *23*, 7345-7354.

B. ESTUDIO DEL EFECTO INHIBIDOR DE PARP PJ-34 SOBRE LA EXPRESIÓN GÉNICA EN HUVEC

B. ESTUDIO DEL EFECTO DEL INHIBIDOR DE PARP PJ-34 SOBRE LA EXPRESIÓN GÉNICA EN HUVEC

Se realizaron extracciones de ARN (Martin–Oliva et al 2006) a partir de células HUVEC control y tratadas con PJ-34 (Figura 1). Se realizó un estudio de expresión con la tecnología de los microarrays de ADN o GeneChip de Affymetrix Human genome U133 plus (Quiles-Perez et al., 2009) en la Unitat Cientifico tècnica de Suport (UCTS) Institut de Recerca Hospital Vall d'Hebron. El resultado d microarray de expresión génica está recogido en las Tabla 1 y 2 microarrays y Figuras HeatMap. En el análisis se han considerado solamente los genes cuya función es conocida y se ha encontrado que globalmente la expresión de 195 genes disminuyeron tras el tratamiento con PJ34 mientras que 168 genes aumentaron su expresión. Para su estudio se han agrupado según su función biológica, en los siguientes grupos mostrados en la Tabla 2.

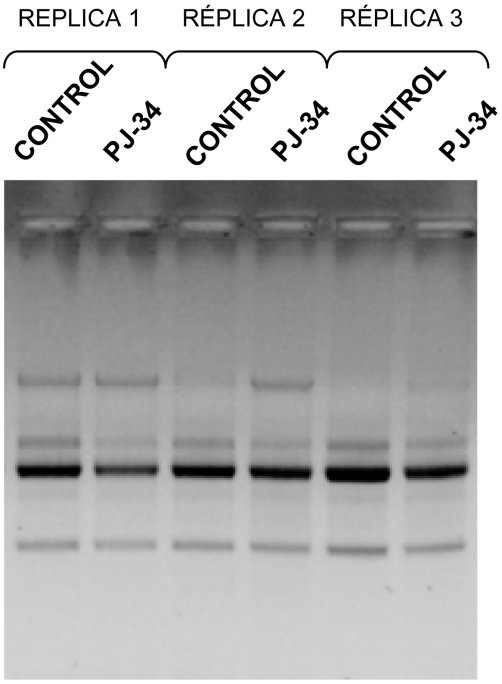


Figura 1. Electroforesis 1% Agarosa TAE 0,5 X, 2 μl de ARN humano (HUVEC) 300 ng/μl.

Tablas 1. Genes grupo control vs grupo tratado con PJ-34 de células HUVEC, usando la tecnología de los microarrays de ADN o GeneChip de Affymetrix (Quiles-Perez 2009)

	(down regulated) disminución de expresión	(up regulated) aumento de expresión
Factores de crecimiento y receptores	0	38
Reparación del ADN y dinámica de la cromatina	41	36
Adhesión celular, migración, citoesqueleto	55	22
Apoptosis y ciclo celular	0	20
Factores de Transcripción	21	13
Metabolismo y respuesta a estrés	37	17
Transporte de membranas	9	10
Procesamiento del RNA	12	0
Homeostasis del hierro hipoxia, óxido-reducción	4	0
Respuesta inmune	1	0

La expresión más aumentada la hemos encontrado para ACTA2 (alfa2- actina, 5,42), E-selectina (5,10), y SLUG (5,04). Cabe destacar que mientras que la activación de SNAIL-1 está regulada por poli(ADP-ribosilación) (Rodríguez et al 2011), la expresión de SLUG (SNAIL-2) se incrementa tras la inhibición de PARP; estos resultados llevan a pensar en una regulación diferencial de las dos formas de SNAIL aunque se deben acometer los estudios necesarios para comprobar cómo se afecta la activación, los niveles de expresión de la proteína y si hay una especificidad dependiendo del tipo celular que condicione el efecto de la inhibición de PARP sobre SLUG. De hecho la expresión de SLUG y la regulación de su promotor tiene características muy diferenciadas de la de SNAIL-1 (Shih and Yang 2011)

La expresión génica más disminuida corresponde a Aurora quinasa B (AURKB)(-6,00), un serina/treonina quinasa implicada en el control de la mitosis y cuya expresión se encuentra frecuentemente aumentada en células

tumorales; la activación de aurora quinasa se ha descrito que depende de PARP (Monaco et al 2005)

Se está analizando en la actualidad la el estudio de la expresión por q-PCR de algunos genes seleccionados por su especial relevancia en biología vascular como es el caso de Cystathionine beta-synthase (CBS) implicada en la disfunción endotelial derivada a la exposición crónica a niveles elevado de homocisteína (Cheng et al., 2011), lámina B1 , un importante componente nuclear implicado en la proliferación y senescencia (Shimi et al., 2011), MCAM o CD146 también conocido como molécula de adhesión de células de melanoma, es una proteína clave en las células endoteliales cuya sobreexpresión está relacionada con la progresión metastásica del melanoma y del cáncer de próstata (Ouhtit et al 2009)

Toda esta alteración en la expresión génica tras la inhibición de PARP implica un profundo cambio en el programa transcripcional de las células endoteliales, quedando de nuevo de manifiesto el papel fundamental que juega la modificación por poli(ADP-ribosilación) en la función de gran número de factores de transcripción. Cabe además destacar el efecto sobre un gran número de genes afectados implicados en señalización intercelular (factores de crecimiento, citoquinas y quimioquinas) en el control del ciclo celular y de la respuesta a daños en el ADN.

Genes cuya expresión disminuye tras el tratamiento con el inhibidor de PARP PJ34:

Tablas 2. Lista de genes agrupados por familia Grupo Control vs grupo tratado con PJ-34de células HUVEC, usando la tecnología de los microarrays de ADN o GeneChip de Affymetrix (Quiles-Perez 2009)

MIGRACIÓN CELULAR, ADHESIÓN CELULAR, CITOESQUELETO

Simbolo	Fold (control/ tratado)	Nombre del gen
76P	-2,23	gamma-tubulin complex component 4-like
ADAMTS18	-3,18	A disintegrin and metalloproteinase with thrombospondin motifs 18
ADAMTSL1	-2,38	ADAMTS-like protein 1

ANK3	-2,12	ANKIRIN3
ANLN	-4,73	Actin-binding protein anillin
AQP1	-2,39	AQUAPORIN
CALU	-2,04	Calumenin
CBS	-2,12	Cystathionine beta-synthase
CBX3	-2,35	chromobox homolog 3
CKAP2L	-5,01	CKAP2L cytoskeleton associated protein 2-like
COL1A2	-2,69	collagen, type I, alpha 2
DIAPH3	-2,29	diaphanous homolog 3
DLG1	-2,23	DLG1 discs, large homolog 1
DNAH14	-3,09	DNAH14 dynein, axonemal, heavy chain 14
DNAH5	-2,38	DNAH5 dynein, axonemal, heavy chain 5
DNAJC9	-2,83	DNAJC9 DnaJ (Hsp40) homolog, subfamily C, member 9
EEF1G	-2,52	EEF1G eukaryotic translation elongation factor 1 gamma
EIF5	-2,07	eukaryotic translation initiation factor 5
EMP2	-2,81	epithelial membrane protein 2
erg1	-2,14	potassium voltage-gated channel, subfamily H (eag-related), member 2
HMMR	-2,56	hyaluronan-mediated motility receptor
ITGB3BP	-2,67	Integrin beta 3 binding protein (beta3-endonexin)
JUP	-2,97	junction plakoglobin FORM COMPLEX WITH CADHERINS
KIFC1	-3,40	kinesin family member 23
KNSL6	-4,91	kinesin family member 2C
MATR3	-2,84	matrin 3
MCAM	-2,74	melanoma cell adhesion molecule
NDP	-2,74	Norrie disease (pseudoglioma)
NRG1	-4,53	neuregulin 1
PCDH7	-2,04	Isoform A of Protocadherin-7
PNN	-2,26	pinin, desmosome associated protei
POSTN	-4,24	periostin, osteoblast specific factor!!!
RPL39	-2,59	ribosomal protein L39
RPS27	-4,09	ribosomal protein S27 pseudogene
SERPIND1	-2,83	serpin peptidase inhibitor, clade D (heparin cofactor), member 1
SMAGP	-2,11	small cell adhesion glycoprotein
SMC2	-2,36	structural maintenance of chromosomes 2
SMC4	-3,96	structural maintenance of chromosomes 4
SMC6	-2,27	structural maintenance of chromosomes 6
SNRPD1	-2,12	small nuclear ribonucleoprotein D1 polypeptide 16kDa
SPAG5	-3,35	sperm associated antigen 5
SR140	-2,85	U2 snRNP-associated SURP domain containing
STEAP1	-3,20	six transmembrane epithelial antigen of the prostate 1
SYN47	-2,27	homer homolog 1
TROAP	-2,09	trophinin associated protein (tastin)
TUBA1	-2,56	TUBULIN A1
TUBA1B	-2,10	TUBULIN 1B
TUBB	-2,79	TUBULIN B
TUBB2	-2,36	tubulin, beta 2C
TUBB2A	-2,06	tubulin, beta 2A
TUBB2C	-2,41	Tubulin beta-2C chain
TUBD1	-2,42	Tubulin delta chain
TUBGCP3	-2,09	tubulin, gamma complex associated protein 3
VRK1	-2,27	vaccinia related kinase 1

REPARACIÓN DEL ADN Y DINÁMICA DE LA CROMATINA

Simbolo	Fold (control/ tratado)	Nombre del gen
ADPRTL2	-2,42	poly (ADP-ribose) polymerase family, member 2 **(PARP-2)**
BARD1	-3,90	BRCA1-associated RING domain protein
BRCA1	-4,13	breast cancer 1, early onset
BRCA2	-4,92	breast cancer 2
CBX3	-2,35	chromobox homolog 3
CBX5	-3,51	chromobox homolog 5
CHAF1A	-2,04	CHAF1A chromatin assembly factor 1, subunit A (p150)
CHC1	-2,14	RCC1 regulator of chromosome condensation 1
CHEK1	-2,99	ATM RESPONSE
DNA2	-4,82	DNA2 DNA replication helicase 2 homolog
FANC1	-3,51	FANCONI ANEMIA 1
FANCB	-2,00	FANCCONI ANEMIA B
FANCD2	-3,04	Fanconi anemia, complementation group D2
FBRNP	-2,53	heterogeneous nuclear ribonucleoprotein A3
FEN1	-4,23	flap structure-specific endonuclease 1
GTSE1	-4,97	G-2 and S-phase expressed 1
H2AX	-2,73	H2A histone family, member X
HMG2	-3,14	high-mobility group box 2
HMG3	-2,39	high-mobility group box 3
HMSH2	-2,58	mutS homolog 2, colon cancer, nonpolyposis type 1
HP1BP3	-3,33	heterochromatin protein 1, binding protein 3
IMPORTIN	-2,04	karyopherin (importin) beta 1
KIF 1	-2,82	kinesin like protein
KPNB1	-2,05	karyopherin (importin) beta 1
LMNB1	-4,30	lamin B1
HMSH2	-2,58	mutS homolog 2, colon cancer, nonpolyposis type 1
MSH6	-2,13	mutS homolog 6
OIP5	-3,93	Opa interacting protein 5
PHF17	-3,14	PHD finger protein 17
rad51	-5,21	RAD51 homolog (S. cerevisiae)
RAD51AP1	-5,10	RAD51 associated protein 1
RAD54L	-2,31	RAD54-like
RCH1	-2,19	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)
SETMAR	-2,12	SET domain and mariner transposase fusion gene
SMC2	-2,36	structural maintenance of chromosomes 2
SMC4	-3,96	structural maintenance of chromosomes 4
SMC6	-2,27	structural maintenance of chromosomes 6
SUV39H2	-3,02	suppressor of variegation 3-9 homolog 2
UNG	-2,47	uracil-DNA glycosylase
XPOT	-3,27	exportin, tRNA
XRCC9	-3,05	Fanconi anemia, complementation group G

METABOLISMO

Simbolo	Fold (control/ tratado)	Nombre del gen
ACAT2	-2,35	cetyl-Coenzyme A acetyltransferase 2
ACSL3	-2,43	acyl-CoA synthetase long-chain family member 3
ALDH1L2	-3,10	aldehyde dehydrogenase
AMAC1	-4,58	acyl-malonyl condensing enzyme 1

AMD1	-2,89	adenosylmethionine decarboxylase 1
ASNS	-3,31	asparagine synthetase
AZIN1	-2,36	antizyme inhibitor 1
CROT	-2,62	CROT carnitine O-octanoyltransferase
CYP1A1	-2,15	P450 FAMILY
CYP51A1	-2,33	cytochrome P450, family 51, subfamily A, polypeptide 1
DHCR7	-2,31	7-dehydrocholesterol reductase
ELOVL	-4,33	ELOVL family member 5, elongation of long chain fatty acids
ELOVL6	-4,18	ELOVL family member 6, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)
FS	-2,12	globoside alpha-1,3-N-acetylgalactosaminyltransferase 1
GART	-2,46	phosphoribosylglycinamide formyltransferase
GMPS	-2,06	guanine monphosphate synthetase
GPAT	-2,23	phosphoribosyl pyrophosphate amidotransferase
HK2	-3,23	hexokinase 2
HMGCR	-2,37	3-hydroxy-3-methylglutaryl-Coenzyme A reductase
LRP8	-3,06	low density lipoprotein receptor-related protein 8, apolipoprotein e receptor
MAC30	-5,26	transmembrane protein 97
MGAT4A	-2,61	mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme A
MTHFD1	-2,49	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1,
MTHFD2	-2,66	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase
NUDT12	-2,52	nudix (nucleoside diphosphate linked moiety X)-type motif 12
PFAS	-2,48	phosphoribosylformylglycinamidine synthase
PHGDH	-2,29	phosphoglycerate dehydrogenase
PPAT	-2,21	phosphoribosyl pyrophosphate amidotransferase
PTPLAD2	-2,43	protein tyrosine phosphatase-like A domain containing 2
SCD	-2,18	stearoyl-CoA desaturase
SQLE	-2,28	squalene epoxidase
SRM	-2,09	spermidine synthase
ST1B2	-2,35	sulfotransferase family, cytosolic, 1B, member 1
UAP1	-2,36	UDP-N-acteylglucosamine pyrophosphorylase 1
UBASH3B	-3,31	Ubiquitin-associated and SH3 domain-containing protein B
UCK2	-2,25	Uridine-cytidine kinase 2
USP1	-4,05	ubiquitin specific peptidase 1

TRANSCRIPCIÓN

Simbolo	Fold (control/ tratado)	Nombre del gen
ASXL1	-5,06	Retina and anterior neural fold homeobox protein 2
FBXO5	-4,66	F-box protein 5
DEPDC1	-8,56	DEPDC1 DEP domain containing 1
BNC2	-2,64	Zinc finger protein basonuclin-2
EZH2	-2,88	enhancer of zeste homolog 2
GAL	-2,26	binding protein transcription factor, beta subunit 1
HOXA3	-2,11	homeobox A3
ILF3	-2,34	interleukin enhancer binding factor 3, 90kDa
MLX	-3,98	MAX-like protein X
RARB	-2,58	retinoic acid receptor, beta
RBBP4	-2,02	retinoblastoma binding protein 4
RBBP8	-4,35	retinoblastoma binding protein 8
RBL1	-4,23	retinoblastoma-like 1

RBL1	-2,19	RETINOBLASTOMA-LIKE
SDP35	-4,59	DEP domain containing 1
TAF1A	-2,41	TATA box binding protein (TBP)-associated factor, RNA polymerase I, A, 48kDa
TAF4B	-3,11	TAF4b RNA polymerase II, TATA box binding protein (TBP)-associated factor, 105kDa
TEAD4	-2,06	TEA domain family member 4
TFDP1	-2,64	transcription factor Dp-1
TTF2	-2,47	transcription termination factor, RNA polymerase II
ZFD25	-2,11	zinc finger protein 107

PROCESAMIENTO DE ARN

Simbolo	**Fold (control/ tratado)**	**Nombre del gen**
DCP2	-2,1	DCP2 decapping enzyme homolog
EXOSC2	-2,15	EXOSC2 exosome component 2
FBRNP	-2,53	heterogeneous nuclear ribonucleoprotein A3
HNRNPU	-3,05	heterogeneous nuclear ribonucleoprotein U
MRTO4	-2,04	mRNA turnover 4 homolog
OIP-2	-2,61	exosome component 8
PMSCL1	-2,13	polymyositisscleroderma autoantigen 1 (75kD)
SFRS2IP	-2,74	SR-related CTD-associated factor 11
SFRS7	-2,2	SPLICING FACTOR
SGOL1	-2,47	SUGOSHINA
SIP1	-2,45	motor neuron protein interacting protein 1
SNRPD1	-2,12	small nuclear ribonucleoprotein D1 polypeptide 16kDa

TRANSPORTE DE MEMBRANAS

Simbolo	**Fold (control/ tratado)**	**Nombre del gen**
ABCA8	-2,13	ATP-binding cassette, sub-family A (ABC1), member 8
ABCD3	-2,64	ATP-binding cassette sub-family D member 3
CHAC2	-2,52	cation transport regulator homolog 2
erg1	-2,14	potassium voltage-gated channel, subfamily H (eag-related), member 2
SCLT1	-4,15	sodium channel and clathrin linker 1
SLC1A4	-3,03	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4
SLC38A5	-2,02	SULUTE CARRIER
SLC4A5	-2,06	solute carrier family 4, sodium bicarbonate cotransporter, member 5
SLC5A3	-2,28	solute carrier family 5

IRON HOMEOSTASIS, HIPOXIA, REDOX

Simbolo	Fold (control/ tratado)	Nombre del gen
TFRC	-3,15	transferrin receptor (p90, CD71)
HPDL	-2,59	4-hydroxyphenylpyruvate dioxygenase-like
MGST1	-2,19	microsomal glutathione S-transferase 1
TMX3	-2,05	thioredoxin-related transmembrane protein 3

RESPUESTA INMUNE

Simbolo	Fold (control/ tratado)	Nombre del gen
HLA-DPA1	-2,23	major histocompatibility complex, class II, DP alpha 1

Genes cuya expresión aumenta tras el tratamiento con el inhibidor de PARP PJ34:

FACTOR DE CRECIMIENTO, RECEPTORES, QUIMIOCINAS, CITOQUINAS

Simbolo	Fold (Control/ Tratado)	Nombre Del Gen
BMP2	2,29	Bone Morphogenetic Protein 2
CCL20	4,78	Chemokine (C-C Motif) Ligand 20
CD69	2,85	Cd69 Molecul
CKLF	3,49	Chemokine-Like Factor
CSF1	2,18	Colony Stimulating Factor 1
CXADR	2,31	Coxsackie Virus And Adenovirus Receptor
CXCL1	2,95	Chemokine (C-X-C Motif) Ligand 1 (Melanoma Growth Stimulating Activity, Alpha)
CXCL2	2,41	Chemokine
CXCL2	3,48	Chemokine (C-X-C Motif) Ligand 2
CXCL5	2,41	Chemokine (C-X-C Motif) Ligand 5
CXCR4	3,90	Hemokine (C-X-C Motif) Receptor 4
DCR1	2,32	Decoy Receptor
EFNA1	2,59	Ephrin-1
EIF2B2	2,59	Eukaryotic Translation Initiation Factor 2b, Subunit 2 Beta, 39kda
ESM1	2,36	Endothelial Cell-Specific Molecule 1
FAS	3,39	Receptor
HSD17	2,25	Hydroxysteroid (17-Beta) Dehydrogenase 2
IL1A	3,38	Interleukin 1, Alpha
IL6	3,81	Interleukin 6
IL8	2,66	Interleukin 8
KIAA0353	4,03	Synemin, Intermediate Filament Protein
CHEMOKINE, CYTOKINE	3,54	Killer Cell Immunoglobulin-Like Receptor, Three Domains, Long Cytoplasmic Tail, 2
MYCN	2,14	N-Myc Downstream Regulated 1
NCR1	2,99	Natural Cytotoxicity Triggering Receptor 1
NDRG1	3,20	N-Myc Downstream Regulated 1
NHLH2	2,79	Nescient Helix Loop Helix 2
NPYR	4,01	Chemokine (C-X-C Motif) Receptor 4
PGF	3,15	Placental Growth Factor
PIG3	2,49	Tumor Protein P53 Inducible Protein 3
SSTR1	2,94	Omatostatin Receptor 1
TGFA	2,72	Transforming Growth Factor, Alpha
THBD	2,09	Thrombomodulin

TMEM178	2,45	Transmembrane Protein 178
TNFAIP3	3,23	tumor necrosis factor, alpha-induced protein 3
TNFRSF10C	2,88	Tumor Necrosis Factor Receptor Superfamily, Member 10c, Decoy Without An Intracellular Domain
TNFRSF21	2,46	Tumor Necrosis Factor Receptor Superfamily, Member 21
TRAF4	2,01	Tnf Receptor-Associated Factor 4
TRAIL	2,21	TNF-related apoptosis-inducing ligand

TRASDUCCIÓN DE SEÑALES

Simbolo	Fold (Control/ Tratado)	Nombre Del Gen
ANXA4	2,33	Annexin4
ANXA4	2,34	Annexin4
ARHGAP6	3,15	Rho Gtpase Activating Protein 6
ARL4C	2,11	Adp-Ribosylation Factor-Like 4c
CAP2	2,09	Cap, Adenylate Cyclase-Associated Protein, 2
DUSP1	2,19	Map Kinase Pathway
DUSP5	2,46	Dual Specificity Protein Phosphatase 5
HRAS	3,04	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
NOG	2,60	Tgf-Beta Inhibitor
OPTN	2,61	Optineurin
RAB28	2,69	Member Ras Oncogene Family
RGL	2,05	Ral Guanine Nucleotide Dissociation Stimulator-Like 1
RGS3	2,20	Regulator Of G-Protein Signaling 3
SYTL2	2,47	Synaptotagmin-Like 2
TNFAIP3	2,65	Tumor Necrosis Factor, Alpha-Induced Protein 3

TRANSCRIPCIÓN

Simbolo	Fold (Control/ Tratado)	Nombre Del Gen
ATF3	4,10	Activating Transcription Factor 3
BHLHE40	2,38	Basic Helix-Loop-Helix Family, Member E40
BTEB2	2,67	Kruppel-Like Factor 5 (Intestinal)
HLX1	2,24	H2.0-Like Homeobox
HOXB8	2,52	Homeobox B8
IKBE	2,00	Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells Inhibitor, Epsilon
MLF1	2,79	Myeloid Leukemia Factor 1
MYCN	2,14	N-Myc Downstream Regulated 1
NHLH2	2,79	Nescient Helix Loop Helix 2
NR4A2	3,14	Nuclear Receptor Subfamily 4, Group A, Member 2
RUNX1	2,34	Runt-Related Transcription Factor 2
SLUG	5,04	Snail Homolog 2
SQSTM1	2,32	Activates Nfkb

REPARACIÓN DE ADN Y DINÁMICA DE LA CROMATINA

Simbolo	Fold (Control/ Tratado)	Nombre Del Gen
ANKRD33	3,30	Ankyrin Repeat Domain 33
ATF3	4,10	activating transcription factor 3
BEX-4	2,03	Brain Expressed, X-Linked 4
BTG2	3,31	Antiproliferative
DDB2	2,08	Damage-Specific Dna Binding Protein 2, 48kda
GADD45B	2,70	Growth Arrest And Dna-Damage-Inducible, Beta
GADD45B	2,73	Growth Arrest And Dna-Damage-Inducible, Beta
GPR155	2,23	G Protein-Coupled Receptor 155
H1F0	3,15	H1 Histone Family, Member 0
H1F2	3,36	Histone Cluster 1, H1c [
H2AFA	2,50	Histone Cluster 1, H2ae
H2AFL	6,73	Histone Cluster 1, H2ac
H2AFO	4,53	Histone Cluster 2, H2aa3
H2AFP	2,90	Histone Cluster 1, H2ag
H2BC	2,47	Histone Cluster 1, H2bc
H2BFA	2,77	Histone Cluster 1, H2bg
H2BFB	4,09	Histone Cluster 1, H2bd
H2BFK	2,13	Histone Cluster 1, H2bi
H2BFL	2,96	Histone Cluster 1, H2bc
H2BFS	2,65	H2b Histone Family, Member S
H3FK	3,00	Histone Cluster 1, H3h
HCFC1R1	2,63	Host Cell Factor C1 Regulator 1 (Xpo1 Dependent)
HDAC9	3,13	Histone Deacetylase9
HES2	2,26	Hairy And Enhancer Of Split 2
HIST1H2BK	3,17	Histone Cluster 1, H2bk
HIST2H2BE	4,51	Histone Cluster 2, H2be
MDM2	4,20	Mdm2 p53 binding protein homolog
MICALL2	2,55	Mical-Like 2
MLF1	2,91	myeloid leukemia factor 1
NCR1	2,99	Natural Cytotoxicity Triggering Receptor 1
NDRG1	3,20	N-Myc Downstream Regulated 1
NID2	2,55	Osteonidogen
RAD51C	2,10	Rad51 Homolog C
RCF1	2,78	Replication Factor C (Activator 1) 1
SPATA18	2,97	Spermatogenesis Associated 18 Homolog
XPCC	2,64	Xeroderma Pigmentosum, Complementation Group C

METABOLISMO Y RESPUESTA A ESTRÉS

Simbolo	Fold (Control/ Tratado)	Nombre Del Gen
ABHD4	2,48	Abhydrolase Domain Containing 4
AMPD3	2,19	Denosine Monophosphate Deaminase 3
APOD	2,11	Apolipoprotein D
CHST2	2,61	Carbohydrate (N-Acetylglucosamine-6-O) Sulfotransferase 2
DDAH1	2,45	Dimethylarginine Dimethylaminohydrolase 1
DPYSL4	2,29	Dihydropyrimidinase-Like 4
FDXR	2,59	Ferredoxin Reductase
GPI	2,04	Glucose-6-Phosphate Isomerase

HSD17	2,25	Hydroxysteroid (17-Beta) Dehydrogenase 2
LASS3	2,30	Lag1 Homolog, Ceramide Synthase 3
NMNAT1	2,01	Nicotinamide Nucleotide Adenylyltransferase 1
NUDT7	2,25	Nudix (Nucleoside Diphosphate Linked Moiety X)-Type Motif 7
OLAH	2,80	Oleoyl-Acp Hydrolase
PLA2G4C	2,26	Phospholipase A2, Group Ivc (Cytosolic, Calcium-Independent)
PLTP	2,65	Phospholipid Transfer Protein
SDC2	2,18	Stearoyl-Coa Desaturase (Delta-9-Desaturase)
SULF2	3,07	Sulfatase 2

TRANSPORTE DE MEMBRANAS

Simbolo	Fold (Control/ Tratado)	Nombre Del Gen
ABCB9	2,02	Atp-Binding Cassette, Sub-Family B (Mdr/Tap), Member 9
AKD2	2,11	Adenylate Kinase Domain Containing 1
CLN2	2,04	Tripeptidyl Peptidase I
CPM	2,79	Carboxypeptidase M
GK1	2,17	Galactokinase 1
GPC1	2,61	Glypican1
PLAU	3,60	Plasminogen Activator, Urokinase
SEB4B	2,02	Rna Binding Motif Protein 38
SLC12A2	2,45	Solute Carrier Family 12
SLC30A1	2,30	Solute Carrier Family 30 (Zinc Transporter), Member 1

MIGRACIÓN, ADHESIÓN CELULAR Y CITOESQUELETO

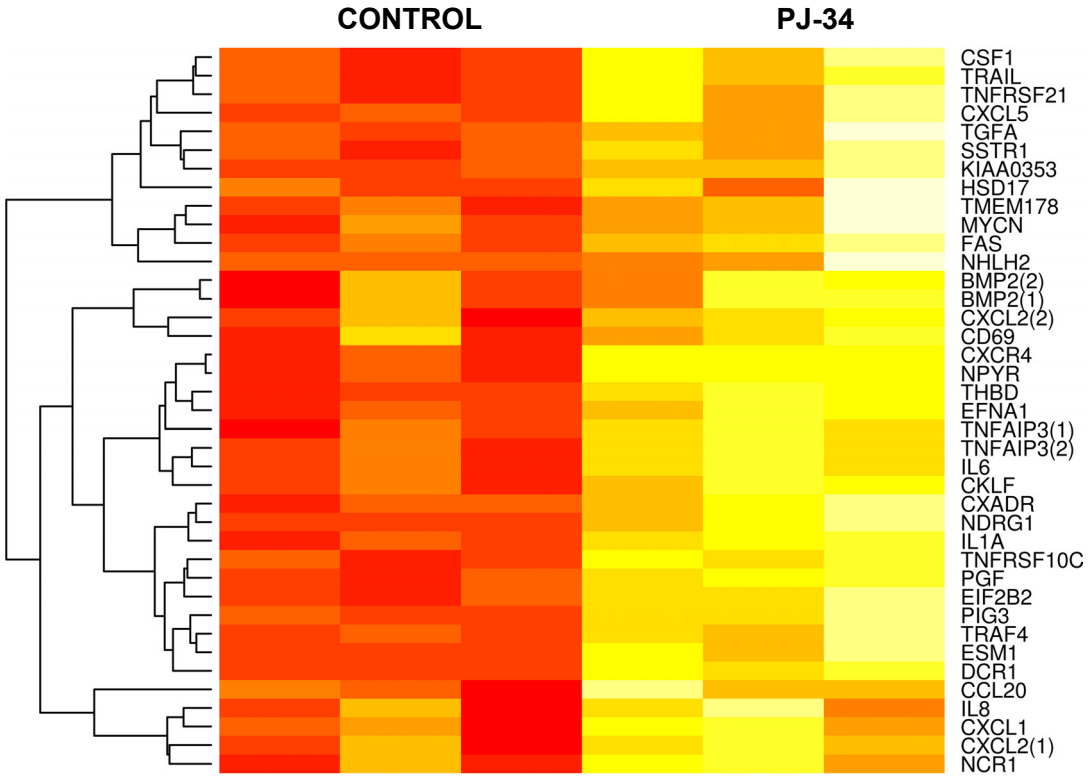
Simbolo	Fold (Control/ Tratado)	Nombre Del Gen
ACTA2	5,41	Actin, Alpha 2, Smooth Muscle, Aorta
ADRB2	2,57	Adrenergic, Beta-2-, Receptor, Surface
CLDN5	2,19	Claudin5
CLDND1	2,20	Claudin Domain Containing 1
EFNA1	2,59	Ephrin-1
EIF2B2	2,59	Eukaryotic Translation Initiation Factor 2b, Subunit 2 Beta, 39kda
ESM1	2,36	Endothelial Cell-Specific Molecule 1
FOXF1	2,19	Fokheadboxf1
IGSF4	2,04	Cell Adhesion Molecule 1
LAMA4	2,31	Laminin A4
MMP1	3,33	Matrix Metallopeptidase 1 (Interstitial Collagenase)
MMP10	2,89	Matrix Metalloprotease 10
NID2	2,55	Osteonidogen
PCDH16	2,29	Protocadherin 17
PDZD2	2,34	Pdz Domain Containing 2
PGF	3,15	Placental Growth Factor
PLAU	3,60	Plasminogen Activator, Urokinase
SAT	3,18	serine acetyltransferase
SELE	5,10	Selectin E
TGFA	2,72	Transforming Growth Factor, Alpha
THBD	2,09	Thrombomodulin
VWF	2,39	Von Willebrand Factor

APOPTOSIS, CICLO CELULAR

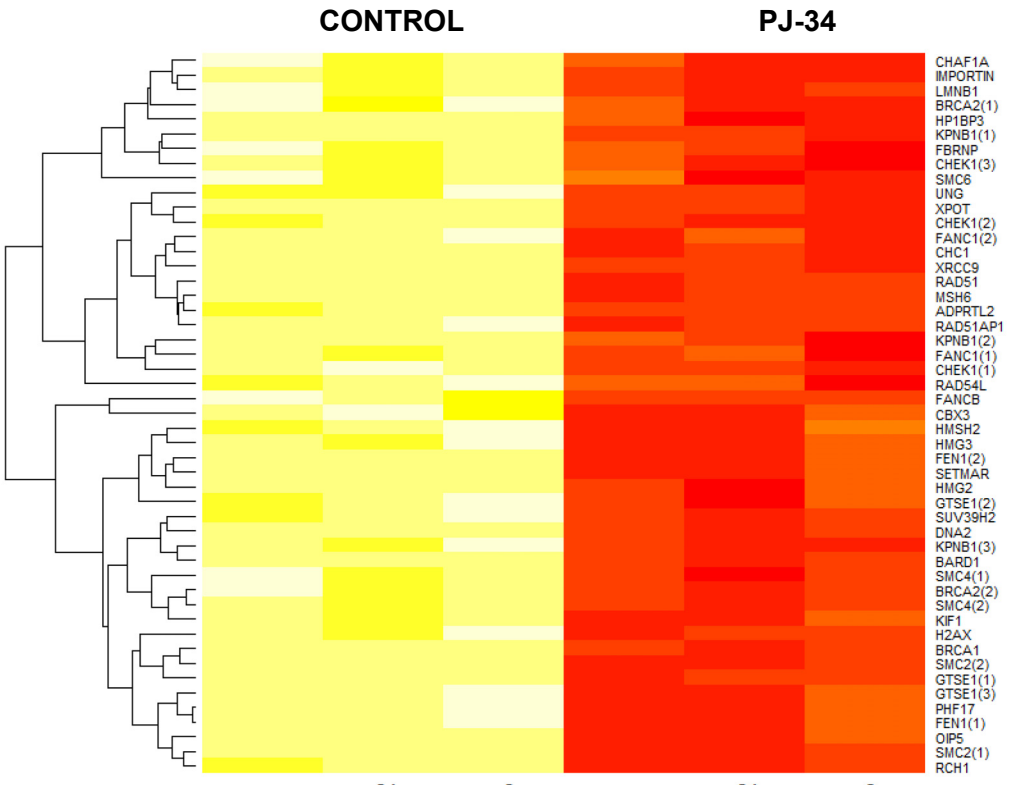
Simbolo	Fold (control/ tratado)	Nombre del gen
ANXA1	2,75	Annexin1
ANXA4	2,33	Annexin4
ANXA4	2,34	Annexin4
BIRC3	2,55	Baculoviral Iap Repeat Containing 3
BRE	2,63	Antiapoptotic
CD95	3,08	Fas
CDKN1A	3,47	Cyclin-Dependent Kinase Inhibitor 1a (P21, Cip1)
DCR1	2,32	Decoy Receptor
DUSP1	2,19	Map Kinase Pathway
FAS	3,39	TNF receptor superfamily, member 6
HTT	2,27	Huntingtin Antiapoptosis P53 Binding
OPTN	2,61	Optineurin
PIG3	2,49	Tumor Protein P53 Inducible Protein 3
PMAIP1	2,04	Phorbol-12-Myristate-13-Acetate-Induced Protein 1
SCIN	3,07	Scinderin
SPIN3	2,05	Spindlin Family, Member 3
TNFRSF10C	2,88	Tumor Necrosis Factor Receptor Superfamily, Member 10c, Decoy Without An Intracellular Domain
TRAF4	2,01	Tnf Receptor-Associated Factor 4
TRAIL	2,21	tumor necrosis factor (ligand) superfamily, member 10
TST	2,29	Thiosulfate Sulfurtransferase (Rhodanese)

HeatMap

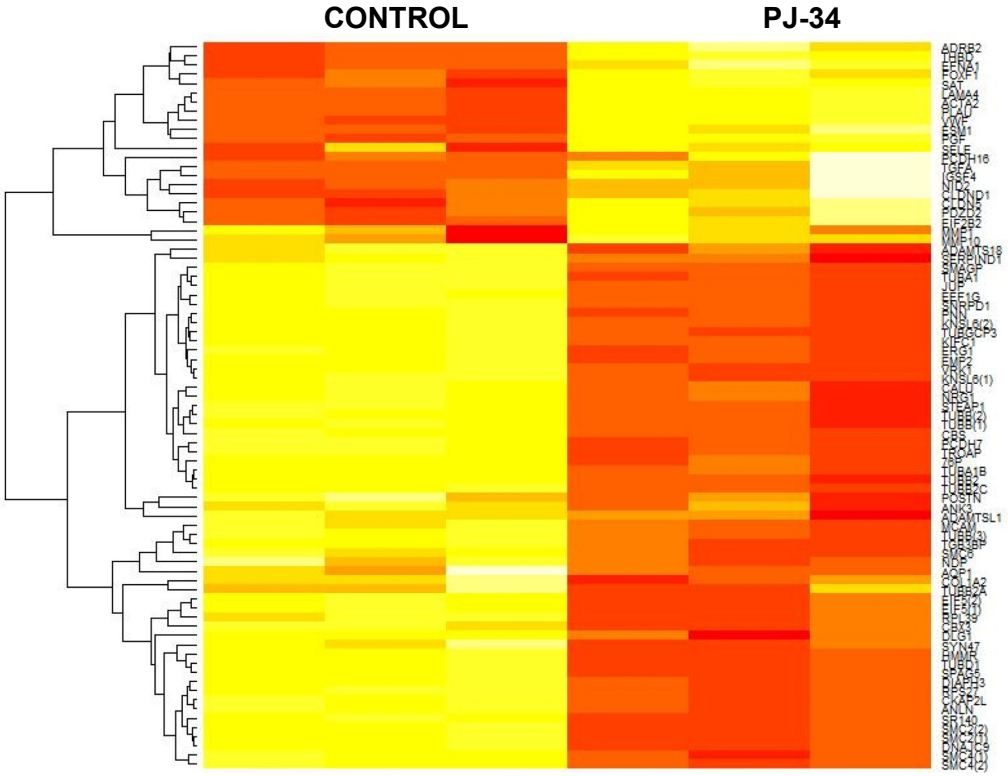
Factores de crecimiento y receptores



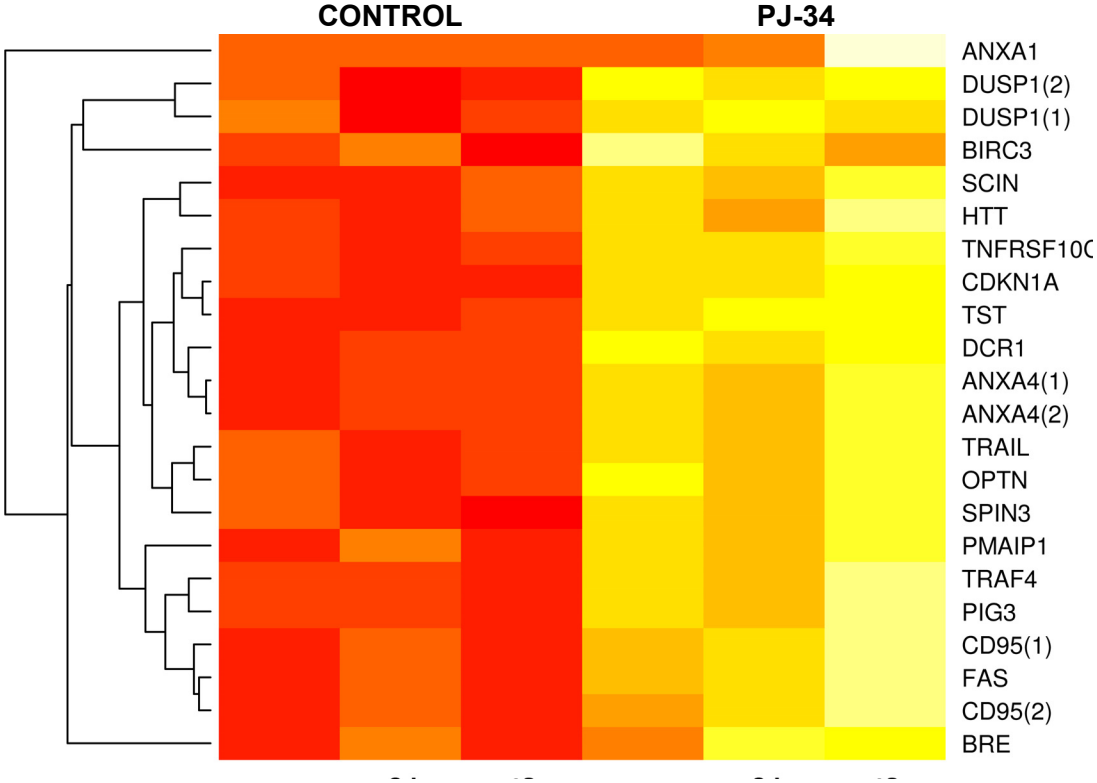
Reparación del ADN y dinámica de la cromatina



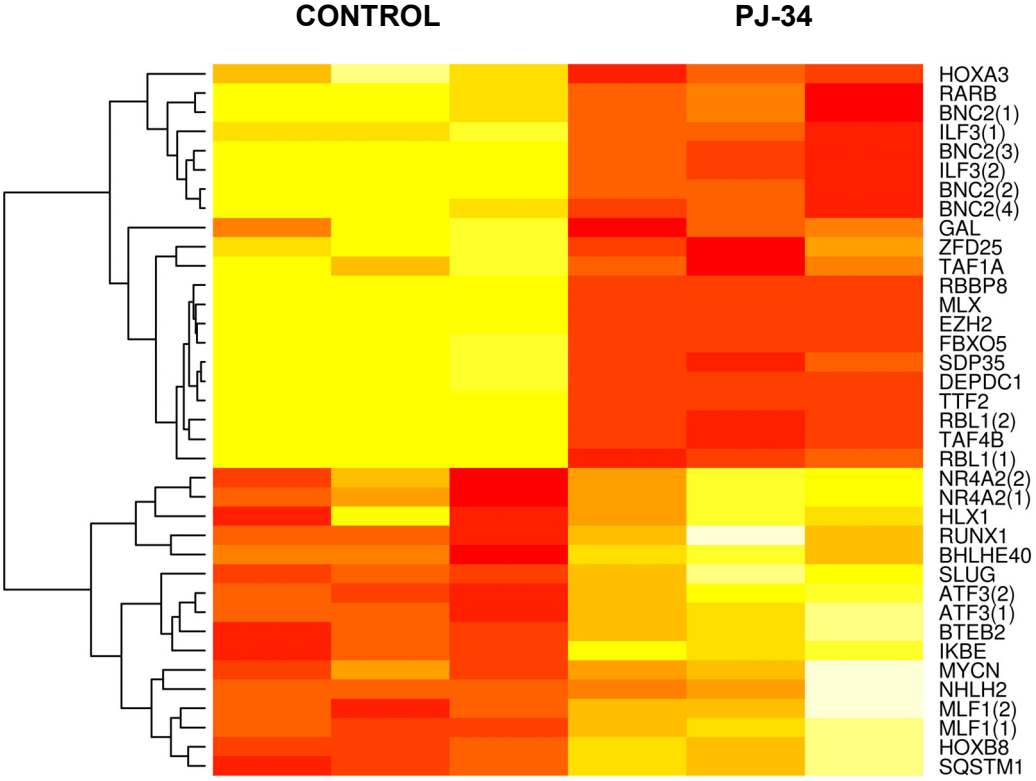
Adhesión celular, migración, citoesqueleto



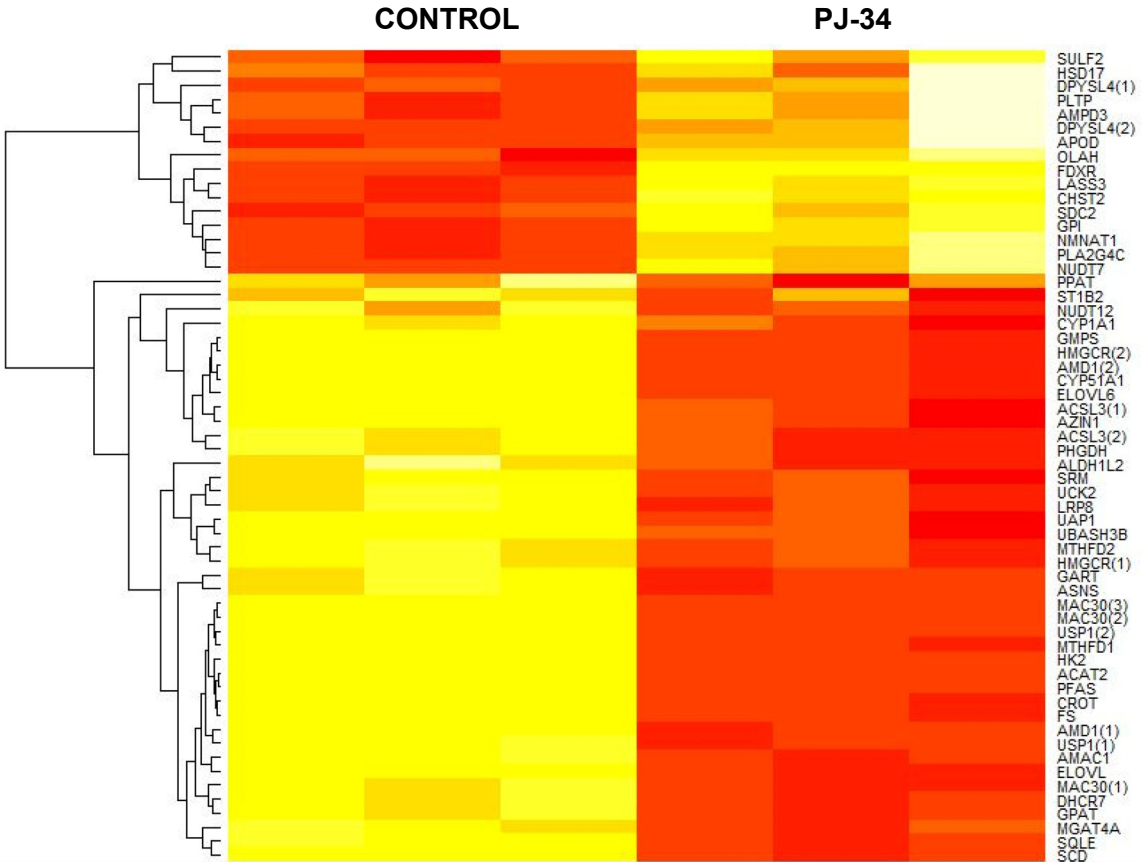
Apoptosis y ciclo celular

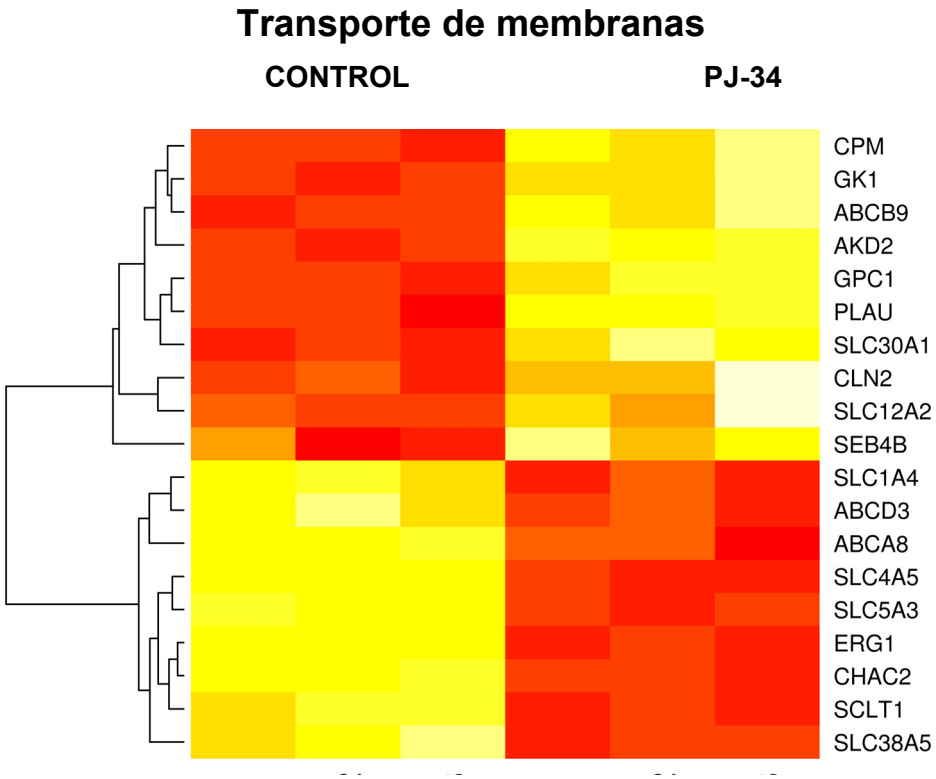


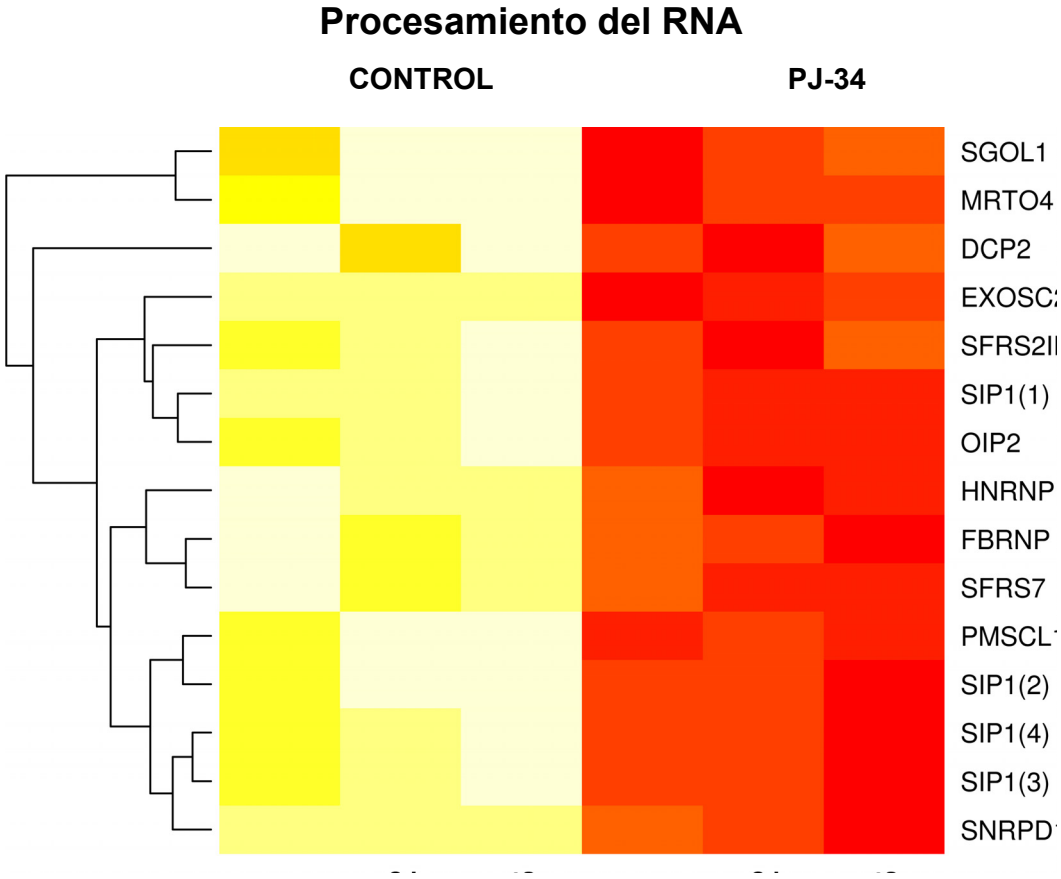
Factores de Transcripción



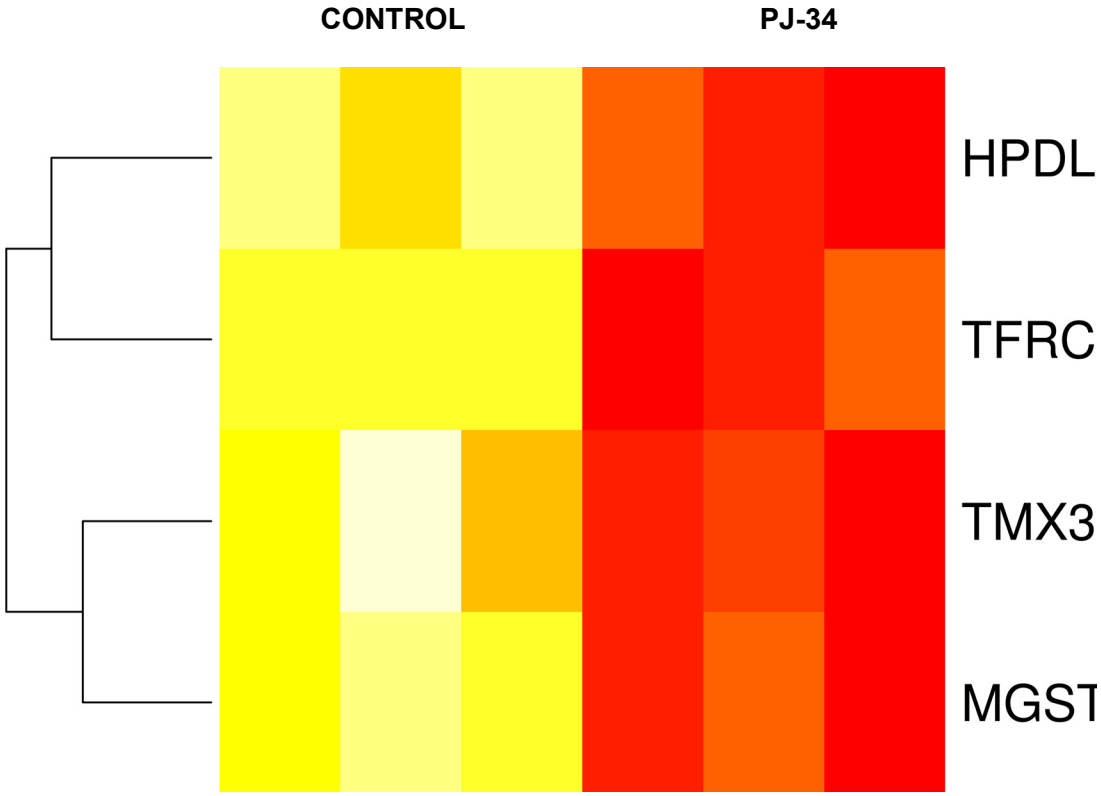
Metabolismo y respuesta a estrés



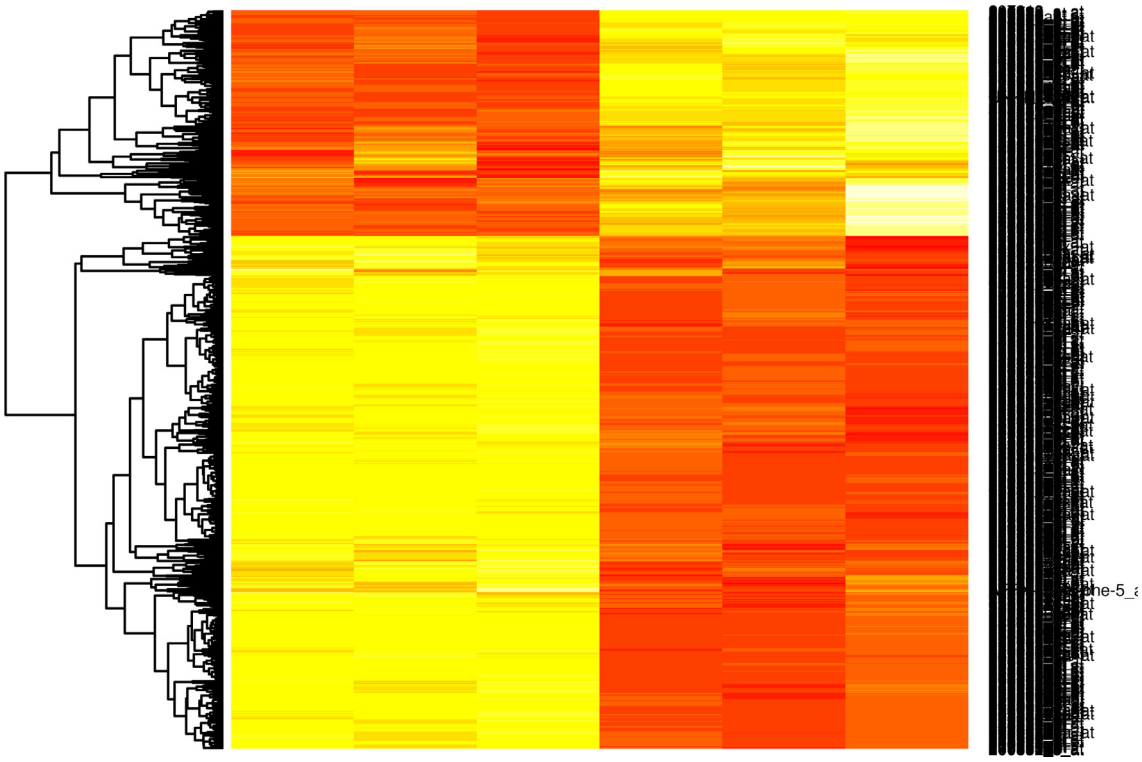

Transporte de membranas
CONTROL
PJ-34
CPM
GK1
ABCB9
AKD2
GPC1
PLAU
SLC30A1
CLN2
SLC12A2
SEB4B
SLC1A4
ABCD3
ABCA8
SLC4A5
SLC5A3
ERG1
CHAC2
SCLT1
SLC38A5


Procesamiento del RNA
CONTROL
PJ-34
SGOL1
MRTO4
DCP2
EXOSC
SFRS2I
SIP1(1)
OIP2
HNRNP
FBRNP
SFRS7
PMSCL
SIP1(2)
SIP1(4)
SIP1(3)
SNRPD

Homeostasis del hierro hypoxia, óxido-reducción



Todos los genes variables



VI. DISCUSIÓN

DISCUSIÓN

Los inhibidores de PARP son una nueva e importante clase de agentes anticancerígenos, y ahora hay más de 40 ensayos clínicos en curso o en desarrollo para estudiar la eficacia de los inhibidores de PARP en el tratamiento de varios tipos de cáncer. Dado el enorme interés en este blanco terapéutico, es importante comprender las funciones de PARP-1 y otras PARPs en la biología de las células tumorales. Hasta hace poco, el desarrollo de inhibidores de PARP-1 se había centrado casi exclusivamente en la función de esta enzima en la reparación del ADN, No obstante, los datos acumulados en los últimos 15 años, fundamentalmente desde el desarrollo de los ratones knockout para PARP-1, han puesto de manifiesto una gran complejidad de funciones de esta proteína, tal como se ha tratado en el apartado correspondiente.

Además de su papel directo en el reconocimiento y la reparación de daños en el ADN, PARP puede regular la función de varios factores de transcripción, incluyendo p53 y NFkB. En el contexto de ciertos tipos de cáncer, PARP-1 interactúa con los factores de transcripción HIF-1α (Martin Oliva et al., 2006) y Snail 1 (Rodriguez et al 2010.). El mecanismo relacionado con los efectos de la inhibición del PARP en la plasticidad vascular y la metástasis sigue siendo relativamente desconocido. En esta Memoria se ha identificado PARP-1 como modulador clave de los cambios en las características funcionales del proceso de la EndMT (implicada en la pérdida de función de los vasos asociados a tumores) y de los cambios fenotípicos que facilitan la adquisición de capacidades prometastásicas de las células tumorales. A través del análisis proteómico de las células endoteliales que han sido tratadas con un inhibidor de PARP se ha identificado la proteína de filamento intermediario como una diana de la inhibición de PARP. Los filamentos intermediarios como la vimentina y queratina influyen en roles no mecánicos del tráfico de proteínas y la señalización intracelular (Ivaska et al., 2007), que a su vez influyen en los procesos celulares, tales como la adhesión celular y la polarización. La vimentina se expresa abundantemente en las células mesenquimales y

juega un papel crítico en la cicatrización de heridas, la angiogénesis y la progresión del cáncer. La vimentina también se ha descrito como marcador tumoral específico en la angiogénesis y en un modelo de tumor de ratón la inhibición de vimentina endotelial reduce de manera significativa el crecimiento tumoral y reduce la densidad de los microvasos (Van Baijnum et al., 2006). La vimentina es un marcador tanto en EndMT como EMT y está también sobre-expresada en muestras de tumores en comparación con tejidos normales. Esta proteína también contribuye al fenotipo tumoral e invasivo (Gilles et al., 2003). Nuestros resultados indican que los inhibidores de PARP reducen el potencial metastásico de las células de melanoma, al menos en parte, a través de su capacidad de regular negativamente la expresión de vimentina.

Recientemente se ha identificado un mecanismo por el cual PARP-1 puede regular la expresión de vimentina en las células de cáncer de pulmón a nivel transcripcional. En este estudio PARP-1 se une y activa el promotor de vimentina independientemente de su dominio catalítico (Chu et al., 2007). Nuestros resultados indican que, o bien la pérdida de PARP-1 o la inhibición de esta proteína resultan en la disminución de la expresión de vimentina. Teniendo en cuenta el efecto de la inhibición de PARP en la expresión de vimentina, es probable que exista un mecanismo adicional distinto de la regulación transcripcional, que requiera la activación de PARP-1. Asimismo la existencia de este mecanismo distinto de la regulación transcripcional podría explicar la disminución de la angiogénesis, siendo esto respaldado por los estudios proteómicos de inhibidores de PARP en HUVEC, donde se observa una disminución de la vimentina y en consecuencia una disminución de la movilidad celular y reduciendo así la formación de nuevas estructuras vasculares. El último eslabón entre la inhibición de PARP en la expresión de la vimentina podría implicar la capacidad de PARP-1 y/o la poli (ADP - ribosa) para modular a Snail 1 y la transcripción dependiente de HIF (Elser et al, 2008.); Rodríguez et al., 2011, Wang et al, 2011). Nuestros resultados también revelan que los niveles de vimentina no son simplemente un sello o marca de la EMT. Mientras que el silenciamiento de vimentina en las células del melanoma puede revertir el fenotipo de la EMT, en parte por la

promoción de la regulación negativa de la proteína quinasa AXL (que participan en la motilidad celular), la expresión forzada de vimentina en las células tumorales que carecen de esta proteína (MCF7, las células del tumor de mama), es suficiente para provocar el cambio de fenotipo epitelial a mesenquima. GSK3b es un regulador aguas arriba de los factores clave que intervienen en EMT como Snail 1 y β-catenina. Planteamos la hipótesis de que la vimentina podría estar implicada en la modulación de GSK3b. De hecho, la expresión de vimentina potencia la inducción de EMT por Li (un inhibidor de GSK3b) por Li aumenta la expresión de vimentina e induce EMT (Fig. 3B); por el contrario, la vimentina impide la acción inhibitoria sobre EMT derivada del silenciamiento de ILK (que conduce a la activación de GSK3b). (Fig. 3D). Hay señales mecánicas que pueden inactivar GSK3b resultando en la estabilización de la β-catenina. Los filamentos intermedios son importantes para permitir que las células individuales, los tejidos y los organismos puedan hacer frente a diversos tipos de estrés, y juegan un papel significativo en el comportamiento mecánico de las células (Bertaud et al.). Es posible que la vía de señalización que integra la activación de PARP con la alteración de la expresión de la vimentina y las fluctuaciones en la actividad de GSK3b está relacionada con la capacidad de los inhibidores de la PARP para desactivar la señalización de AKT (Tapodi 2005, resultados no mostrados en nuestro laboratorio), que daría lugar a la activación de GSK3b y la modulación de la señalización corriente abajo, hasta culminar con la reversión de la EMT. Dentro de las células tumorales, este mecanismo podría ser apoyado por la capacidad de PARP-1 para modular los factores de transcripción relacionados con la metástasis, como Sanil1 y HIF.

El mimetismo vasculogénico, como patrón de la microcirculación del tumor de novo, difiere de la descripción clásica de la angiogénesis dependiente del endotelio. Este es un proceso característico de células de melanoma muy agresivo donde se ha encontrado que pueden expresar genes que anteriormente se consideraban exclusivos de células endoteliales y que son característicos de células tumorales de melanoma agresivas. Los factores de transcripción HIF-1α and HIF-2α, que están estabilizados durante hipoxia, son los principales reguladores de VE-cadherina. La regulación

transcripcional mediada por HIF durante hipoxia es crítica para la adaptación de las células tumorales a condiciones de hipoxia. Como resultado la expresión de genes diana de HIF está asociada a un aumento de la malignidad tumoral. Aunque la expresión de VE-cadherina no está regulada por hipoxia, HIF-2α (pro no HIF1) activan al promotor de VE-cadherina uniéndose a los sitios HRE hipoxia (hypoxia responsive element) en condiciones de normoxia [41].

Oct-4 es un factor de transcripción esencial para el mantenimiento de la pluripotencia de células madre. Su expresión es inducida con el propósito de estimular una respuesta de diferenciación. En tumores Knock-in para HIF-2α, se ha observado un aumento de la fracción de células indiferenciadas, y este fenotipo podría se puede invertir por anulación (Knok-down) de Oct-4. Estos hallazgos identifican un papel de HIF-2a mediando la diferenciación endotelial, y dado el incremento en la expresión de esta proteína en los tumores agresivos, HIF-2a puede ser un gen candidato clave en el estudio de la VM.

Utilizando un modelo murino de metástasis de melanoma de pulmón, nuestros resultados in vivo indican que el PARP reduce fuertemente la diseminación metastásica de las células del melanoma, al menos en parte induciendo una reducción en la densidad de los microvasos del tumor junto con los cambios en el patrón de expresión de marcadores de la EMT (Snail 1, vimetina y E-cadherina) dentro del tumor.

Snail es el regulador principal en la EMT, y la activación de esta proteína puede regular la invasión del tumor a través de la represión de la transcripción de la E-cadherina. La inhibición de PARP regula la actividad del represor de E-cadherina, representando una estrategia potencialmente beneficiosa para luchar contra la progresión del cáncer, Los mecanismos por los cuales la PARP-1 influye en la activación de Snail están siendo investigados en nuestro laboratorio.

Los resultados del array en tejidos humanos de melanoma sugieren una compleja interacción entre la expresión de PARP-1 y la progresión del melanoma. Es difícil de comprobar experimentalmente la EMT en vivo

debido a la naturaleza reversible y dinámica del proceso. Aunque las células de melanoma no son de naturaleza epiteliales, la EMT para este tumor está bien caracterizada y la relevancia del cambio de cadherina se ha descrito anteriormente usando varios métodos experimentales, se ha demostrando que en líneas de células de melanoma transfectadas con N-cadherina se observa una transformación morfológica de forma parecida a la epitelial y forma parecida a fibroblasto (Krengel et al., 2004). Asi mismo se ha reportado una re-expresión adenoviral de la E-cadherina en células de melanoma reguladas hacia abajo en el endógeno N-cadherina y una reducción del potencial maligno de estas células (Krengel et al., 2004).

En los últimos años PARP ha emergido como un blanco importante y de eficacia demostrada en primera línea en las terapias anticáncer. Debido a su capacidad para regular una serie de funciones celulares (de la reparación del ADN a la muerte celular y la transcripción), la inhibición de PARP pueden afectar a múltiples facetas del metabolismo del tumor. Estos resultados indican claramente que las varias actividades nuevas de PARP-1 pueden contribuir a los efectos de la terapia anticáncer dirigidas a esta proteína, al interferir con la fisiología del tumor y el microambiente tumoral. Dados estos resultados, es de vital importancia el dilucidar los mecanismos de regulación de las funciones nuevas de PARP-1 y el poli (ADPribose) en la biología del tumor por lo que los inhibidores de PARP, en última instancia, podrían hacer la transición hacia el uso clínico de rutina. Asimismo es de gran interés continuar investigaciones en PARP-1 y su relación con EMT y EndMT, ya que estos procesos son claves en el desarrollo de vasos angiogénicos asociados a tumores y el proceso de metástasis.

Para profundizar en el mecanismo por el que la inhibición de PARP afecta a la dinámica de las células endoteliales hemos llevado a cabo un estudio global de alteración de la expresión génica. El análisis global de la expression génica tras el tratamiento con el inhibidor der PARP reveló diferencias muy importantes entre los dos grupos (tabla 1, resultados, heat map) afectando a familias de genes implicados en diversas funciones celulares: factores de crecimiento y receptores, reparación del ADN y

dinámica de la cromatina, adhesión celular, migración y citoesqueleto, apoptosis y ciclo celular, factores de transcripción, metabolismo y respuesta a estrés, transporte de membranas, procesamiento del RNA, homeostasis del hierro, hipoxia y óxido-reducción, respuesta inmune. El grupo más numeroso cuya expresión resulta disminuida tras la inhibición de PARP, es el de genes implicados en la adhesión, migración y citoesqueleto. El aumento más importante en la expresión génica corresponde al grupo de genes que codifican para factores de crecimiento y receptores de estos. La confirmación de la alteración en la expresión de genes candidatos por q-PCR está en la actualidad en progreso y serán objeto de trabajos posteriores en nuestro laboratorio.

VII. CONCLUSIONES

CONCLUSIONES

1. Los inhibidores de PARP disminuyen la angiogénesis *in vitro* en células HUVEC, en in vivo en "*plugs"* subcutáneos en ratón, y atenúa la capacidad de migración celular.

2. La pérdida de PARP-1, o su inhibición, resultan en la disminución de la expresión de la proteína de filamentos intermediarios vimentina, implicada en la transformación endotelio mesénquima y epitelio mesénquima.

3. La inhibición de PARP conlleva un cambio global en el patrón de expresión génica de células endoteliales HUVEC don se ha encontrado que la expresión de 195 genes está disminuida mientras que 168 genes aumentaron su expresión tras el tratamiento con PJ34.

4. La inhibición de PARP disminuye el desarrollo tumoral en un modelo de xenógrafos subcutáneos en ratón, afectando además a la formación de estructuras de tipo vascular por la células de melanoma (mimetismo vasculogénico),

5. La inhibición de PARP inhibe el proceso de metástasis in vivo, disminuyendo los focos metastáticos en pulmón y otros órganos diana y aumentando la supervivencia.

6. Los inhibidores de PARP reducen la expresión de marcadores de la transición endotelio mesénquima en células endoteliales a través de su capacidad (al menos en parte) de regular negativamente la expresión de vimentina, la disminución de Snail1 y aumento de VE-cadherina.

7. Los inhibidores de PARP reducen la transición epitelio mesénquima de células de melanoma a través de su capacidad de regular

negativamente la expresión de vimentina, la disminución de Snail y aumento las E-cadherina. El aumento de la expresión de vimentina por sí misma es capaz de inducir la transición EMT afectando a la señalización de la ruta ILK/GSK3-b/Snail1.

Conclusión final:

La transformación metastásica de las células de melanoma puede ser atenuada a distintos niveles mediante el tratamiento con inhibidores de PARP tanto *in vitro* como *in vivo*, abriendo un potencial terapéutico para estos compuestos no sólo para la eliminación local del tumor sino para impedir su diseminación.

VIII. PERSPECTIVAS

PERSPECTIVAS

1. Regulación diferencial del promotor de VE-cadherina en células endoteliales y de melanoma metastático.

2. Profundizar en el estudio de la señalización a través de ILK/GSk3 beta en células de melanoma y endoteliales tras la inhibición de PARP o la sobreexpresión de vicentina.

3. Estudio proteómico de poli(ADP-ribosilación) durante la angiogénesis y el mimetismo vascular.

4. Microarray de genes inducidos en melanoma maligno durante el desarrollo de mimetismo vascular.

5. Efecto in vivo de la sobreexpresión de viementina en un modelo de metástasis murina.

6. Estudiar la asociación de marcadores de EMT con la expresión de PARP-1 en distintos estadiajes de melanoma humano.

IX. BIBLIOGRAFÍA

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X. PUBLICACIONES

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Research article

Interaction between ATM and PARP-1 in response to DNA damage and sensitization of ATM deficient cells through PARP inhibition

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Abstract

ATM and PARP-1 are two of the most important players in the cell's response to DNA damage. PARP-1 and ATM recognize and bound to both single and double strand DNA breaks in response to different triggers. Here we report that ATM and PARP-1 form a molecular complex *in vivo* in undamaged cells and this association increases after γ-irradiation. ATM is also modified by PARP-1 during DNA damage. We have also evaluated the impact of PARP-1 absence or inhibition on ATM-kinase activity and have found that while PARP-1 deficient cells display a defective ATM-kinase activity and reduced γ-H2AX foci formation in response to γ-irradiation, PARP inhibition on itself is able to activate ATM-kinase. PARP inhibition induced γ H2AX foci accumulation, in an ATM-dependent manner. Inhibition of PARP also induces DNA double strand breaks which were dependent on the presence of ATM. As consequence ATM deficient cells display an increased sensitivity to PARP inhibition. In summary our results show that while PARP-1 is needed in the response of ATM to gamma irradiation, the inhibition of PARP induces DNA double strand breaks (which are resolved in and ATM-dependent pathway) and activates ATM kinase.

Background

The ATM protein kinase is centrally involved in the cellular response to ionizing radiation (IR) and other DNA double-strand-break-inducing insults. In persons affected with ataxia-telangiectasia (A-T), associated mutations in the ataxia-telangiectasia mutated (*atm*) gene render cells unable to cope with the genotoxic stresses from ionizing radiation and oxidative damage, thus resulting in a higher concentration of unrepaired DNA. Functional inactivation of the ATM gene product and Atm-null mice, which were created by disrupting the Atm locus, recapitulate the human A-T phenotype and display growth retardation, mild neurological dysfunction, male and female infertil-

ity, extreme predisposition to thymic lymphomas, and acute sensitivity to ionizing radiation [1-3].

ATM, the product of the ATM gene, is a member of a family of large proteins found in various organisms that share a COOH-terminal PI3 kinase-like domain. ATM has serine/threonine protein kinase activity and mediates the activation of multiple signal transduction pathways reviewed in [4-6].

Although it has been well established that IR exposure activates the ATM kinase domain, the actual mechanism by which ATM responds to damaged DNA has remained enigmatic until recently. Initial evidences indicated that ATM activation might involve autophosphorylation. A breakthrough in our understanding of this process came in a landmark publication by Bakkenist and Kastan [7]. They found that ATM molecules are inactive in undamaged cells, being held as dimers or higher-order multimers. In this configuration, the kinase domain of each molecule is blocked by the FAT domain of the other. Following DNA damage, each ATM molecule phosphorylates the other on a serine residue at position 1981 within the FAT domain, a phosphorylation that releases the two molecules from each other's grip, turning them into fully active monomers.

Poly(ADP-ribose) polymerase (PARP-1) is a nuclear enzyme which is activated in response to genotoxic insults by binding damaged DNA and attaching polymers of ADP-ribose to nuclear proteins at the expense of its substrate NAD+. The protein respond to DNA damage by transferring 50 to 200 molecules of ADP-ribose to various nuclear proteins, including transcription factors, histones and PARP-1 itself [8]. This poly(ADP-ribosyl)ation activity of PARP-1 appears to be important for maintaining genomic integrity [9] and it has been associated with longevity. Furthermore, PARP-1 is activated by agents infringing single stranded DNA damage such as alkylating agents, ionizing radiation, and oxidative damage.

A function of PARP-1 as a nick sensor has been proposed [10]. Its rapid activation upon DNA damage may result in poly (ADP-ribosyl)ation of key enzymes such as transducers of DNA damage, or alternatively, PARP-1 automodification could result in the recruitment of transducers to the damaged site. In this regard, a link between ATM and PARP-1 is supported by recent findings. One of these studies has demonstrated a sustained PARP-1 activation in ATM-/- cells due to the persistence of DNA damage [11,12]. Moreover, Menissier-de Murcia *et al*. [12], have reported that ATM and PARP-1 double deficient mice have a severe synergistic phenotypes leading to early embryonic lethality due to the effects of these proteins on signalling DNA damage and/or on distinct pathways of DNA repair. Furthermore, a recent study has shown that in vitro, PARP-1 inhibited the activation a subset of ATM substrates such as phosphorylation of p53 on serine 15 [13].

The aim of this study has been to elucidate the interaction between PARP-1 and ATM and how this partnership is involved in regulation of DNA repair pathways. We present evidences showing a physical association between ATM and PARP-1 in response to DNA damage as well as a poly (ADP-ribosyl)ation of ATM. The biological consequence of this interaction is a diminished activation of ATM-kinase in the absence of PARP-1. Surprisingly, preventing poly(ADP-ribosyl)ation with PARP inhibitors results in an increased constitutive ATM-kinase related to the PARP inhibitor's ability to induce DNA double strand breaks (DSBs) which were resolved in an ATM-dependent manner.

Results

PARP-1 interacts with ATM in vivo *and ATM is modified by poly(ADP-ribosyl)ation*

Previous studies have shown that PARP-1 and ATM double deficient mice are embryonic lethal very early during development, suggesting that the two proteins together are needed for the every day life of the animal [12]. In the present study our principal aim was to test whether these two proteins interact (both physical and functionally) in the response to γ-radiation. Figure 1A shows an ATM co-immunoprecipitation study using G361 cells (HT44, an ATM deficient cell line, was used as a negative control). ATM complexes were immunoprecipitated from nuclear extracts using an antibody against ATM (SYR 10G3/1) and the presence of PARP-1 was tested by immunoblot analysis using an anti-PARP-1 antibody. ATM form a tight complex with PARP-1 (1A, upper panels). Reciprocal immunoprecipitation experiments confirmed the previous observation (not shown). Interestingly, this complex was much more evident after DNA damage infringed with either the single alkylating agent N-methyl-N-nitrosourea (MNU, 2 mM) or 10 Gy of γ-irradiation. The interaction was direct and not mediated by DNA since the presence of ethidium bromide was not effective in abolishing the formation of the complex and the specificity of the pull-down was confirmed by the lack of co-immunoprecipitation using an IgG control (not shown). These results were confirmed by co-localisation studies with confocal microscopy, where after γ-irradiation the number of co-localised ATM/PARP-1 foci (yellow) increased respect to untreated cells. Both ATM and PARP-1 are localised in foci after DNA damage.

In order to check whether ATM was modified or not by PARP-1 following DNA damage, the modification of ATM by PARP-1 was analysed in a time course experiment using

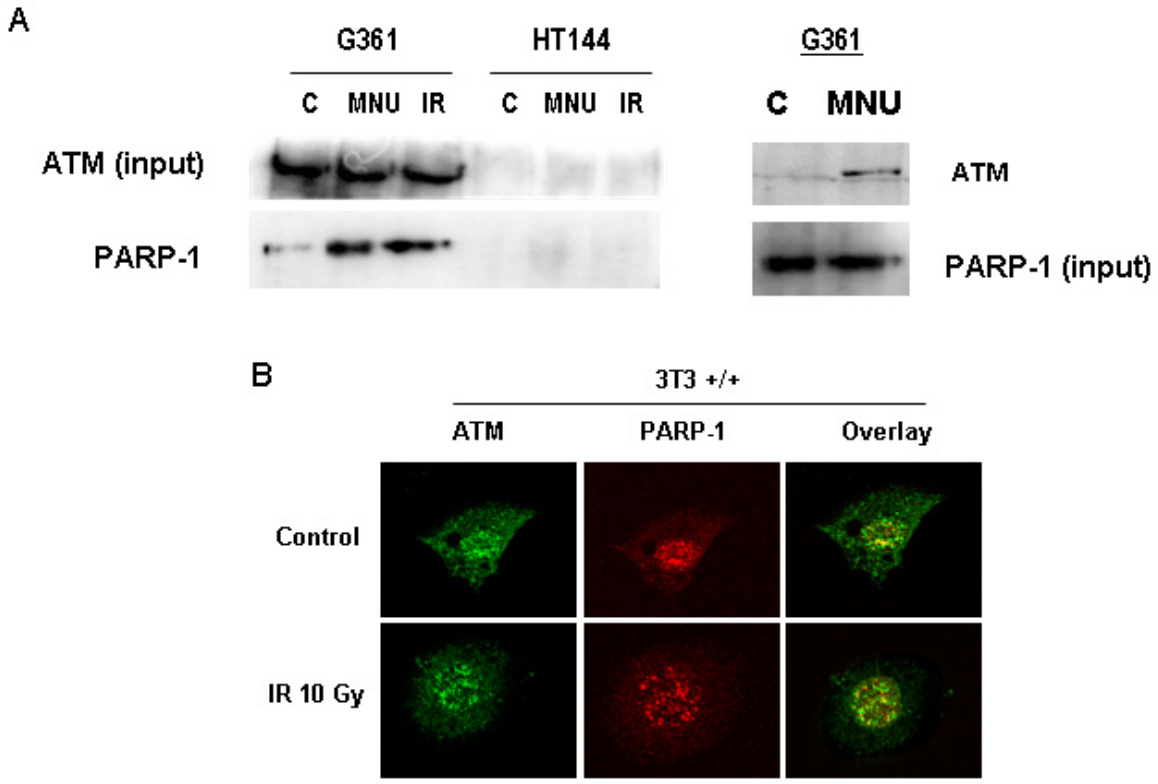

Figure 1
ATM form a complex with PARP-1 in DNA that is much more evident after treatment with DNA damaging agents. A: ATM was immunoprecipitated in human melanoma cell lines G361 and HT144 (ATM deficient) as explained under methods and the presence of PARP-1 was tested by immunoblot analysis. Cells were treated with 10 Gy IR or 2 mM MNU for 30 min. In the left panel, PARP-1 was immunoprecipitated from G361 cells treated or not with 2 mM MNU and western blot was performed to reveal ATM. B: Double indirect immunofluorescence in 3T3 fibroblasts (*parp-1*+/+)of PARP-1 (red signal) and ATM (green). Yellow signal correspond with co-localization of both proteins.

the same antibody to immunoprecipitate ATM (figure 2A). ATM is, indeed, modified by poly (ADP-rybosil)ation and this modification increases during DNA damage, reaching a maximum after 30 min and then start to decrease. Polymer signal correspond to ATM molecular weight. Again, confocal microscopy confirmed the co-localisation of ATM and poly (ADP) ribose after ionizing radiation (figure 2B). In conclusion, these results are the first indication that ATM is physically associated to PARP-1 and is a substrate for this enzyme, co-localizing in the same foci after DNA damage.

PARP-1 is needed for optimal activation of ATM

One key question derived from the previous results concern the functional consequences of the interaction between PARP-1 and ATM and between poly (ADP-ribosyl)ation and ATM on ATM activation. To address this question we have measured ATM kinase activity in wild type and PARP-1 deficient cells and in the presence and absence of the PARP inhibitor 4-amino,1–8,naphtalimide (ANI). Splenocytes from *parp-1*+/+ and *parp-1*-/- mice or G361 cells (a human melanoma cell line) were irradiated at 10 Gy. ATM was immunoprecipitated 30 minutes after the IR treatment and the ATM kinase assay performed (figure 2C). In these conditions ATM was strongly activated in response to ionizing radiation in *parp-1*+/+ splenocytes

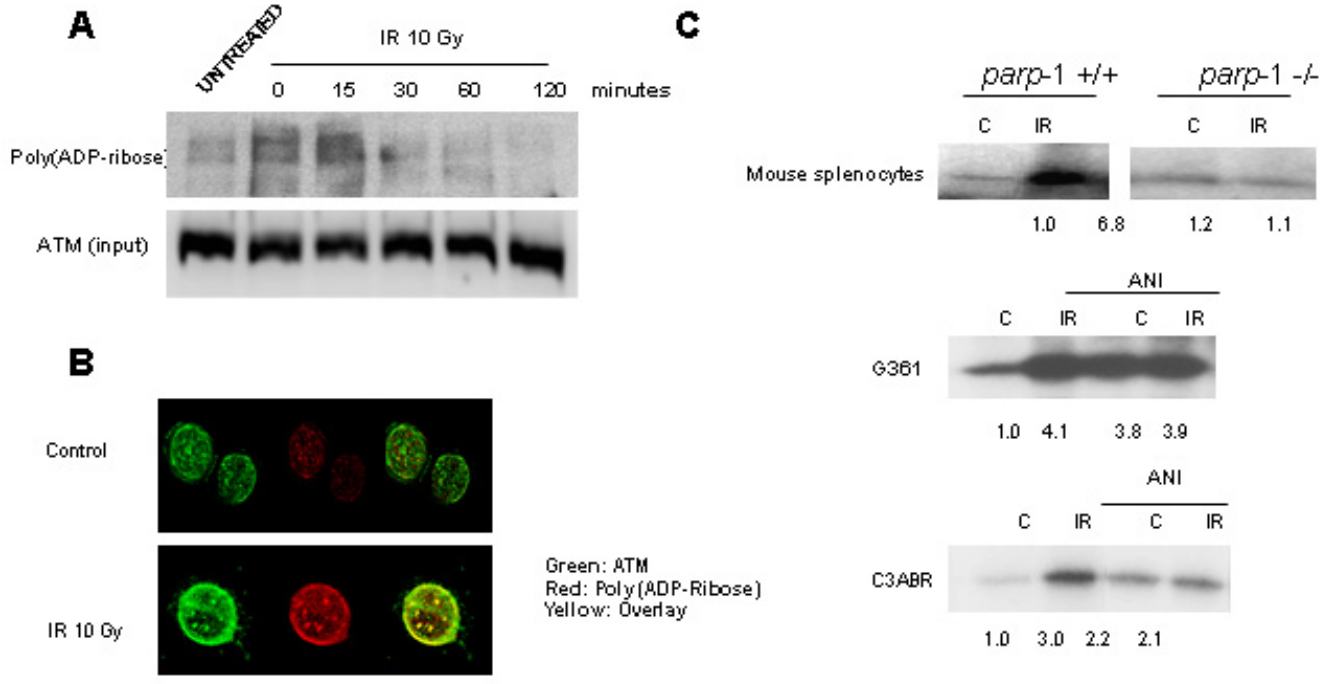

Figure 2
ATM is modified by poly(ADP)ribose. A: ATM was immunoprecipitated in G361 cells and the presence of poly(ADP ribose) was tested by immunoblot analysis in a time course, reaching a maximum after 15 min of IR (10 Gy). Equal loading was normalized by the input of ATM. B: Double indirect immunofluorescence in 3T3 fibroblasts (*parp-1*+/+) of poly(ADP-ribose) (red signal) and ATM (green). Yellow signal correspond with poly(ADP-ribosyl)ation of ATM. C: PARP-1 is needed for optimal activation of ATM. In vitro ATM-kinase assay. ATM activity increases after γ-irradiation in wild-type mouse splenocytes, but not in *parp*-1 knockout mouse splenocytes, where ATM is activated in control (upper panels). In G361 cells and C3ABR (middle and lower panel) irradiated with or without ANI co-treatment, ATM kinase activity increases after γ-irradiation and/or PARP inhibitors. Equal loading was checked with coomasie blue staining. Normalized signal respect to coomassie blue is shown below. Results are representative of three independent experiments.

but not in *parp*-1-/- (Figure 3A). In G361 and C3ABR cells IR also activates ATM kinase but this increase was not observed if prior to IR there was a pre-incubation with the PARP inhibitor, ANI (figure 2C, middle and lower panel); surprisingly, the simple presence of the PARP inhibitor was able to activate ATM kinase.

PARP inhibitors promote ATM activation through induction of DSBs

To gain insight about the increased basal ATM-kinase activity after the inhibition of PARP-1 with ANI (figure 2C), we performed an indirect inmunofluorescence against H2AX in its phosphorylated form, after the incubation with the PARP inhibitor in order to detect any DNA damage response. ATM wild type and deficient cells (G361 and HT144 respectively) were incubated with ANI at different times. Remarkably the sole fact of the incubation with ANI was able to elicits H2AX phosphorylation in wild type but not in ATM deficient cells. This effect was transient and reached a peak in 2 hours, declining afterwards (figure 3). γ-Irradiation was used as positive internal control (figure 3, upper panels). Previous results from our group have shown that PARP-1 null cells have a deficient p53 ser15 phosphorylation in response to ionising radiation [15], confirming with a different ATM substrate that ATM activation is compromised in the absence of PARP-1.

Recent results have shown that inhibition of PARP leads to stalled replication fork and the formation of DNA double strand breaks that are resolved by homologous recombination [17]. In order to test this possibility in our system we performed neutral comet assay to detect double strand breaks (DSB). DSB were produced by treatment with the PARP inhibitor in both ATM proficient (G361) and deficient (HT144) cells but only ATM wild type cells were able to completely resolve double strand breaks (figure 4A). PARP inhibition activates ATM through the induction of DSBs which are repaired by HR; since ATM deficient cells were less efficient in resolving DNA strand breaks that

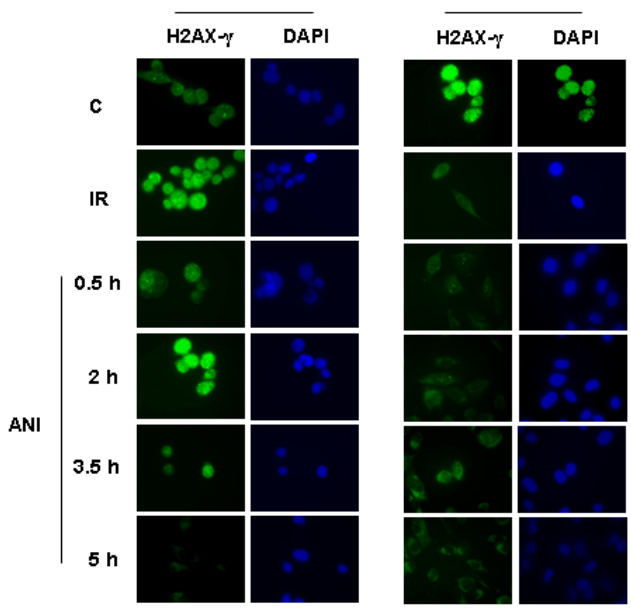

Figure 3
ATM is activated by PARP inhibitors. Immunostaining for γ-H2AX in G361 (ATM wild type) and HT144 (ATM deficient) cells exposed to γ-irradiation (positive control) or ANI (time course after drug exposure). H2AX (green signal) is phosphorilated by ATM after ANI treatment in absence of γ-irradiation in G361 cells (ATM-proficient). DAPI is shown in blue and both views are in coincidence.

result from PARP inhibition we next examined their sensitivity to ANI in the presence and absence of IR. Results in figure 4B clearly show that ATM deficient cells are much more sensitive to PARP inhibition with or without further DNA damage.

Discussion

Cells have evolved various sophisticated pathways to sense and overcome DNA damage as a mechanism to preserve the integrity of the genome. Environmental attacks like radiations or toxins, or spontaneous DNA lesions, trigger checkpoint activation and consequent cell cycle arrest leading to DNA repair or apoptosis. Two key proteins that coordinate recognition of DNA damage and signal transduction to p53 are ATM and PARP-1. ATM and PARP-1 participate in distinct forms of DNA repair that partially compensate for each other. PARP-1 and ATM participate in base excision repair (BER) and homologous recombination (HR), respectively. It is normally assumed that ATM signals for double strand breaks while PARP-1 participates in signalling from single DNA strand lesions. Here we report that these two proteins form a molecular complex that co-localizes in DNA damage foci.

Considerable evidence from *in vitro*, cell culture and *ex vivo* studies shows that poly(ADP-ribosyl)ation plays a critical role in the survival and maintenance of genomic stability of proliferating cells exposed to low or moderate levels of DNA-damaging agents [18]. The data presented in this study strongly support a role for PARP-1 and poly (ADP-ribose) in ATM activation: in the absence of PARP-1 there is a deficient ATM-kinase activation in response to ionizing radiation as measured by intrinsic kinase activity and H2AX phosphorylation. These results are in agreement with previous data showing that PARP-1 deficient mice are extremely sensitive to low doses of γ-radiation (as is the case for ATM-null mice), and this phenotype could be adscribed to a deficient ATM-kinase activation in tissues such as the intestine epithelium [9]. Also, results from our group have shown that p53 accumulation and p53-dependent gene activation are compromised in *parp*-1 knockout cells after γ-irradiation [15]. The insight of the consequences of the poly(ADP-ribosyl)ation of ATM are not clear yet since the inhibition of PARP induced indirectly DNA DSB, initiating new responses to DNA damage that interfere with elucidation of the activation of ATM.

PARP inhibitors have been used as radio and chemo-sensitizers in a number of experimental settings and a mechanism for DSB induction through the collision of unrepaired single DNA strand lesions with replication forks has been suggested [19,20]. Early reports claimed that the PARP inhibitor 3-aminobenzamide was a radiosensitizer only in rodent cells [21] however more recently ANI (1000-fold more potent at inhibiting PARP activity compared with 3-aminobenzamide (3-ABA)) has been found to be radiation sensitizer to both rodent and human tumor cells [9,15,22]. The novel PARP inhibitor AG14361 has shown to increase the specificity and *in vivo* activity to enhance radiation therapy of human cancer through vasoactive effects and not directly in the cells in culture [23]. Therefore the question still remains open as to how human tumors could benefit from PARP inhibition during radiotherapy.

The second main conclusion in this study is that inhibition of PARP-1 activity leads to DSBs induction and activation of ATM and, at the same time, prevents IR-induced ATM-kinase activity. From the results presented here, there is a clear duality in the effect of PARP inhibition on ATM: while the lack of response to IR in ANI treated cells indicates that poly (ADP-rybosylation) of ATM is probably needed for optimal ATM activation, long term exposure to PARP inhibitor results in the generation of DSBs and secondarily in the activation of ATM kinase. DSBs generated in this way are due to stalled replication fork [24] and they are resolved by homologous recombination (HR), providing a therapeutic opportunity to specifically

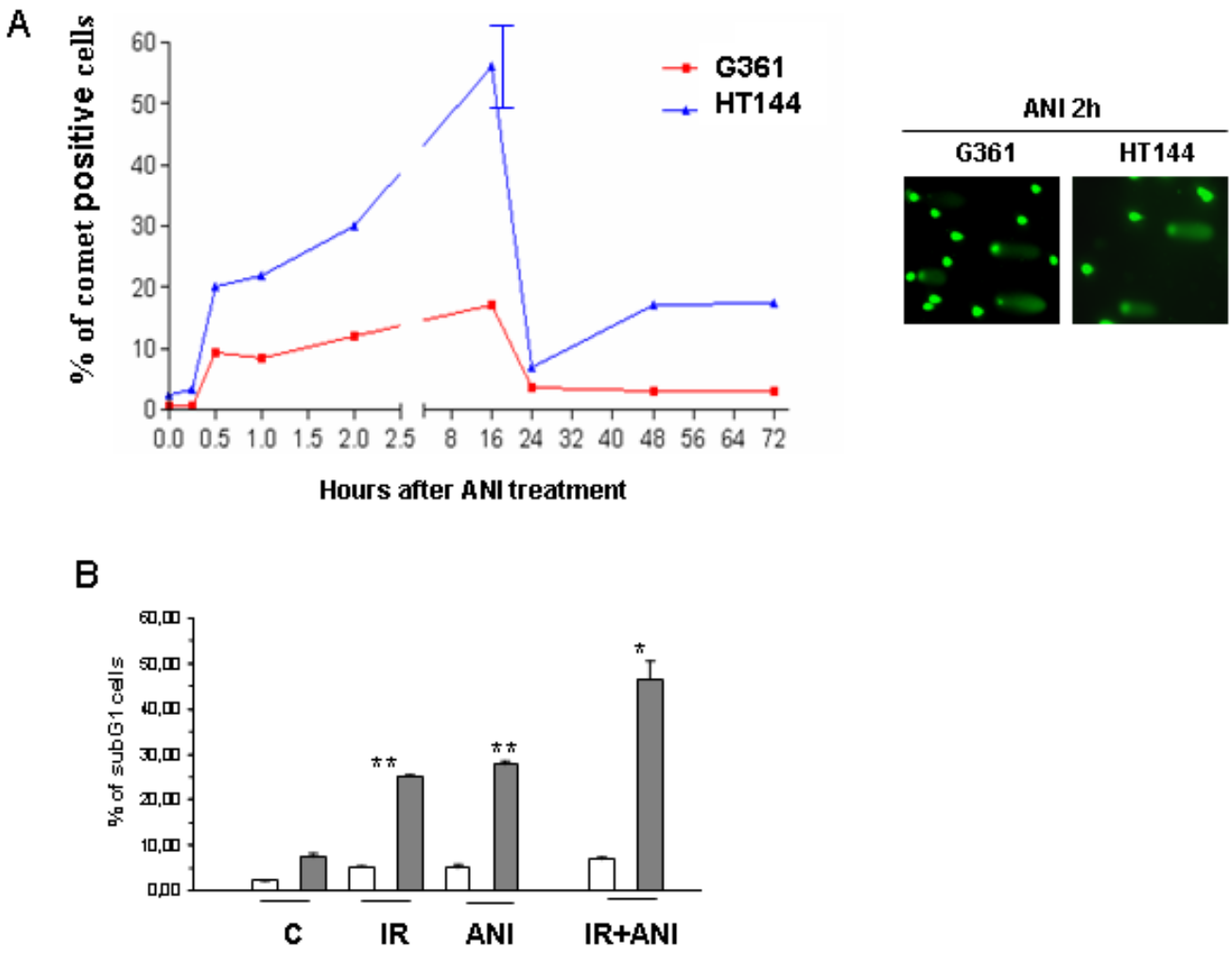

Figure 4

PARP inhibitors activate DSB repair by homologous recombination. A: neutral comet assay for detection of double strand breaks. For scoring the comet pattern, 800 nuclei from each slide were counted. HT144 ATM-deficient cells display more DSBs than G361 ATM-proficient cells after ANI treatment and only ATM wild type cells were able to completely resolve DSBs. Experiment is one representative of three similars. B: Analysis of cell death. Sub-G1 analysis was performed by flow cytometry using the propidium iodide (PI) DNA-staining method. Increased cell death with ANI is observed after long times of exposure (72 hours) in ATM-deficient cells (HT144, grey bars and ATM-proficient cells, G361, white bars) treated either with ANI alone or in combination with γ-irradiation respect ATM-proficient cells. Results represent the average ± SEM of three independent observations. * $p < 0,05$; **$p < 0,01$ respect to untreated HT144.

kill HR deficient tumor cells, as has been previously shown by different laboratories [25,26].

In summary, our study demonstrates a strong association between ATM and PARP-1 during the response to ionizing radiation, being PARP-1 and its activity needed for optimal activation of ATM kinase. On the other hand, inhibition of PARP leads to the activation of ATM kinase as result of the generation of DSBs, making ATM deficient cells particularly sensitive to PARP inhibitors.

Conclusion

In this study we demonstrate the physical interaction between PARP-1 and ATM in response to ionizing radiation, the modification of ATM by poly(ADP-ribose) and the functional consequences of this interaction in PARP-1 deficient cells, where the activation of ATM kinase is compromised in response to IR. Additionally, PARP inhibition induces DNA double strand breaks who are resolved in an ATM-dependent manner. As result of that, ATM kinase is activated by PARP inhibition and ATM deficient cells are

much more sensitive to PARP inhibition than ATM proficient cells.

Methods

Cell culture and treatments

We have used immortalised (3T3) murine embryonic fibroblasts expressing or lacking PARP-1 from *parp-1* +/+ and -/- mice. G361 and HT144 are respectively wild type and A-T cell lines from a melanoma patient [14] (a kind gift from Dr. Y. Shiloh, Sackler School of Medicine, Tel Aviv).

Cells were in exponential growth at the time of IR treatment and the PARP inhibitor ANI was dissolved in culture medium at a concentration of 10 μM. It was added 60 min prior to IR. Irradiations, with or without ANI co-treatment, were performed using a ^{60}Co source at a dose rate of 1.67 Gy/min. The used in all experiments was 10 Gy, unless otherwise stated.

Indirect immunofluorescence

Immunostaining forATM, γ-H2AX, PARP-1 and PAR (poly(ADP-ribose)) were performed on cells plated onto coverslips and grown for 24 h before treatments. The medium was removed, the coverslips rinsed twice in PBS (37°C) and fixed in ice-cold methanol-acetone (1:1) for 10 minutes in the experiments with ATM, γ-H2AX, and in formaldehyde 4% for 10 minutes in the experiments with PARP-1 or PAR. The coverslips were rinsed twice in PBS-T (PBS containing 0,1% Tween-20) prior to incubation with primary antibody for 16 h at 4°C. The primary antibodies used in these experiments were: rabbit polyclonal IgG anti-phospho-H2AX (Ser139) (Upstate, Lake Placid, NY), mouse monoclonal IgG anti-PARP (Ab-2, Oncogene). anti-ATM mouse monoclonal antibody SYR 10G3/1 was a kind gift from Y. Shiloh (Tel Aviv University); anti-PAR rabbit polyclonal antibody was purchased from Biomol, Plymouth Meeting, PA. The coverslips were rinsed 3 times in PBS-T followed by a 45 min incubation at room temperature in the dark and then rinsed 4 × 5 min in PBS-T. The secondary antibodies used in this study were FITC-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO) and Cy3-conjugated sheep anti-mouse IgG (Sigma, St. Louis, MO) at a concentration of 1:400. Antibodies were diluted in PBS containing 1% bovine serum albumin and 5% goat serum. Nuclear counterstaining with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) was performed after removal of excess secondary antibody. Slides were prepared using the Vectashield mounting medium (Vector Lab., Inc., Burlingame, CA 94010), coverslipped and stored in the dark at 4°C. Immunofluorescence images were obtained linear range of detection to avoid signal saturation using a Leica confocal microscopy.

Immunoprecipitation and western blotting assay

Cell extracts, SDS-PAGE electrophoresis and western blotting were performed as previously described [15]. For immunoprecipitation, cell lysates were precleared by constant mixing for 2 hours with protein A-Sepharose (Pharmacia). The beads were removed by centrifugation, and the supernatant was mixed constantly overnight with a monoclonal antibody against ATM (SYR 10G3/1) or PARP-1 (Anti-PARP-1 Ab-2, Oncogene). Immune complex were adsorbed onto protein A-Sepharose, boiled and electrophoresed on polyacrylamide gels. The membranes were probed with antibodies directed against ATM (SYR 10G3/1, Tel Aviv University), PARP-1 (Anti-PARP-1 Ab-2, Oncogene) and Poli (ADP-Ribose) (Biomol).

In Vitro ATM-kinase assay

ATM kinase assays were conducted using the protocol described by Sarkaria *et al*. [16]. ATM was immunoprecipitated from G361 cell extracts and from *parp-1* +/+ and *parp-1* -/- murine spleen extracts. Briefly cell extracts were prepared by resuspending cells in lysis buffer (20 mM HEPES pH = 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, with protease inhibitors and 0.2% Tween during 20 minutes at 4°C. The lysates were clarified by centrifugation and immunoprecipitations were carried out with SYR 10G3/1 anti-ATM mouse monoclonal antibody. Then immune complex were adsorbed onto protein A-Sepharose and washed twice with kinase buffer (10 mM HEPES pH = 7.4, 50 mM NaCl, 10 mM $MgCl_2$) and once with a high salt buffer (0.1 M Tris-HCl pH = 7.4, 0.6 M NaCl). Kinase reactions were initiated with the addition of an equal volume of kinase buffer containing PHAS-I (20 ng/ml), 10 mM $MnCl_2$, 1 mM DTT and 10 mM [^{32}P]ATP. Kinase reactions were performed at 30°C during 20 minutes and terminated by the addition of 6×SDS loading buffer (1:1), and reaction products were resolved by SDS-PAGE. Incorporation of ^{32}P into the PHAS-I substrate was evaluated by phosphorimaging. All kinase reactions were performed under linear reaction conditions. Equal loading in each lane was guaranteed by coomasie blue staining.

Abbreviations

ATM, ataxia telanagiectasia

BER, base excision repair

DAPI, 4',6'-diamidino-2-phenylindole dihydrochloride

DSB, double strand break

DTT, 1,4-dithiothreitol

EGTA, ethylene glycol tetraacetic acid

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

HR, homologous recombination

MNU, N-methyl-N-nitrosourea

PAR, poly(ADP-ribose)

PARP, poly(ADP-ribose) polymerase

SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Authors' contributions

RA-Q carried out poly(ADP-ribosylation) studies, immunofluorescence, ATM kinase, comet assay and apoptosis studies; JAMG contributed to apoptosis studies; DMO helped with ATM kinase assay; AP, MTV, RMR and RQP contributed to immunoprecipitation and comet assays studies. JMM, GdM, MRdA conceived and participated in design the study. FJO conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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Modulation of Transcription by PARP-1: Consequences in Carcinogenesis and Inflammation

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Abstract: Post-translational modification of proteins by poly(ADP-ribosyl)ation is involved in the regulation of a number of biological functions. While an 18 member superfamily of poly(ADP-ribose) polymerases (PARP)s has been described PARP-1 accounts for more than 90% of the poly(ADP-ribosyl)ating capacity of the cells. PARP-1 act as a DNA nick sensor and is activated by DNA breaks to cleave NAD(+) into nicotinamide and ADP-ribose to synthesize long branching poly(ADP-ribose) polymers (PAR) covalently attached to nuclear acceptor proteins. Whereas activation of PARP-1 by mild genotoxic stimuli facilitate DNA repair and cell survival, severe DNA damage triggers different pathways of cell death including PARP-mediated cell death through the translocation of apoptosis inducing factor (AIF) from the mitochondria to the nucleus. PAR and PARP-1 have also been described as having a function in transcriptional regulation through their ability to modify chromatin-associated proteins and as a cofactor of different transcription factors, most notably NF-κB and AP-1. Pharmacological inhibition or genetic ablation of PARP-1 not only provided remarkable protection from tissue injury in various oxidative stress-related disease models but it result in a clear benefit in the treatment of cancer by different mechanisms including selective killing of homologous recombination-deficient tumor cells, down regulation of tumor-related gene expression and decrease in the apoptotic threshold in the co-treatment with chemo and radiotherapy. We will summarize in this review the current findings and concepts for the role of PARP-1 and poly(ADP-ribosyl)ation in the regulation of transcription, oxidative stress and carcinogenesis.

INTRODUCTION

PARP-1 is the founding member of the PARP family that contains as many as 18 distinct proteins in humans [1]. PARP-1 is an abundant nuclear protein found in most eukaryotes apart from yeast. It binds to DNA strand breaks and concomitantly synthesizes oligo- or poly(ADP-ribose) chains using NAD+ as substrate and covalently coupled to various acceptor proteins or to itself resulting in the attachment of linear or branched polymer of poly(ADP-ribose). PARP-1's full activation is strictly dependent on the presence of strand breaks in DNA and is modulated by the level of automodification [1, 2] (Fig. (**1**)). PARP family members share a conserved catalytic domain that contains the "PARP signature" motif, a highly conserved sequence (100% conserved in PARP-1 among vertebrates) that forms the active site [1]. Some PARP family members identified solely on homology, however, have not yet been shown to possess intrinsic PARP enzymatic activity [3]. In addition to a catalytic domain, PARP family members typically contain one or more additional motifs or domains, including zinc fingers, "BRCA1 C-terminus-like" (BRCT) motifs, ankyrin repeats, macro domains, and WWE domains (a protein–protein interaction motif), conferring specific properties to the different PARP members [4].

PARP-1 has a highly conserved structural and functional organization including an N-terminal double zinc finger DNA-binding domain (DBD) [1], a nuclear localization signal [2], a central automodification domain [3], and a C-terminal catalytic domain [4] (Fig. (**2**)). PARP-1's basal enzymatic activity is very low, but is stimulated dramatically in the presence of a variety of allosteric activators, including damaged DNA, some undamaged DNA structures, nucleosomes, and a variety of protein-binding partners [3, 5]. The targets of PARP-1's enzymatic activity include PARP-1 itself, which is the primary target *in vivo*, core histones, the linker histone H1, and a variety of transcription-related factors that interact with PARP-1 [3, 5-7] (Fig. (**1**)).

The DNA binding domain contains a repeated sequence (residues 2-97 and 106-207) in which 35 amino acids are duplicated. Interestingly these residues are strictly conserved during evolution and are crucial for DNA interaction. These crucial residues for DNA-binding consist in two zinc finger residues (FI and FII) [8] (Fig. (**2**)). The automodification domain of PARP-1 is rich in glutamic acid residues, consistent with the fact that poly(ADP-ribosy)lation occurs on such residues. This domain also comprises a BRCT motif that is present in many DNA damage repair and cell-cycle checkpoint proteins [5, 8-9]. Globally, the structure and activities of PARP-1 suggest important roles for this in a variety of cell functions. The activities and functions of the other PARP family members have not been studied to the same extent as PARP-1, although a clearer picture for some of the PARP family members has been emerging, as noted below and reviewed in more detail elsewhere [1, 4].

PARP in Genome Integrity

Although the most recent findings challenge the concept that the obligatory trigger of PARP-1 activation are the nicks and breaks in the DNA strand [4], this stimulus remains the most studied and well known. DNA damage can be induced by a variety of environmental stimuli including free radical oxidation, alkylation, and ionizing radiation. The binding of PARP-1 to damaged DNA, including single-strand breaks (SSBs) and double-strand breaks (DSBs), through its double zinc finger DNA-binding domain potently activates PARP-1 enzymatic activity (as much as 500-fold) [10, 11]. As such,

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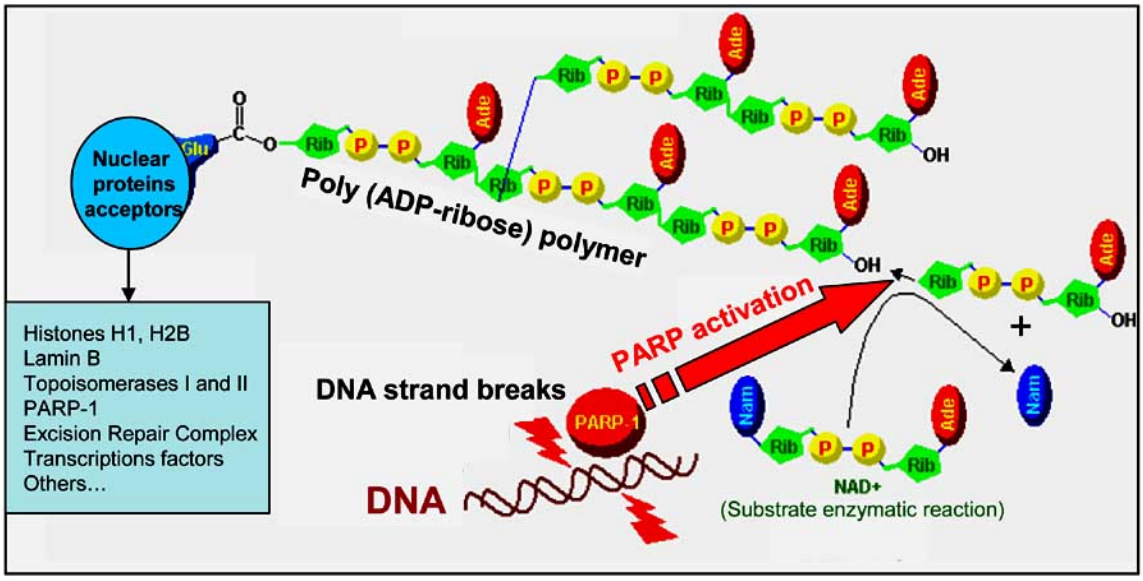

Fig. (1). DNA damage induces PARP -1 activation. A number of genotoxic agents that produces DNA strand breaks activates PARP -1 leading to poly(ADP -ribose) polymer formation from NAD+ and consequent covalent modification by poly(ADP -ribosyl)ation of different nuclear acceptor proteins.

PARP-1 can function as a DNA damage sensor (Fig. (**1**)). With low levels of DNA damage, PARP-1 acts as a survival factor involved in DNA damage detection and repair. In contrast, with high levels of DNA damage, PARP-1 promotes cell death [12]. PARP-1 has been implicated in multiple DNA repair pathways, including the SSB, DSB, and base excision repair (BER) pathways [13]. PARP-1 interacts physically and functionally with various proteins involved in these DNA repair pathways, and may recruit the repair proteins to sites of DNA damage [14]. PAR itself, as a covalent attachment of automodified PARP-1, may also act to recruit repair proteins to sites of DNA damage. PARP-2, the only other PARP enzyme whose catalytic activity is known to be stimulated by damaged DNA, has also been implicated in BER through interactions with XRCC-1 and PARP-1 [15, 16].

The DNA damage response is currently viewed as a signal-transduction pathway involving sensors that activates signal transducers upon detection of damaged DNA. These transducers in turn modulate the activity of effectors that redirect cellular functions while the damage is being repaired. Cellular responses to genomic insults include activation of DNA repair pathways, cell cycle arrest, and initiation of cell death processes [17]. A function of PARP-1 as a nick sensor has been proposed [18]. Its rapid activation in response to DNA strand breaks may result in the poly(ADP-ribosyl)ation of key enzymes such as transducers of the DNA damage. Alternatively, PARP-1 auto-poly(ADP-ribosyl)ation could result in the recruitment of transducers to the damaged site (Fig. (**1**)). How PARP and poly(ADP-ribosyl)ation participate in the initial cell's response to DNA damage and their interaction with key players of this pathway (such as p53 and ATM) has been extensively reviewed previously [13].

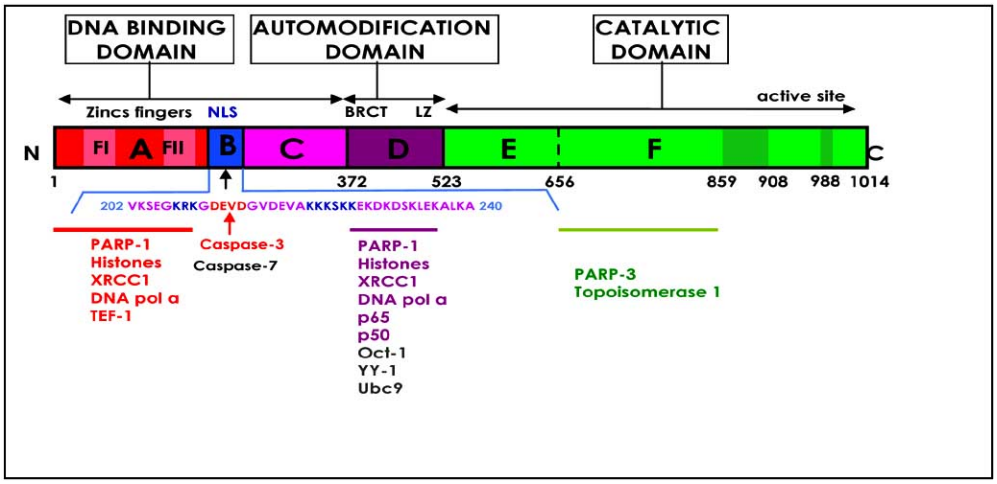

Fig. (2). Structural domains of human PARP-1. DNA binding domain (DBD) contains two zinc finger structures (FI and FII), the nuclear localization signal (NLS) and caspases -3 and -7 cleavage site; Automodification domain contains BRCT (*BRCA1* like C -terminus) motif and Leucine Zipper motif (LZ); and C -terminal Catalytic domain. Also, the interactions of PARP domains with others proteins are showed.

In the present review we will focus on different aspects of PARP's role in in the regulation of transcription and the consequences in carcinogenesis and inflammation.

PARP-1 AND TRANSCRIPTION

A very important aspect of PARP-1 is its involvement in the modulation of chromatin structure and transcription [17]. PARP-1's enzymatic activity is stimulated dramatically by the binding of PARP-1 to damaged DNA and hence, most studies of PARP-1 have focused on its role in DNA repair and cell death pathways [4]. Considerably less is known about the chromatin-dependent gene regulatory activities of PARP-1 under physiological conditions where the integrity of the genome is maintained. As mentioned above, the PARP-1's literature is replete with the view that PARP-1 enzymatic activity is strictly dependent on damaged DNA as an allosteric activator [5]. However, studies identifying other allosteric activators, including certain undamaged DNA structures [18] and PARP-1 binding proteins [19], have challenged this view. Nucleosomes, for example, are potent activators of PARP-1 auto(ADP-ribosyl)ation, more than damaged DNA [20].

Two modes of PARP-1 regulatory activity of transcription have been proposed: (1) a histone-modifying enzymatic activity that can regulate chromatin structure and (2) an enhancer/promoter binding cofactor activity that can act in conjunction with other transcription-related factors [17] (Fig. (**3**)). In summary, PARP-1 can function both as a structural component of chromatin and as a modulator of chromatin structure through an intrinsic enzymatic activity.

PARP-1-Dependent Histone-Modifying Activity and Transcription

PARP-1 exerts its effects in modulating chromatin by directly (ADP-ribosyl)ating core histones and chromatin associated proteins, thereby promoting the dissociation of nucleosomes and the decondensation of chromatin [5, 17, 21, 23]. Although it cannot be excluded that trans-modification is not necessary for PARP-1-dependent regulation of chromatin structure.

PARP-1 is an abundant nuclear protein supporting the idea that either itself or PARP-1-related proteins (with functional redundancy) can function as a structural component of chromatin *in vivo* [22]. In this model, PARP-1, when incorporates into compact transcriptionally repressed chromatin structures, is poised for NAD+-dependent activation, automodification, and subsequent release from chromatin, facilitating chromatin decondensation and transcription by Pol II [22]. DNA binding transcriptional activators could provide the trigger for PARP-1 by recruiting NAD+-synthesizing enzymes. Automodification of PARP-1 is acutely sensitive to small changes in ATP concentration. Thus, the numerous transcription-related factors that consume ATP have the potential to reduce local ATP concentrations and increase PARP-1 enzymatic activity. On the other hand, the incorporation of PARP-1 protein into chromatin promotes the formation of higher-order chromatin structures that localize to discrete chromatin domains *in vivo* and this incorporation has a repressive effect on Pol II transcription *in vitro* [20].

A specific example of histone-modifying PARP-1 activity is puff formation in Drosophila polytene chromosomes, which presents PARP-1-dependent accumulation of PAR at decondensed, transcriptionally active loci [22]. PARP protein is widely distributed in Drosophila polytene chromosomes but is normally inactive. However, upon exposure to a heat shock stimulus, PARP accumulates rapidly at heat shock gene loci, where it develops intense poly(ADPribosyl)ation activity. Similarly, PARP accumulates at sites of ecdysone-induced puffs in polytene chromosomes of fruit fly larvae shortly before pupation. Inactive PARP is recruited, presumably by certain transcription factors, to target genes where it becomes activated. PARP then adds long ADP-ribose tails to the histone proteins of nucleosomes around which the DNA is wrapped. Nucleosomes containing poly-ADP-ribosylated histones are unable to remain tightly packed, resulting in "loosening" or decondensation of

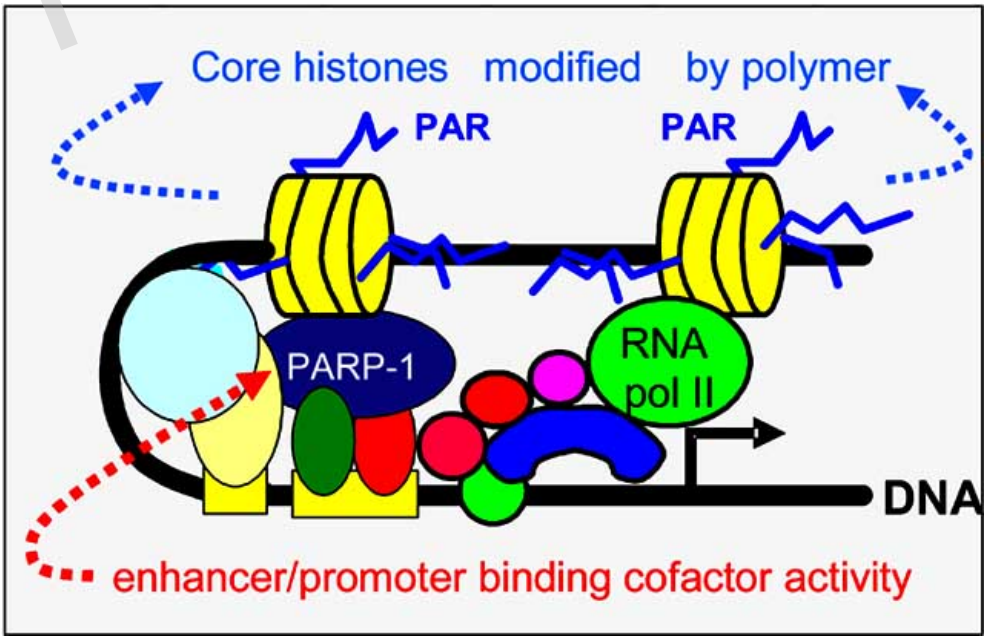

Fig. (3). Models of regulation of transcriptional activity by PARP -1. Two modes of PARP -1-dependent transcription regulation have been proposed. First, a histone -modifying by PARP -1 that can regulate chromatin structure; second, as transcriptional cofactor PARP -1 activity can enhancer the transcription with other transcription -related factors (adapted of Krauss WL and Lis JT, 2003).

the chromatin. *In vitro* experiments suggest that transcription is initially facilitated by PARP, but as soon as transcription factors dissociate from the DNA, they too become inactivated through poly-ADP-ribosylation, thus preventing repeated cycles of transcription [23]. In this way, PARP ensures a strong but transient transcriptional response to a heat shock or ecdysone stimulus. Ultimately, PARP poly-ADP-ribosylates itself and dissociates from the DNA. The mechanism of PARP action seems adapted to facilitate sudden bursts of transcriptional activity in response to transient environmental signals [24].

Further support for the importance of PARP-1-mediated poly(ADP-ribosyl)ation of chromatin modulators in the regulation of DNA-dependent processes [25] is the functional link between PARP-1 and FACT. FACT (facilitates chromatin transcription) is a heterodimer composed of hSpt16 and SSRP1 [27] that allows RNA polymerase II to proceed along the chromatin template. Biological functions of FACT are regulated by poly(ADP-ribosyl)ation [26] and in this way, both, PARP-1 and FACT, are involved in the global regulation of chromatin architecture. hSpt16 but not SSRP1 is poly(ADP-ribosyl)ated *in vivo* especially following genotoxic stress, and additionally, there is a direct interaction between hSpt16 and PARP-1 [26]. Nucleosome-binding activity of hSpt16 (and FACT) is decreased after poly(ADP-ribosyl)ation. Whether poly(ADP-ribosyl)ated FACT dissociate from nucleosome through the disruption of its interaction with the histones remains to be determined. These results suggest that pol II dependent transcription may be regulated through the modulation of chromatin-binding property of FACT by PARP-1 [28].

A recent report of Ju *et al*. [29] show that estrogen-dependent transcription of pS2 requires a promoter intermediate containing a double-strand break (DSB) that is generated by a protein complex containing topoisomerase II (TopoIIβ) and PARP-1. TopoIIβ and PARP-1 collaborate in an interesting way that alters the molecular composition and structure of the pS2 promoter during an estradiol (E2)-dependent transcriptional response. E2 not only rapidly induces an increase in binding of estrogen receptor α (ERα) to the promoter but also causes a concomitant rapid exchange of the co-repressors for what appears to be an activation complex containing PARP-1 and TopoIIβ. Both PARP-1 and TopoIIβ enzymatic activities are critical for the activation of pS2 transcription, although the definitive target of this PARP-1 enzymatic activity has yet to be determined [30]. Prior to treatment with E2, PARP-1 is associated with three adjacent nucleosomes in the promoter region (NucE and a nucleosome on each side, NucU and NucT), possibly acting as part of a repression complex [29] or as a direct nucleosome binding factor [20]. Upon estrogen treatment, PARP-1 departs from NucU and NucT, perhaps as a consequence of auto poly(ADPribosyl)ation, resulting in a loss of nucleosome binding activity [20]. In addition, a PARP-1/TopoIIβ activation complex containing the nuclear receptor coactivator ASC2 becomes concentrated on NucE. The factor dynamics on the three nucleosomes are accompanied by changes in chromatin structure. H1 is lost from NucE and is replaced with HMGB1/2, a non histone structural protein. H1 could be a target for poly(ADP-ribosyl)ation by PARP-1, causing its release. Alternatively, PARP-1's ability to compete with H1 for binding to nucleosomes could function in the dissociation of H1 from NucE [20]. In this system, a transient DSB occurs in promoter DNA adjacent to a nucleosome containing the DNA binding sequence for Erα (NucE) and the formation of this DSB requires TopoIIβ enzymatic activity and participates in the subsequent exchange of factors at the promoter. A possible role for the TopoIIβ-dependent DSB in stimulating PARP-1 enzymatic activity has yet to be addressed. In addition to pS2, Ju *et al*. [29] observed recruitment of TopoIIβ and PARP-1 and other components of the complex to the PSA, RARβ, Dio1, and MMP12 promoters upon gene activation, as well as promoter cleavage.

PARP-1 as an Enhancer/Promoter Binding Cofactor

With respect to the second mode of PARP-1 regulatory activity, the role of PARP-1 in transcription is well established with several independent studies revealing its potent effect on activators like AP-2 (activator protein 2), p53, NF-κB, B-Myb, TEF-1/Max, SP-1, YY-1 and STATs [31-40]. PARP-1 modulates the activity of key transcription factors involved in tumor promotion such as AP-1 (whose defective activation by either PARP inhibition or genetic deletion of PARP-1 results in an effective blockage of gene expression, [41]) and HIF-α [70]. In addition, PARP-1 was identified among the constituents of positive cofactor-1 complex [42], essential for the activity of transcription factors such as NF-κB, Sp1 and Oct-1. Nevertheless, the exact mechanism by which PARP-1 affects transcription lacks clarity and the ambiguity is evident in some cases.

PARP-1 has been shown to play different roles on transcription factors, depending on the presence of specific binding partners, the proliferative status of the cell, the concentration of NAD+ and the presence of DNA strand breaks. In the presence of NAD+, PARP-1 dependent silencing of transcription involves poly(ADP-ribosyl)ation of specific transcription factors like p53 and fos [43, 44], which prevents both their binding to the respective DNA consensus sequences and the formation of active transcription complexes [23, 45-48], reporting a negative role for PARP-1 in transcription regulation. Direct interaction of PARP-1 protein with its own gene promoter resulted in suppression of transcription [48]. However, in response to DNA damage, PARP-1 catalytic activity was stimulated and automodification of PARP-1 subsequently prevented its interaction with the promoter. This relieved the PARP-mediated block on the promoter and allowed for transcription of PARP-1 and other genes suppressed by PARP-1. In the absence of NAD, PARP-1 promotes activator-dependent transcription by interacting with RNA polymerase II-associated factors [46, 49], transcription enhancer factor 1 (TEF1) and an increasing number of transcription factors, including AP-2, B-Myb, YY-1, Oct 1, NF-kB, and p53 [32, 33, 36, 46, 50-53]. Other example is HSF-1, which requires nuclear presence of PARP-1, but not its catalytic activity, for the DNA binding. On the other hand , PARP-1, either alone or in a heterodimeric complex with Ku protein, has been shown to specifically bind internal sequences of matrix attachment regions (MARs) [54] that are required for extending chromatin domains and enhancer-distal positions accessible to transcription factors.

PARP-1 may have a dual regulatory role with opposing effects and it is possible that PARP-1 studies on some transcription factors had characterized and interpreted only one of the two effects. The case of AP-2α-dependent transcription [55] is an example of dual regulation, where the C-terminal enzymatic domain of PARP-1 strongly interacts with AP-2α to poly(ADP-ribosyl)ate it affecting negatively its DNA binding and thereby its transcriptional activation. However, the low-affinity interaction of the middle region has an enzymatic activity-independent positive effect on AP-2α transcription and it is possible that PARP-1 connect AP-2α to the general transcriptional machinery. Griesenbeck *et al*. [56] revealed that automodification of PARP-1 plays a crucial role in choosing partners to interact with. It is possible that the state of automodification determines the time of interaction with AP-2α and histones. The structural overlap of the automodification region with the co-activator domain may represent an important built-in regulatory mechanism. This may also explain the

existence of a weaker interaction of AP-2α with this region. In view of this, PARP-1 has an important biological function beyond its enzymatic activity and warrants a new look at this molecule as a multifaceted protein rather than as one with a single catalytic function with multiple effects.

On the contrary, PARP-1 does not exert a dual effect on E2F-1 transcriptional activation because E2F-1 is not a substrate for modification by PARP-1 [57]. PARP-1 binds E2F-1 through the automodification domain of PARP-1 and together, as a complex, augments binding to the E2F-1 promoter region and expression of E2F-1-responsive genes (including E2F-1 itself) [58] thus verifying that PARP-1 acts as a positive co-activator of E2F-1-mediated transcription. That PARP-1 neither binds internal sequences of the E2F-1 promoter nor modifies E2F-1 by poly(ADP-ribosyl)ation is consistent with the fact that PARP-1-E2F-1 binding does not require the DNA-binding domain or the catalytic active site of PARP-1. PARP-1 also enhances the transactivation of B-Myb independently of PARP-1 enzymatic activity [33]. Given that binding sites for E2F-1 are also present in b-myb promoters [59], it is possible that, in addition to direct binding of PARP-1 to B-Myb, PARP-1 can induce b-myb transcription by its coactivation of E2F-1. In this case, PARP-1 does not play a direct role in the transcription of E2F-1-responsive genes by binding to internal E2F-1 promoter sequences and acting as a transcription factor itself, unlike its sequence-specific interaction with other DNA elements such as MCAT1 elements, the Reg gene promoter, the IUR element in the CXCL1 gene, and HTLV-1 TxREs [46, 60-62]. Within the CXCL1 promoter, the IUR element binds PARP-1 [61]. In normal melanocytes, PARP-1 activity is silent, leading to binding of PARP-1 to the promoter of CXCL1 and preventing NF-kB from binding to the promoter. However, in cancer cells exhibiting bioenergetic malfunction, this balance is shifted, resulting in more auto-poly(ADP-ribosylation) of PARP-1, dissociating PARP-1 from the promoter and allowing for an increased binding of NF-kB to the promoter and activated transcription. Here, aberrant activation of PARP-1 in melanoma cells regulates the transcriptional activity of NF-kB. Thus, it appears that the physical interaction of PARP-1 with the CXCL1 promoter asserts a negative effect in transcription, whereas the activity of PARP-1 is important in promotion of CXCL1 transcription. PARP-1 regulates CXCL1 gene expression both negatively and positively, once more having a dual role as a transcriptional modulator [63], where a fine balance exists between the inactive and active state of PARP-1. Moreover, the cell/tissue- and pathway-specific roles of PARP-1 in transcription have been clearly demonstrated [40].

PARP-1 has been suggested to act as a promoter-specific coactivator [65]. PARP-1 has been identified as an interaction partner not only of NF-κB but also of several sequence specific transcription factors and cofactors including Oct-1, and PC3/topoisomerase-I [65] and has been shown to increase the transcriptional activity of these transcription factors [65].

PARP-1 in the NF-κB, HIF and TCF-4/β-Catenin Pathways: Implications in Carcinogenesis

PARP-1 can act both as an inhibitor and activator of NF-κB-dependent transcription. In the context of NF-κB target gene transcriptional regulation, Chang and Alvarez-Gonzalez [64] reported that direct PARP-1 interaction with NF-κB inhibits the binding of NF-kB to its element and this inhibition is relieved by the auto-poly(ADP-ribosylation) of PARP-1. Several reports demonstrated that coactivator activity of PARP-1 for NF-κB-dependent gene expression seems to be dependent on the stimuli and cell type [65]. PARP-1 directly interacted with both subunits of NF-κB (p65 and p50) *in vitro* and *in vivo* [66]. Remarkably, neither the DNA binding nor the enzymatic activity of PARP-1 was required for full activation of NF-κB in response to various stimuli *in vivo* [66]. Note that PARP-1 is an important regulator of skin carcinogenesis and this is due, at least in part, to its ability to modulate the response through NF-κB [41]. In addition, PARP-1 directly interacted with p300/CBP and synergistically coactivated NF-κB-dependent transcription [51]. NF-κB-dependent trans-activation of PARP-1-dependent promoters not only requires the enzymatic activity of the co-activator of NF-κB p300/CBP but also that PARP-1 itself is acetylated *in vivo* in response to inflammatory stimuli. However, the exact molecular mechanism by which acetylation of PARP-1 regulates the co-activator activity of PARP-1 in the context of chromatin remains to be investigated. Acetylation of PARP-1 is required for the interaction with p50 and the transcriptional activation of NF-κB in response to inflammatory stimuli. Acetylation of PARP-1 might be mainly required for the NF-κB-dependent promoter activity. However, it remains to be further investigated whether acetylation of PARP-1 could also strongly influence other sequence-specific transcription factors or cofactors under certain conditions. The synergistic coactivation of PARP-1, p300/CBP, and also the Mediator complex was dependent on acetylation of PARP-1. Based on the multistep interaction model of transcriptional activation proposed by Malik *et al*. [67], PC1/PARP-1 might facilitate together with other structural/architectural positive cofactors the co-operative interactions between sequence-specific activators and different co-activator complexes such as p300/CBP and Mediator, thereby providing an architectural function in stabilizing the pre-initiation complex [65].

PARP-1 interacts *in vivo* with the Mediator complex under physiological conditions and directly interacted *in vitro* with the Mediator subunits MED14 and CDK8 as well as the TFIIF subunit RAP74 but not with TFIIB, TATAbox-binding protein (TBP) and the tested TBP-associated factors. PARP-1 might only function during assembly of the pre-initiation complex [49]. Whether acetylation of PARP-1 might regulate the PARP-1 activity at this level in the context of chromatin remains to be investigated. However, it seems unlikely since the Mediator subunits DRIP150 and CDK8 did not bind to the acetylated domain in PARP-1. Acetylation of PARP-1 might be mechanistically required for the stabilization of preformed PARP-1 containing transcriptional coactivator-cofactor complexes and this modification is important for its role as transcriptional coactivator and the different physiological functions of PARP-1 might be in general regulated by post-translational modifications in a stimulus-dependent manner [68].

Expression and stabilization of Hipoxia Inducible Factor-alpha (HIF-α) differs drastically between wild type and *parp-1*-deficient cells and also in the presence of the PARP inhibitor DPQ [41]. In some types of cancer models it has been reported that PARP-1 deletion contribute to a defective activation of transcription factors that play a key role in tumor development such as NF-κB, AP-1 and HIF [41, 70]. In the case of skin carcinogenesis the decreased susceptibility of *parp*-1-/- mice could also be attributed to the reduced inflammatory/oxidative stress component in *parp*-1-/- mice. In the absence of PARP-1, the oxidative cell damage produced during the inflammatory response in the initial steps of skin neoplasia is prevented through the inactivation of key transcription factors involved in carcinogenesis [70] (Fig. (**4**)). Thus, inhibition or genetic elimination of PARP-1 interferes with the promotion of tumors of epithelial origin, in which inflammatory processes play a critical role [71]. Finally, another example of PARP-1 as transcriptional cofactor is TCF-4/β-catenin-evoked gene transactivation. PARP-1 physically interacts with the transcription factor TCF-4 and augments its transcriptional activity evoked by oncogenic β-catenin, participating in the

transcriptional regulation of target genes. In addition, PARP-1 may be indirectly regulated by the TCF-4/β-catenin complex establishing a positive feedback loop that enhances PARP-1 expression [69].

These findings together with the fact that monotherapy with PARP inhibitors is effective in BRCA-1 -/- and BRCA-2 -/- cancer cells and tumors (due to the inability of these cells to repair by homologous recombination the stalled replication fork damages induced by PARP inhibitors) [72, 73] and the radio and chemopotentiation with the use of PARP inhibitors (Fig. (**4**)), place the PARP's field in the cutting edge in the pre-clinical advances in cancer treatment.

PARP-1 IN INFLAMMATION, OXIDATIVE STRESS AND CELL DEATH

It has been demonstrated in the last years that PARP-1 might play a significant role in the regulation of the inflammatory response. A considerable number of studies on either PARP-1 deficient mice or PARP inhibitors have revealed that the inactivation of PARP-1 improves the outcome of a variety of patho-physiological conditions associated with an exacerbated tissue or systemic inflammation. Different mechanisms have been proposed to explain the role of PARP-1 in the inflammatory response.

PARP-1 in Inflammatory Diseases

Inflammation is the first response of the immune system to infection, irritation or other injury, which occurs as defensive response. Inflammation is characterised by the immediate infiltration at the site of injury or infection with immune system components and is manifest by increased blood supply and vascular permeability which allows chemotactic peptides, neutrophils, and mononuclear cells to leave the intravascular compartment. Peroxynitrite is a labile, toxic oxidant species produced from the reaction of superoxide and nitric oxide (NO) [74]. Peroxynitrite, as well as hydroxyl radical, are the key pathophysiologically relevant triggers of direct DNA single strand breakage [75]. Moreover, several studies have demonstrated that peroxynitrite produces mitochondrial injury as well as an increase in mitochondria-derived reactive oxygen species generation [76, 77]. Endogenous production of peroxynitrite and other oxidants by immunostimulated macrophages and neutrophils induce prolonged DNA damage in neighboring cell [78-80]. Likewise, in brain slices (upon activation of NMDA receptors that trigger for enhanced NO, superooxide and peroxynitrite production) and smooth muscle cells, led to oxidant species-mediated DNA single strand breakage and PARP related cell injury [81, 82]. A considerable number of studies have revealed a crucial role of PARP-1 in cell death after various inflammation processes like ischemia-reperfusion damage, haemorrhagic shock, septic shock, lung inflammation, diabetes mellitus and chronic inflammatory disorders such as arthritis and inflammatory bowel diseases (ulcerative colitis and Crohn's disease), diseases of the central nervous system, such as allergic encephalomyelitis and multiple sclerosis, uveitis, periodontal inflammation, meningitis, asthma and possibly in various forms of dermal inflammation [83]. Experimental evidence supports that Reactive Oxygen Species (peroxynitrite, hydrogen peroxide,

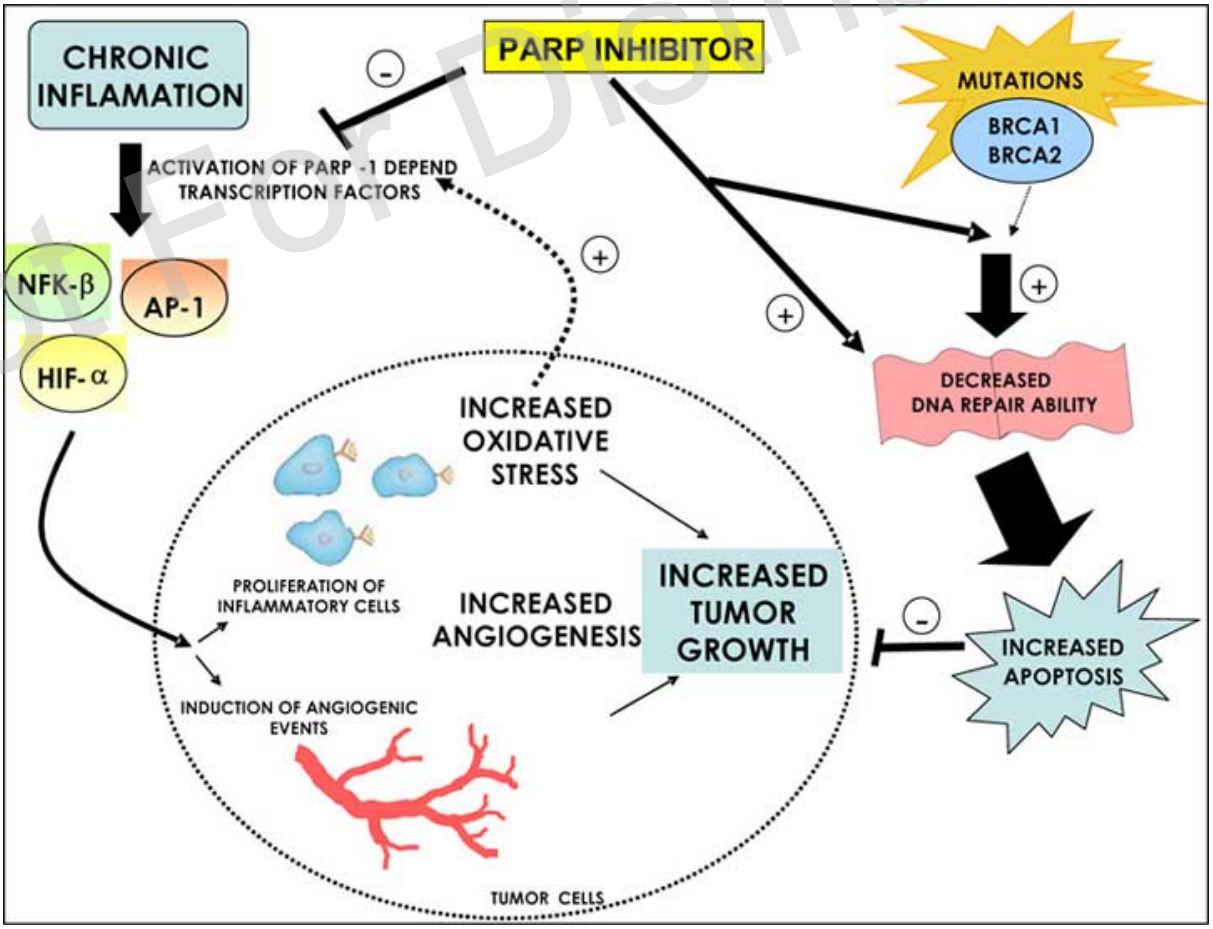

Fig. (4). Proposed model of action of PARP inhibitors in tumors. PARP inhibitors might interfere with tumor growth at different levels counteracting with the transcriptional activation of tumor-related transcription factors, angiogenesis process and in promotion of apoptosis in combination with chemotherapy, and by induction of cell death in homologous recombination deficient tumor cells (BRCA-1 and 2 deficient cells).

nitric oxide, etc) generated during inflammation response, induce DNA strand breakage and PARP activation [83, 84] (Fig. (**4**)). This activation significantly contributes to the pathophysiology of various forms of inflammation and its inactivation reduced the onset and progression of these illnesses. As will be outline below, different mechanisms have been proposed to explain that the inactivation of PARP-1 (either pharmacologically or using genetically engineered mice lacking PARP-1), improve the outcome of a variety of pathophysiological conditions associated with an exacerbated tissue or systemic inflammation [83].

Mechanisms to Explain the Role of PARP-1 in the Inflammatory Response

The first mechanism described is "The suicide hypothesis" in which Berger proposed that the excessive DNA damage induces massive PARP-1 activation that leads to a rapid depletion of NAD+ and ATP, to an irreversible cellular energy failure, to a drastic reduction of energy dependent processes and to necrotic-type cell death consequent to disruption of oxidative metabolism [84]. This suicide model gained new support in the mid-1990s because after an inflammatory stress (LPS, ischemia-reperfusion injury, etc), different cell types, including macrophages and endothelial cells, activate a massive synthesis of nitric oxide (NO), which is in turn converted into a genotoxic derivative, peroxynitrite that generates single-strand ed DNA breaks and hyperactivation of PARP-1 and depletion of cellular NAD+ and ATP. Moreover, the peroxynitrite also induces mitochondrial free radical generation that produces more DNA damage and PARP-1 activation and finally causes cell necrosis [85, 86]. It has also been described the PARP-1 activation rapidly modulates the mitochondrial functioning and triggers mitochondrial dysfunction [87-89]. The level of PARP-1 activation has been considered as a molecular switch between necrosis versus apoptosis [90] and the NAD+ as a metabolic link between DNA damage and cell death [91]. The treatment with NADH or pyruvate blocks PARP-1-mediated cell death [92, 93]. The pathophysiological significance of PARP- 1 hyperactivation is well exemplified by the remarkable therapeutic efficacy of PARP-1 inhibitors in experimental models of disorders characterized by DNA damage such as ischemia, diabetes, shock, inflammation and cancer [36, 83] (Fig. (**4**)).

Recently a key observation on the mechanism by which PARP-1 activation and NAD+ consumption could lead, under overwhelming DNA damage, to cell death is the pathway that involves the mitochondrial release of apoptosis inducing factor (AIF) and cytochrome c, directly link to the massive synthesis of poly (ADP-ribose), and the activation of a caspase-independent cell death pathway [87, 94-97].

However, several lines of evidence suggest that under specific conditions the beneficial effects of PARP-1 inhibition are independent from the prevention of energy failure [98]. The suicide hypothesis, therefore, might be valid only in conditions of massive DNA rupture and intense PARP-1 activation.

PARP-1 may also influence the stress/inflammation response through regulation of transcription factors and associated gene transcription. PARP-1 has been reported either to activate or repress transcription activity [5]. Nuclear factor-κB (NF-κB)/Rel transcription factors play a central role in the regulation of genes involved in the immune and inflammatory response. NF-κB regulates the expression of TNF-α, iNOS, interleukins IL-1β, IL-2, IL-6 and IL-8 as well as the adhesion molecules ICAM-1 and E-selectin. As has been treated above, reports by different groups, including ours, have shown that PARP-1 inhibitor have minimal or no effect on NF-κB activation, while cells and mice lacking PARP-1 display a dramatic deficiency in this transcription factor activation [34, 36, 40, 41, 51] (Fig. (**4**)).

These interesting findings have implicated PARP-1 in upstream events of inflammatory signalling (Fig. (**4**)). PARP inhibitors may affect the signalling pathways and they might play a key role in an inflammatory model as well as a significant role in cell survival. Recent data showed that PARP inhibition-induced Akt activation is dominantly responsible for the cytoprotection in pathophysiological conditions associated with oxidative stress and inflammation [99, 100]. Furthermore, PARP inhibitors down-regulated two elements of the MAP kinase system, ERK 1/2 and p38 mitogen-activated protein (MAP) kinase but not JNK in a tissue-specific manner [40, 101]. However, the exact nature of the regulation of phosphatidylinositol 3-kinase-Akt/protein kinase B and MAP kinase by PARP-1 remains to be elucidated.

These beneficial effects of PARP inhibitors probably result from improvement of cellular energetic status leading to cell survival and from inhibition of signal transduction leading to suppressed expression of inflammatory mediators. The contribution of these two mechanisms to the effect of PARP inhibitors in various disease models may likely differ to a great extent.

The marked beneficial effect of PARP inhibitor in many animal models of various diseases suggests that they can be exploited to treat human inflammatory diseases. However, crucial safety experiments must be done, due to the fact that PARP has been involved in DNA repair and maintenance of genomic integrity and its log-term inactivation could increase the possible risk of mutation rate and cancer formation (Fig. (**4**)).

CONCLUSIONS

Here we have summarised several independent lines of evidence that are all supporting an involvement of members of the PARP-1 in transcription, carcinogenesis and inflammation (Fig. (**4**)). As genotoxic stress – mainly induced by ROS – is believed to be the major driving force for tissue damage and inflammation-related carcinogenesis, mechanisms that counteract it or reverse its consequences should be crucial for maintaining genetic integrity. The role of PARP and PAR in cellular physiology has greatly diverged in the last decade. For many years PARP (and more exactly, PARP-1) has been envisaged solely (and importantly) as a protein involved in detection and signalling of DNA damage. The number of PARP/PAR-associated cellular functions currently goes from DNA damage detection and repair to cell death pathways, telomeric function, transcription, chromatin structure, etc., with important consequences in the physiology and pathophysiology of processes such as the control of genome integrity, carcinogenesis, the inflammatory response and neuronal function. The next challenges for this exciting field have to address how these ubiquitous factors can have so many different functions, the insights of the PARPs activation to synthesize the polymer in the absence of DNA damage, the role of the polymer as a signalling molecule in the nucleus and cytosol, and a better understanding of cellular poly(ADP-ribosyl)ation duties of other PARP family members, may help to build up a theoretical body of knowledge that will improve the translational applications of PARP(s) as a therapeutic target.

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EDUCATIONAL SERIES Green Series*

Poly(ADP-ribose)polymerase-1 (PARP-1) in carcinogenesis: potential role of PARP inhibitors in cancer treatment

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Abstract Poly(ADP-ribose)polymerase-1 (PARP-1) is a nuclear, zinc-finger, deoxyribonucleic acid (DNA)-binding protein that detects specifically DNA strand breaks generated by different genotoxic agents. Whereas activation of PARP-1 by mild genotoxic stimuli facilitates DNA repair and cell survival, severe DNA damage triggers different pathways of cell death, including PARP-mediated cell death through the translocation of apoptosis inducing factor (AIF) from the mitochondria to the nucleus. Pharmacological inhibition or genetic ablation of PARP-1 results in a clear benefit in cancer treatment by different mechanisms, including selective killing of homologous recombination-deficient tumor cells, downregulation of tumor-related gene expression, and decrease in the apoptotic threshold in the cotreatment with chemo- and radiotherapy. We summarize in this review the findings and concepts for the role of PARP-1 and poly(ADP-ribosylation) in the regulation of carcinogenesis and some of the preclinical and clinical data available for these agents, together with the challenges facing the clinical development of these agents.

Keywords PARP-1 · Carcinogenesis · DNA repair · Antineoplasic therapy

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Introduction

PARP-1 in signaling from DNA damage and cellular injury

Poly(ADP-ribose)polymerase-1 (PARP-1) is a deoxyribonucleic acid (DNA) nick-sensor that signals the presence of DNA damage and facilitates DNA repair (as shown in Figs. 1 and 2). The polymerase catalyzes the addition of ADP-ribose units to DNA, histones and various DNA repair enzymes, which affects many different cellular processes as diverse as transcription, DNA replication, differentiation, gene regulation, protein degradation, and spindle maintenance. The first PARP enzyme was discovered more that 40 years ago [1], and is the prototype for a superfamily of 17 members [2]. PARP-1 is a nuclear protein whose zinc-finger DNA-binding domain localizes PARP-1 to the site of DNA damage. In knockout mouse models, deletion of PARP-1 impairs DNA repair but is not embryonically lethal [3]. The residual PARP activity [~10%] is due to PARP-2 [4]. Double PARP-1 and PARP-2 knockout mice die during early embryogenesis [5], suggesting the critical role for polymer synthesis in critical cellular functions beyond the well-established role in DNA repair.

PARP-1 has a highly conserved structural and functional organization (Fig. 1) including: (1) an N-terminal double zinc-finger DNA-binding domain (DBD), (2) a nuclear localization signal, (3) a central automodification domain, and (4) a C-terminal catalytic domain. PARP-1's basal enzymatic activity is very low but is stimulated dramatically in the presence of a variety of allosteric activators, including damaged DNA, some undamaged DNA structures, nucleosomes, and a variety of protein-binding partners. The targets of PARP-1's enzymatic activity include PARP-1 itself, which is the primary target *in vivo*, core histones, the

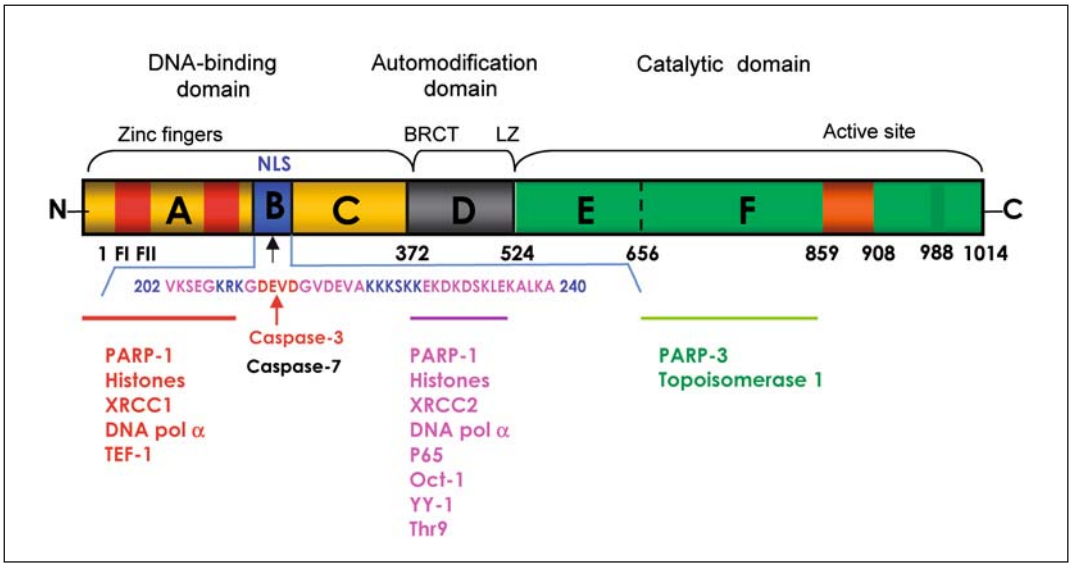

Fig. 1 Structural domains of Poly(ADP-ribose)polymerase-1 (PARP-1). The DNA-binding domain (DBD) at its N-terminus (*N*) from amino acids 1 to 372 contain two zinc-finger motifs and a bipartite nuclear localization signal (NLS). Between amino acids 372 and 524 of PARP-1, there is an automodification domain comprising a breast cancer (BRCA) C-terminus-like (*BRCT*) interaction domain, which mediates its interactions with protein partners. At its C-terminus (C) between amino acids 524 and 1014, PARP-1 has a catalytic domain, within which is contained its PARP signature motif

linker histone H1, and a variety of transcription-related factors that interact with PARP-1 [6].

The zinc-finger domain of PARP binds to single-strand DNA (ssDNA) breaks, whereas the N-terminus contains the nicotinamide adenine dinucleotide (NAD)-binding site responsible for the catalytic activity that cleaves NAD+ and attaches poly-ADP-ribose to the target protein, including PARP-1 itself (Fig. 1). This results in a highly negatively charged polymer, which in turn, leads to the unwinding and repair of the damaged DNA through the base excision-repair pathway. Overactivation of PARP results in NAD+ and energy stores depletion, leading to cellular demise by necrosis or antipoptosis inducing factor (AIF)-dependent cell death [7]. Consequently, multiple mechanisms exist to prevent overactivation of PARP. Auto-poly(ADP-ribosyl)ation negatively regulates PARP activity [8]. Degradation of these polymers is catalyzed by poly(ADP-ribose) glycohydrolase, which cleaves ribose–ribose bonds, and ADP-ribosyl protein lyase, which removes the protein proximal to the ADP-ribose monomer. Once the polymer is cleaved, PARP is released and inactivated, allowing it to bind another site of DNA damage. PARP-1 is also known to bind double-stranded DNA (dsDNA) breaks (DSBs). The enzyme activates several proteins involved in the homologous recombination repair and the nonhomologous end-joining pathways [9]. It is believed that PARP-1 is an antirecombinogenic factor that prevents accidental recombination of homologous DNA. PARP-1 has also been implicated in breast cancer 1 (BRCA1)- and BRCA2-dependent homologous recombination repair [10]. When PARP-1 is inhibited, single DNA breaks (SSBs) persist and result in stalled replication forks and DSBs. In BRCA1- and BRCA2-deficient cells, these lesions are not repaired through homologous recombination repair, which leads to cell-cycle arrest and apoptosis. Comparatively, less is known about the contribution of PARP-2 to DNA repair, although it seems that PARP-1 and PARP-2 both participate in overlapping DNA-damage-signaling processes and may partially compensate for one another [11].

Role in angiogenesis

PARP-1 has also been implicated in angiogenesis. Complementary DNA (cDNA) microarray analysis during skin carcinogenesis showed that PARP-1 modulates expression of genes involved in angiogenesis, such as Hif-1 alpha, Pecam-1, and OPN [12]. In particular, inhibition of PARP-1 seems to result in decreased activation of the hypoxia-inducible factor. Hypoxia-inducible factor is thought to regulate tumor-cell adaptation to hypoxia through transactivation of genes involved in glucose transport, glycolysis, erythropoiesis, angiogenesis, vasodilation, and respiratory rate, including vascular endothelial growth factor [13]. Decreased hypoxia-inducible factor-1 function in tumors may contribute to tumor cell death.

Development of PARP inhibitors

It has been claimed for decades that inhibitors of PARP activity have implications in the treatment of many diseases, including cancer, cardiac infarct, stroke, and diabetes [14].

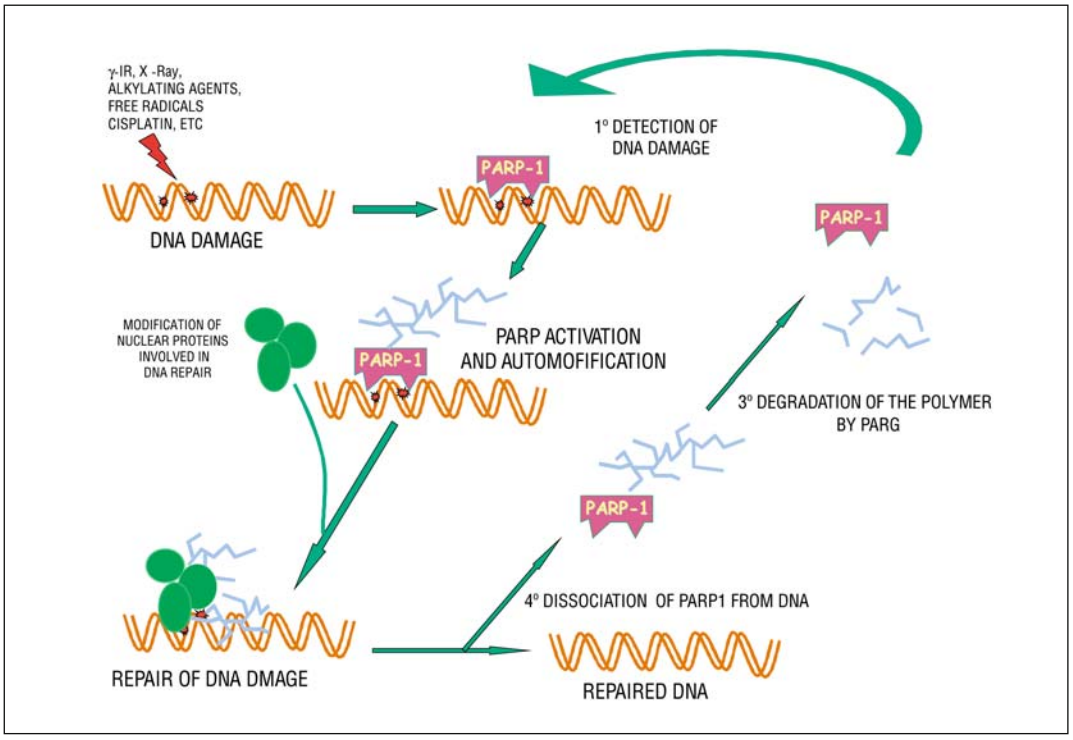

Fig. 2 Mechanism of activation and regulation of poly(ADP-ribose)polymerase-1 (PARP-1). (*1*) PARP-1 binds to damaged DNA and activates polymer synthesis; (*2*) once it is activated, PARP-1 produces the modification of different acceptors proteins (including histones, p53, ATM, base excision-repair proteins, and different transcription factors) and its automodification; (*3*) automodification of PARP-1 renders this protein inactive and it loses its DNA-binding affinity, allowing entry of the repair complex; (*4*) the polymer is rapidly degraded by PARG action, and the cycle is completed, leaving PARP-1 ready for the next cycle
ATM: ataxia telangiectasia mutated; *PARG:* poly (ADP-ribose) glycohydrolase

PARP inhibitors first emerged in the 1980s as potential chemotherapeutic targets in cancer [15], showing an exquisite cytotoxicity on proliferating cells but only after treatment with genotoxic agents. As mentioned previously, PARP-1 and PARP-2 have a role in DNA repair, and PARP inhibitors can be used to suppress DNA repair and promote apoptosis in cells that are treated with certain anticancer agents.

Early studies on PARP inhibition used nicotinamide and 3-aminobenzamide and other substituted benzamides, which had specificity for PARP in the low micromolar range [16]. PARP inhibitors examined to date are competitive inhibitors of NAD+. The relatively low potency of these agents has led to development of more potent and specific PARP inhibitors. Optimization of PARP inhibitor structures to increase potency and specificity have been published by some of the leading industry groups. An excellent review of different chemical classes of PARP inhibitors has been written by Southan and Szabo [17].

PARP inhibition in cancer therapy: chemotherapeutics and radiation

Increased PARP activity is one mechanism by which tumor cells avoid apoptosis caused by DNA-damaging agents. Because PARP plays an important role in the repair of DNA strand breaks, including those induced by radiation and chemotherapeutic drugs, inhibitors of this enzyme have potential to improve cancer chemotherapy or radiotherapy [17, 18].

A number of PARP inhibitors have been shown to enhance radiation-induced cell killing, the effect of which was particularly pronounced when present in the postirradiation period [19]. Earlier, commercially available PARP inhibitors, such as 3-aminobenzamide or nicotinamide, are characterized by incomplete inhibition of PARP activity and, moreover, exhibit various nonspecific effects [20], and as such they have limited utility as radiosensitizers. PARP-1 and 2 are essential for the repair of SSBs (through the base excision-repair pathways [21]. Inhibition of PARP sensitizes tumor cells to cytotoxic therapy [22], which induces DNA damage that would normally be repaired through the base excision-repair system.

As stated before, an intriguing area of use of PARP-1 inhibitors is in the potentiation of radiotherapy. Treatment with ionizing radiation is the most widely used anticancer intervention after surgery. Radiotherapy damages cells by causing both SSB and DSB and inducing apoptotic cell death. DNA repair mechanisms within cancer cells attempt to minimize this damage and are therefore a cause of re-

sistance. Once again, there are causes of resistance. Tumor cells have shown increased sensitivity to gamma- and X-radiation in the presence of PARP inhibitors [23–26]. Radiosensitization by PARP inhibition seems to have greater effect on cells in the S and G2 phases of the cell cycle, and noncycling cells exhibit minimal sensitivity [25, 26]. Although radiosensitization is partly due to the inhibition of SSB repair, it is likely that DSB repair, which may be more cytotoxic, is also affected. Although PARP activity seems important for repair of both SSBs and DSBs caused by ionizing radiation, it has been reported that a PARP-1-deficient cell line is resistant to the radiosensitization effects of PARP-1 inhibitors at low doses of radiation [24]. It is thought that, in this instance, PARP-2 compensates for the lack of PARP-1, whereas at high doses of radiation, and consequently greater DNA damage, PARP-2 is unable to do so. Other studies have also shown that the combination of DNA-dependent protein kinase inhibitors and PARP inhibitors have an additive effect on radiation-induced DNA damage [26, 27]. Both enzymes are involved in the binding and repair of DSBs, and the combination seems to prevent 90% of DSB repair.

Monotherapy in cancer

In recent years, exciting data have emerged suggesting that PARP inhibitors might be beneficial in cancer treatment as a single agent. This would certainly have an advantage in terms of toxicity, as one of the concerns of chemotherapy combination studies is that, as well as enhancing the anticancer effect, systemic PARP inhibition during exposure to a cytotoxic agent might increase myelosuppression and other organ toxicities. Germ-line mutations in BRCA1 and BRCA2 indicate high risk for breast, ovarian, and other cancers. Paired papers were published [28, 29] demonstrating hypersensitivity of both BRCA1 and BRCA2 homozygous mutant cell lines and xenografts to a PARP inhibitor alone. In these cells, PARP inhibition results in cell-cycle arrest and apoptosis. This suggests a role for PARP inhibitors as single agents in cancers exhibiting BRCA1 and BRCA2 mutations. A recent preclinical study has shown that BRCA2-deficient cells are sensitive to the PARP inhibitor NU1025. The proposed mechanism of action for this effect is that inhibition of PARP induces spontaneous SSBs, collapses replication forks, and triggers homologous recombination (HR) for repair. However, in cells with background deficiency in HR (e.g., BRCA2-deficient cells) PARP inhibitor induces cell death, as the collapsed replication forks are not repaired. This new approach offers an exciting possibility of selectively targeting tumors that are BRCA2-/- in BRCA2± patients [29]. These findings were questioned when a study using the human Caucasian, pancreas, adenocarcinoma-1 (CAPAN-1) pancreatic cancer cell line carrying a loss-of-function BRCA2 mutation did not seem to be sensitive to the older generation of PARP inhibitors, 3-aminobenzamide and NU1025 [30]. However, McCabe et al. [31], after evaluating CAPAN-1 with the more potent inhibitor KU0058948, suggested that the sensitivity of BRCA1- and BRCA2-deficient cells to PARP inhibition seems to be dependent on the potency and/or specificity of the PARP inhibitor. Recent studies from the Ashwoth group have identified the mutations in BRCA2 gene responsible from PARP inhibition sensitivity in the CAPAN-1 cell line [32] and new gene target mediating lethal sensitivity to PARP inhibition [33].

These results suggest that the concept of synthetic lethality, in which two pathway defects that alone are innocuous but combined become lethal, may be a relevant approach to inhibiting DNA repair in tumors. PARP inhibitors may be more effective in patients with tumors with specific DNA repair defects, such as BRCA mutations, or with other therapeutics that inhibit other DNA repair pathways.

PARP inhibition in clinical trials

After three generations of inhibitors, increased potency and suitable pharmacokinetic properties have allowed preclinical trials in animal models to evaluate the benefit of these inhibitors in different pathologies, such as cancer and inflammation. From these data, different pharmaceutical companies are conducting human clinical trials with PARP inhibitor (Table 1). However, these inhibitors target the catalytic site of PARP enzymes, which is highly similar among different PARP family members, and no isoform-specific PARP inhibitors are yet available [34]. A few compounds (KU0058684 and KU0058948) have been shown to be specific inhibitors of PARP-1 and PARP-2 and exhibit between one and three orders of magnitude selectivity in comparison with other PARP members, such as PARP-3, vault PARP, and tankyrase [28]. It is important to mention here that putative PARP-2-selective inhibitors have already been identified in yeast-growth-inhibition studies [35]. Likewise, the additional loop identified in the crystal structure of the catalytic fragment of PARP-2 compared with that of PARP-1 will inform the design of specific PARP-2 drugs [36]. In malignant melanoma, disruption of PARP pathways by inhibitors of PARP, and thus inhibition of base excision repair, has been shown to potentiate the cytotoxicity of DNA-damaging agents such as temozolomide [37]. PARP induces resistance to chemotherapy drugs such temozolomide by repairing the DNA damage and tumor-killing effects caused by chemotherapy drugs. There is evidence that by blocking this protein, cancer cells may be more sensitive to chemotherapy, and this may improve its effects on tumors. There are several candidates of PARP inhibitors, all of which have entered preclinical or very early clinical testing. A phase I dose-escalating study using he PARP inhibitor AG-014699 in combination with temozolomide demonstrated that a full dose of temozolomide could

Table 1 Poly(ADP-ribose)polymerase-1 (PARP-1) inhibitors in cancer therapy

Agent	Company	Single/combination therapy	Toxicity doses	Route of administration	Disease status	Clinical
AG014699	Pfizer (New York, NY)	Single agent/combination with temozolomide	No dose-limiting toxicities	IV	Locally advanced or metastatic breast cancer and advanced ovary cancers in known carriers of a BRCA1 or BRCA2 mutation, malignant melanoma	Phase 1 in solid tumors complete, phase 2 in melanoma complete
KU59436	AstraZeneca/KuDOS (London, United Kingdom)	Single	Minimal toxicity	Oral	Advanced solid tumors Women with advanced breast cancer	Phase 1 Phase 2
ABT-888	Abbott Laboratories (North Chicago, IL)	Single	Was toxic to both oxic and hypoxic cells	Oral	Refractory solid tumors and lymphoid malignancies	Phase 0 ongoing
BSI-201	BiPar (Brisbane, CA)	Single		IV	Advanced solid tumors	Phase 1 ongoing
INO-1001	Inotek/Genentech (Beverly, MA)	Combination with temozolomide, single		IV	Metastatic melanoma, glioblastoma Acute ST-segment elevation myocardial infarction	Phase 1 Phase 2
GPI 21016	MGI Pharma (Bloomington, MN)	Combination with temozolomide		Oral	Solid tumors	Phase 1 planned

be given in the presence of AG-014699 [38]. Phase II studies are ongoing. A phase I trial coordinated at the MD Anderson Cancer Center evaluating the intravenous PARP inhibitor INO-1001 plus oral temozolomide in terms of tolerability, safety, and pharmacokinetics in 18 patients with newly diagnosed or recurrent unresectable stage III and IV melanoma is ongoing.

Conclusions

Pharmacological inhibition of key proteins involved in the response to DNA damage has emerged as an effective tool for cancer treatment, as the resistance of cancer cells to DNA-damaging agents originates from the modulation of DNA repair pathways. PARP has important prosurvival and protective functions in terms of DNA repair. A multitude of novel pharmacological inhibitors of PARP has entered clinical testing either as adjunct antitumor therapeutics or as monotherapy in familiar breast and ovarian cancer. As with all new therapeutic areas, the usefulness of this target is unproven, but the potential effect of this class of agents is large. Whereas the clinical benefit of PARP inhibitors is being tested, additional new areas of research are also opening up in the preclinical front, which should eventually help in the consecution of new and more effective PARP inhibitors able to regulate the fine tuning of this pathway, including the specific inhibition of different PARP members, the association of PARP-1 in different complexes, and the activation/inactivation of poly(ADP-glycohydrolase).

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PARP-1 Modulates Deferoxamine-Induced HIF-1α Accumulation Through the Regulation of Nitric Oxide and Oxidative Stress

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Abstract Poly(ADP-ribose) polymerase-1 (PARP-1) is a nuclear protein that, once activated by genotoxic agents, modulates the activity of several nuclear proteins including itself. Previous studies have established that PARP-1 inhibition may provide benefit in the treatment of different diseases, particularly those involving a hypoxic situation, in which an increased oxidative and nitrosative stress occurs. One of the most important transcription factors involved in the response to the hypoxic situation is the hypoxia-inducible factor-1 (HIF-1). The activity of HIF-1 is determined by the accumulation of its α subunit which is regulated, in part, by oxidative stress (ROS) and nitric oxide (NO), both of them highly dependent on PARP-1. Besides, HIF-1α can be induced by iron chelators such as deferoxamine (DFO). In this sense, the therapeutical use of DFO to strengthen the post-hypoxic response has recently been proposed. Taking into account the increasing interest and potential clinical applications of PARP inhibition and DFO treatment, we have evaluated the impact of PARP-1 on HIF-1α accumulation induced by treatment with DFO. Our results show that, in DFO treated cells, PARP-1 gene deletion or inhibition decreases HIF-1α accumulation. This lower HIF-1α stabilization is parallel to a decreased inducible NO synthase induction and NO production, a higher response of some antioxidant enzymes (particularly glutathione peroxidase and glutathione reductase) and a lower ROS level. Taken together, these results suggest that the absence of PARP-1 modulates HIF-1 accumulation by reducing both NO and oxidative stress. J. Cell. Biochem. 104: 2248–2260, 2008.

Key words: PARP-1; HIF-1α; deferoxamine; nitric oxide; oxidative stress

Poly(ADP-ribose) polymerase-1 (PARP-1) is a nuclear, zinc-finger, DNA-binding protein that detects specifically DNA-strand breaks generated by different genotoxic agents, such as ROS and peroxynitrite [D'Amours et al., 1999]. Once activated, it modulates the activity of different nuclear proteins, including itself, by catalysing the attachment of ADP-ribose units. Previous studies have established that PARP-1 inhibition may provide benefit in the treatment of different diseases, particularly those involving a hypoxic situation such as ischemia or cancer, in which an increased oxidative and nitrosative stress occurs [Eliasson et al., 1997; Zingarelli et al., 1997; Bowes and Thiemermann, 1998; Ding et al., 2001; Martin-Oliva et al., 2006]. Although the molecular mechanisms underlying this protection are not completely known, it has been reported that PARP-1 genetic ablation or pharmacological inhibition decreases the oxidative and nitrosative stress associated with those pathological situations [Oliver et al., 1999; Zingarelli et al., 2003; Cuzzocrea, 2005; Siles et al., 2005].

One of the most important transcription factors involved in the physiological responses to hypoxia is HIF-1, a heterodimeric DNA-binding complex composed of α and β subunits [Wang et al., 1995]. HIF-1β is constitutively expressed, so that HIF-1 activity depends on HIF-1α subunit level. In normoxic conditions, and with the presence of iron, HIF-1α is

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hydroxylated by HIF-1 prolyl hydroxylases (PHD), ubiquitinated by the von Hippel–Lindau protein and rapidly degraded by the proteosome [Epstein et al., 2001; Metzen et al., 2003a]. However, under hypoxic conditions or iron chelation, HIF-1α hydroxylation does not take place, its level is up-regulated, and consequently it can dimerize with the β subunit, creating the functional complex.

HIF-1α accumulation can be modulated by certain factors, such as oxidative and nitrosative stress, although their exact implication is controversial and probably linked [Kohl et al., 2006]. In this sense some authors have proposed that nitric oxide (NO) blocks HIF-1α stabilization [Sogawa et al., 1998; Agani et al., 2002; Hagen et al., 2003] while others contend that it causes HIF-1α accumulation [Brune and Zhou, 2003; Metzen et al., 2003b]. Similarly, ROS formation has also been linked to both HIF-1α induction and destabilization [Fandrey et al., 1994; Huang et al., 1996; Chandel et al., 2000; Kietzmann et al., 2000; Yang et al., 2003; Callapina et al., 2005a].

Iron chelators, such as deferoxamine (DFO), are widely used in the literature with two main different purposes. On the one hand, they are used as antioxidants as they prevent the iron from redox cycling and thereby inhibit hydroxyl formation by the Fenton or Haber–Weiss reaction [Williams et al., 1991; Saad et al., 2001]. However, and surprisingly, recent research has established that in certain situations DFO may also increase the oxidative status by decreasing the GSH cellular level [Seo et al., 2006] or inducing reactive oxygen species (ROS) production [Cadenas and Davies, 2000]. On the other hand, iron chelators are also used as a hypoxic-mimetic agent as they have been shown to mimic the effect of oxygen deprivation by inducing a number of hypoxia-response genes [Gleadle and Ratcliffe, 1998; Bianchi et al., 1999; Zhu and Bunn, 1999]. Based on this background, some authors have proposed the therapeutical use of DFO to ameliorate the hypoxic damage or to strengthen the post-hypoxic response [Palmer et al., 1994; Hurn et al., 1995; Groenendaal et al., 2000; Mu et al., 2005; Freret et al., 2006].

Taking into account the involvement of both NO and ROS in the activation of HIF-1α, and considering that both parameters are regulated by PARP-1 (both by the protein and the activity), we propose to evaluate the impact of PARP-1 on HIF-1α accumulation by DFO treatment.

MATERIALS AND METHODS

Cell Culture and Treatments

Immortalized murine embryonic fibroblasts (MEFs) and primary MEFs derived from day-13.5 embryos, expressing or lacking PARP-1 (*parp-1*$^{+/+}$ *and parp-1*$^{-/-}$), were grown in 10% foetal bovine serum supplemented Dulbecco's modified Eagle's medium (FBS–DMEM, Sigma, St. Louis, MO, USA) and incubated at 37°C in a humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2. Fibroblasts were treated for different periods of time with the iron chelator deferoxamine DFO at a subcytotoxic dose (200 μM). The PARP inhibitor 3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone (DPQ; Alexis Biochemicals, San Diego, CA; 40 μM), dissolved in culture medium immediately before use, was employed to corroborate the results obtained in *parp-1* knockout cells. DPQ solutions also contained <1% DMSO to improve solubility as it is sparingly soluble in water. When used, DPQ was added simultaneously to DFO and thereafter present in the culture throughout the experiment.

Western Blot

For Western blot analysis, equal amounts of denatured total-protein extracts were loaded and separated in 7.5% or 12% SDS-polyacrylamide gel (HIF-1α and Mn-SOD, respectively). Proteins in the gel were transferred to a PVDF membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) and then blocked. Polyclonal antibodies to HIF-1α (1/1000, Bethyl Lab, Inc., Montgomery, TX) and Mn-SOD (1/8000, StressGen Biotechnologies Corp., Victoria B.C., Canada) and a monoclonal antibody to α-tubulin (Sigma, St. Louis, MO, USA), as internal control, were used for detection of the respective proteins. Antibody reaction was revealed with chemiluminescence detection procedures according to the manufacturer's recommendations (ECL kit, Amersham Corp., Buckinghamshire, UK).

Real-Time RT-PCR

Gene expression of adrenomedullin (AM) was quantitatively assessed by real-time PCR using β-actin as the normalizing gene. Total RNA was isolated from cell extracts using TRIzol reagent

(Invitrogen) according to the manufacturer's instructions. After treating with DNase, cDNA was synthesized from 1.5 μg total RNA using reverse transcriptase (SuperscriptTM III RT, Invitrogen) with oligo-(dT) 15 primers (Promega). Real-time PCR was performed on the Stratagene MxPro 3005P qPCR system using the DyNAmo HS SYBR Green qPCR kit (Finnzymes, Espoo, Finland). Nucleotide sequences of the primers were as follows: 5′-CAGCAATCAGAGCGAAGC-3′ (forward) and 5′-ATGCCGTCCTTGTCTTTGTC-3′ (reverse) for AM; 5′-TGAGGAGCACCCTGTGCT-3′ (forward) and 5′-CCAGAGGCATACAGGGAC-3′ (reverse) for β-actin. Experiments were performed with triplicates, and the values were used to calculate the ratio of AM to β-actin, with a value of 1 used as the control.

PARP Activity Assay

PARP activity after DFO treatment was assayed, in wild-type cells, using a colorimetric kit according to the manufacturer's instructions (Universal Colorimetric PARP Assay Kit with Histone-Coated Strip Wells, Trevigen).

NO Measurement

Nitric oxide (NO) production was indirectly quantified by determining nitrate/nitrite and *S*-nitroso compounds (NO_x), using an ozone chemiluminescence-based method. For an estimation of the NO_x level, at the end of each treatment time, cells were collected and lysed by three freeze–thaw cycles. After centrifugation at 14,000*g* for 30 min, supernatants were collected and protein was quantified [Bradford, 1976]. Samples were deproteinized in deproteinization solution (0.8N NaOH and 16% $ZnSO_4$). The total amount of NO_x in the deproteinized samples was determined by a modification [López-Ramos et al., 2005] of the procedure described by Braman and Hendrix [1989] using the purge system of Sievers Instruments, model NOA 280i. NO_x concentrations were calculated by comparison with standard solutions of sodium nitrate. Final NO_x values were referred to the total protein concentration in the initial extracts.

iNOS Confocal Microscopy

The iNOS expression was evaluated by confocal microscopy. Briefly, cells were grown on slides and treated for 24 h either with DFO (*parp-1*$^{+/+}$ and *parp-1*$^{-/-}$) or DFO+DPQ (*parp-1*$^{+/+}$). Afterwards, cells were washed three times in PBS, fixed in fresh cold 4% paraformaldehyde for 10 min, washed again with PBS, permeabilized with PBS/0.2% Triton X-100 for 5 min, and blocked with 5% bovine serum albumin. Cells were incubated o/n at 4°C with iNOS monoclonal antibody (1/100 in PBS/0.2% Triton X-100 and 1% bovine serum albumin; Transduction Lab., Lexington, KY) and then washed three times in PBS/0.2% Triton X-100. The secondary antibody, linked to the Cy2, was diluted 1/1000 in PBS/0.2% Triton X-100 and incubated for 2 h at room temperature in the dark. Finally cells were washed three times in PBS/0.2% Triton X-100 and stained with DRAF 5 (1/2500) for 15 min. After mounting, slides were coverslipped and stored in the dark at 4°C. Results were compared with those found in control cells.

Measurement of Intracellular Generation of ROS

Flow-cytometric analysis of the intracellular generation of ROS was performed using 2′, 7′-dichlorofluorescein diacetate (DCFH) as a probe. ROS in the cells oxidize DCFH, yielding highly fluorescent 2′,7′-dichlorofluorescein (DCF). Cells were cultured in six-well plates and treated with DFO (200 μM) for different experimental times. One hour before the end of the experiment, DCFH (2 μg/ml) was added. Once the incubation was finished, cells were harvested, washed, centrifuged, resuspended in DMEM medium, and analysed by flow cytometry (excitation at 504 nm and fluorescence detection at 530 nm). Fluorescence was analysed in viable cells characterized by forward scatter *versus* side scatter. Data were normalized to the control values.

ROS production was also evaluated by confocal microscopy. Briefly, cells were grown on slides and treated for 24 h either with DFO (*parp-1*$^{+/+}$ and *parp-1*$^{-/-}$) or DFO+DPQ (*parp-1*$^{+/+}$). One hour before the end of the experiment DCFH (2 μg/ml) was added. Afterwards, cells were washed three times in PBS, fixed in fresh cold 4% paraformaldehyde for 10 min, washed again with PBS and stained with DRAF 5 (1/2500) for 15 min. After mounting, slides were coverslipped and stored in the dark at 4°C. Results were compared with control.

Antioxidant Enzyme Assays

At the end of each incubation period, cells were collected, washed with cold PBS and lysed

for 20 min at 4°C in EBC buffer (20 mM Tris–HCl pH 8; 150 mM NaCl, 1 mM EDTA, 0.5% NP-40) with protease inhibitors. After centrifugation at 14,000*g* for 15 min at 4°C, supernatants were collected and protein was quantified [Bradford, 1976].

Glutathione transferase (GST) activity towards the 1-chloro-2,4-dinitrobenzene (CDNB) was measured spectrophotometrically, as described by Habig et al. [1974]. Catalase (CAT) activity was studied by monitoring the decomposition of H_2O_2 at 240 nm, according to the method described by Beers and Sizer [1952]. Glutathione reductase (GR) activity was measured by following the rate of NADPH oxidation at 340 nm [Carlberg and Mannervik, 1985]. Superoxide dismutase (SOD) activity was assayed by measuring the rate of inhibition of cytochrome *c* reduction by superoxide anions generated by a xanthine/xanthine oxidase system [Flohé and Ötting, 1984]. As a means of discriminating between Cu/Zn-SOD and Mn-SOD activities, the assay was additionally performed after incubation in the presence of KCN, which selectively inhibits Cu/Zn-SOD isoform. Glutathione peroxidase (GPX) activity was determined in a coupled assay with GR using H_2O_2 as a substrate [Flohé and Günzler, 1984].

Statistical Analysis

Data are expressed as means ± S.D. Statistical comparisons between the different experimental times of DFO treated *parp-1*$^{-/-}$ and *parp-1*$^{+/+}$ cells and their corresponding controls were made by one-way ANOVA with a *post hoc* Student's *t*-test, accepting $P < 0.05$ as the level of significance. The effect of PARP-1 was evaluated by a two-way ANOVA followed by a *post hoc* Student's *t*-test.

RESULTS

HIF-1α Stabilization is Modulated by PARP-1

To investigate the effect of PARP-1 on DFO-mediated HIF-1α stabilization, we monitored the amount of this transcription factor in wild-type and *parp-1*$^{-/-}$ immortalised MEFs. As shown in Figure 1a, after 24 h of treatment HIF-1α was significantly more expressed in DFO-treated wild-type cells than in their counterpart *parp-1*$^{-/-}$. This result was corroborated in primary *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ MEFs (Fig. 1b). Moreover, the pharmacological inhibition of PARP in wild-type cells also decreased HIF-1α induction after DFO treatment (Fig. 1c).

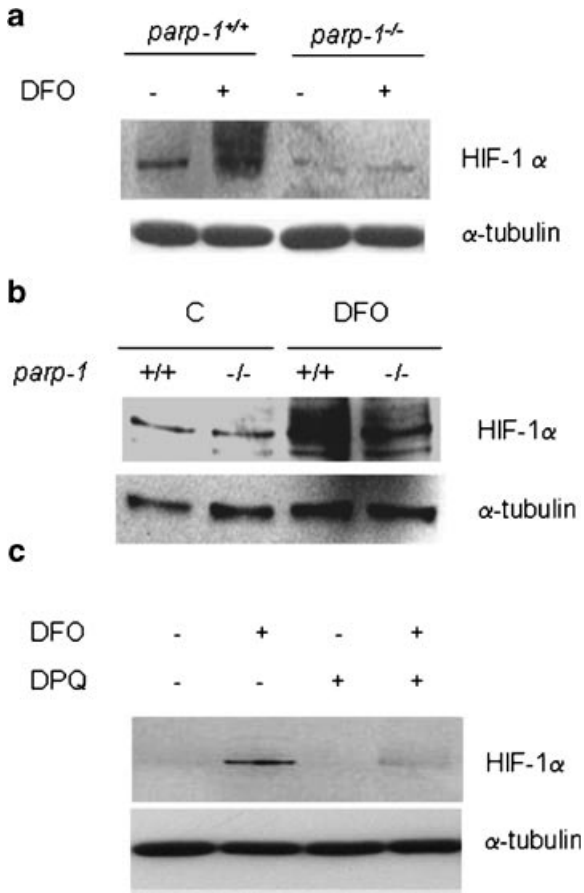

Fig. 1. Western blot analysis of the effect of PARP-1 on DFO-mediated HIF-1α stabilization after 24 h of treatment. Representative immunoblot of HIF-1α expression in control and DFO-treated wild-type and *parp-1*$^{-/-}$ immortalised **(a)** or primary **(b)** MEFs. **(c)** Representative Western blot of HIF-1α expression in control and DFO-treated wild-type cells in the presence or absence of the PARP inhibitor DPQ. α-Tubulin immunodetection was also included as a protein-loading control.

To analyze HIF-1 activation in response to DFO we observed the transcription of the typical HIF-1 target gene AM [Garayoa et al., 2000]. The real-time PCR results (Fig. 2) show that the expression of AM is greatly influenced by the presence of PARP-1. Particularly, after 24 h of DFO treatment AM mRNA levels were significantly higher in *parp-1*$^{+/+}$ cells.

Altogether, these results suggest a role of PARP-1 in HIF-1α accumulation and activity.

The Absence of PARP-1 Promotes an Altered NO Response

NO is a free radical reportedly involved in HIF-1α stabilization. In our experimental model, the absence of PARP-1 induced altered NO production, which could be linked to the

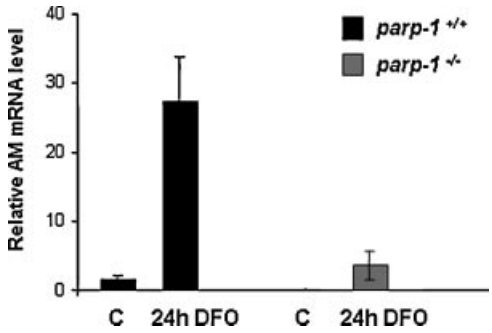

Fig. 2. Effect of DFO treatment on AM mRNA expression in *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ cells. The results are expressed as mRNA expression relative to control wild-type cells after normalization against beta-actin. Each sample was analysed in triplicate. The mean ± SD of three RNA extracts for each experimental condition is represented.

differential HIF-1α accumulation previously observed in *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ *cells*.

As shown in Figure 3a, basal NO_x level in control cells was significantly decreased in *parp-1*$^{-/-}$ cells ($P < 0.001$). Moreover, the absence of PARP-1 promoted a different NO response to DFO ($P < 0.02$). Particularly, in wild-type cells the treatment induced an initial decrease in the NO_x level, which was followed by the recovery of the basal level (12 and 16 h) and a sharp increase ($P < 0.001$) after 24 h of treatment. In the absence of PARP-1, this rise also occurred, but was considerably lower than in *parp-1*$^{+/+}$ cells.

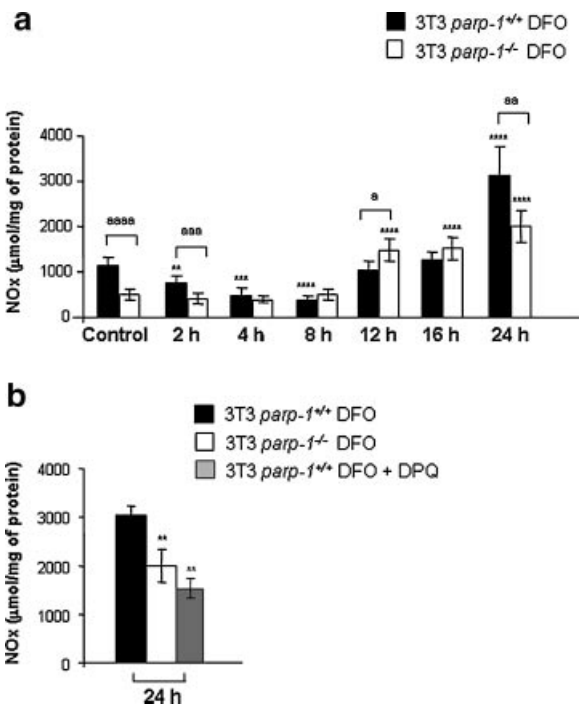

Fig. 3. **a**: Effect of DFO treatment on NOx levels (µmol/mg of protein) in *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ cells. **b**: NOx levels in *parp-1*$^{+/+}$, *parp-1*$^{-/-}$ and PARP inhibited cells (DPQ) after 24 h of DFO treatment. Values represent the mean ± SD from three independent experiments. Statistically significant differences from the corresponding control group: $^{**}p < 0.02$; $^{***}p < 0.01$ and $^{****}p < 0.001$. Statistically significant differences between *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ cells: $^{a}p < 0.05$, $^{aa}p < 0.02$, $^{aaa}p < 0.01$, and $^{aaaa}p < 0.001$.

To confirm the involvement of PARP-1 on NO production, we cotreated wild-type cells with DFO and with the PARP inhibitor DPQ for 24 h. Our results (Fig. 3b) showed that the NO peak detected in wild-type cells after a 24 h treatment was significantly lowered by inhibiting PARP ($P < 0.02$). Moreover, the NO production in PARP inhibited cells reproduced that found in *parp-1*$^{-/-}$ cells. These results strongly suggest that PARP-1 is related to the final NO burst observed in wild-type cells.

DFO-Induced iNOS Expression is Decreased in PARP-1 Null Cells and after PARP Inhibition

Although NO can be generated by different mechanism, the sharp increase of NO detected in wild-type cells after 24 h of DFO treatment seemed to indicate the implication of the iNOS isoform. Consequently we analysed, after this time, the iNOS expression by confocal microscopy in both *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ cell lines and in PARP inhibited cells. As reflected in Figure 4, iNOS level was considerably higher in wild-type cells and the inhibition of PARP mimicked the expression observed in *parp-1* knock-out cells.

DFO Induces ROS Production only in the Presence of PARP-1

Oxidative stress seems to be one of the factors involved in HIF-1α stabilization. Moreover, it is known that PARP-1 presence induces a higher oxidative status in cells [Groenendaal et al., 2000; Zhou et al., 2006]. To test whether, in our experimental model, PARP-1 affected the oxidative status, we next analysed ROS levels before and after DFO treatment in wild-type and *parp-1* knock-out cells (Fig. 5). The results (Fig. 5a) demonstrate that ROS production significantly differed in the two cell lines ($P < 0.001$) after treatment. In fact, although a progressive increase in ROS production, which became significant after 12 h ($P < 0.05$), was observed in wild type cells, no changes were detected in the *parp-1*$^{-/-}$ ones. PARP-1 involvement in oxidative stress generation was confirmed by simultaneously treating

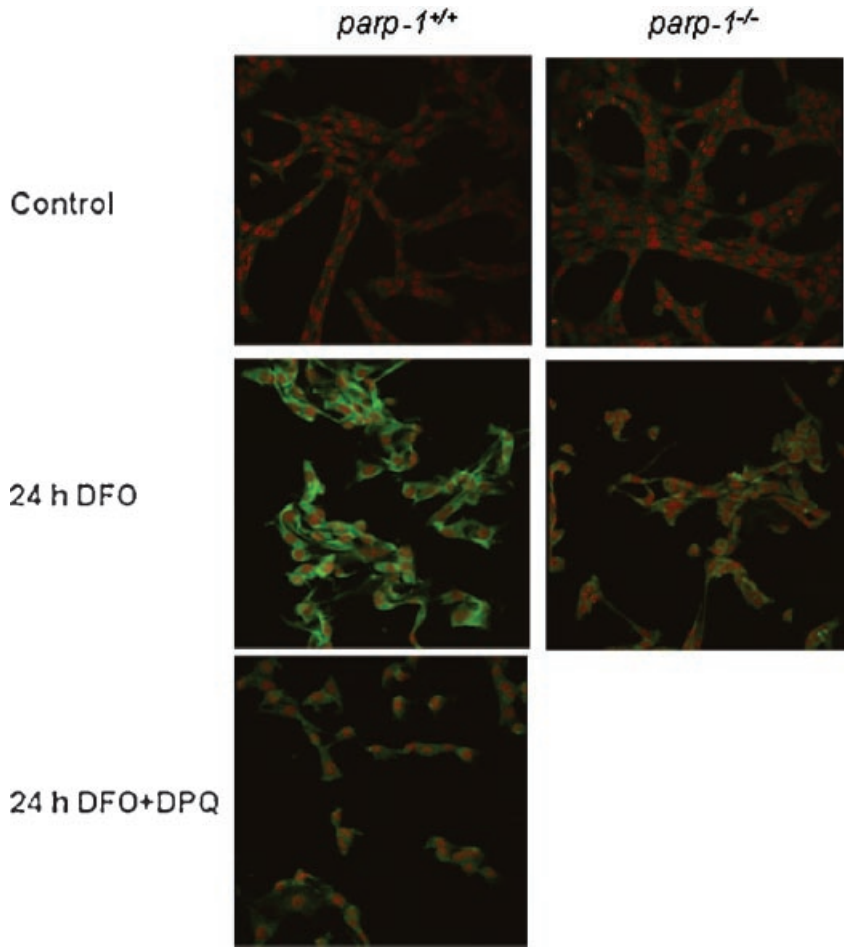

Fig. 4. Confocal immunofluorescence of iNOS in *parp-1*$^{+/+}$, DPQ-treated, and *parp-1*$^{-/-}$ cells after 24 h of DFO exposure. Control cells of the two different genotypes are also shown.

wild-type cells with DFO and DPQ for 24 h, the experimental time in which the oxidative stress was highest. As shown in Figure 5b, PARP-1 inhibition significantly prevented ROS production. These results by flow cytometry were confirmed by confocal microscopy (Fig. 6). Therefore, we conclude that PARP-1 involves higher oxidative stress after DFO treatment.

PARP-1 Activity is Induced after DFO Treatment

Genotoxic agents, such as ROS, induce PARP-1 activation [D'Amours et al., 1999]. Considering the effect of the presence of PARP-1 on ROS production, we evaluated whether DFO treatment induced PARP activity. We chose the 16 and 24 h experimental times in which a patent ROS increase had been previously observed only in wild-type cells. The results shown in Figure 7 revealed a significant PARP activation after DFO treatment ($P < 0.05$).

Mn-SOD is Differently Expressed in *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ Cells

ROS are produced mainly in mitochondria. Mn-SOD, due to its mitochondrial location, represents an important initial component in the cellular defence against ROS. In this context, we hypothesized that DFO treatment may induce a different Mn-SOD level in *parp-1*$^{+/+}$ *and parp-1*$^{-/-}$ cells. As shown in Figure 8, Mn-SOD expression was clearly higher in wild-type cells, although no changes were detected after treatment in any of the cell lines.

Parp-1 Abrogation Decreases the Activity of the Main Antioxidative Enzymes but Allows a Higher Antioxidant Response after the Treatment

Finally, to complete the overall view of the oxidative status, we tested the activity of

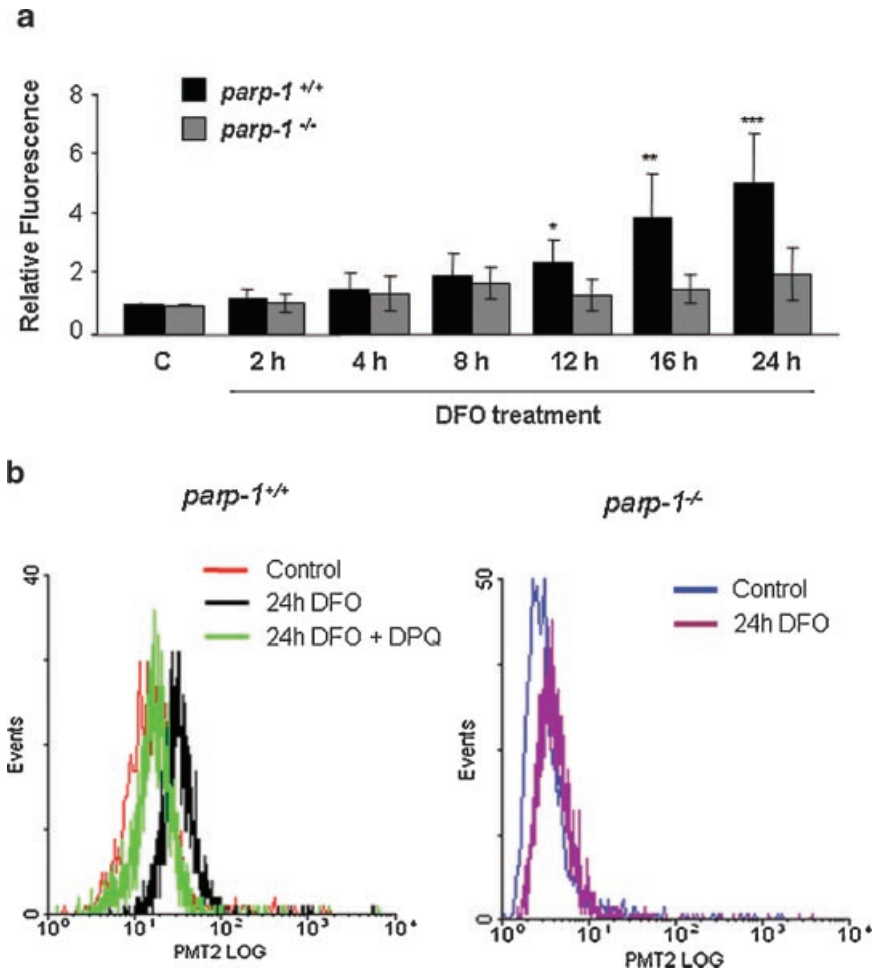

Fig. 5. **a**: Time course of ROS generation in DFO-treated cells. DCF fluorescence as a measurement of ROS production was determined by flow cytometry. Mean values of the log fluorescence for *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ cells at different times after DFO treatment. Results were normalized to those in control cells in the different genotypes. Values are given as the means ± SD from three independent experiments. Statistically significant differences from the corresponding control group: $^{*}p < 0.05$, $^{**}p < 0.02$ and $^{***}p < 0.01$. **b**: Effect of pharmacological PARP inhibition on ROS production after DFO treatment. The increased ROS level observed in *parp-1*$^{+/+}$ cells after a 24 h DFO incubation was reduced by co-treatment with DPQ. Representative traces displayed by flow cytometry in *parp-1*$^{+/+}$ cells, with and without DPQ, and in *parp-1*$^{-/-}$ cells after 24 h of DFO treatment are shown. Results for control cells are also displayed.

the main antioxidant enzymes (GST, CAT, GR, Mn-SOD, Cu/Zn-SOD, GPX) in the presence or absence of PARP-1 (Fig. 9). PARP-1 presence sharply boosted the basal activity of all the enzymes assayed. However, DFO treatment in wild-type cells promoted no further rise in any of the enzymatic activities analysed. Moreover, Cu/Zn-SOD activity even decreased after 16 and 24 h, in parallel with the augment in the ROS production previously described. On the contrary, in *parp-1*$^{-/-}$ cells, a significant increase was observed in Cu/Zn-SOD after 8 h and in Se-GPX and GR activity after 8, 12, and 16 h of DFO treatment.

DISCUSSION

DFO is an iron chelator which has been shown to ameliorate the damage induced by hypoxia-ischemia [Palmer et al., 1994; Hurn et al., 1995; Groenendaal et al., 2000; Mu et al., 2005; Freret et al., 2006]. One of the mechanisms proposed for its therapeutical use is the induction of HIF-1α accumulation, a transcription factor involved in the expression of several genes that facilitate the adaptation to hypoxia, in which PARP-1 activation appears to play a pivotal role. The activity of the transcription factor HIF-1 seems to be regulated by different factors

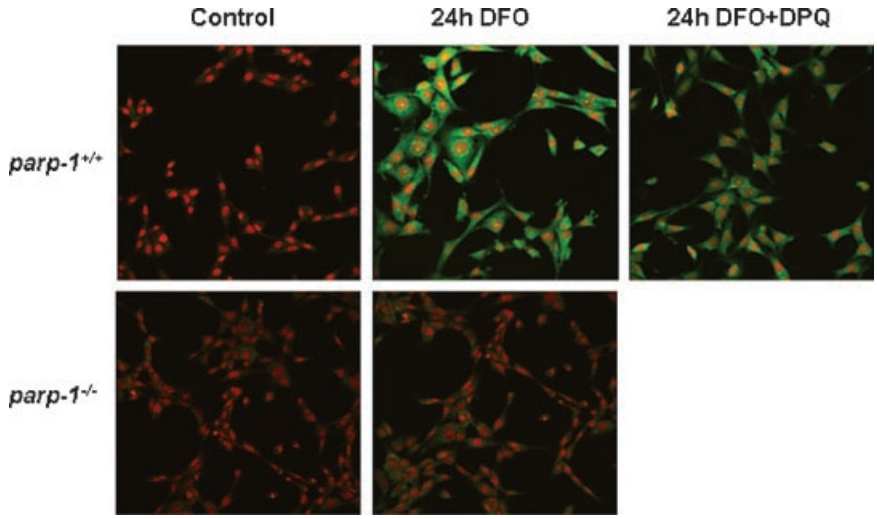

Fig. 6. Confocal immunofluorescence of DCF fluorescence in *parp-1*$^{+/+}$ cells, with and without DPQ, and in *parp-1*$^{-/-}$ cells after 24 h of DFO treatment. Photographs of cells without treatment are also shown.

such as ROS and NO, both related to PARP-1. Taking into account the increasing interest and potential clinical applications of PARP inhibition and DFO treatment, and considering the pathophysiological role of ROS and NO, we have analysed the effect of PARP-1 on HIF-1α accumulation induced by DFO.

Our results show that in DFO-treated cells, PARP-1 gene deletion or inhibition decreases HIF-1α accumulation. This deficient HIF-1α stabilization is parallel to a decreased oxidative stress, iNOS induction and NO production, suggesting that PARP-1 absence modulates HIF-1 accumulation by reducing both ROS and NO level (Fig. 10).

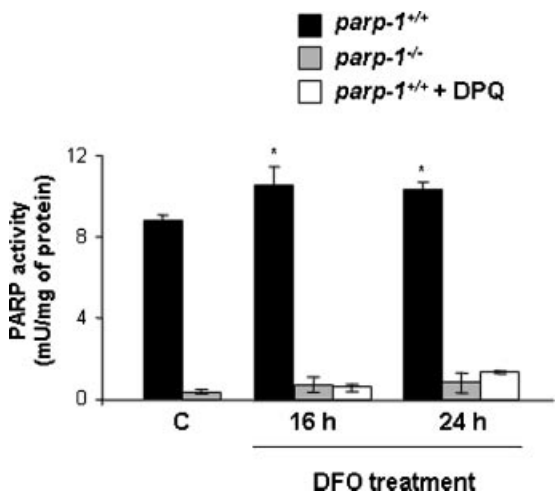

Fig. 7. PARP activity in control, 16 and 24 h DFO-treated *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ cells. Data obtained in DPQ+DFO (16 and 24 h) *parp-1*$^{+/+}$ cells are also included. Values represent mean ± SD from three independent experiments. Unit definition: 1 U PARP incorporates 100 pmol poly(ADP) from NAD^+ into acid-insoluble form in 1 min at 22°C. Statistically significant differences from the control group: $^*p < 0.05$.

Inhibition of PHD through iron chelation is recognized as the main mechanism responsible for the stabilization of HIF-1α after DFO treatment. However, ROS have also been proposed as signalling molecules which take part in HIF-1α regulation, and, vice versa, HIF-1α induction has also been associated with the protection of cells against certain forms of oxidative stress [Zaman et al., 1999; Siren et al., 2000; Digicaylioglu and Lipton, 2001]. Concerning the first point, some authors have proposed that ROS production decreases HIF-1α accumulation probably by restoring PHD activity, favouring HIF-1α degradation [Huang et al., 1996; Callapina et al., 2005a]. However, according to our results, others suggest that free radicals mediate HIF-1α accumulation [Chandel et al., 1998; Agani et al., 2000; Quintero et al., 2006]. In this sense, the results presented here show that a higher HIF-1α accumulation is observed only in those cells (*parp-1*$^{+/+}$) in which ROS production after DFO treatment steadily increases. Moreover the implication of PARP-1 in this process is corroborated by the results obtained in *parp-1* deleted cells or after PARP inhibition.

Oxidative stress can be generated by the rise in ROS formation, by the restricted/insufficient antioxidative defence mechanisms or by the combination of the two. The analysis of the main

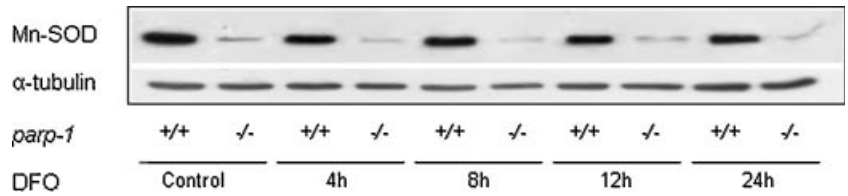

Fig. 8. Analysis of Mn-SOD expression in control and DFO-treated *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ cells. A representative immunoblot is shown. α-tubulin immunodetection was also included as a protein-loading control.

antioxidant enzymes revealed that the higher ROS level found in wild-type cells is concomitant to a higher antioxidant activity, suggesting a cellular adaptation to ROS. In addition, we have demonstrated that Mn-SOD, a mitochondrial enzyme which scavenges superoxide and converts it to H_2O_2, is significantly more expressed in wild-type cells. In this sense, it has been published that exposure to low ROS concentrations may act as a preconditioning factor which induces the enzymatic antioxidant system [Ravati et al., 2000]. In fact, the lower oxidative stress in cells with PARP-1 genetic deletion correlates with a lower, but quite efficient, activity of the main antioxidant enzymes. The role of DFO on oxidative stress is somewhat controversial, however its effect on the antioxidative enzymes

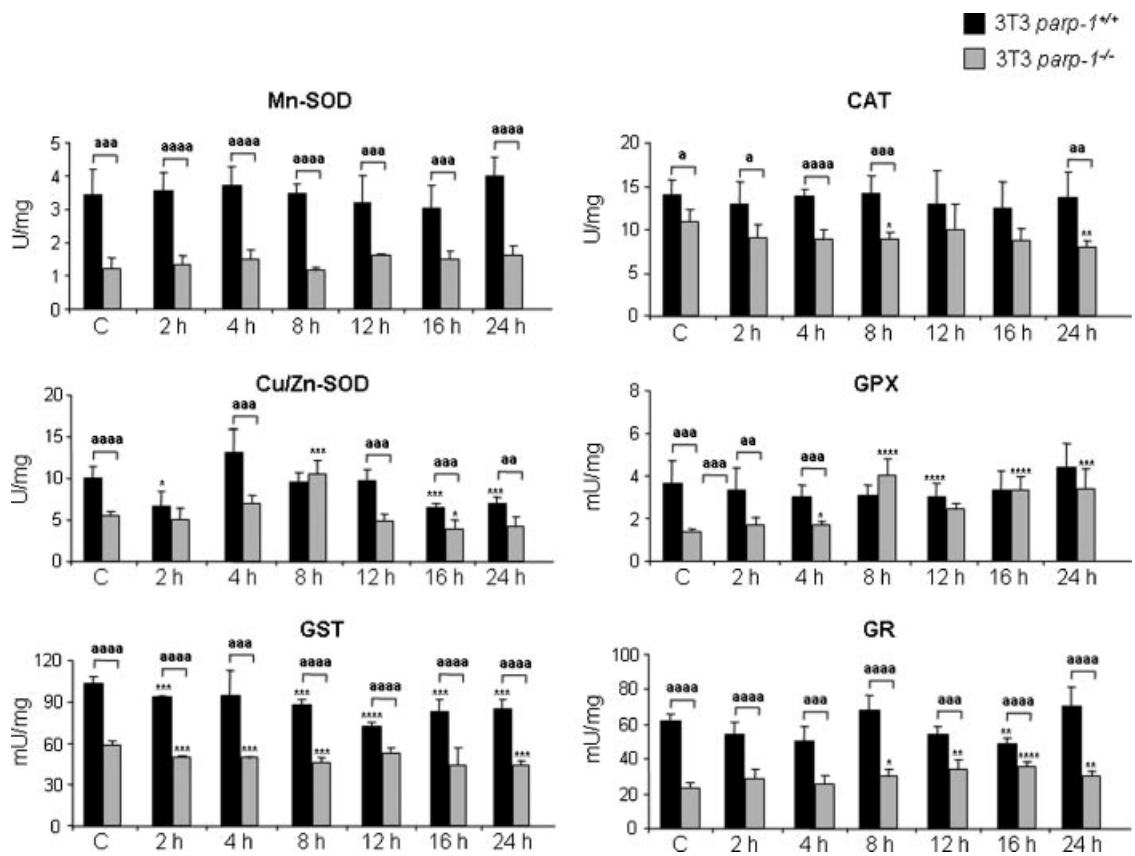

Fig. 9. Effect of DFO treatment on Mn-SOD, Cu/Zn-SOD, GST, CAT, GPX and GR activity. Data are expressed as mean ± SD for n = 4 per group in U/mg or mU/mg of protein. One SOD unit is defined as the amount of enzyme that inhibits the rate of cytochrome *c* reduction by 50%. One unit of GST activity is defined as the amount of enzyme needed to form 1 μmol of GSH-CDNB conjugate per min. One CAT unit is defined as the amount of enzyme that catalyses the decomposition of 1 μmol of H_2O_2 per min. One unit of GPX or GR activity is defined as the amount of enzyme that catalyses the oxidation of 1 μmol of NADPH per min. Statistically significant differences from the corresponding control group: $^{*}p < 0.05$, $^{**}p < 0.02$, $^{***}p < 0.01$, $^{****}p < 0.001$. Statistically significant differences between *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ cells: $^{a}p < 0.05$, $^{aa}p < 0.02$, $^{aaa}p < 0.01$ and $^{aaaa}p < 0.001$.

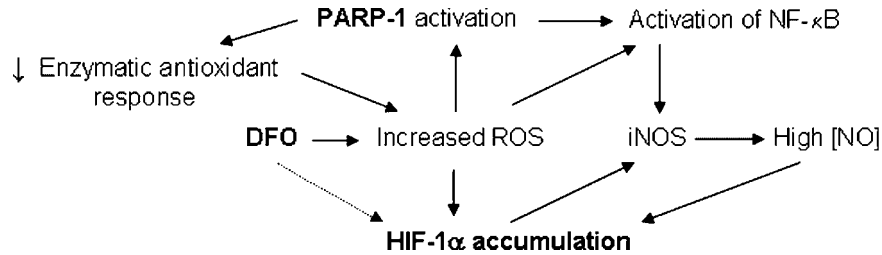

Fig. 10. Proposed model of action of PARP-1 in DFO-induced HIF-1α accumulation. DFO induces HIF-1α accumulation directly (. . .) or indirectly (—). PARP-1 plays a pivotal role, through its effect on ROS and NO production, in the indirect pathway. For details, please see the text.

is very weak [Kadikoylu et al., 2004]. In agreement with those findings, we have observed no increased enzymatic antioxidant activity after DFO treatment in wild-type cells, but a decrease in Cu/Zn-SOD at the end of the experiment, which correlates with the higher ROS production. However *parp-1* deletion favours the response of some enzymes, particularly GPX and GR. GR is important for the maintenance of GSH level which is used by other antioxidant systems, such as the GPX. These results appear to indicate that, although it has been proposed that DFO may increase the oxidative status by reducing GSH cellular level [Seo et al., 2006], the absence of PARP-1 ameliorates this effect. Moreover, it has been reported that the activity of Mn-SOD affects HIF-1α accumulation [Huang et al., 1996; Wang et al., 2005]. In our experimental model, Mn-SOD activity and expression is considerably reduced in *parp-1*-deleted cells. However, the fact that neither the activity nor the expression of this enzyme is affected by DFO, independently of PARP-1 presence, makes it difficult to establish a relationship between Mn-SOD and HIF-1α induction.

NO is a free radical which has also been shown to play a role on the stabilization of HIF-1α [Callapina et al., 2005a; Callapina et al., 2005b; Kohl et al., 2006; Quintero et al., 2006; Berchner-Pfannschmidt et al., 2007]. In this sense, we have observed that, according to the HIF-1α results, DFO treatment induced a different NO response in wild-type and PARP inhibited or PARP-1 null cells. NO has been described either to inhibit or to enhance HIF-1α accumulation depending on the experimental conditions. It has been proposed that under normoxic conditions, such as those in DFO-treated cells, NO inhibits PHD activity, inducing HIF-1α stabilization by competing for O_2 at the central iron of the active site [Zhang et al., 2002; Quintero et al., 2006]. In our study, the higher HIF-1α accumulation detected in *parp-1*$^{+/+}$ cells points to a link between higher NO levels and the HIF-1α induction. In fact, we have demonstrated that, in those cells and in the same experimental conditions, an ameliorated NO increase achieved by co-treatment with a PARP inhibitor coincides with a lower HIF-1α induction. This result argues in favour of both the implication of PARP-1 in NO production and the importance of NO on HIF-1α accumulation, suggesting that PARP-1 inhibition, by a slight induction of NO, decreases DFO-induced HIF-1α accumulation.

Iron chelators, such as DFO, can reportedly induce transcription of iNOS [Weiss et al., 1994; Dlaska and Weiss, 1999], although this effect does not always occur [Woo et al., 2006]. The sharp increase of NO detected in wild-type cells after 24 h of DFO treatment pointed to the induction of iNOS, the isoform which generates the highest concentrations of NO and which, in turn, is up-regulated by HIF-1 [Palmer et al., 1998; Semenza, 2005]. In fact, we have observed that the DFO treatment induced iNOS expression, although the response was quantitatively reduced after genetic deletion of PARP-1. In this sense, it is well known that PARP-1 is a co-modulator of NF-κB, and PARP-1-deficient cells and mice display reduced iNOS induction [Oliver et al., 1999; Conde et al., 2001]. Moreover, it is known that ROS, and especially O_2^-, are major modulators of NO activity [Grisham et al., 1999]. In this context, it can be assumed that the increased ROS production observed in wild-type cells after DFO treatment could enhance NF-κB activation [Schreck et al., 1991], promoting iNOS induction and the consequent final burst in NO production detected in these cells. Moreover, the increased HIF-1α accumulation observed particularly in *parp-1*$^{+/+}$ cells may also cooperate in the

induction of iNOS. Finally, as we have shown that DFO treatment activated PARP-1, it can be assumed that both DFO and PARP-1 activation cooperate, through iNOS induction, to prompt the final rise in NO production detected in wild-type cells.

In conclusion, the data presented here and summarized in Figure 10, suggest that PARP-1 inhibition decreases DFO-induced HIF-1α accumulation by modulating the NO level and by decreasing oxidative stress.

ACKNOWLEDGMENTS

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Review Article

PARP inhibitors: New partners in the therapy of cancer and inflammatory diseases

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ABSTRACT

Poly(ADP-ribose) polymerases (PARPs) are defined as cell signaling enzymes that catalyze the transfer of ADP-ribose units from NAD^+ to a number of acceptor proteins. PARP-1, the best characterized member of the PARP family, which currently comprises 18 members, is an abundant nuclear enzyme implicated in cellular responses to DNA injury provoked by genotoxic stress. PARP is involved in DNA repair and transcriptional regulation and is now recognized as a key regulator of cell survival and cell death as well as a master component of a number of transcription factors involved in tumor development and inflammation. PARP-1 is essential to the repair of DNA single-strand breaks via the base excision repair pathway. Inhibitors of PARP-1 have been shown to enhance the cytotoxic effects of ionizing radiation and DNA-damaging chemotherapy agents, such as the methylating agents and topoisomerase I inhibitors. There are currently at least five PARP inhibitors in clinical trial development. Recent in vitro and in vivo evidence suggests that PARP inhibitors could be used not only as chemo/radiotherapy sensitizers, but also as single agents to selectively kill cancers defective in DNA repair, specifically cancers with mutations in the breast cancer-associated genes (BRCA1 and BRCA2). PARP becomes activated in response to oxidative DNA damage and depletes cellular energy pools, thus leading to cellular dysfunction in various tissues. The activation of PARP may also induce various cell death processes and promotes an inflammatory response associated with multiple organ failure. Inhibition of PARP activity is protective in a wide range of inflammatory and ischemia–reperfusion-associated diseases, including cardiovascular diseases, diabetes, rheumatoid arthritis, endotoxic shock, and stroke. The aim of this review is to overview the emerging data in the literature showing the role of PARP in the pathogenesis of cancer and inflammatory diseases and unravel the solid body of literature that supports the view that PARP is an important target for therapeutic intervention in critical illness.

Contents

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Introduction

The enzymatic activity responsible for the synthesis of poly(ADP-ribose) (PAR) polymers was originally described in the early 1960s in Paul Mandel's laboratory in Strasbourg [1]. This initial observation generated significant biological interest and started a rather competitive field of research in which laboratories from various disciplines involved in studies on genomic stability, posttranslational modification of proteins, transcription, cell cycle, cell survival, and cell death are still heavily involved.

In the course of the years the enzymes responsible for PAR synthesis have been given different acronyms but currently are called PAR polymerases (PARPs), a rather generally accepted term. In the past decade, genomic approaches have allowed the identification of 18 putative PARP sequences in the human genome, and a significant amount of information is available for at least 5 enzymes [2]: PARP-1 (113 kDa), PARP-2 (62 kDa), PARP-3 (60 kDa), PARP-4 (193 kDa), and PARP-5 (142 kDa) or Tankyrase 1 (TRF1-interacting, ankyrin-related ADP-ribose polymerase).

PARP-1 is the founding member and the most commonly studied of these enzymes. It is an abundant nuclear protein in which it is possible to distinguish three domains: first, a DNA-binding region able to recognize DNA strand breaks; second, a central automodification region rich in glutamic acid and containing a breast cancer-associated protein C-terminal motif; and third, a NAD-binding region with all the catalytic activities of the full-length enzyme. DNA strand breaks remarkably increase PARP-1 basal activity (up to 500 times) [3]. Compelling evidence suggests that PARP-1, through its physical association with or by the poly(ADP-ribosyl)ation of partner proteins, regulates chromatin structure, DNA metabolism, and gene expression [4]. PARP-1 accounts for at least 85% of maximally activated cellular PARP activity.

The development of PARP inhibitors

Most of the PARP inhibitors in development mimic the nicotinamide moiety of NAD^+. PARP catalyzes the cleavage of NAD^+ into ADP and ADP-ribose and attaches several molecules of the latter to the target protein in a process called poly(ADP-ribosyl)ation. Therefore, molecules that mimic NAD^+ block the binding of the NAD^+ to the enzyme, inhibiting PARP activity. The discovery of inhibitors of PARP was initially based on empirical, high-throughput screening, followed by optimization by chemical modifications based on structure-based design (see [5] for an excellent review). First-generation inhibitors were developed 30 years ago: nicotinamide, benzamide, and substituted benzamide, in particular 3-aminobenzamide (3-AB), were shown to be competitive inhibitors of PARP (Fig. 1). Initial research demonstrated that all the benzamides are more potent inhibitors than nicotinamides or nicotidamide [6]. However, these classical inhibitors lacked specificity and potency. The benzamides and 3-AB are not specific PARP inhibitors. They affect cell viability, glucose metabolism, and DNA synthesis, and in the case of 3-AB used in combination with chemotherapy or radiotherapy, millimolar concentrations are needed, which have a toxic effect [7]. The derivatives of benzamide highlight the importance of the carboxy group, as N-alkylation or replacement with a thioamide greatly reduces

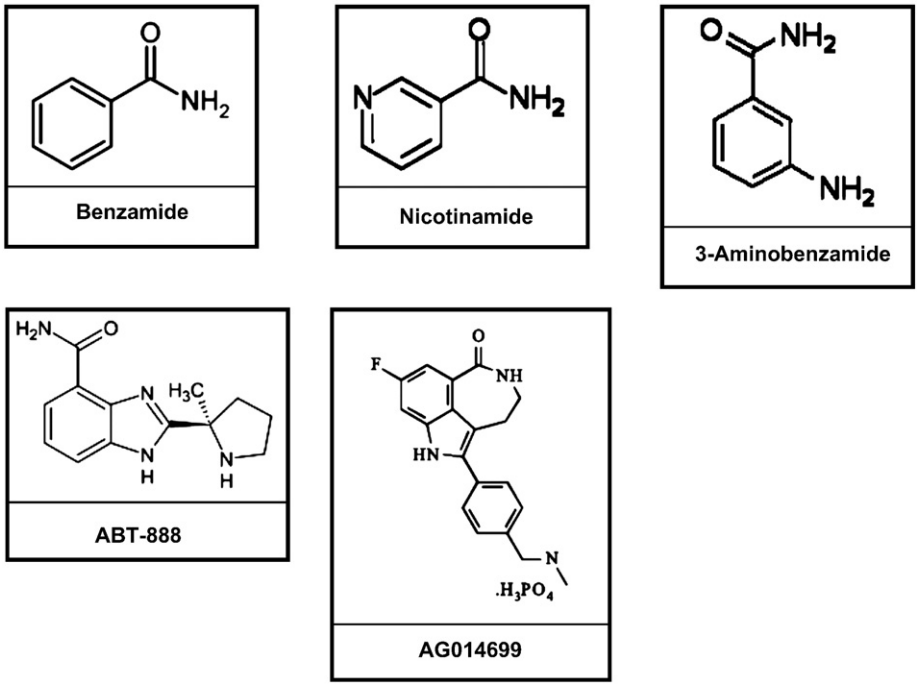

Fig. 1. Inhibitors of PARP. The classical inhibitors are nicotinamide, benzamide, and substituted benzamide, in particular 3-aminobenzamide. Important inhibitors in clinical trials include ABT-888 and AG014699.

activity. The benzamides inhibit PARP by interfering with the binding of NAD to the enzyme's active site, but the benzamides also bind to DNA and thereby prevent the recognition of DNA breaks by PARP, inhibiting the activation of PARP [8]. A second generation of very potent PARP inhibitors was developed in the 1990s, producing 170 specific inhibitors. All these inhibitors may be classified as analogs of benzamide. A common structural feature shared by all very strong inhibitors is a carbonyl, either attached to an aromatic ring as a carbamoyl group or built into a polyaromatic skeleton as an N-substituted carbamoyl or C-extended acetyl group. The carbonyl group, however, is not indispensable for the inhibitory action, for example, quinazoline and isoquinolines do not have a carbonyl group, but have a C = N double bond in the analogous position. Likewise, the aromatic ring also contributes to the inhibition, but is not absolutely required. All these inhibitors act in the reaction between PARP and NAD^+ and they are used in the micromolar range [9]. A third generation of inhibitors, benzamidazoles, not only had potency but also allowed the elucidation of the PARP inhibitor structure–activity relationship. Other compounds developed had cyclic structures [10], including AG014699, an important inhibitor used in clinical trials [11]. Many agents are derived from a basic 3-aminobenzamide structure and include libraries of benzimidazoles (Abbott), pthalazinones (Kudos), ideno[1,2-*c*]isoquinolinones (Inotek), and tricyclic indoles (Pfizer). This has created a multitude of potent inhibitors with various solubility and pharmacokinetic characteristics. Some of these agents with favorable attributes have entered clinical trials, including ABT-888 (Fig. 1), indeno-isoquinolinone (INO-1001), BSI-201, and AG-014699 (Table 1). These drugs differ in chemical structure and bioavailability but tend to have a short half-life and require frequent doses [12].

This review highlights the main mechanisms and pathways that underlie the rationale for the development of various PARP inhibitors for the treatment of neoplasia and diverse inflammatory diseases, emphasizing the potential of these inhibitors as single therapeutic agents in specific DNA-repair-deficient tumors as well as in chronic and acute inflammatory diseases.

PARP and DNA repair

The genome is under constant mutational stress through exposure to exogenous and endogenous agents that damage DNA. In mammalian cells, more than 150 proteins have been described that are involved in the response to DNA damage [13]. These proteins coordinate the repair of DNA lesions by different repair pathways [14], although there are also considerable overlap and interactions between mechanisms. In the absence of adequate detection and repair of DNA damage, these alterations can result in genome instability, which is considered a hallmark of tumor cells [15].

PARP-1 is a molecular sensor of DNA breaks and it has a key role in the spatial and temporal organization of their repair [4,16]. PARP-1 is activated by single- and double-strand breaks (SSB and DSB, respectively), being one enzyme critical in the base excision repair (BER) pathway [17].

As PARP-1 actively participates in DNA repair, inhibition of its activity can lead to enhanced cell death either alone or in combination with DNA-damaging agents [18]. In fact, the development of DNA-repair inhibitors initially focused mainly on their use as potentiators of cytotoxic agents. As such, PARP-1 inhibitors have been discussed as potential chemotherapeutic agents that can potentiate other cytotoxic treatments [18,19]. The use of PARP-1 inhibitors in combination with standard chemotherapeutic agents also seems attractive in the sense that by sensitizing tumor cells to cytotoxic agents one might be able to give a lower dose and maintain the same relative efficacy while at the same time reducing the toxic side effects [20]. Several studies have already shown the effectiveness of PARP inhibition in combination with either radiotherapy or chemotherapy in a range of human tumor mouse xenograft models [21,22].

Loss-of-function mutations in a significant number of DNA-damage-response genes predispose to a variety of familial cancers. In addition to gene mutation, epigenetic silencing of DNA repair genes has also been associated with tumorigenicity [13]. Tumor defects in DNA damage response may not only be causative of disease but can also be exploited therapeutically, through sensitivity of tumor cells to agents that cause DNA damage. However, the majority of DNA-damage reagents are not specific and can cause similar extents of damage in both mutant and wild-type cells. In contrast, PARP inhibitors have emerged as a new regime with high therapeutic benefit for tumors that are deficient in these specific proteins.

PARP inhibitors in the treatment of DNA repair deficiency-related cancers

Inhibition of PARP induces accumulation of large numbers of unrepaired SSBs, leading to the collapse of replication forks during S phase and the consequent generation of DSBs. Single-strand breaks are usually repaired by the base excision repair pathway; therefore, inhibition of this pathway greatly increases the number of unrepaired single-strand breaks, which subsequently leads to DSBs at replication forks. Therefore, cells deficient in DNA DSB repair are highly sensitive to chemical inhibitors of PARP [23–25]. In contrast, cells with intact

Table 1

Agent	Company	Single/combination therapy	Disease	Clinical status	Reference
AG014699	Pfizer (New York, NY, USA)	Single agent/combination with temozolomide	Locally advanced or metastatic breast cancer and advanced ovarian cancers in known carriers of a BRCA1 or BRCA2 mutation; malignant melanoma	Phase 1 in solid tumors complete, phase 2 in melanoma complete	Plumer et al. [163]
KU59436	AstraZeneca/KuDOS (London, UK)	Single	Advanced solid tumors	Phase 1	Fong et al. [164]
			Women with advanced breast cancer	Phase 2	http://clinicaltrials.gov
			Measurable BRCA1- or BRCA2-positive advanced ovarian cancer	Phase 2	
ABT-888	Abbott Laboratories (North Chicago, IL, USA)	Single	Refractory solid tumors and lymphoid malignancies	Phase 0 ongoing	
BSI-201	BiPar (Brisbane, CA, USA)	Single	Advanced solid tumors (ovarian, uterine, lung, and other)	Phase 1 complete; phase 2 ongoing	http:www.biparsciences.com
INO-1001	Inotek/Genentech (Beverly, MA, USA)	Combination with temozolomide, single	Metastatic melanoma; glioblastoma	Phase 1	http://clinicaltrials.gov
		Combination with doxorubicin	p53-deficient breast cancer	Preclinical	Mason et al. [165]
		Single	Cardiovascular indications; prevention of postoperative aortic aneurysm repair complications	Phase 2	Jagtap and Szabo [5] Pacher and Szabo [88]
GPI 21016	MGI Pharma (Bloomington, MN, USA)	Combination with temozolomide	Solid tumors	Phase 1 planned	

DNA DSB-response pathways repair damage with high fidelity and accordingly show very little sensitivity to PARP inhibitors (Fig. 2).

Mutations in the breast cancer susceptibility genes BRCA1 and BRCA2 are responsible for the majority of hereditary breast cancers [26]. Tumors in patients with heterozygous BRCA1 or BRCA2 germline mutations typically show somatic loss of heterozygosity at the BRCA1 or BRCA2 locus, respectively, resulting in loss of the wild-type allele [27,28]. BRCA1 and BRCA2 encode proteins that are required for efficient homologous recombination (HR), an error-free form of DSB repair [29,30]. Because both BRCA1- and BRCA2-deficient cells have such elevated utilization of error-prone repair pathways [31], the loss of BRCA function causes genome instability [32], which renders viable cells susceptible to acquiring additional cancer-initiating genetic lesions such as activation of oncogenes and inactivation of tumor-suppressor genes. The tumors that arise are BRCA1 or BRCA2 deficient ($BRCA1/2^{-/-}$), whereas the remaining somatic cells still have functional BRCA proteins ($BRCA1/2^{+/-}$). BRCA1- and BRCA2-deficient cells are highly sensitive to agents that cause replication forks to stall. For example, cells with dysfunctional BRCA2 are characterized by an increased sensitivity to γ-irradiation or DNA-damaging agents [33–36] because of the absence of error-free DSB-repair mechanisms. PARP inhibitors are lethal in BRCA-deficient cells as inhibition of PARP leads to the persistence of DNA lesions that would normally be repaired by BRCA-mediated homologous recombination. Repair of DSBs by alternative error-prone DSB-repair mechanisms causes large numbers of chromatid breaks and aberrations, leading to cell cycle arrest and apoptosis. Cell lines lacking BRCA1 or BRCA2 are extremely sensitive to PARP inhibitors, whereas the $BRCA^{+/-}$ and $BRCA^{+/+}$ cells are relatively nonresponsive to the treatment [23,24]. Indeed, in vivo experiments demonstrate highly efficient and selective deletion by PARP inhibition of nonneoplastic $BRCA2^{-/-}$ cells, with no apparent deleterious effect on the surrounding BRCA2-functional cells or whole-animal physiology [37], and complete growth arrest of xenografted tumors derived from BRCA2-deficient cells with no effect on BRCA2-wild-type tumors [23,24]. Despite these observations of selective cytotoxicity against nontumor cells with engineered BRCA2 mutations, relatively few data are available about the effects of PARP inhibitors on BRCA2-deficient mammary tumor cells. However, a recent report shows a potent growth arrest induced by PARP inhibition in BRCA2-deficient mammary cancer cell lines [38]. If we exploit the fact that the PARP-1 pathway becomes essential only in homologous recombination-deficient cells, we could develop a treatment highly specific for the BRCA-deficient tumor cells [23]. The aim is to generate DNA damage that, under normal circumstances, is repaired with high fidelity but in a BRCA-dependent fashion [31]. These observations suggest that PARP inhibitors might be an effective

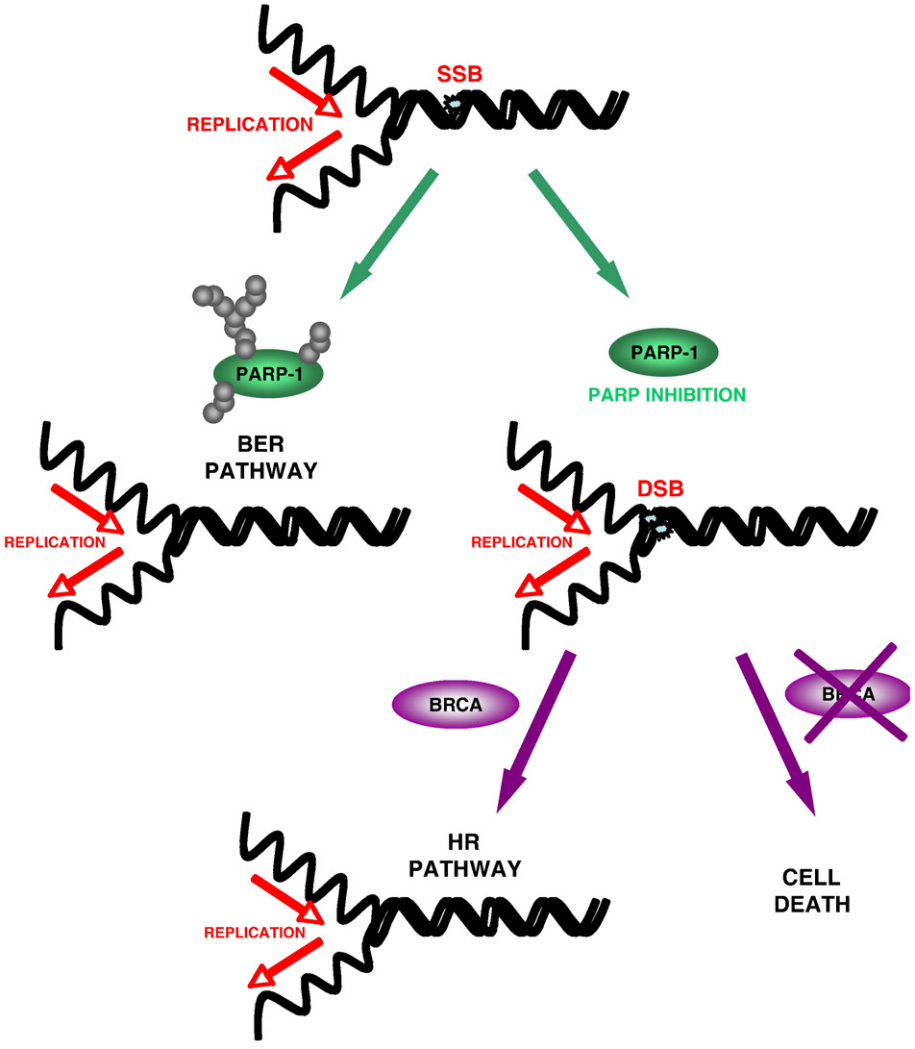

Fig. 2. PARP inhibitors in the treatment of DNA repair deficiency-related cancer. Inhibition of PARP induces the accumulation of large numbers of unrepaired SSBs usually repaired by the BER pathway, leading to the collapse of replication forks and the consequent generation of DSBs. PARP inhibitors are lethal in BRCA-deficient cells as inhibition of PARP leads to the persistence of DNA lesions that would normally be repaired by BRCA-mediated homologous recombination. In contrast, cells with intact DNA DSB-response pathways repair damage with high fidelity and accordingly show very little sensitivity to PARP inhibitors.

therapy for cancers arising in carriers of BRCA1 or BRCA2 mutations and have led to the assessment of these agents as monotherapy for cancer in BRCA mutation carriers [39]. PARP inhibition may also be considered as a potential prophylactic treatment of nonneoplastic BRCA2-deficient cells in women heterozygous for BRCA2, as well as a primary follow-up treatment after surgery in patients in whom BRCA2 deficiency has led to tumorigenesis. It is one of the few examples in which the defect causing the cancer is exploited for targeted therapy.

In contrast, another report concluded that PARP inhibition is not a viable therapeutic approach based upon the insensitivity of one cell line treated with two weak small-molecule inhibitors of PARP [40]. However, the selective effect of PARP inhibition on BRCA deficiency is a property of only very potent PARP inhibitors [24], though, clinically speaking, it is not the potency of a drug that is most important but the efficacy, such as some authors point out [41]. Moreover, their studies indicate that the use of PARP inhibitors in mouse BRCA1$^{-/-}$ mammary cancer cells seems to show only mild specificity in comparison with their controls in the xenografted nude mice. On the other hand, PARP inhibitors seem to uniformly inhibit the growth of human breast cell lines regardless of the BRCA1 genotype [41]. Earlier observations indicate that BRCA1$^{-/-}$ mammary cancer cells are indeed much more resistant to PARP inhibition than BRCA1$^{-/-}$ nontumor cells in the allograft model. These authors believe that the noncancerous cells are generally naïve, whereas tumor cells represent cells that have had multiple genetic mutations and that in a tumor there may be numerous fundamentally different cancer cells when classified by the type and frequency of individual genomic changes [20]. Another independent study has also not demonstrated inhibition of tumor growth with the chosen PARP inhibitor, although there was reduced but detectable polymer in this samples and the lack of response may be a result of incomplete PARP inhibition [42]. These observations cast doubt on the uniformity of the specific cell killing of BRCA-deficient cells upon PARP inhibition. Maybe, some specific mutations during tumor progression due to genetic instability associated with BRCA1 and BRCA2 deficiency block the sensitivity of BRCA1/2$^{-/-}$ cancer cells to PARP inhibition. In fact, it has been suggested that mutation in BRCA2 and sensitivity to therapeutics in BRCA2 mutation carriers can be suppressed by intragenic deletion [43]. In the absence of functional BRCA2, mechanisms such as single-strand annealing and nonhomologous end joining compensate and repair DSBs in the absence of HR, by aligning short regions of homology flanking the DSB, deleting the intervening sequence [30,44]. As a consequence of this same HR deficiency, new BRCA2 proteins with deletions are capable of restoring HR, leading to resistance. In summary, BRCA-associated tumorigenesis may be divided into two phases regarding sensitivity to PARP inhibition. In the early stages of tumorigenesis, BRCA-mutant cells are sensitive to PARP inhibition. However, in the second stage of tumorigenesis, BRCA-deficient tumor cells may become insensitive to PARP inhibition owing to additional mutations. Thus, the use of this strategy for therapeutic treatment of hereditary breast cancers is dependent on the continued susceptibility of BRCA mutant cells to PARP inhibitors [20].

PARP inhibitors have now entered clinical trials and initial results are promising, with frequent sustained responses in BRCA-mutation carriers. Furthermore, PARP inhibitors are likely to produce few side effects, because PARP-1-knockout mice are viable and healthy [45,46] and inhibitors that are specific for PARP are relatively nontoxic and do not directly damage DNA; also, this approach is more specific than standard cytotoxic chemotherapy. Initial observations in patients with known BRCA-associated cancers, or those with a strong family history of disease suggestive of BRCA mutation, indicate that a novel, potent, and orally bioavailable PARP inhibitor [47] shows low toxicity, and there are suggestions of significant antitumor activity, as assessed by radiography and by measurement of tumor biomarkers. This PARP inhibitor is being tested for efficacy as a monotherapy in BRCA-associated ovarian and breast cancer in phase II clinical trials [13,48]. Many of the side effects are independent of the mechanism of action of these drugs and it seems that short-term inhibition of PARP is well tolerated and that PARP inhibition can be achieved at reasonable doses. Perhaps more debatable is the suggestion that the low toxicity of potent PARP inhibitors may enable their prophylactic use in women heterozygous for BRCA mutations [49]. There is still no clinical experience regarding the tolerability of long-term PARP inhibition. Prolonged inhibition of DNA repair could enhance the mutation rate and the potential for secondary malignancies. In fact, a recent study reveals an important role for PARP-1 in suppressing mammary tumorigenesis in mice and, importantly, it suggests that long-term inhibition of PARP activity may bring increased risk for breast cancer formation [50]. Nevertheless, short periods of prophylactic use may have utility but must obviously be weighed against the actual risk a BRCA mutation carrier has of developing malignancy [51].

Very recently an interesting report has settled the basis of the resistance to PARP inhibition acquired by a specific mutation on BRCA2 [52]. Mutations in BRCA2 and sensitivity to therapeutics in BRCA2-mutation carriers can be suppressed by intragenic deletion. Multiple mechanisms have been described for the reversion of mutations in humans, but the size and nature of the deletions they observe are uncommon. Variants of BRCA2 lacking a significant fraction of the protein are competent in mediating PARP resistance and, surprisingly, in HR. The profound sensitivity of BRCA2-deficient cells to PARP inhibitors is determined by their defect in error-free repair and deletions may also arise because of this same HR deficiency.

Despite the clinical promise of PARP inhibitors in the treatment of BRCA-related cancer, extending the utility of these agents to other cancers is challenging. Sensitivity to PARP inhibition depends on homologous recombination deficiency and not on inherited BRCA1 or BRCA2 deficiency per se. Therefore, this approach may be more widely applicable in the treatment of other impairments of the HR pathway. The identification of novel mediators of cellular response to PARP inhibitors may highlight additional patient populations that might benefit from this therapeutic approach. In this way, a high-throughput RNAi screen has been used to identify new determinants of sensitivity to a PARP inhibitor [53].

Other pathways involved in HR

Loss of PARP-1 has been shown to cause an increase in Rad51 foci and sister chromatid exchanges [54] as a result of an increase in the number of lesions normally repaired by HR. The sensitivity of cells deficient in proteins involved in HR to PARP inhibition suggests that treatment with PARP inhibitors maybe a useful therapeutic strategy for tumors displaying defects in the HR pathway other than BRCA defects. The observation that ATM and CHK2 depletion resulted in sensitivity to PARP inhibitors further suggests that PARP inhibition would be beneficial for a wide variety of cancers with dysfunction in genes involved in the DNA-damage response [25]. The effects of deficiency of several proteins involved in HR on sensitivity to PARP inhibition have been examined and it has been shown that a deficiency in RAD51, RAD54, DSS1, RPA1, NBS1, ATR, ATM, CHK1, CHK2, FANCD2, FANCA, or FANCC induces such a sensitivity. These results suggest that PARP inhibition might be a useful therapeutic strategy not only for the treatment of BRCA mutations but also for the treatment of a wider range of tumors bearing a variety of deficiencies in the HR pathway.

Particular attention has been paid to the interaction between PARP and ATM. These two proteins, together with DNA-PK, are involved in responding to DNA damage by activating pathways responsible for cellular survival. A defective activation of ATM has been described in PARP-1-deficient cells; on the other hand, ATM-null cells display an extraordinary sensitivity to PARP inhibitors [55,56], suggesting that ATM and the collapse in the replication fork induced by the PARP inhibitor function upstream of HR to repair certain types of DSBs [55].

PARP-1 seems to be important for HR levels; however, it is not directly involved in this process, and the increase in HR seen in PARP-1-deficient cells is probably due to an accumulation of recombinogenic substrates in these cells. It is important to note that this treatment differs from previous therapies in that no additional genotoxic drug is needed to cause cell death, and in this respect the it would be expected that the treatment would have few side effects.

Mismatch-repair (MMR)-deficient cells

Mutations in genes involved in MMR or in their expression result in increased risk of tumor development and in increased resistance to many anticancer therapies [51,57]. PARP inhibitors have been shown to sensitize resistant cells to alkylating agents [52,58]; moreover, a PARP inhibitor (AG1461) enhanced temozolomide activity in MMR-proficient cells, but intriguingly, it was more effective in MMR-deficient cells [57]. It is likely that in MMR-deficient cells there is a switch in toxic lesion and that this new lesion is repaired in a PARP-dependent way. Temozolomide causes a mixture of lesions and in MMR-deficient cells these lesions can be repaired by BER. As PARP participates in BER, PARP inhibitors would be expected to enhance specifically the cytotoxicity of temozolomide in MMR-deficient cells.

PARP inhibitors as antiangiogenic agents

As has been described in the previous section, PARP inhibitors are being developed for the treatment of cancer, both in monotherapy and in combination with radiation and chemotherapeutic agents in humans. Recently, a number of reports from various laboratories, including ours, have led to a novel and unexpected effect of PARP inhibitors, showing a relationship between PARP and angiogenesis, and to the proposition of PARP inhibitors as antiangiogenic agents.

So far at least five PARP inhibitors have been efficiently used in vitro [58–61] to inhibit vascular endothelial growth factor (VEGF)-induced proliferation, migration, and tube formation in human umbilical vein endothelial cells (HUVECs) and in tumor models [62]. 3-AB and PJ-34 have been shown to do this in HUVECs in a dose-dependent manner [59]. In a second report, the same group showed a dose-dependent reduction in VEGF-a and basic fibroblast growth factor-induced proliferation, migration, and tube formation in HUVECs in vitro by two potent PARP inhibitors, 5-aminoisoquinolinone hydrochloride (5-AIQ) and 1,5-isoquinolinediol (IQD). Moreover, PARP inhibitors prevented the sprouting of rat aortic ring explants in an ex vivo assay of angiogenesis. [60].

In vitro Matrigel plug assays have also been used to demonstrate that in *parp*-knockout mice or in the presence of PARP inhibitors, there is a drastic reduction in the hemoglobin content in the plug compared to the wild-type control mice [61]. The PARP inhibitor PJ-34 was also shown to efficiently inhibit the chicken chorioallantoic membrane model of angiogenesis when used at low concentrations [58]. More recently, in a study of the in vivo role of PARP-1 in melanoma aggressiveness by means of silencing *parp-1* expression, a striking difference was reported when the *parp-1*-silenced melanoma cells were injected into mice; they showed a nearly complete absence of tumor vascularization compared to the wild-type melanoma tumor [63]. Similar results were found in a transplanted lung cancer model, showing that X-ray treatment combined with the PARP inhibitor ABT-888 resulted in a 50% decrease in von Willebrand factor staining of the tumor compared to X-ray treatment alone [64].

The molecular mechanisms underlying the effects of PARP on angiogenesis are still unclear. We have shown in a model of skin carcinogenesis that concomitant treatment of the mouse skin with the PARP inhibitor 3,4-dihydro-5-[4-1-(1-piperidinyl)butoxy]-1-(2*H*)-isoquinolinone (DPQ) in addition to 7,12-dimethyilbenz[a]anthracene (DMBA) and 12-O-tetradecanoylphorbol acetate (TPA) reduced by 80% the number of lectin-positive blood vessels in the tumor compared to the tumor promoted by DMBA and TPA alone [62]. Further, in this study we showed that PARP activity has the ability to modulate the expression of genes involved in angiogenesis, particularly the hypoxia inducible factor (HIF), whose activity is impaired when tumors are induced either in presence of DPQ or in *parp-1*-knockout mice. HIF-α has been largely involved in tumor progression by promoting a global response to hypoxia, including new vessel formation. There are results suggesting that the absence of PARP-1 modulates HIF-α accumulation by reducing both NO and oxidative stress [65]; however, the ultimate molecular link between HIF-α and

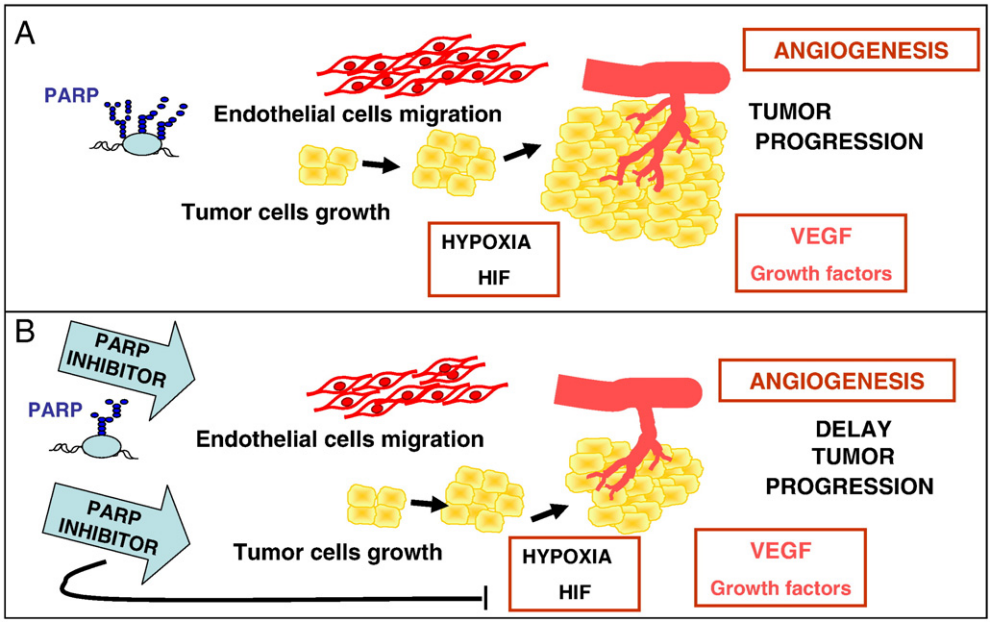

Fig. 3. (A) Tumor progression. Hypoxia stimulates the expansion and remodeling of the existing vasculature to enhance blood flow to oxygen-deprived tissues. This process is accomplished primarily through the activation of HIF target genes involved in various steps of angiogenesis, such as vascular endothelial growth factor (VEGF) and other growth factors. (B) PARP inhibitors promote a delay in tumor formation and a dramatic reduction in tumor size. PARP inhibitors have an antiangiogenic effect, and they might be an interesting target for the treatment of cancer.

PARP-1 has to date not been established clearly and further work will be necessary to unravel this mechanism.

The main conclusion arising from these publications dealing with PARP and angiogenesis is the requirement of the PARP pathway integrity to have a proper angiogenic network development, making PARP an attractive target for reducing angiogenesis in cancer and other diseases caused by an angiogenic dysfunction (Fig. 3). Nonetheless, we have to mention that the antiangiogenic properties of PARP inhibition are still controversial: biological activity of VEGF has been shown to be downregulated by poly(ADP-ribosyl)ation attributed to the PAR activity (but not to PARP inhibition), an antiangiogenic property [66]; IGF-1, which is known to promote angiogenesis, downregulates PARP function by phosphorylation, and this effect would contribute to increasing the VEGF gene transcription [67]. These discrepancies in the antiangiogenic activity of PARP inhibitors may be due to the pleiotropic effects of PARP-1, which works as an enzyme that can alter both positively and negatively a high number of transcription factor activities by covalent attachment of PAR polymer or by direct interaction with transcription factors.

Role of poly(ADP-ribose) polymerase in oxidative/nitrosative stress-related pathologies

It must be stressed that PARP-1 functions as a double-edged sword: on one hand, moderate activation of PARP can be of physiological importance via enhancement of DNA repair. On the other hand, overactivation of PARP represents an important mechanism of tissue damage in various pathological conditions associated with oxidative and nitrosative stress, including myocardial reperfusion injury [68,69], heart transplantation [70], and autoimmune β-cell destruction associated with diabetes mellitus [52,53,71].

In various pathological conditions, an imbalance between the production and the elimination of reactive oxygen- and reactive nitrogen-containing intermediates (ROS and RNS, respectively) has been described and referred to as oxidative/nitrosative stress [72]. Many of these species (e.g., peroxynitrite and hydroxyl radical) can cause DNA damage, which results in the activation of PARP [69,73]. Continuous or excessive activation of PARP produces a depletion of NAD^+ and ATP, slowing the rate of glycolysis and mitochondrial respiration, eventually leading to cellular dysfunction and necrotic cell death (Fig. 4).

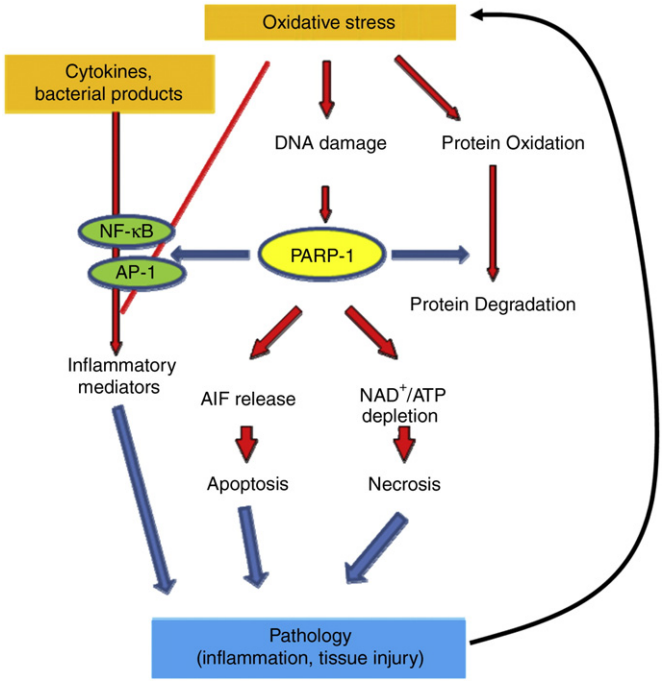

Fig. 4. The central role of PARP-1 in oxidative/nitrosative stress-related pathology.

Moreover, PARP-1 regulates the expression of various proteins implicated in inflammation at the transcriptional level (e.g., inducible nitric oxide synthase (iNOS), intercellular adhesion molecule-1, and major histocompatibility complex class II) [68,74–76]. The absence of functional PARP-1 (either genetic or pharmacological) decreased the expression of a host of proinflammatory mediators, including cytokines, chemokines, adhesion molecules, and enzymes (e.g., iNOS). Nuclear factor-κB (NF-κB) is a key transcription factor in the regulation of these proteins and PARP has been shown to act as a coactivator in NF-κB-mediated transcription [77,78,62]. Recently, it has also been reported that PARP-1 regulates other transcription factors implicated in stress/inflammation, such as AP-1, Oct-1, SP-1, HIF, and Stat-1 [79].

Poly(ADP-ribosyl)ation inhibitors: promising drug candidates for a wide variety of pathophysiologic conditions

The "suicide hypothesis" is considered a key mechanism explaining the protective effects of PARP inhibition in various oxidative stress-induced diseases, involving overwhelming DNA damage (as result of ROS and RNS release during inflammation) and excessive activation of PARP leading to NAD and ATP depletion and necrotic cell death. The production of both NO (via the route of formation of peroxynitrite) and ROS induces damage in DNA and consequently leads to the activation of PARP-1 and PARP-2 and starts a cycle of consumption of cellular energy, resulting in a drop in levels of NAD and ATP, slowing the rates of glycolysis and mitochondrial respiration, and the cell may enter into general dysfunction and a process of cell death by necrosis [77, 80–83].

In addition to this mechanism, the role of PARP-1 in inflammatory diseases has been suggested by de Murcia's initial observation identifying PARP-1 as a coactivator of NF-κB, the key inflammatory transcription factor [77,84–87]. De Murcia's group identified deficient NF-κB activation as the underlying mechanism of the endotoxin resistance of $PARP^{-/-}$ mice. Thus it was proposed that PARP-1 is required for NF-κB-mediated transactivation. The NF-κB/Rel transcription factors play a central role in the regulation of genes involved in the immune and inflammatory response. Various groups, including ours, have shown that PARP inhibitors have minimal or no effect on NF-κB activation, whereas cells and mice lacking PARP-1 display a dramatic deficiency in this transcription factor activation [88].

Inhibition of PARP provides remarkable therapeutic benefits in various acute, often life-threatening diseases (e.g., reperfusion injury, septic and hemorrhagic shock, stroke) as well as in chronic inflammation (e.g., arthritis, experimental allergic encephalomyelitis, asthma).

PARP-1 inhibitors in cardiovascular diseases

Activation of PARP and the beneficial effects of various PARP inhibitors have been demonstrated in various forms of endothelial dysfunction such as those associated with circulatory shock, hypertension, atherosclerosis, preeclampsia, and aging [88]. Chronic treatment with the PARP inhibitors PJ-34 and INO-1001 for 2 months in a rodent model has been demonstrated to improve endothelial and cardiac dysfunction associated with aging [88,89], showing the involvement of the nitro-oxidative stress–PARP pathway in the pathophysiology of cardiac and vascular aging. Furthermore, recent data suggest that activation of PARP importantly contributes to the development of endothelial dysfunction in various experimental models of diabetes and also in humans [88] and the pharmacological inhibition of PARP improves endothelium-dependent relaxation in these pathological conditions.

PARP-1 activation has recently been involved in the pathogenesis of ischemia and reperfusion-induced myocardial injury. Reperfusion injury is the principal cause of tissue damage occurring in conditions such as myocardial infarction, cardiopulmonary bypass, aortic reconstructive surgeries, stroke, and organ transplantation, as well as being a major mechanism of end-organ damage complicating the course of circulatory shock. Pharmacological inhibition of PARP with various inhibitors (3-AB, nicotinamide, ISQ, 5-AIQ, IQD, BGP-15, 1,11*b*-dihydro-[2*H*]-benzopyrano-[4,3,2-*de*]-isoquinolin-3-one (GPI-6150), PJ-34, and INO-1001) or PARP-1 genetic inactivation attenuates myocardial necrosis in the acute and delayed stages of myocardial infarction (reviewed in [68,90]). In addition, in vivo studies have indicated that these beneficial effects were accompanied by suppression of neutrophil infiltration and a decrease in inflammatory cytokines [91]. The reduction of myocardial necrosis is associated with diminished morphological alterations and improvement of ventricular contractility [92]. Several recent human studies have investigated the role of peroxynitrite, known to be an obligatory trigger of oxidative DNA damage and consequent PARP activation, in myocardial ischemia–reperfusion in patients undergoing open heart surgery [93]. Cardioprotection afforded by PARP-1 inhibitors was more evident when these agents were given at the onset of the reperfusion process, suggesting that ROS formation during this phase is the principal source of cardiac damage [94]. These data encourage novel approaches based on PARP-1 inhibition for the protection against myocardial infarction. Thus, animals treated with PARP-1 inhibitors or PARP-1-deficient mice showed decreased tissue damage and reduced levels of inflammatory mediators in several models of ischemia–reperfusion and heart transplantation.

Heart failure

It is important to note that activation of PARP-1 has been reported in various models of heart failure, cardiomyopathies, and myocardial hypertrophy. A novel peroxynitrite decomposition catalyst, FP15 [95], and PARP inhibitors PJ-34 [96] and INO-1001 [94–97] attenuated the development of cardiac dysfunction and myocardial nitrotyrosine formation and increased the survival in doxorubicin-induced mouse cardiomyopathy models [96,98] and also in a rat model of chronic heart failure induced by permanent left anterior coronary artery ligation in rats [96,98]. In addition, PARP inhibition with PJ-34 or INO-1001 resulted in improved heart failure-associated decreased endothelial function and decreased myocardial hypertrophy and adverse remodeling [99]. Importantly, recent studies have also demonstrated overexpression of PARP-1 and increased PARP activity in biopsies from human subjects with heart failure [100]. The PARP inhibitor INO-1001 was found to induce a tendency to reduce the plasma levels of C-reactive protein and the inflammatory marker IL-6, without reducing plasma markers of myocardial injury. No drug-related serious adverse events were observed in the patients receiving the drug during the study period [98] (Table 1).

Administering the PARP inhibitor PJ-34 after ligation improved the cardiac dysfunction and vascular relaxation but still yielded the same amount of nitrotyrosine [101]. In a model of heart transplantation [102], PARP inhibition before excision of the donor heart and transfer of it to the recipient improved the oxidative stress effects, like DNA strand breaks, lipid peroxidation, and drop in NAD^+ and ATP levels. The size of the necrotic area was reduced, whereas the number of apoptotic cells was increased. So, it remains unclear whether PARP inhibition is beneficial in heart transplantation.

Cardiovascular aging

Accumulation of macromolecular damage, including damage to DNA and genomic instability, is considered a driving force for the aging process and age-related diseases. The prematuring aging disease, Werner syndrome, is characterized by genomic instability and hypersensitivity to DNA-damaging agents and defective poly (ADP-ribosyl)ation There are reports that chronic administration (2 months) of the PARP inhibitor PJ-34 improved cardiac function and augmented endothelial function in the aorta of aged (22 months old) Wistar rats [103]. Also, treatment with the PARP inhibitor INO-1001 (for 2 months) was shown to improve both the diastolic and the systolic cardiac function in aged (24 months old) F344 rats. Nonetheless, the vascular levels of PARP-1do not seem to change substantially in aging. One interesting observation is the increased apoptosis-related PARP-1 cleavage and the increased 89-kDa cleaved fragment in aged coronary arteries [104]. PARP-1 may contribute to a number of proinflammatory mechanisms, and NF-κB-dependent transcriptional activation upon TNFα challenge is partially inhibited in *parp-1*$^{-/-}$ endothelial cells [105–107]. Future studies are likely to give additional clues to the vascular effects of the pharmacological disruption of PARP-1, including a decrease in vascular inflammation with antiaging effects.

PARP-1 inhibitors in the treatment of diabetes

Insulin-dependent diabetes mellitus (IDDM; juvenile diabetes or type 1 diabetes) originates from β-cell destruction in the islets of Langerhans. The quality of life of patients and the expectations of life for them are determined by the various complications of the disease itself. Once the disease is established, increased levels of blood glucose (hyperglycemia) are accompanied by an endothelial dysfunction and various vascular disorders in organs such as retina, brain, kidney, or heart, which are the main determinants of diabetes-related mortality [89,108–111]. The pathogenesis is associated with endothelial dysfunctions, including alteration of cellular redox state, increase in the formation of diacylglycerol, and activation of protein kinases C, among others. Many of these alterations lead to the formation of ROS and RNS, such as superoxide anion or peroxynitrite, which play an important role in endothelial dysfunction and other complications associated with diabetes [112,113].

Streptozotocin is a compound that induces diabetes, causing a massive and selective killing of pancreatic β cells, through a mechanism of DNA alkylation and induction of the production of NO. This type of induction of disease is used as a model to study the disease in animals [104,114,115]. In response to the streptozotocin, β cells enter apoptosis, but recent studies have shown that the main route of destruction of these cells for IDDM is necrosis. When cell death occurs by necrosis, the release of cytokines, NO, and ROS leads to inflammation and an increase in protein glycation, which would result in increased levels of blood glucose.

There is the possibility of using chemical inhibitors of PARP in the treatment of diabetes; this is exemplified by treatment with the pharmacological PARP inhibitor PJ-34: treatment 1 week after the induction of diabetes ameliorated vascular PARP accumulation and restored normal vascular function, without altering systemic glucose levels, plasma glycated hemoglobin levels, or pancreatic insulin content [116].In addition, treatment with PJ-34 restored endothelium-dependent vasodilation and cellular energy levels and reduced the levels of cytokines and inflammatory response. Recent data indicate that pharmacological inhibition of PARP might suppress NF-κB activation and the expression of adhesion molecules both under constant high-glucose conditions in cultured endothelial cells in vitro [115].

Another aspect of diabetes is the possibility of regeneration of the islets of Langerhans. It was demonstrated that PARP-1 is a component of the active transcription complex for the Reg gene and regulates its DNA binding by poly(ADP-ribosyl)ation [113]. The Reg protein is important for the proliferation of β cells and is induced by the concerted action of dexamethasone and IL-6. Thus, PARP-1 inhibition in combination with dexamethasone and IL-6 leads to increased transcription of Reg and accelerated regeneration of pancreatic islets cells.

There is the possibility of using inhibitors of PARP-1, such as the PJ-34, in the pathogenesis of diabetic complications such as retinopathy, nephropathy, and diabetic neuropathy.

Retinopathy

There is now evidence that PARP-1 activation occurs in microvessels and the ganglionic layer of the diabetic retina [117–119]. This role is supported by one independent interventional preclinical study. In this long-term (9-month) study [120,121], the role of PARP-1 was investigated in hyperglycemia-induced cell death in vitro and in the development of diabetic retinopathy in vivo. Streptozotocin-diabetic Lewis rats were treated with vehicle or PJ-34. Diabetes was found to increase the activity of PARP in retina measured at 2 months, and PJ-34 prevented this increase. PARP activation was detectable in the nuclei of retinal capillary endothelial cells and pericytes; diabetes of 9 months' duration increased the number of both TUNEL-positive capillary cells and acellular capillaries (a marker of degenerate capillaries) and PJ-34 significantly inhibited these alterations without influencing glycemic control. Moreover, PARP inhibition prevented cell death induced by high glucose levels in retinal capillary endothelial cells.

Nephropathy

Recently one group [122,123] demonstrated that PARP-1 activation occurs in the tubule of streptozotocin-induced diabetic rats. This PARP activation is attenuated by two structurally unrelated PARP inhibitors, 3-AB and 1,5-isoquinolenidiol, which also counteracted the overexpression of endothelin-1 (ET-1) and ET receptors in the renal cortex.

Neuropathy

Recent studies suggest that the oxidative/nitrosative stress/PARP-1 pathway also plays a key role in the development of diabetic neuropathy: the progressive loss of sensory and motor ability in mice with diabetes can be prevented by pharmacological inhibition of PARP-1 [124–129].

PARP-1 inhibitors in rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by inflammation of the synovial lining and destruction of the adjacent bone and cartilage. Although its etiology is unknown, evidence indicates that the recruitment and activation of neutrophils, macrophages, and lymphocytes into joint tissues and the formation of the pannus are hallmarks of the pathogenesis of RA. Inflammatory mediators, such as cytokines and free radicals, produced by infiltrating inflammatory cells play a critical role in joint damage [126]. The fact that the inflammatory process in RA is chronic suggests that immune regulation in the joint is disturbed. Available therapies based on immunosuppressive agents inhibit the inflammatory component of RA and have the potential to slow progressive clinical disability by delaying erosions and deformity [124–127,130]. However, they neither reduce the relapse rate nor delay disease onset, and a continued treatment is required to prevent the inflammatory and autoimmune components of the disease.

To test potential therapeutic agents, the collagen-induced arthritis (CIA) model, a murine experimental disease model that shares a number of clinical, histologic, and immunologic features with RA, is used. In this model, treatment with 5-AIQ reduced the frequency of arthritis, ameliorated symptoms, and inhibited joint damage. AIQ offers therapeutic advantages over other PARP inhibitors, such as benzamide and the phenanthridinone derivate PJ-34, used in several autoimmune models [131]. Thus, PJ-34 showed a protective effect in experimental autoimmune encephalomyelitis, CIA, and experimental type I diabetes only when administered on a prophylactic regime before the disease onset, losing its therapeutic effect in animals with established clinical signs [132]. In addition, in contrast to AIQ, the protective effects of these PARP-1 inhibitors disappeared once treatment was terminated [133]. Thus, AIQ downregulated the production of the proinflammatory/cytotoxic cytokines (TNFα, IFN-γ, IL-6, IL-1β, and IL-12) and increased the levels of the anti-inflammatory cytokine IL-10, which ameliorates the disease [134].

PARP-1 inhibitors in the treatment of shock

Septic shock is the most common cause of death in intensive care units and it is usually the result of a systemic gram-negative bacterial infection, for example *Pseudomonas aeruginosa,* resulting in hypotension and failure of a number of organ and systems, such as liver, kidney, and heart. The major cause of these failures is the action of an endotoxin formed by various bacterial species that affect these systems. In particular, the endotoxin is the bacterial membrane component lipopolysaccharide (LPS); this component is able to activate the NF-κB/Rel family of transcription factors, inducing the expression of a number of genes involved in the pathogenesis of endotoxic shock: TNFα, IL-1β, IL-2, IL-6, IL-8, adhesion molecules, cyclooxygenase-2, and inducible NO synthase [77,135]. Blood leukocytes, including monocytes, macrophages, and neutrophils, are exquisitely responsive to microbial infection and play important roles in the induction of sepsis [136]. During the initial phase of bacterial infection, a transient induction of proinflammatory cytokines, as TNFα, IL-1β, and IL-2, occurs in the blood of septic patients. At this moment the leukocytes enhance the production of anti-inflammatory cytokines.

As described above, two models might be operating to explain the relationship between PARP-1 and septic shock induced by LPS. Endotoxin and free radicals elicit DNA strand breaks, which activate PARP-1 and deplete NAD and ATP. Extensive DNA damage caused by ROS triggers PARP-1 overactivation and causes necrotic cell death. This is the main mechanism underlying the PARP-1 suicide model [137]. Additionally, in the absence of PARP-1, NF-κB-dependent transcription is impaired and the release of inflammatory mediators is downregulated. As a consequence PARP-1-deficient mice are extremely resistant to endotoxic shock induced by LPS [138]. Several pharmacological inhibitors for different purposes have been used, such as 3-AB and PJ-34, in animal models.

The traditional PARP-1 inhibitor, 3-AB, was used as a treatment in sepsis induced by LPS in mice and pigs, but with poor results [139] in the mechanism of septic shock. However, 3-AB is considered a poor inhibitor of PARP-1 and it has pronounced toxicity in vivo.

The novel and potent PARP-1 inhibitor PJ-34 was found to protect against LPS-induced tissue damage [73,140]. Administration of PJ-34 immediately before intraperitoneal implantation of *Escherichia coli* reduced peritonitis and bacteremia and increased survival rates (83% survival after 24 h compared to only 12% within the control group), accompanied by a significant reduction in serum levels of TNFα.

Hemorrhagic shock (HS) and resuscitation trigger the expression of a cascade of inflammatory mediators, resulting in tissue damage, multiple organ dysfunction, and eventually death. The major feature of HS is blood loss, which causes deterioration in organ microcirculation. This damage is associated with the formation of free radicals and oxidant species [135]. Upon resuscitation, reperfusion and reoxygenation in this tissues lead to the production of reactive radical species such superoxide anions, hydrogen peroxide, hydroxyl radicals, peroxynitrite, and nitric oxide [141], which can cause cellular damage by interference with signal transduction pathways and peroxidation of cellular membranes. Previous studies of HS have implicated PARP-1 as a mediator of organ injury in hemorrhage and resuscitation by showing the beneficial effects of pharmacological inhibition or by employing mice that are genetically deficient in PARP [142]. During HS NO is synthesized from L-arginine by a family of isoenzymes termed NO synthases. Overproduction of NO has been demonstrated in a variety of inflammatory disorders. In these disorders, NO-derived

oxidant species trigger DNA single-strand brakes and initiate PARP activation, leading to the rapid depletion of cellular NAD and levels of ATP. So, in tissue damage resulting from HS, there are optimal conditions for the activation of PARP. Various groups have studied the effects of different PARP inhibitors or gene silencing of *parp-1* on HS and its effects on tissues.

Parp-1$^{-/-}$ mice were protected from the rapid cardiovascular decompensation that develops soon after resuscitation and showed a clear survival in comparison with control animals [143]. In PARP-knockout mice all the pathophysiological alterations, like reperfusion, barrier failure, lung neutrophil recruitment, or vascular hypocontractility, were reduced significantly. Furthermore, this group observed PARP-1 activation in the necrotic intestinal epithelial and stromal cells of mice subjected to HS.

Other studies have focused on the pharmacological inhibition of PARP-1. Pretreatment with 3-AB lessened the increase in permeability of the ileum and reduced the presence of liver enzymes in the plasma observed after hemorrhage and resuscitation [144]. The use of 3-AB slowed the metabolic acidosis and lactate accumulation observed during HS and resuscitation. In contrast, posttreatment with 3-AB was ineffective in this model. Since 2006, some groups have been using water-soluble inhibitors, such as 5-AIQ. 5-AIQ treatment in male rats attenuated shock/resuscitation-induced increase in intrahepatic leukocyte–endothelial cell interaction, with a marked reduction in both sinusoidal leukostasis and venular leukocyte adherence [145]. Treatment with 5-AIQ improved the liver microcirculation and function, establishing a future as a palliative treatment for hemorrhagic patients.

Stroke

Over the past decade, poly(ADP-ribosyl)ation has emerged as a crucial event in the pathogenesis of ischemic stroke. A large body of evidence unambiguously demonstrates that activity of PARP-1 significantly increases during brain ischemia and that inhibition of this enzymatic activity affords substantial neuroprotection from ischemic brain injury. The World Health Organisation defines stroke as a clinical syndrome caused by acute cerebral deficit and lasting for more than 24 h. Three main types of stroke have been classified: ischemic (accounting for 80% of all cases), hemorrhagic (15%), and subarachnoid hemorrhage (5%). Ischemic neuronal death is mediated, at least in part, by augmentation of NO caused by *N*-methyl-D-aspartate (NMDA) receptor-dependent activation of neuronal NO synthase (nNOS) activity. Indeed, ischemic neurodegeneration is reduced in mice null for nNOS [146] or in animals receiving nNOS inhibitors [147]. PARP-1 hyperactivity is causative in postischemic brain damage. In the intricate cascade of events that leads to ischemic neurodegeneration, PARP-1 is thought to be localized downstream of DNA damage induced by reactive oxygen and nitrogen species. Several lines of evidence support this assumption. For instance, using the comet assay to evaluate single- and double-strand DNA breaks, it has been demonstrated that NMDA receptor antagonists reduce DNA ruptures and ischemic brain injury, whereas PARP-1 inhibitors decrease infarct volumes without reducing the extent of DNA damage [148]. Furthermore, PARP-1 activation is significantly reduced in the ischemic brain of mice deficient in nNOS [70]. PARP-1-null mice are less sensitive to ischemia than nNOS$^{-/-}$ mice [149], suggesting that mechanisms in addition to NO co-occur with PARP-1 activation. It is also worth noting that hyperactivation of PARP-1 in the ischemic brain is not a single, time-limited event in the complex and prolonged process of ischemic neuronal death. Indeed, a vicious cycle triggered by intraneuronal Ca^{2+} accumulation leads to multiple waves of ROS formation in the ischemic brain tissue [150,151], thereby eliciting prolonged DNA damage and sustained PAR formation. This can also be due to self-amplifying positive forward cycles of ischemia-induced immune cell recruitment and ROS production [151] and/or to direct Ca^{2+}-dependent PARP-1 activation [150]. Remarkably, repression of poly(ADP-ribosyl)ation provides impressive protection from ischemic brain injury. Significant reduction of brain infarcts in PARP-1-knockout mice has been first reported by one pioneering study [151]. In particular, Dawson and her group report that brain infarct is 80% smaller in PARP-knockout mice compared to wild type 22 h after 2 h MCAO. Likewise, oxygen and glucose deprivation (OGD)-induced neuronal death is highly reduced in mixed cortical cultures from PARP-1-knockout mice with respect to that occurring in cortical cultures from wild-type counterparts [152,153]. Numerous studies showing the neuroprotective effects of chemical inhibitors of PARP-1 in in vitro and in vivo models of brain ischemia have been reported (Table 1). For instance, the PARP-1 inhibitor 3-AB affords significant neuroprotection when preinjected into mice and rats subjected to transient [151,154] or permanent [155] brain ischemia. Similarly, DPQ is a potent PARP-1 inhibitor capable of preventing neuronal death in in vivo [156] and in vitro [157] models of brain ischemia. Other compounds with different molecular moieties but potent inhibitory activity on PARP-1, such as PJ-34 [158], INO-1001, 5-chloro-2-[3-(4-phenyl-3,6-dihydro-1-(2*H*)-pyridinyl)propyl]-4-(3*H*)-quinazolinone (FR247304) [159], and 2-methyl-3,5,7,8-tetrahydrothiopyrano-[4,3-*d*]-pyrimidine-4-one (DR2313) also protect the brain tissue from the ischemic insult. A recent report on the neuroprotective effects of the PARP-1 inhibitor GPI-6150 in a model of global hypoxia in rat pups infers that neuroprotection is dependent on drug-induced hypothermia [156].

The therapeutic potential of PARP-1 suppression in ischemic stroke is also emphasized by studies that use different experimental approaches. For instance, RNA interference-dependent PARP-1 repression induces resistance to OGD in differentiated neuroblastoma cells [160]. In addition, transfection of PARP-1 into the brain of PARP-1-knockout mice by means of Sindbis virus increases the animal's sensitivity to ischemic stroke relative to LacZ-injected PARP-1-knockout mice. Notably, cerebral injection of Sindbis virus carrying PARP-1 worsens ischemic brain damage in wild-type animals, and treatment with DR2313 2 h after ischemia still reduces the progression of infarct in rats [154]. INO-1001 improves neurological functions with a time window of 6 h in a transient ischemia model in the rat [161]. These findings are in keeping with those showing that thieno[2,3-c]isoquinolin-5-one (TIQ-A) rescues cultured neurons from OGD-induced death even when added to the incubation medium 30 min after the ischemic insult [162]. With longer post-treatment time windows, in vitro ischemic neuroprotection by TIQ-A is lost. The efficacy of posttreatment is related not only to the length of the time window but also to the inhibitor used. For example, 3-AB loses its neuroprotective effects when injected in rats as early as 15 min after the ischemic insult. To date, the considerable amount of information on poly(ADP-ribosyl)ation and stroke undoubtedly indicates a pathogenetic role for PAR formation in ischemic brain injury. However, critical issues still wait to be resolved. For instance, current drugs do not exhibit significant selectivity toward PARP-1. This is of particular significance given the role of PARP-2 in DNA repair and the mutagenic potential of nonselective PARP-1 inhibitors.

Perspectives

Pharmacological inhibition of key proteins involved in the response to DNA damage has emerged as an effective tool for cancer treatment, as the resistance of cancer cells to DNA-damaging agents originates from the modulation of DNA repair pathways. PARP has important prosurvival and protective functions in terms of DNA repair. A multitude of novel pharmacological inhibitors of PARP has entered clinical testing either as adjunct antitumor therapeutics or as monotherapy in familiar breast and ovarian cancer. In addition to the antineoplastic action of PARP inhibitors, the evidence summarized above strongly supports a crucial role for the ROS/NF-κB/PARP pathway in mediating multiple inflammatory diseases, including type 1 diabetes and associated endothelial dysfunction, cardiovascular injury and heart failure, sepsis, rheumatoid arthritis, and stroke. The

information available to date supports the view that PARP overactivation during inflammatory damage is a pivotal feature of tissue damage in various inflammatory-based pathologies and that the pharmacological inhibition of PARP may provide significant benefits in these conditions by salvaging affected tissues from necrosis and by reducing, as well as by downregulating, the inflammatory responses. As with all new therapeutic areas, the usefulness of this target is unproven, but the potential effect of this class of agents is large. Whereas the clinical benefit of PARP inhibitors is being tested, additional new areas of research are opening up in the preclinical front, which should eventually help in the discovery of new and more effective PARP inhibitors able to regulate the fine-tuning of this pathway, including the specific inhibition of the various PARP members, the association of PARP-1 in different complexes, and the activation/inactivation of poly(ADP-glycohydrolase).

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ROS-induced DNA damage and PARP-1 are required for optimal induction of starvation-induced autophagy

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Abstract: In response to nutrient stress, cells start an autophagy program that can lead to adaptation or death. The mechanisms signalling form starvation to the initiation of autophagy are not fully understood. In the current study we show that the absence or inactivation of PARP-1 strongly delays starvation-induced autophagy. We have found that DNA damage is an early event of starvation-induced autophagy as measured by γ-H2AX accumulation and comet assay, with PARP-1 knockout cells displaying a reduction in both parameters. During starvation, ROS-induced DNA damage activates PARP-1, leading to ATP depletion (an early event after nutrient deprivation). The absence of PARP-1 blunted AMPK activation and prevented from the complete loss of mTOR activity, leading to a delay in autophagy. PARP depletion favours apoptosis in starved cells, suggesting a pro-survival role of autophagy and PARP-1 activation after nutrient deprivation. In vivo results show that neonates of PARP-1 mutant mice subjected to acute starvation, also display deficient liver autophagy, implying a physiological role for PARP-1 in starvation-induced autophagy. Globally, we have found that PARP signalling pathway is a key regulator of the initial steps of autophagy commitment following starvation.

Introduction

Nutrient starvation alarms eukaryotic cells to adjust metabolism to survive. An early response of the cellular metabolic adjustments involves inhibition of growth and induction of macroautophagy (referred to as autophagy) to optimize the usage of limited energy supplies. Autophagy, as a cellular process mobilizing intracellular nutrient resources, plays an important role in contributing to survival during these growth unfavorable conditions. Is a highly conserved self-eating process in which intracellular membrane structures engulfed a portion of cytoplasmic organelles for lysosomal degradation. Eukaryotic cells have developed a mechanism through which autophagy induction is tightly coupled to the regulation of cell growth. Disruption of autophagic pathways is associated with multiple disease states, including neurodegenerative diseases, cancer, infection, and several types of miopathy (Levine & Kroemer, 2008). Autophagy is also a major mechanism by which starving cells reallocate nutrients from unnecessary to more essential processes (Levine & Kroemer, 2008). During autophagy, a cytosolic form of light chain 3 (LC3; LC3-I) is cleaved and then conjugated to phosphatidylethanolamine to form the LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes (Kabeya et al, 2000). Poly(ADP-ribose) polymerase (PARP) enzymes catalyze the conversion of NAD^+ to polymers of poly(ADP-ribose) (PAR) (Schreiber et al, 2006). Although its role in the DNA-damage response has long been recognized, recent works indicate that PAR itself acts to directly induce cell death through stimulation of apoptosis-inducing factor (AIF) release (David et al, 2009; Wang et al, 2009).

A recent study from our group have also implicated PARP-1 in autophagy induced by DNA damage and oxidative stress (Munoz-Gamez et al, 2009). There are, however, important issues that remain unresolved, i.e., the involvement of PARP signalling in a physiologic model of autophagy as is the case for nutrient deprivation and the

connection of PARP activation with the autophagic components. Among the numerous factors involved in the regulation of autophagy and growth, mTOR (target of rapamycin) is a key component that coordinately regulates the balance between growth and autophagy in response to physiological conditions and environmental stress.

In the current study we have found that starvation-induced autophagy results in ROS production, DNA damage (as measured by comet assay and γ-H2AX accumulation) and PARP-1 activation, leading to the inhibition of mTOR. Moreover, *parp-1-/-* neonates display a deficient autophagy response following acute starvation. Altogether these results place PARP-1 and PAR formation as key players in the decision of the cell to engage autophagy.

Results

The absence or inhibition of PARP-1 delays starvation-induced autophagy

Starvation or nutrient deprivation is a physiological cellular stress to induce autophagy in eukaryotic cells. To study the role of PARP-1 in starvation-induced autophagy, we transfected transiently *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ MEFs 3T3 cells with GFP-LC3, and we starved these cells with HANK buffer for 1, 2 and 4,5 hours; the percentage of cells with punctated pattern of GFP-LC3 was counted under fluorescence microscopy. In the non-starved cells, GFP-LC3 was diffusely distributed in the cytosol and nucleus, but after treatment with HANK's there was a punctated pattern, indicative of an accumulation of autophagosomes (Fig.1A). The number of GFP-LC3 vesicles was higher in *parp-1*$^{+/+}$ MEFs, approximately 20 vesicles/cell in *parp-1*$^{+/+}$ MEFs and 8-9 vesicle/cells in *parp-1*$^{-/-}$ MEFs at 2 hours of starvation (Fig.S1). At different times of starvation, a decreased number of cells with GFP-LC3 punctated pattern was observed in *parp-1*$^{-/-}$ MEFs (Fig. 1A). Rapamycin, as an inhibitor of mTORC1, was used as internal positive control of an autophagy-inducing agent; parp-

1-/- cells were also less sensitive to rapamycin-induced autophagy than parental cells (figure 1A). The conversion of LC3-I to LC3-II through proteolytic cleavage and lipidation is a hallmark of mammalian autophagy; we measured the LC3 translocation during starvation in *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ cells, and this translocation decreased in *parp-1*$^{-/-}$ MEFs (Fig.1B). These data indicated a pronounced delay of autophagy in the absence of PARP-1.

To further evaluate the implication of autophagy in this model, we used the chemical inhibitors of autophagy 3-Methyladenine (3-MA), an inhibitor of class III phosphatidylinositol 3-kinase (Blommaart et al, 1997) , as well as the siRNA-based knockdown of one essential autophagy protein, Atg7. 3-MA or siRNA of Atg7 elicited an important reduction in the number of cells with GFP-LC3 punctated pattern in *parp-1*$^{+/+}$ MEFs 2 hours of starvation, blocking effectively the starvation-induced GFP-LC3 translocation and the development of autophagy (Fig.1C and D respectively). In starved *parp-1*$^{-/-}$ MEFs, 3-MA or Atg7 siRNA completely prevented autophagy (Fig.1C, 1D and Fig. S2). These data suggest that the translocation of GFP-LC3 observed in *parp-1*$^{+/+}$ MEFs is due to autophagy and reflect the functional role of autophagy during starvation. The absence of PARP-1 synergize with 3-MA or ATG7 siRNA to maintain very low levels of autophagy during starvation (Fig. 1C and 1D). Lysosome fusion with autophagosomes was not affected in *parp-1* $^{-/-}$ cells. Treatment with chloroquine to inhibit lysosome fusion result in a similar accumulation of LC3 vesicles in parp-1 $^{+/+}$ and parp-1 $^{-/-}$ (figure S3A).

To evaluate more in depth the role of PARP-1 in starvation-induced autophagy, we tested the effect of the PARP-1 inhibitor DPQ and siRNA-based depletion of PARP-1 on the levels of autophagy in *parp-1*$^{+/+}$ MEFs; cells were transfected with GFP-LC3 and starved with HANK buffer at different times. Inhibition of PARP-1 with 40 μM DPQ reduced the number of cells with a typical GFP-LC3 punctated pattern in starvation in *parp-1*$^{+/+}$ MEFs but had no effect in *parp-1*$^{-/-}$ MEFs (Fig 2A). Similar results were also

obtained using two other different PARP inhibitors, PJ34 and olaparib (figure S3B). PARP-1 silencing induced a reduction in the number of autophagic cells after 2 hours of starvation (Fig.2B), maintaining similar levels to those obtained with *parp-1*$^{-/-}$ cells (Fig. 2A). Further, siRNA of PARP-1 reduced the endogenous LC3 translocation during starvation at levels very similar to non-starved in *parp-1*$^{+/+}$ MEFs (Fig. 2C). The non-specific siRNA had no effect on the levels of autophagy MEFs *parp-1*$^{+}$/+. These data suggest that PARP-1 and PARP activation play an active role in the commitment to autophagy in situations of nutrient deprivation. To corroborate this finding, we reconstituted PARP-1 in *parp-1*$^{-/-}$ MEFs with pBC-PARP-1 cDNA (Fig2D and Fig. S4) and we co-transfected with GFP-LC3. Cells transfected with the empty pBC vector were used as negative control. The reconstitution of PARP-1 in *parp-1*$^{-/-}$ MEFs during starvation increased the rate autophagic cells respect to *parp-1*$^{-/-}$ MEFs transfected with the empty vector (Fig.2D and Fig.S4). These data suggest that PARP-1 participates in the commitment of starvation-induced autophagy and may be indirectly involved in the formation of autophagosomes, since its inhibition or silencing leads to a delay in the autophagic response, including LC3 translocation and autophagosomes formation (Fig.2C). Indeed, autophagy was delayed but not abrogated after PARP-1 ablation since increasing starvation time to 8 hours resulted in an equivalent autophagic rate (not shown).

Starvation induces ROS production, DNA damage and activation of PARP-1,

PARP-1 is a nuclear enzyme activated by DNA damage; following genotoxic stress PARP-1 synthesizes a branched polymer of poly(ADP-ribose) or PAR which participates in the regulation of the nuclear homeostasis (Krishnakumar & Kraus, ; Munoz-Gamez et al, 2009; Schreiber et al, 2006)**.** Many different cellular insults that cause DNA damage activate PARP-1 and induce PARP-1-dependent cell death.

During starvation there is an important production of Reactive Oxygen Species or ROS which are involved in triggering autophagy (Chen et al, 2009; Scherz-Shouval et al, 2007). We hypothesized that ROS production during starvation could induce activation of PARP-1 and play an important role in the regulation of PARP-1-dependent autophagy.

Starvation induces activation of PARP-1 in *parp-1*$^{+/+}$MEFs, measured as PAR synthesis (Fig.3A). To correlate the production of PAR with the deprivation of nutrients we used the PARP inhibitor PJ34 during starvation and a positive control of activation of PARP-1 (H_2O_2); we indeed confirmed the synthesis of PAR by western blot (Fig.3A) and immunofluorescence (Fig.S5).

To demonstrate the production of ROS during starvation we used 2'7'-Dichlorofluorescein diacetate (DCFDA) as a probe to measure ROS (in particular, this probe detect H_2O_2) in *parp-1*$^{+/+}$MEFs and *parp-1*$^{-/-}$MEFs by flow cytometry (LeBel et al, 1992). PARP-1 deficient cells displayed a reduced production of ROS even at very short times following starvation (Fig.3B). This finding agrees with previous results showing a reduced ROS production in lymphocytes challenged with exogenous oxidative stress and treated with PARP inhibitors (Virag et al, 1998a). Assuming that ROS synthesis and their nuclear diffusion to induce DNA damage is very fast, we choose 30 minutes of starvation as the time to measure DNA damage with COMET assay and γ-H2AX, which phosphorylation is essential for the initial recognition of DNA breaks. COMET assay showed that 30 minutes after starvation, DNA damage was much more pronounced in *parp-1*$^{+/+}$ MEFs (Fig.3C); the tail moment of the comets is much higher in *parp-1*$^{+/+}$ cells, and almost fully repair is achieved after 1 hour, indicating that DNA-repair machinery is active (Fig.3C); on the contrary, in *parp-1*$^{-/-}$ cells the level of DNA damage is clearly reduced respect to parental cells at 30 minutes, but the DNA-repair machinery is not as efficient as in parental cells, resulting in a residual damage after 60 min of starvation (Fig.3C).

At the same time, *parp-1*$^{+/+}$ MEFs had higher levels of phosphorylation of H2AX, suggesting an increased number of DNA lesions due to the boost in ROS production (Fig.3D). γ-H2AX signal peaks at 1 hour of starvation in *parp-1*$^{+/+}$ MEFs while in PARP-1 deficient cells γ-H2AX continues to accumulates, similar to what we showed above for the COMET assay. Furthermore, indirect IF analysis reveals that the number of *parp-1*$^{+/+}$ cells with positive staining against γ-H2AX after 1 hour of starvation was elevated respect to *parp-1*$^{-/-}$ cells (Fig.S6).

To further confirm the implication of ROS in the initiation of autophagy we used the antioxidant n-acetylcysteine (NAC). Cells exposed to this compound showed a decrease in γ-H2AX accumulation and LC3II lipidation, indicating that ROS release is key in triggering DNA damage and subsequent autophagy (fig. 3E).

Globally, these data indicate that during starvation there is an important production of ROS in *parp-1*$^{+/+}$ MEFs, these ROS induce DNA damage and PARP-1 activation leading to PAR synthesis and triggering the initiation of autophagy associated to nutrient deprivation. Although parp-*1*$^{-/-}$ cells do produce ROS during starvation, this production do not lead to massive DNA damage and PARP-1 activation; consequently this cells display an impaired autophagy.

Lack of PARP-1 reduces ATP depletion, AMPK activation and mTOR inhibition during starvation-induced autophagy

Energy depletion, measured as a balance of AMP/ATP rate, is the main sensor for AMPk to induce autophagy. To investigate whether PARP-1 is implicated in AMPk dependent autophagy, we measured the levels of ATP in *parp-1*$^{+/+}$MEFs and *parp-1*$^{-/-}$ MEFs after different times of starvation. The levels of ATP in *parp-1*$^{+/+}$ MEFs after 60 minutes of starvation decreased to less than 50% of the initial level, while in *parp-1*$^{-/-}$

MEFs ATP levels decreased significantly more slowly (Fig.4A). Treatment with 3-MA during starvation blocked ATP depletion in *parp-1*$^{+/+}$ cells, indicating that this energy drop was due to autophagy induced by nutrient deprivation (Fig.4A). 3-MA also prevented ATP depletion in MEFs *parp-1*$^{-/-}$ cells, that have delayed autophagy. The depletion of ATP levels, corresponded with a sustained activation, through phosphorylation, of AMPk in *parp-1*$^{+/+}$ MEFs (Fig. 4B). This activation was strongly inhibited in *parp-1*$^{-/-}$ MEFs (Fig.4B).

mTOR is a serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription (Hay & Sonenberg, 2004). mTOR is a sensor of the cellular energy status. For this action it is regulated by the kinase AMPk, that is an important activator of autophagy (Gwinn et al, 2008; Hoyer-Hansen & Jaattela, 2007; Meley et al, 2006; Shaw et al, 2004). We have thus, evaluated the status of mTOR (which turn-off autophagy when it is activated) by determining the phosphorylation of its substrate p70s6 kinase. Short after starvation (30 minutes) *parp-1*$^{+/+}$ cells attained a complete inhibition of mTOR (Fig.4C), indicating that parental cells were engaged in autophagy, while in *parp-1*$^{-/-}$ cells mTOR was only partially inhibited even after 1 and 2 hours of nutrient starvation; 4 hours after starvation mTOR activation started to recover in the absence of PARP-1 (Fig.4C). Antioxidant treatment with NAC prevented from starvation-induced mTOR inactivation (figure 4D). These data suggested that PARP-1 may control mTOR activity during starvation and functions as a positive regulator of autophagy when cellular energy declines.

To confirm the previous results, we analyzed the role of PARP-1 in the pathway of activation of mTOR, during starvation in a tumoral model. In cancer cells the energy balance is critical to adapt the cell to the tumoral microenvironment, including low nutrient and oxygen availability (Gogvadze et al, 2008; Tomitsuka et al). We have performed assays with a tumoral cell line of human breast cancer cells stably

transfected with GFP-LC3, MCF7-GFP-LC3. During starvation these cells show endogenous LC3 translocation and the typical punctated pattern of GFP-LC3 counted under fluorescence microscopy (Figures 4E). MCF7 GFP-LC3 cells were used to determine the level of activation of mTOR during starvation in presence or absence of PARP-1 (Fig.4F). In these cells, mTOR is rapidly inhibited after starvation; 15 min. after nutrient deprivation the phosphorylation levels of p70s6 kinase decreased drastically and the cells had entered in autophagy. Treatment with 3MA prevented from starvation-induced mTOR inactivation and down-regulated AMPK (Fig.S7). In MCF7 GFP-LC3 depleted of PARP-1 using siRNA (Fig. 4F), mTOR inhibition was strongly delayed. mTOR activation was maintained over the time, similar to the results obtained in *parp-1*$^{-/-}$ MEFs (Fig.4C). The delayed inhibition of mTOR in PARP-1 knockout cells and after PARP-1 silencing, demonstrated that PARP-1 disabling regulated autophagy by preventing from complete mTOR inactivation.

To further confirm the implication of efficient DNA damage in the initiation of autophagy we used HT144 melanoma cells (an ATM deficient cell line) which is unable to properly repair γ-irradiation-induced DNA damage (not shown). These cells displayed very high levels of DNA damage under basal conditions according to the elevated constitutive γ-H2AX (figure S8A). G361 cells (ATM wt melanoma cells) accumulated DNA damage following starvation and inhibition of mTOR/p70S6k was achieved after 15 minutes. mTOR/p60S6k decreased also very rapidly in ATM mutant cells, but a residual activation was still detected after 30 min. of starvation (Figure S7A). The rate of autophagy determined as LC3 processing (Figure S8B) and quantitation of LC3 punctated cells (figure S8C) was delayed in ATM mutant cells, further supporting the mechanistic implication of DNA damage repair in the cell's ability to engage autophagy.

Impaired autophagy by PARP-1 disabling, leads to increased apoptotic cell death

Autophagy is an adaptation and survival pathway against adverse conditions, but if these conditions are maintained for a long time, can lead to cell death, ordinarily called Autophagic Cell Death or ACD (Kroemer & Levine, 2008). Autophagic cell death is morphologically defined as a type of cell death that occurs in absence of chromatin condensation, but it is accompanied by massive autophagic vacuoles in the cytoplasm (Levine & Yuan, 2005). In contrast with the typical apoptotic cell death, in ACD there is no nuclear fragmentation, plasma membrane blebbing, caspase activation or engulfment by phagocytes, in vivo (Baehrecke, 2005). Cells undergoing autophagy under adverse conditions can recover to their optimum physiological state after changing of the surrounding conditions, and the adverse stimulus disappears. However, cells that have autophagy inhibited or cells with a disrupted mitochondrial transmembrane potential, inexorably died even if optimal conditions are recovered (Boya et al, 2005). To determine what is the predominant cell death process in starvation-induced autophagy after ablation/inactivation of PARP-1, we investigated both autophagy and apoptotic cell death features by prolonging the time of starvation. Time course of total cell death following starvation showed accelerated cell death rate in the absence of PARP-1 as determined by propidium iodide (PI) incorporation (Fig. 5A). PARP-1 inhibition with PJ34 or PARP-1 knockdown gave similar results of increased cell death after nutrient deprivation (Fig. 5A). PARP-1 inhibited/silenced *parp-1*$^{+/+}$ MEFs 3T3 died at similar rate than *parp-1*$^{-/-}$ MEFs 3T3 at 8 hours of starvation (Fig. 5A); therefore, the absence of PARP-1 during starvation accelerates cell death. These data were confirmed by other different methods of cell death assays as Trypan Blue Dye Exclusion Staining and MTT Cell Survival Assay and with a different PARP-1 inhibitor as DPQ 40 μM (data not shown).

Autophagy has a pro-survival function after cellular stress like nutrient withdrawal. This difference in cell demise following starvation could be due to the

decreased levels of autophagy after disabling PARP-1. To determine whether autophagy has a cytoprotective role in parental cells, we inhibited autophagy with 3MA and we determined cell viability 8 hours after nutrient deprivation (Fig. 5B). Blocking autophagy with 3MA protected *parp-1*$^{+/+}$ MEFs against cell death but not PARP-1 deficient cells, suggesting that wild type cells died mainly as consequence of autophagy after starvation; on the contrary, the inactivation/blockade of PARP-1 lead to non-autophagic cell death. Silencing of the autophagic gene ATG7 strongly delayed cell death in MCF-7 cells subjected to nutrient starvation while the treatment with the PARP inhibitor PJ34 still increased cell death of ATG7 silenced/autophagy deficient cells, suggesting that autophagy-derived cell death is not involved in the accelerated cell demise produced by PARP inhibition (figure 5C) .

To analyze the type of cell death that takes place in the absence or after inhibition of PARP-1 during starvation, we used a double-knockout cell line for Bax and Bak. Bax and Bak are two essential proteins in the apoptotic process and these cells cannot undergo mitochondrial outer membrane permeabilization (MOMP) during apoptosis (Caro-Maldonado et al, 2010). Additionally, this cell line is stably transfected with GFP-LC3. To determine the levels of autophagy, we starved these cells for different times up to 120 minutes (Fig.5D). Bax/Bak double mutant cells displayed LC3 processing during starvation (Fig. 5D) and the percentages of cells with the typical GFP-LC3 pattern (Figure 5E) was similar to *parp-1*$^{+/+}$ MEFs. Moreover, the co-treatment with 3MA or with the PARP inhibitor DPQ reduced the percentage of autophagic cells, as we observed for *parp-1*$^{+/+}$ MEFs (Fig.5 E, and 2A). To determine to what extent the apoptotic component contributes to the increased cell death in the absence of PARP-1, we silenced PARP-1 in *Bax*$^{-/-}$/*Bak*$^{-/-}$GFP-LC3 MEFs by siRNA; knockdown of PARP-1 in a context of *bax/bak* knockout decreased the levels of cell death during nutrient starvation (Figure 5F) , suggesting that the gain in cell death after inactivation of PARP-1 (in a bax/bak wild type context (figures 5A-C)) has an apoptotic component. To

confirm this result we have measured caspase 3/7 and caspase-8 activation by fluorimetric assay and western blot respectively. Time course of caspase 3/7 activation was significantly increased in parp-1 -/- cells (figure5G); caspase-8 processing did not take place in *parp-1*$^{+/+}$ MEFs (Figure 5H). Moreover, by quantitation of pycnotic nuclei (not shown) as well as double staining with annexin V and PI (Figure 5I), apoptotic cells were determined to be significantly increased in the absence of PARP-1. These data confirm that *parp-1*$^{-/-}$ MEFs with delayed autophagy enter in apoptosis after several hours of starvation; thus, the presence and activation of PARP-1 allows a cytoprotective autophagy, where the cell try to compensate the lack of nutrient and energy depletion by undergoing controlled autophagy. Thus, PARP-1 wild-type cells die by autophagy-dependent cell death while PARP-1 knockout cells and die mainly by apoptosis.

Impaired starvation-induced autophagy in PARP-1 deficient mice

In order to analyse the in vivo consequences of the impaired autophagy after invalidation of PARP-1, we starved for 4 hours pups from both *parp-1* $^{+/+}$ and *parp-1*$^{-/-}$ mice. TEM (transmission electron microscopy) analysis showed that liver from fed PARP-1 proficient mice displayed characteristics abundant, well structured mitochodria as well as lipid droplets. After starvation, autophagy-derived ultrastructural changes include concentrical membranes structures engulfed in autophagosomes, ER dilation and also lipid droplets accumulation, which were already found in non starved liver (figure 6A). Concentrical membranes structures reflect degradation of membranous cellular components that rearrange in membranous whorls called myelin figures. To further support this observation, we measured changes in LC3-I levels (no LC· lipidadiion was detected in this experiments) in liver samples from *parp-1* $^{+/+}$ and *parp-*

1$^{-/-}$ mice pups subjected or not to starvation. Reduction in LC3-I was much more pronounced in livers from *parp-1* $^{+/+}$ mice (figure 6B).

The intracellular storage and utilization of lipids are critical to maintain cellular energy homeostasis. Cellular lipids are stored as triglycerides in lipid droplets and hydrolysed into fatty acids for energy in the fed liver and is also one of the initial responses to starvation. A second cellular response to starvation is the induction of autophagy, which delivers intracellular proteins and organelles sequestered in double-membrane vesicles to lysosomes for degradation and use as an energy source. Thus, in the absence of PARP-1, liver from neonates has impaired autophagic response as has been reported in knockout mice for genes involved in the core complex of autophagy (Komatsu et al, 2005) (Lee et al, 2008).

Discussion

Genetic and environmental factors modulate the response of multicellular organisms to stress and the maintenance of tissue homeostasis and a highly integrated response patterns are found in many organisms, but the means by which so many diverse pathways, critical for cellular, tissue and ultimately, for organism survival, are coordinated, has yet to be elucidated**.** In this study, we show that optimal induction of autophagy induced by nutrient deprivation requires PARP-1 activation. Our results clearly demonstrate the lack of AMPk activation after PARP-1 disabling. These findings are in agreement with previous results showing an interaction between PARP-1 and AMP metabolism related to the hydrolysis od ADP-ribose (Formentini et al, 2009). In the absence of PARP-1 or after its inhibition/silencing, ATP levels are not reduced as much as in wild type cells (Fig. 4A); consequently AMPk remains inactive and do not signal for mTOR inactivation, leading to impaired autophagy. At present the link

between PARP-1 activation and AMPk has not ben stablished. One speculative possibility is that ROS-induced DNA damage and PARP-1 over-activation caused mitochondrial dysfunction and Ca^{2+} release, thereby activating AMPk (Hoyer-Hansen & Jaattela, 2007) . Other possibility that may explain how PARP-1 connects with the AMPk/mTOR signalling (besides the maintenance of the energy status) is that PARP inactivation leads to unefficient ATM response which has been reported to be involved in the activation of AMPk (Aguilar-Quesada et al, 2007; Haince et al, 2007) .

Our data also suggest that the PARP-1 is an important in vivo regulator of autophagy and provide a link between PARP-1 function and the overall cellular response to nutrients shortage. The results described here demonstrate that nutrient starvation, ROS production and DNA damage leads to PARP-1 activation, needed for the cells to engage starvation-induced autophagy.

We also show that the role of PARP-1 in starvation-induced autophagy is related to its ability to sense DNA damage and deplete energy stores after its over-activation, but we cannot exclude perturbation in Ca^{2+} flux after PARP-1 ablation upstream of the mitochondria leading to altered ATP synthesis and AMPK activation (Cardenas et al). Moreover, we have also analysed the in vivo consequences of PARP-1 inactivation in starvation-induced autophagy. Starvation induces hepatic autophagy and increases delivery to the liver of fatty acids from adipose tissue lipolysis. Electron microscopy revealed that starvation increased the frequency of lipid droplets with areas of increased density and asymmetrically localized multi-membrane structures. The crucial role of neonatal autophagy was clearly demonstrated by targeted inactivation of the autophagy-related genes Atg5 and Atg7 (Komatsu et al, 2005; Kuma et al, 2004). Mice deficient in these genes were apparently normal at birth, except for a slightly lower body weight than control (about -10% in Atg5 null and -18% in Atg7 null mice), but died within 1day after birth. One of the initial observation of the phenotype of *parp-1* knockout mice was that average litter size was smaller (about

40%) than those of $PARP^{+/+}$ (de Murcia et al, 1997); although it is speculative, this convergent phenotype between ATG-7, ATG-5 and PARP-1 knockout mice could in the basis for this reduced size of PARP-1 knockout neonates; obviously, more indepth experimental evidence is needed to confirm this hypothesis.

ROS have been reported to be a hallmark of autophagy in a number of cell types and experimental settings, including nutrient starvation (Scherz-Shouval et al, 2007). Although autophagy after nutrient deprivation has a pro-survival function, our findings support that PARP-1 is necessary for the cell to persist in autophagy following starvation when nutrient deprivation is maintained for a long time (Fig. 5B).. An interesting finding in our study is that suppression of PARP activation by different means leads to impaired autophagy and eventually to increased cell death. Indeed, exposure of PARP deficient cells to a longer starvation period (6 and 8 hours) resulted in an equivalent incidence of autophagy with increased cell death in PARP-1 deficient cells (results not shown). Additionally we have defined the mechanism by which PARP-1 suppression accelerates cell death using the apoptotic deficient cell line double knockout for *bax* and *bak* and we have found that starvation leads $bax^{-/-}bak^{-/-}$ cells to autophagy and cell death, suggesting that apoptosis is not the main pathway of cell demise triggered by starvation. Nonetheless, suppression of PARP-1 in this $bax^{-/-}$ /$bak^{-/-}$ context substantially decreased cell death, contrary to what we observed in apoptosis proficient cells. One major observation in this study is that a physiological trigger of autophagy, such as nutrient deprivation, is able to induce DNA damage, through the generation of ROS. Genotoxic stress has been reported to repress mTOR in response to oxidative stress produced by ROS through a cytoplasmic signaling node for LKB1/AMPK/TSC2 activation in response to oxidative stress (Nakada et al, 2010). Comet assay and γ-H2AX confirmed the persistence of damaged DNA and the level of initial damage correspond with the cell's ability to initiate autophagy. Antioxidant treatment with NAC prevented from DNA damage, mTOR inactivation and slowed

down autophagy. Why PARP-1 deficient cells are prone to die by apoptosis following nutrient deprivation? One interesting possibility is suggested by the results in figure 3C-E: PARP-1 mutant cells display a defective DNA repair during starvation; thus, the cells choose to undergo apoptosis to avoid the harm of bearing unrepaired DNA; in a tumor context, where oxygen and nutrients are limited, this delayed autophagy (together with the tendency to die by apoptosis in the absence of PARP-1), might have benefits to prevent necrosis, ROS and inflammatory burst from tumor growth.

The ultimate reason why PARP-1 deficiency or inactivation leads to inhibition of ROS release is not completely understood. Interestingly, early reports have also shown that alterations in mitochondrial function during oxidant-mediated cytotoxicity, are related to PARP-1 activation rather than to direct effects of the oxidants on the mitochondria (Virag et al, 1998a). ER and mitochondrial Ca^{2+} signalling are also a key mediator of cell's bioenergetic regulation and cell death. Constitutive InsP(3)R Ca^{2+} signaling is required for autophagy suppression in cells under non starving conditions. It has been shown that after nutrient deprivation cells become metabolically compromised due to diminished mitochondrial Ca^{2+} uptake (Cardenas et al). Very recently PARP-1 and PARG (poly(ADPribose)glicohydrolase) have been reported to regulate Ca^{2+} influx through TRPM2 (Blenn et al) and a reduction in Ca^{2+} has been observed after abrogation of PARP-1. In this study by Blenn and col., they reported that Ca^{2+} increased flux leads to caspases activation and cleaving of mitochondrial AIF, which then translocates to the nucleus to cause DNA fragmentation, chromatin condensation, and cell death, following H_2O_2 treatment. Clearly, the level of ROS-derived cyotoxicity and PARP-1 activation differs in both settings: while H_2O_2 treatment produces an overwhelming burst in oxidant mediators, starvation leads to a mild ROS release allowing the cell not to undergo direct cell death but to engage the pro-survival autophagy. In support of that, Scherz-Shouval et al. have demonstrated that ROS in starvation-induced autophagy has a pro-survival function (Scherz-Shouval et al, 2007):

They have shown that oxidative conditions are essential for autophagy, and that the rise in ROS is both local and reversible during starvation, it is not deleterious to cells and serves to oxidize a specific target. On the contrary, massive ROS production during oxidative stress, will lead to ROS-derived cell death by autophagy (Yu et al, 2004). Once starvation has initiated the commitment to autophagy, cells abrogated of PARP-1 undergo a "slow" autophagy which is resolved eventually by the activation of increased cell death, particularly (but not exclusively) apoptosis. It has been previously shown that PARP inhibition can shift the necrotic cell death toward apoptosis, after exposure to oxidative stress (Virag et al, 1998b). In agreement with that, our results show that the gain of cell death observed in the absence of PARP-1 after starvation (leading to oxidative stress), is due to increased apoptosis. In this context PARP-1 is needed for the cell to undergo pro-survival autophagy.

Our model is presented in figure 7 and could be summarized as follows: after nutrient deprivation mitochondrial metabolism is rapidly shifted leading to ROS production and ATP drop. An elevation in the AMP/ATP ratio will activate the nutritional sensor kinase AMPK, whose activation leads to mTORc1 inhibition, allowing the commitment to autophagy. In parallel, ROS production induces DNA damage and PARP-1 overactivation, contributing to the feedback loop to decrease ATP through the consumption of NAD+. In this scenario, the axis ROS/AMPK/mTOR and ROS/DNA damage/PARP-1 activation synergize to optimize the cell's response to nutrient deprivation by inducing prosurvival autophagy (figure 7A). In the absence of PARP-1 ROS production, energy drop and AMPK activation are diminished shuting off the feedback loop responsible for massive energy depletion, and eventually slowing-down autophagy. Alternatively, the cells respond dying by apoptosis due to suboptimal autopagy commitmernt (figure 7B).

Autophagy is a potent tumour-suppressive mechanism, presumably due to its essential contribution to the maintenance of genomic stability (Karantza-Wadsworth et

al, 2007) the avoidance of excessive ROS generation (Mathew et al, 2009) and its participation in cellular senescence (Young et al, 2009), which constitutes a barrier against oncogenesis. Accordingly, multiple genes that are required for the induction/execution of autophagy are potent tumour suppressors, including PTEN, TSC1, TSC2, LKB1, ATG4, Beclin-1, UVRAG, and BH3-only proteins of the Bcl-2 family (Maiuri et al). Here, we revealed the importance of PARP for the autophagic process in a physiologic setting following nutrient starvation. PARP-1 inhibitors are entering clinical trials for different types of cancer. Whether the ability of PARP inhibitors to favour apoptotic cell death during cellular stress, as shortage of nutrients (which very often the case in tumor microenvironnement) could also be exploited as in antitumor therapy by its contribution to autophagy, remains an intriguing possibility for further investigation.

Methods

Cell Culture and Treatments. Immortalized mouse embryonic fibroblast (MEFs 3T3), derived from both wild-type and PARP-1 KO mice, *Bax*$^{-/-}$*Bak*$^{-/-}$GFP-LC3 MEFs 3T3 , g361 and HT144 cell lines were cultured in Dulbeco's Modified Eagle's Medium (DMEM) supplemented with 10 % inactive fetal bovine serum (FBSi, Gibco Invitrogen), at 37 ºC in a humidified 5% CO_2 atmosphere. MCF7 GFP-LC3 cells were cultured in RPMI 1640 GlutaMAX (GIBCO Invitrogen) with 6% FBSi. Cells were starved with Balanced HANK Buffer without Aminoacids (ClNa 140 mM, KCl 5 mM, Cl_2Mg-$6H_2O$ 1.3 mM, Cl_2Ca-2 H_2O 2 mM, HEPES 10 mM, D-glucose 5 mM) , at different times.

For western blot, cells were plated in 6-well plates with a density of 4 x 10^5 cells per plate and treated the next day with HANK buffer. For the assessment of cell death, cells were plated in 24-well plates with a density of 3.5 x 10^4 cells per well and in

6-well plates with 2.5 x 10^5 cells per well. To count the number of vesicles per cell, cells were plated in 6-well with cell density of 4 x 10^4 cells per well on coverslips and starved in the next day at different times. Fluorescence microscopy analysis was performed with a Zeiss Microscope.

The autophagy inhibitor 3 Methyl-Adenine (3MA M9281, Sigma, St Louis, Mo) was dissolved in culture medium and stored at -20ºC (10 mM). PARP-1 inhibitor DPQ (3,4-Dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinolinone]) and PJ34 were from Alexis Biochemicals. DPQ was dissolved in DMSO and stored at -20ºC. For treatments, cell were pre-treated with 40 µM DPQ or 10 µM PJ34, for 1.5 hours before starvation and maintained all the time of the experiment.

Rapamycin (553210 Calbiochem, Germany) and Concanamycin A (C9705 Sigma-Aldrich, St Louis, Mo) were used as autophagy inducers; Rapamycin was used at 100 nM for 4 hours and Concanamycin A at 4 nM for 4 hours.

Cell Viability Assays. The levels of cell death in *parp-1*$^{+/+}$ MEFs 3T3 and *parp-1*$^{-/-}$ cell lines were determined using Trypan Blue Exclusion Assay (93595, Fluka, St Louis, Mo) (a), MTT (b) and Propidium Iodide (c). (a) Trypan blue: 3T3 cells were seeded at 3.5 x 10^4 cells per well in 24-well plates and incubated overnight at 37 ºC. After starvation with HANK Buffer, cells were washed and trypsinized. The cellular pellet was dissolved in 50 µl of Trypan Blue Solution and the number of viable cells was counted in a normal microscope. (b) MTT assay (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide,) was performed using Cell Proliferation Kit I (MTT, 1-65-007,Roche, Mh Germany) following manufacturer's instructions. (c) Propidium Iodide was used as exclusion staining, using a FACScalibur flow cytometer with the Cell Quest software (BD Biosciences).

Inmunofluorescence. Immunostaining for poly (ADP-ribose) (PAR) were performed on cells grown on glass cover-slips and fixed in ice-cold methanol-acetone (1:1) for 10 minutes. Poly (ADP-ribose) was detected by Immunofluorescence, using monoclonal antibody (Trevigen) and FITC-conjugated goat anti-mouse immunoglobulin (Sigma, St Louis Mo). Foci of γH2AX we visualized with a monoclonal antibody for H2AX histone (UPSTATE, #05-636, clone JBW103) and FITC-conjugated goat anti-mouse. Nuclear counterstaining with DAPI was performed after removal of excess secondary antibody. Immunostaining was visualized with a Zeiss Fluorescence Microscope.

Quantification of lipid droplets by fluorescence microscopy. *Parp-1*$^{+/+}$ MEFs 3T3 and *parp-1*$^{-/-}$ seeded (4 x 10^4 cells per well) in 6 wells plates on glass cover-slips; cells were starved for 1 and 2 hours and fixed with Paraformaldehyde Solution (4%, wt/vol in PBS1x with 2% Sucrose) for 10 minutes at room temperature. Lipids droplets were labelled with BODIPY^R 493/503 (Invitrogen), 5 minutes at room temperature and visualised in a Zeiss Fluorescence Microscope. Nuclear counterstaining with DAPI was performed after the probe. 20 μM of Chloroquine for 2 hours was used as positive control of lipids droplets accumulation, during 2 hours.

Western Blot Analysis. After the nutrient starvation with HANK buffer, cells were washed twice with PBS and resuspended in 70 μl of Lysis Buffer (50 mM Tris-HCl pH 8, 0.1 mM EDTA, 0.5% Triton X-100, 12,5 mM β-Mercaptoethanol) for 45 minutes on ice. Pellet was eliminated and the supernatant was stored at -20ºC. Proteins were resolved on SDS-10% polyacrylamide gels and transferred onto PVDF Membrane (Biorad). The blot was blocked with 5% milk powder in PBS1X with 0.1% Tween-20 for 60 minutes, washed with PBS/Tween, and incubated overnight with the different

antibodies anti-poly(ADP-ribose) (PAR) (TREVIGEN, 20591E10, My, USA), anti-PARP-1(C2-10 mouse, ALEXIS, LA), anti-LC3 (NanoTools, clone 5F10, Ref 03231-100/LC3-5F10), anti-ATG7 (Cell Signaling Beverly, MA), anti- phospho-p70s6 kinase (Cell Signaling Technology Beverly, MA), anti-p70s6k total (Cell Signaling, Beverly, MA), anti-phospho-AMPkα (Thr172) (Cell signalling Beverly, MA), anti-AMPkα (Cell Signaling, Beverly, MA), anti-Caspase 8 (BD Pharmingen). α-tubulin (Sigma, St Louis Mo), β-Actin (Sigma Aldrich,) and GAPDH (Santa Cruz Biotechnology) were used as loading control. Bands were visualized by ECL-PLUS (Amersham Biosciences) and the pictures were taken with the imaging system ChemiDoc XRS System (BIO-RAD) and medical X-ray films (AGFA).

ATP determination. Intracellular ATP was measured using a luciferin/luciferase-based assay (Adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit, Sigma-Aldrich, St Louis, USA) following manufacturer's guidelines. A standard curve was generated rather than known concentrations of ATP and used to calculate sample ATP concentrations. Protein concentration was determined using Bradford protein assay reagents (Bio-Rad). The content of ATP was normalized for protein content.

Autophagy Assays. GFP-LC3-expressing cells have been used to demonstrate induction of autophagy. We used the GFP-LC3 expression vector kindly supplied by Dr. T. Yoshimori (National Institute for Basic Biology, Okazaki, Japan), in *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ 3T3 cells transiently transfected with this vector with jetPEITM (Polyplus transfection, Illkirch, France), according to the manufacturer's protocol. The assay was performed on cells grown on glass cover-slips (4 x 10^4 cells per well) in 6-well plates and after the different treatment with HANK buffer, these cells were washed 3 times with sterile PBS and fixed with Paraformaldehyde Solution (4%, wt/vol in

PBS1x with 2% Sucrose) for 10 minutes at room temperature. To determine LC3 localization GFP-LC3-transfected cells were observed under a Zeiss Fluorescence Microscope and to determine LC3-II translocation in *parp-1*$^{+/+}$ MEFs 3T3 and *parp-1*$^{-/-}$ cells we performed a western blot of LC3-I and its proteolytic derivative LC3-II (18 and 16 kDa, respectively), using a monoclonal antibody against LC3, from NanoTools (NanoTools, clone 5F10, Ref 03231-100/LC3-5F10).

Rapamycin and Concanamycin A were used as autophagy inductors, positive controls. Rapamycin was used at 100 nM for 4 hours and Concanamycin A at 4 nM for 4 hours.

Apoptosis Assays In addition to caspase 8 cleavage, apoptosis was determined by two different methods:

a) Pyknotic nuclei: Cells were fixed by Paraformaldehyde (4%, wt/vol in PBS1x with 2% Sucrose) for 10 minutes at room temperature and the number of cells with nuclear apoptotic morphology was determine after 6 and 8 hours of starvation, by DAPI staining using a Zeiss Fluorescence Microscope. Treatment with cycloheximide (CHX C4859, Sigma-Aldrich, St Louis, Mo, USA) 0.5 µg/ml for 8 hours was used as positive control of nuclear apoptotic morphology.

b) Annexin V / IP: *Parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ MEFs 3T3 cells (5 x 10^5 cells per well) were cultured in 6 wells plate and starved for 6 hours. After starvation, cells were washed twice with PBS, trypsinized and centrifuged at 1600 rpm for 5 minutes. The pellets were re-suspended in AnnexinV/IP solution (Roche Applied, Mh Germany), according to the manufacturer's instruction and maintained in the dark at 37ºC for 15 minutes. Apoptotic cells were evaluated in a FACScalibur flow cytometer with the Cell Quest software (BD Biosciences, NJ, USA). Staurosporin (6942, Sigma-Aldrich, St Louis, USA) (3 µM) for 8 hours was used as positive control of apoptosis induction.

Caspase 3/7 activity in 3T3 MEFs: parp-1+/+ 3T3 MEFs and parp-1-/- 3T3 MEFs cells were starved for 4.5 , 6 and 8 hours to induce apoptosis. The Caspase - Glo reagent was added directly to cells in 96-well plates; the final volume was 200 µl per well. The assays were incubated at room temperature for 45 minutes before recording luminiscence in a TECAN infinite 200 Luminometer. Each point represents the average of 3 wells per condition to 3 independent experiments. The "no-cell" blank control value has been substracted from each point. STS 2 µM 3 hours was used as positive control of caspase activation, data generated in apoptotic cells with STS are not shown. ** $p<0.01$ as compared to starved parp-1+/+ 3T3 MEFs.

RNA interference. Cells were transfected with the indicated siRNAs at 50 nM using Dharmafect transfection agent (Dharmacon Research, CO, USA) according to the manufacturer's guide. siRNA corresponding to the cDNA sequences were: ATG7 no.1 from Invitrogen, Human PARP-1 from Ambion Applied Biosystems and Mouse PARP-1 from Santa Cruz Biotechnology.

After 48 hours after of transfection, cells were treated as described and observed under a Zeiss Fluorescence Microscope or the proteins were extracted.

ROS and DNA damage determination. Reactive Oxygen Species production was measured through flow cytometry in an Epics Elite ESP cytometer, Coulter, using DCFDA (35845, FLUKA), 8mg/ml in DMSO, a specific probe to reactive oxygen species. 3T3 cells were seeded at 3.5 x 10^5 cells per well in 6-well plates and incubated overnight at 37 ºC. Cells were incubated for 30 min with the probe before the end of the deprivation and they were washed twice with PBS, trypsinized and the ROS production was analyzed by flow cytometry.

DNA damage was analyzed using two different methods:

a) COMET ASSAY: DNA damage was quantified using Comet Assay kit (R&D Systems, Trevigen, MD) with some modifications. 1 × 10^5 cells/ml were combined with

molten LM Agarose at 37 °C at a ratio of 1:10 vol/vol and pipetted onto a Comet Slide. The slides were placed for 10 min in the dark at 4 ºC and next they were immersed in pre-chilled Lysis solution. The slides were then removed from lysis buffer, washed in TBE buffer and transferred to a horizontal electrophoresis chamber. Voltage (1 V/cm) was applied for 20 min. After washing in distilled water, the slides were immersed in 70% ethanol for 5 min and allowed to air dry. Slides were stained with SYBR Green and then analyzed by fluorescence microscopy. 70–90 cells were evaluated in each sample using the Comet Assay Software Project (CASP software). DNA damage was quantified by measuring the tail moment (TM) calculated as percentage of DNA in the tail × tail length.

b) phospho-γH2AX: To visualize foci of γH2AX we used immunofluorescence with a monoclonal antibody for H2AX histone (UPSTATE, #05-636, clone JBW103) and FITC-conjugated goat anti-mouse. Nuclear counterstaining with DAPI was performed after removal of excess secondary antibody. Immunostaining was visualized with a Zeiss Fluorescence Microscope. Western blot analysis of H2AX phosphorylation was measured with the same antibody. Bands were visualized by ECL-PLUS (Amersham Biosciences) and the pictures were taken with the imaging system ChemiDoc XRS System (BIO-RAD) and medical X-ray films (AGFA).

Electron microscopy. Animal experimental protocols were reviewed and approved by the Ethical Committee of the Spanish Council of Scientific Research (CSIC). Parp-1 wild type and knockout (de Murcia et al, 1997) neonatal mice were used to determine the differences in autophagy induction during starvation in hepatic tissue. Neonatal mice were separated from the mother in the first 4 hours after birth.Livers of wild-type and parp-1 knockout mice were extracted and washed with PBS, prefixed for 30 minutes in a Fixation Solution with Cacodilate Buffer 0.1 M pH 7.4

and Osmium Tetraoxyde for 60 minutes at 4ºC. After this treatment, tissues were washed with miliQ water and the samples stained with Uranil Acetate. The ultrathin sections were performed with a diamond knife in an ultramicrotome (Reichert Ultracut S). The samples were analyzed in a TEM Zeiss 902 with 80 Kv of voltage acceleration (CIC-UGR).

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Figure 1: Starvation-induced autophagy is delayed in PARP-1 knockout cells

(A) *Parp-1*$^{+/+}$ MEFs 3T3 and *parp-1*$^{-/-}$ were transfected with GFP-LC3; 24 hours following transfection, cells were starved with HANK buffer at 1, 2 and 4,5 hours; percentages of LC3 translocation are shown. 100 nM rapamycin for 4 hours was used as positive control of autophagosomes accumulation. The pictures in the right panel show representative images with the subcellular distribution of the autophagic vesicles marker LC3.

(B) Immunoblot analysis of endogenous LC3 translocation in control and starved cells at different times of starvation. Rapamycin was used as positive control of LC3 translocation and β-actin as loading control. Similar results were obtained in 3 independent experiments. * p<0.05 as compared to starved parp-1+/+ 3T3 MEFs and **p<0.01 rapamycin treated parp-1+/+ 3T3 MEFs . # p<0.05 as compared to rapamycin treated parp-1-/- 3T3 MEFs.

(C) Effect of 2 mM 3MA on autophagy of *parp-1*$^{+/+}$ MEFs 3T3 and *parp-1*$^{-/-}$ during starvation. 3MA was added, as pre-treatment 1.5 hours before HANK buffer and maintained during the starvation in both cells lines to slow down autophagy.

(D) Effect of ATG7 silencing on starvation-induced autophagy. *Parp-1*$^{+/+}$ MEFs 3T3 and *parp-1*$^{-/-}$ were transfected with ATG7 siRNA (60nM) and 48 hours later they were transfected with GFP-LC3. 24 hours later cells were starved with HANK buffer for 30 min, 1 and 2 hours. SIMA or non-specific siRNA was used as negative control, using the same protocol as for siRNA transfection. The right panel show the siRNA-mediated suppression of ATG7 expression in MEFs 3T3 48 hours after transfection. GAPDH was used as loading control. *** p<0.001 as compared to starved parp-1+/+ 3T3 MEFs. ## p<0.01 as compared to starved parp-1-/- 3T3 MEFs.

In A, C and D figures, at least 250 cells were counted in a Zeiss fluorescent microscope in both cell lines, in 3 independent experiments. * p <0.01, * * p <0.001 and # p <0.001 as compared to starved *parp-1*$^{+/+}$ MEFs 3T3.

Figure 2: PARP-1 inhibition or silencing interferes with starvation-induced autophagy.

(A) Effect of the inhibition of PARP-1 with DPQ on starvation-induced autophagy. *Parp-1*$^{+/+}$ MEFs 3T3 and *parp-1*$^{-/-}$ were transfected with GFP-LC3; 24 hours later, cells were pre–treated with 40 μM DPQ for 1.5 hours. During the different starvation times, 40 μM DPQ was present with HANK buffer to maintain PARP-1 inhibited Percentages of cells with LC3 translocation are shown.

(B) Effect of PARP-1 silencing on starvation-induced autophagy. *Parp-1*$^{+/+}$ MEFs 3T3 were transfected with murine PARP-1 siRNA (50 nM) and 48 hours later cells were transfected with GFP-LC3, 24 hours after were staved during 2 hours and the

percentage of cells with the typical GFP-LC3 punctuated pattern was compared with the percentage in non-silencing *parp-1*$^{+/+}$ MEFs 3T3 starved at the same times. SIMA or non-specific siRNA was used as negative control at 50 nM, using the same protocol as for siRNA transfection. The right panel show the levels of silencing obtained for PARP-1 48 hours after the transfection. α–tubulin was used as loading control.

(C) Western Blot analysis of the effect of silencing of PARP-1 on endogenous LC3 translocation in *parp-1*$^{+/+}$ MEFs 3T3 starved during 2 hour with HANK buffer. α– tubulin was used as loading control. Similar results were obtained in 3 independent experiments. ** p<0.01 as compared to starved parp-1+/+ 3T3 MEFs.

(D) Reconstitution of PARP-1 in *parp-1*$^{-/-}$ MEFs 3T3 and effect on the levels of starvation-induced autophagy. *Parp-1*$^{-/-}$ MEFs 3T3 were transfected with pBC-PARP-1 to reconstitute transiently PARP-1 and 24 hours later cells were transfected with GFP-LC3; 24 hours after transfection cells were starved with HANK buffer during 30 min and 2 hours. pBC empty plasmid was used as negative control. The right panel show the expression level of PARP-1 24 hours after reconstitution in *parp-1*$^{-/-}$ MEFs 3T3 and compared with the levels expression of PARP-1 in wild type and knockout cells. α–tubulin was used as loading control. p<0.05, ** p<0.01 and *** p<0.001 as compared to starved parp-1+/+ 3T3 MEFs. # p<0.05 and ## p<0.01 as compared to starved parp-1-/- 3T3 MEFs.

In A, B and D figures, at least 250 nuclei were counted in a Zeiss fluorescent microscope in 3 independent experiments. * p <0.01 as compared to starved *parp-1*$^{+/+}$ MEFs 3T3. #p <0.01 as compared to starved *parp-1*$^{+/+}$ MEFs 3T3.

Figure 3: Poly(ADP-ribose) (PAR) synthesis and DNA damage during starvation-induced autophagy.

(A) Western Blot analysis of PAR formation during starvation. *Parp-1*$^{+/+}$ MEFs 3T3 were starved during 30 min, 1 and 2 hours with HANK buffer. Whole cell extracts were subjected to PAGE and PAR was measured with a specific antibody. Where indicated, cells were pre-treated with PJ34, a PARP-1 inhibitor, during 1,5 hours and maintained during the nutrient deprivation. 10 mM H_2O_2 for 10 minutes was used as a positive control PARP-1 activation and PAR synthesis. α - tubulin was used as loading control.

(B) ROS production during starvation. *Parp-1*$^{+/+}$ MEFs 3T3 and *parp-1*$^{-/-}$ were subjected at short times of nutrient deprivation with HANK buffer in the presence of the DCFDA probe (8mg/ml), specific to measure ROS with a flow cytometer. Figure shows DCFDA - fluorescence obtained as arbitrary units in 3 independent experiments in triplicate.

(C) COMET Assay during starvation. *Parp-1*$^{+/+}$ MEFs 3T3 and *parp-1*$^{-/-}$ were starved with HANK buffer at 15, 30 and 60 minutes and then were treated according with the manufacturer´s instruction. Tail moment of 90 nuclei per condition in 3 independent experiments, were analysed by the specific software CASP (Panel C right). In the right panel images of the COMETs extracted from the software to each cell line in the different times of starvation are shown.

(D) Histone γH2AX phosphorylation during starvation. *Parp-1*$^{+/+}$ MEFs 3T3 and *parp-1*$^{-/-}$ were subjected to starvation at the stated times. Total extract were obtained and the levels of phospho- γH2AX were measured by immunoblotting. Similar results were obtained in 3 independent experiments. α - tubulin was used as loading control.

* p <0.01 and * *p <0.001 as compared to starved *parp-1*$^{+/+}$ MEFs 3T3 in figure C left.

(E) Treatment with the antioxidant NAC results in a strong delay in mTOR inactivation during starvation-induced autophagy. * p<0.05, ** p<0.01 and *** p<0.001 as compared to starved *parp-1*+/+ 3T3 MEFs. ## p<0.01 as compared to starved *parp-1-/-* 3T3 MEFs.

Figure 4: PARP-1 modulates AMPk activation and mTOR inhibition during starvation-induced autophagy.

(A) Effect of nutrient starvation on ATP levels. *Parp-1*$^{+/+}$ MEFs 3T3 and *parp-1*$^{-/-}$ were starved with HANK buffer at 15, 30 and 60 minutes. Concentrations of ATP were normalized with total proteins in each sample and referred to the control (100%). 2mM 3MA was added 1,5 hours before nutrient deprivation and was maintained during the experiment in order to inhibit autophagy. Error bars represent SE of the mean (SEM) of 5 independent experiments. #p <0.01 as compared to starved *parp-1*$^{+/+}$ MEFs 3T3.

(B) Immunoblot analysis of AMPk activation in control and starved cells at different times of starvation. The levels of phospho-AMPk were measured with a specific antibody from whole cell extracts in each cell lines after nutrient deprivation. Total AMPk was used to normalize for the non-phosphorylated protein and β- actin as loading control. Similar results were obtained in 3 independent experiments.

(C) Immunoblot analysis of mTOR inhibition during starvation. The levels of phosphorylation of the mTOR substrate, p70s6 kinase, were measured by western blotting in whole cell extracts of *Parp-1*$^{+/+}$ MEFs 3T3 and *parp-1*$^{-/-}$ after different times of starvation. Concanamycin A (2 nM) during 4 hours was used as control of mTOR-independent autophagy while Rapamycin (100 nM) during 6 hours was used as control of mTOR-dependent autophagy. Total p70s6 kinase was used to normalize for the non-phosphorylated protein and β– actin as loading control. Similar results were obtained in two independent experiments.

(D) Treatment with the antioxidant N-acetylcysteine (NAC) strongly delays starvation-induced loss of mTOR activation measured as phospho-p70s6 kinase.

(E) Induction of autophagy in MCF7 GFP-LC3 during starvation. Rapamycin was the positive control of autophagy and 10 mM 3MA was used to . A western blot is shown in parallel to visualize the lipidation of LC3-II. * p <0.01 as compared to non starved MCF7 GFP-LC3 in 3 independent experiments.

(F) PARP-1 knockdown prevents from autophagy-induced mTOR inhibition in a tumoral model. LEFT PANEL: MCF7 GFP-LC3 cells were starved at different times with HANK buffer and the levels of phospho-p70s6 kinase was measured by western blotting. Concanamycin A and Rapamycin were the different controls of mTOR activation and

p70s6 kinase and GAPDH were used to normalize for protein loading. These results were obtained in 3 independent experiments. RIGHT PANEL: MCF7 GFP-LC3 cells were transfected with PARP-1 siRNA (50 nM) (First Line) and 48 hours after transfection, cells were starved with HANK buffer; the levels of phospho-p70s6 kinase was measured by western blot (Second Line). Again Concanamycin A and Rapamycin were used as controls of activation of mTOR and total p70s6 kinase and GAPDH to normalize for protein loading. Similar results were obtained in 3 independent experiments. Quantitation using densitometry is shown on the left; * $p<0.05$, ** $p<0.01$ comparing MCF7 GFPLC3 feeded and starved cells. # $p<0.05$ and ## $p<0.01$ comparing between starved PARP-1 silenced mock transfected MCF7 GFPLC3 cells.

Figure 5: Prosurvival autophagy is switched to apoptosis after PARP-1 ablation

(A) Effect of PARP inhibition and PARP-1 silencing in cell death during autophagy induced by nutrient deprivation. *parp-1*$^{+/+}$ MEFs 3T3 were transfected with PARP-1 siRNA (60 nM) and 48 hours after transfection, cells were pre-treated or not with PJ34(20 μM). Cells were starved during 8 hours with HANK buffer and cell death was analyzed IP incorporation using flow cytometry in 3 independent experiments with 4 replicates per condition.

(B) Effect of autophagy inhibition with 3MA on the survival of *parp-1*$^{+/+}$ MEFs 3T3 and *parp-1*$^{-/-}$ during starvation. 3MA was added, as pre-treatment 1,5 hours before the HANK buffer and kept during starvation in both cells lines to maintain the autophagy inhibited. Percentage of survival was obtained by MTT Survival Assay. Similar survival rates were obtained in 3 independent experiments with 4 replicates per condition.

(C) Effect of knockdown of ATG7 and PARP inhibition on cell death in parp-1 MEFs 3T3. Percentage of survival was obtained by MTT Survival Assay. Similar survival rates were obtained in 3 independent experiments with 4 replicates per condition. While ATG7 silencing prevented from loss of cell viability, PARP inhibition increased cell death even in cell with limited ability to engage autophagy.

(D) Cell death in MEFs *Bax*$^{-/-}$ / *Bak*$^{-/-}$ GFP-LC3 during starvation**:** levels of cell death measured by PI incorporation using flow cytometry during different times of nutrient deprivation. 3 independent experiments with 3 replicates per condition are represented.

(E) Induction of autophagy in MEFs *Bax*$^{-/-}$ / *Bak*$^{-/-}$ GFP-LC3 during starvation. Western Blot of LC3 translocation and percentage of autophagic cells **(D)**, using Rapamycin (100 nM) 4 hours as positive control. DPQ (40 μM) and 3MA (2 mM) were added as pre-treatment 1,5 hours before the HANK buffer and maintained during the assay of starvation. These experiments were repeated 3 times with similar results.

(F): reduction of the percentage of cell death with PARP-1 siRNA (50 nM) during starvation. The right panel show the silencing of PARP-1 48 hours after transfection. Similar results were obtained in 4 experiments with 4 replicates per condition.

(G) Caspase 3/7 activity in 3T3 MEFs: parp-1+/+ 3T3 MEFs and parp-1-/- 3T3 MEFs cells were starved for 4.5 , 6 and 8 hours to induce apoptosis. The Caspase - Glo reagent was added directly to cells in 96-well plates; the final volume was 200 μl per well. The assays were incubated at room temperature for 45 minutes before recording luminiscence in a TECAN infinite 200 Luminometer. Each point represents the average of 3 wells per condition to 3 independent experiments. ** $p<0.01$ as compared to starved parp-1+/+ 3T3 MEFs.

(H) Activation of caspase 8 in *Parp-1*$^{-/-}$ MEFs 3T3 under nutrient deprivation. *Parp-1*$^{+/+}$ MEFs 3T3 and *parp-1*$^{-/-}$ were starved with HANK buffer during 6 hours. The fragment of 38 KDa of caspase 8 processed was visualized by western blot, using a Jurkat cell total lysates as positive control of activation of caspase 8. α- tubulin was used as loading control.

(I) Percentage of apoptotic cells under starvation. *Parp-1*$^{+/+}$ MEFs 3T3 and *parp-1*$^{-/-}$ were starved for 6 and 8 hours and apoptosis evaluated by double staining of annexin V and IP by flow cytometry. Results are from 3 independent experiments with 2 replicates per condition. * $p<0.01$ comparing *parp-1+/+* and *parp-1-/-* starved MEFs.

Figure 6: Deficient liver autophagy in PARP-1 knockout starved mice. 2 hours after birth pups were separated from their mother and starved for 4 hours according to procedures approved by the bioethic committee of the CSIC. Pups were sacrified and livers were removed from and fixed for TEM or lysed for WB analysis. A. Autophagosomes showing concentrical membranes structures; ER, endoplasmic reticulum; L, lipid droples; Ly, lysosomes; N,

Figure 7: **A. PARP-1 is a positive modulator of starvation-induced autophagy**. Following nutrient starvation cells activate autophagy through the activation of AMPk/ inhibition of mTOR. Upstream events involve energy depletion, ROS production and DNA damage. Under this conditions, PARP-1 overactivation leads also to ATP depletion, acting as a feedback loop to reactivate autophagy. This stress signal when maintained leads eventually to cell death with autophagy. **B**. **PARP inactivation delays autophagy and favors apoptosis.** In the absence of PARP-1 or after inactivation of PARP, ROS levels decreases and ATP drop is reduced. As consequence, the feedback loop reactivated by PARP-1 do not take place, and apoptosis is triggered as a mode of cell death.

Figure 1

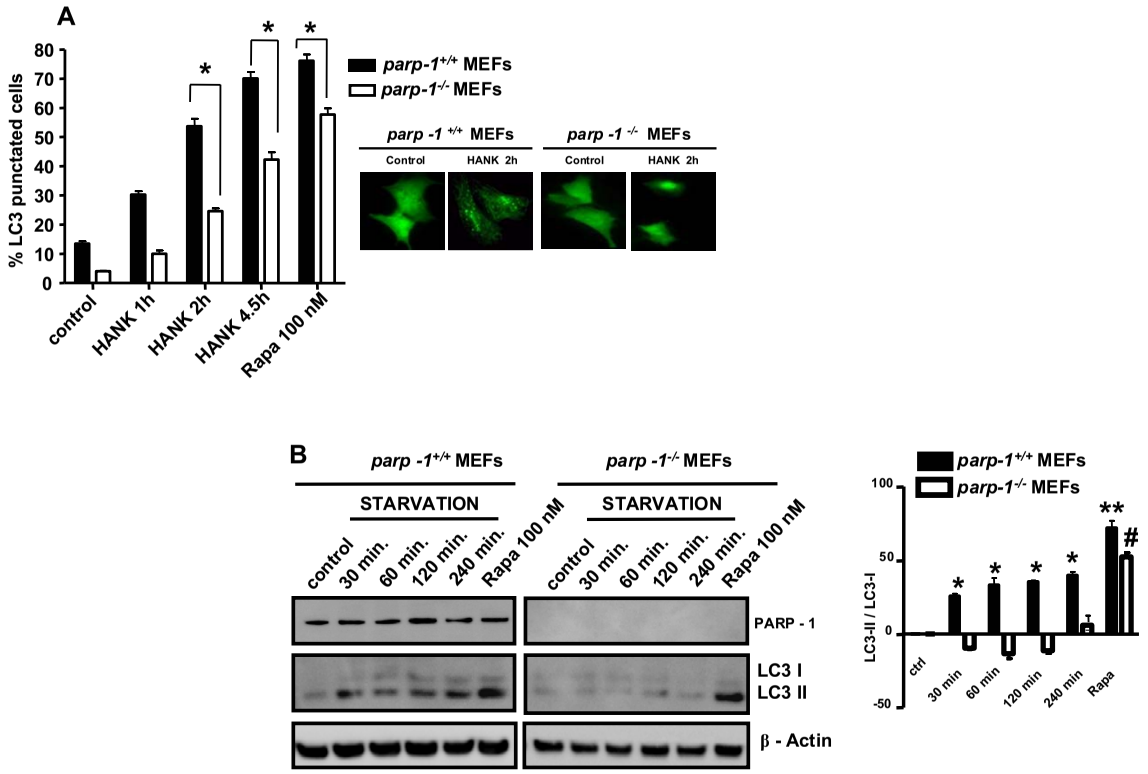


Figure 1

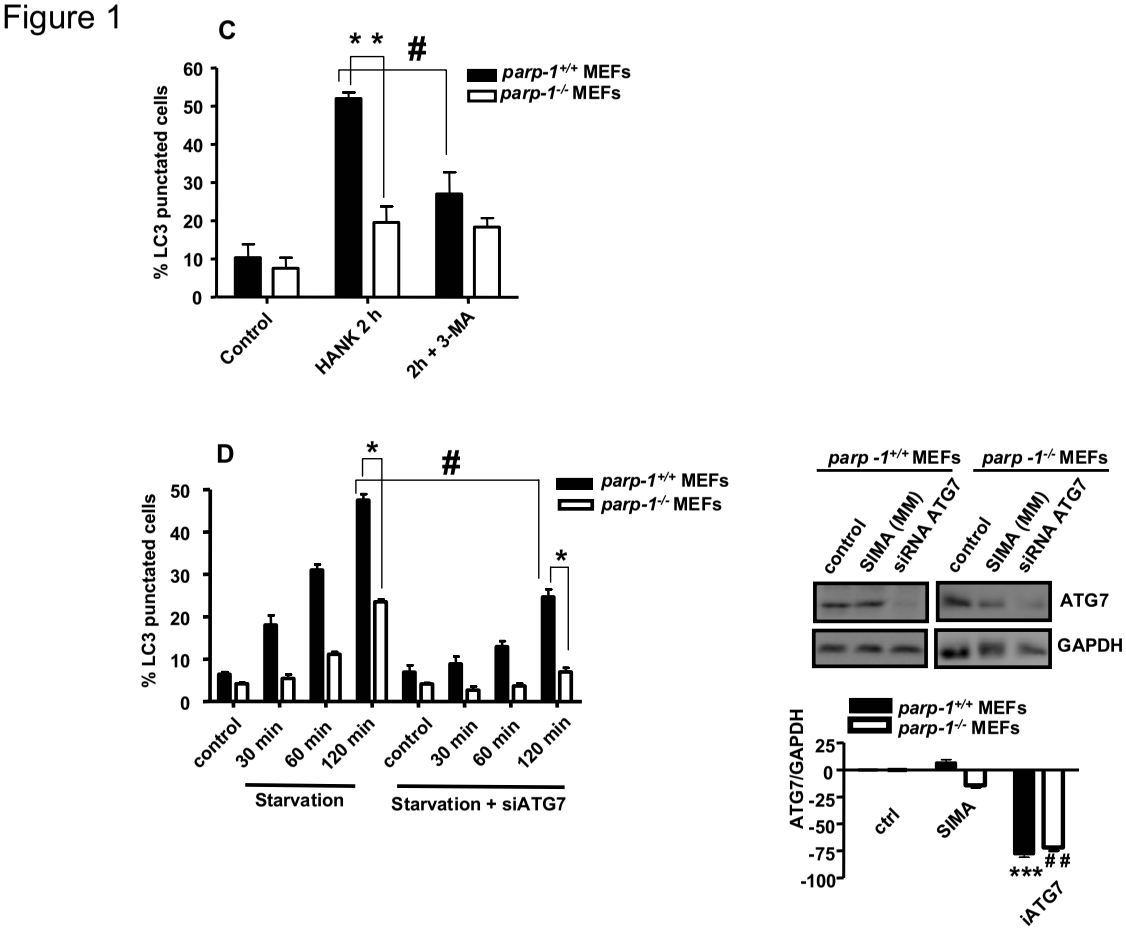


Figure 2

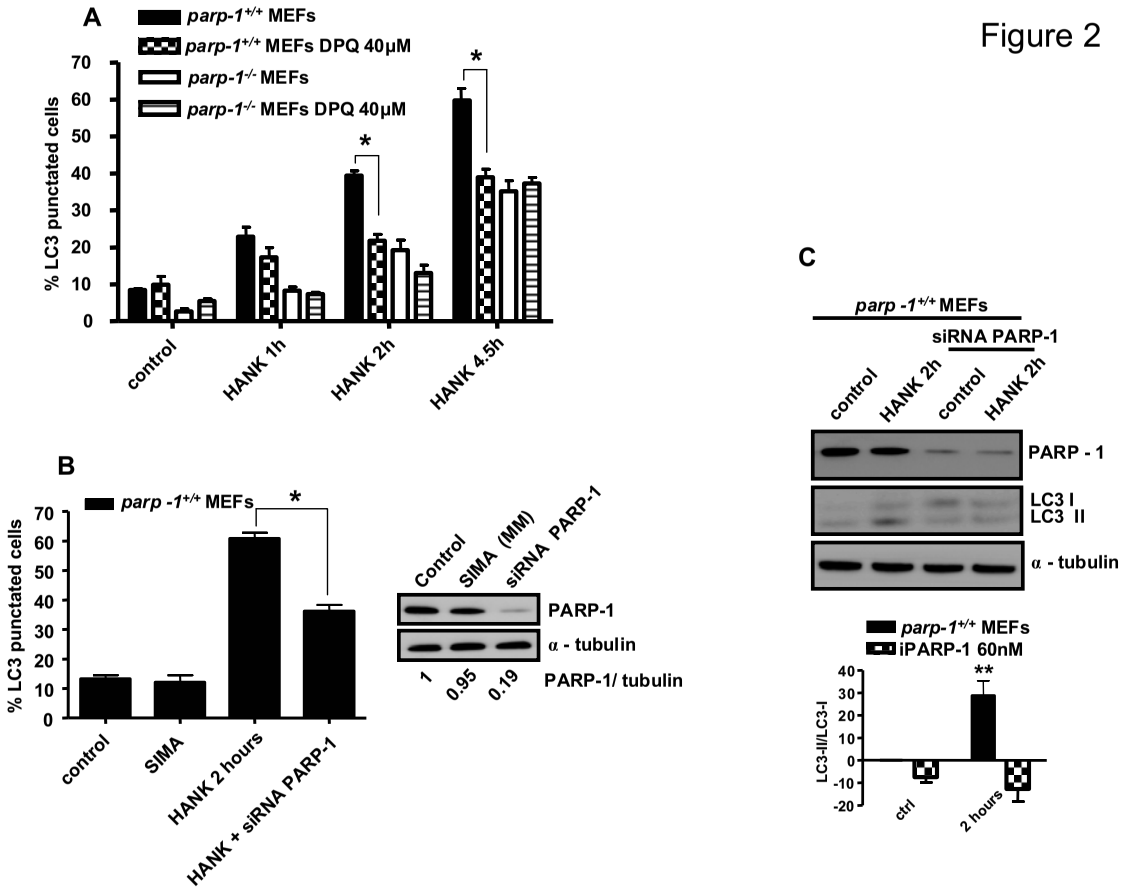
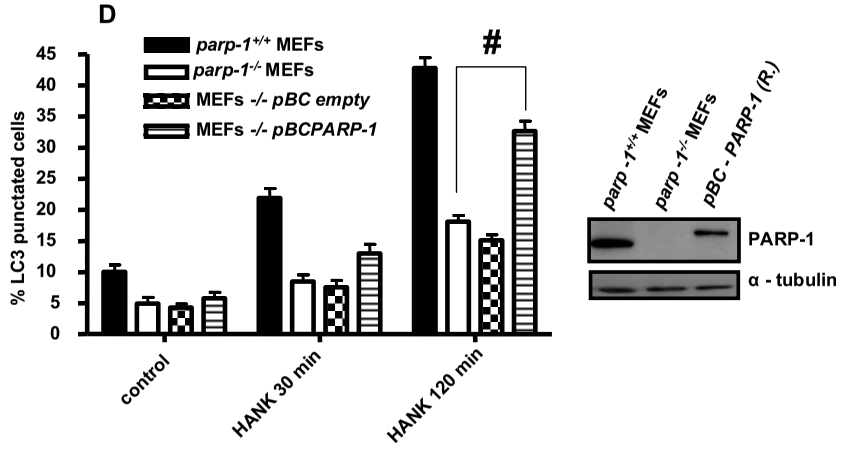


Figure 2



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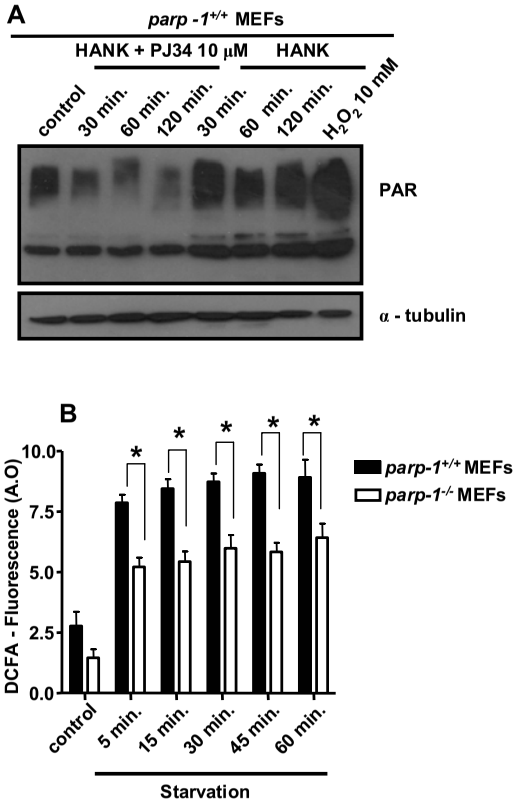


Figure 3

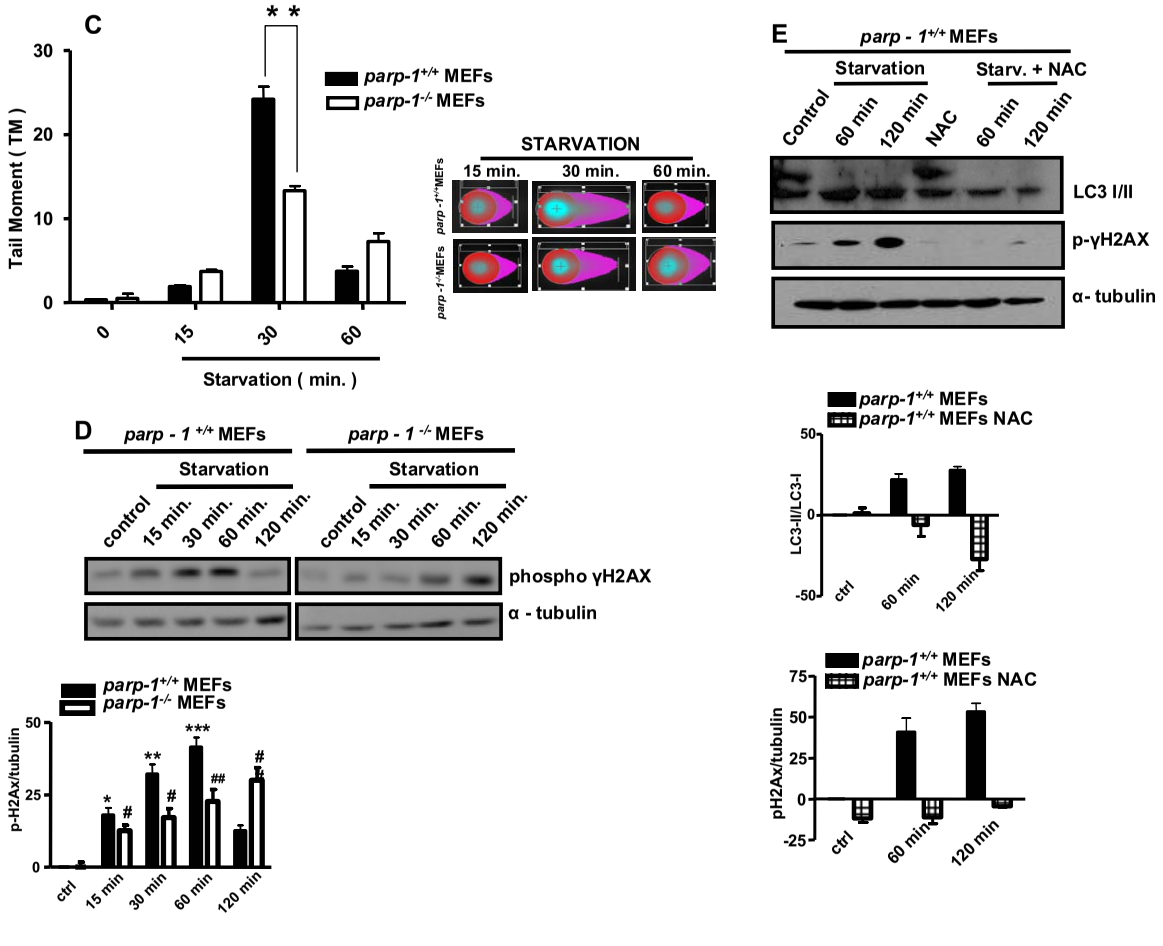


Figure 4

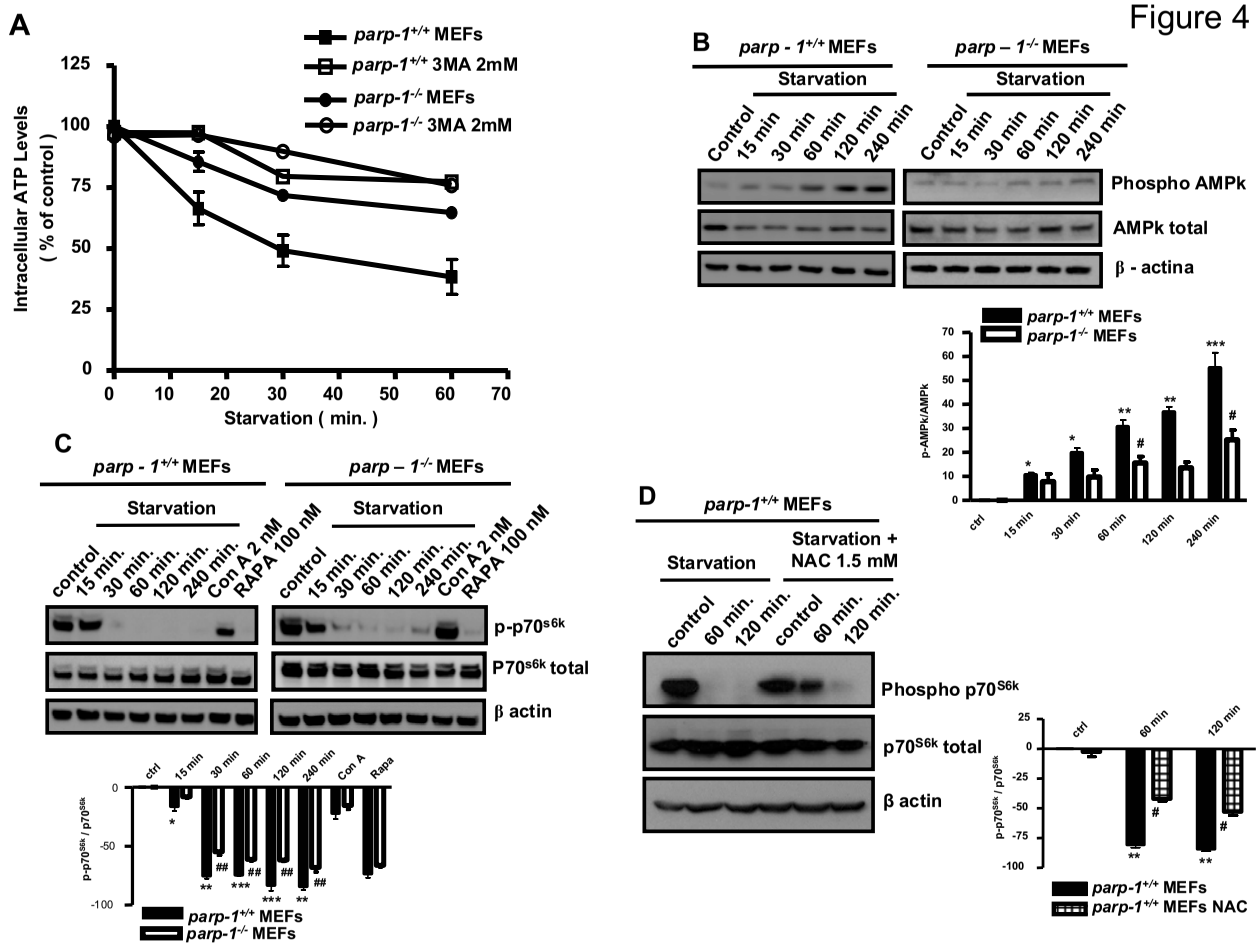
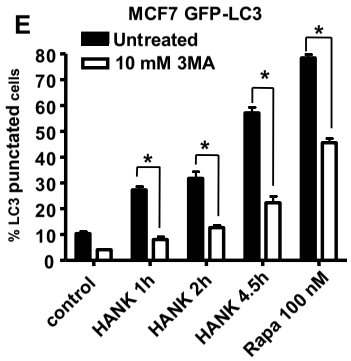


Figure 4



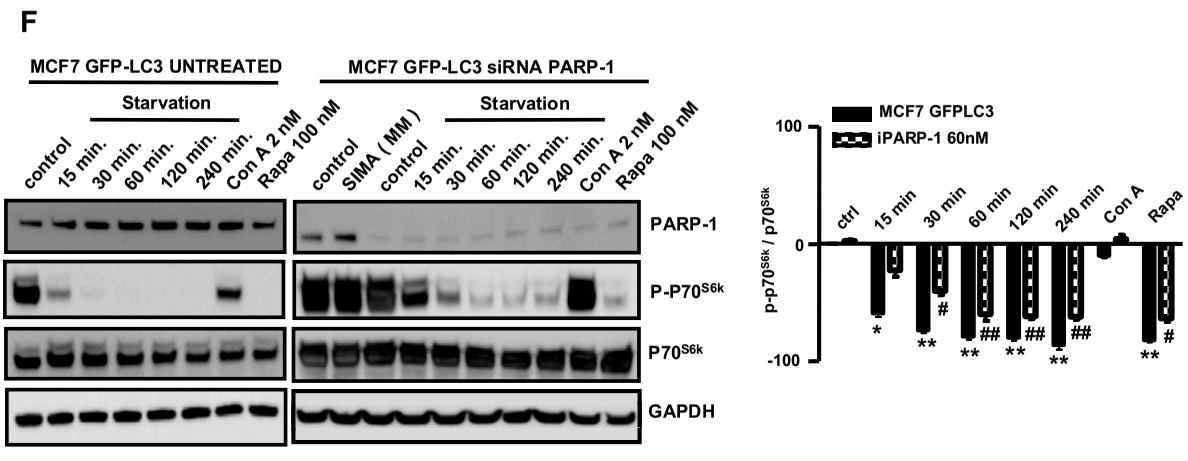
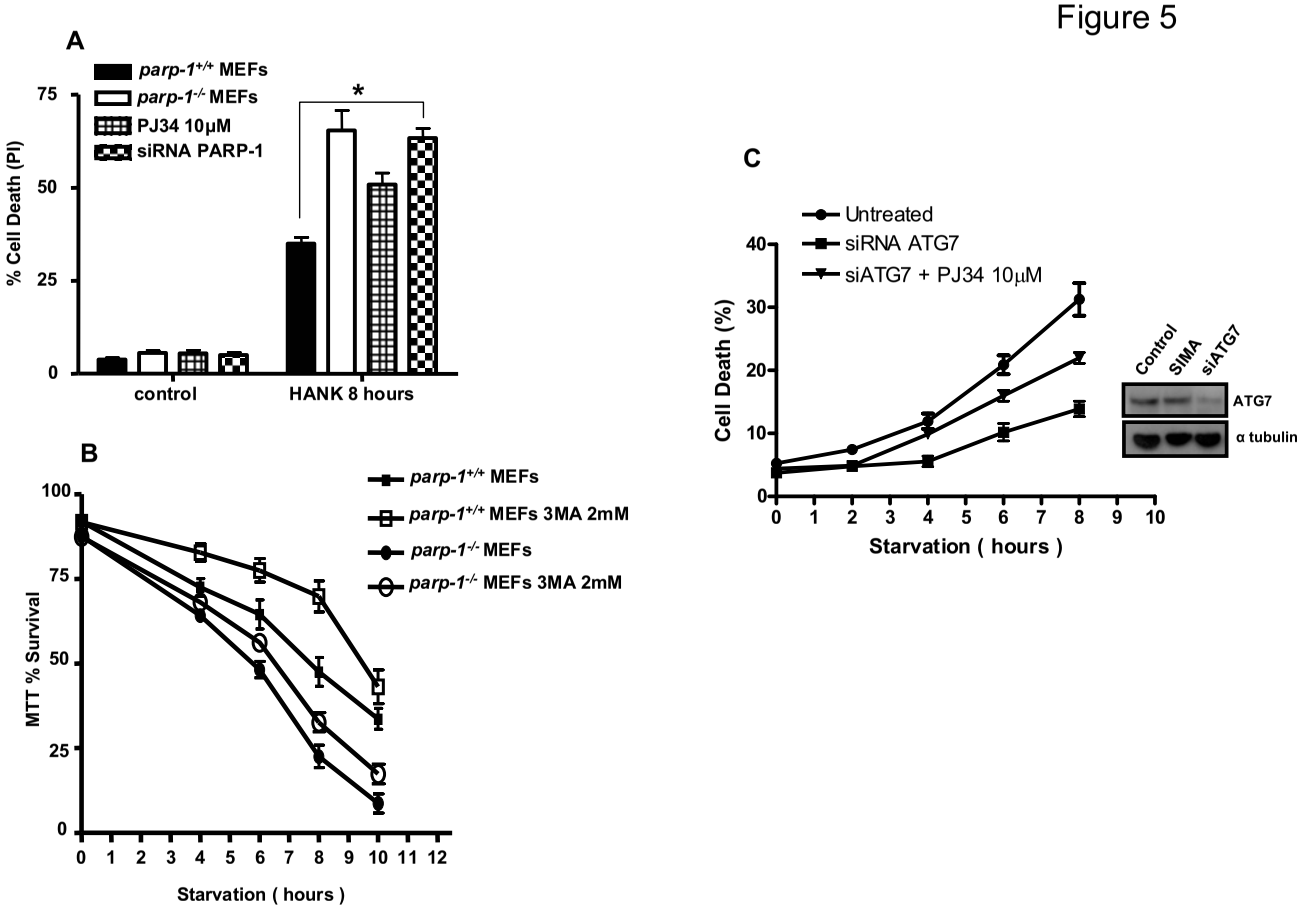
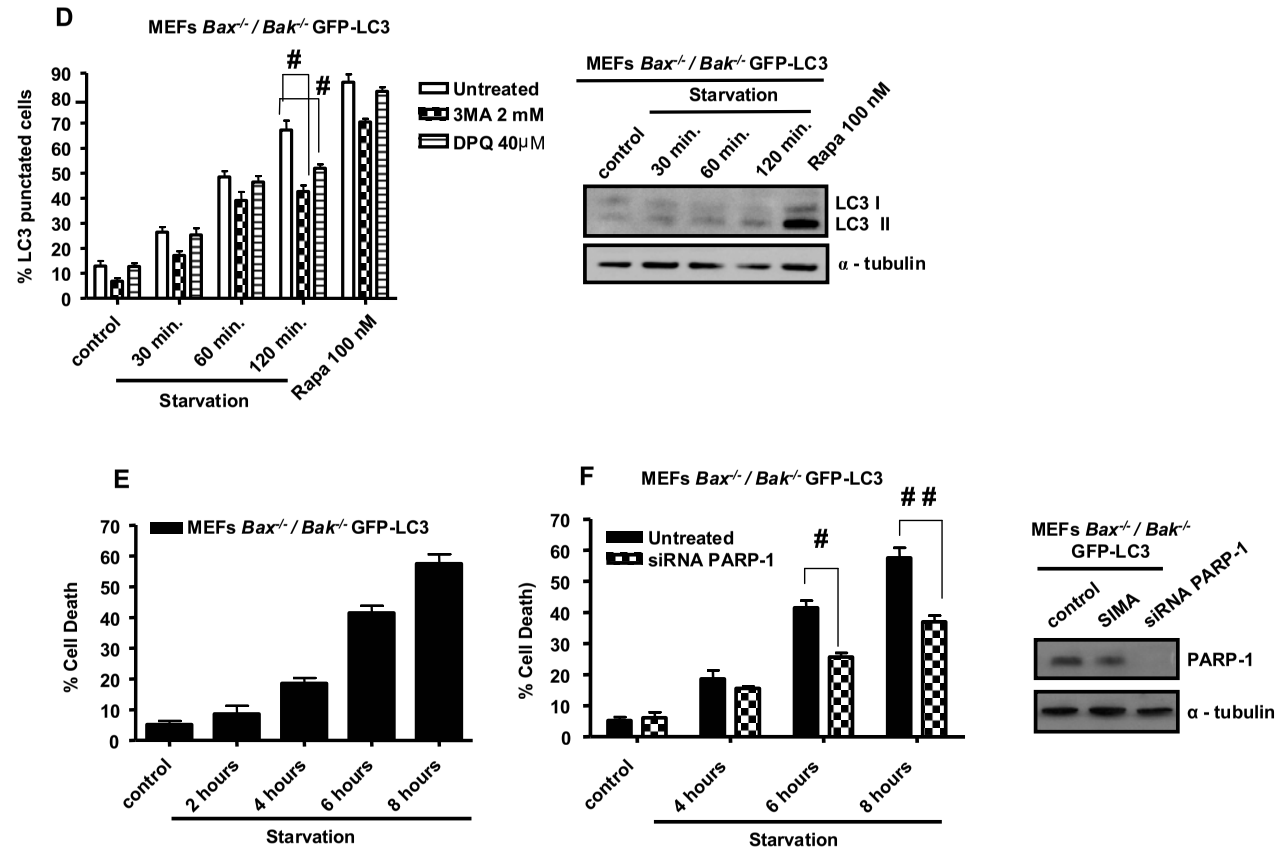
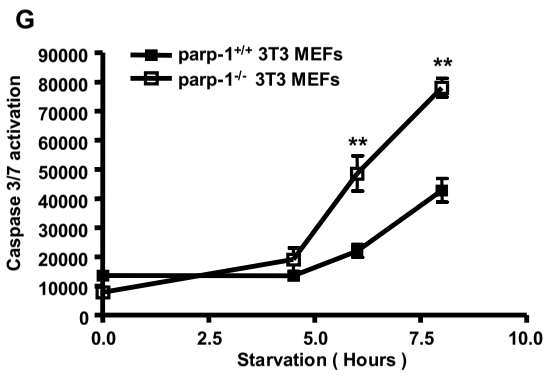
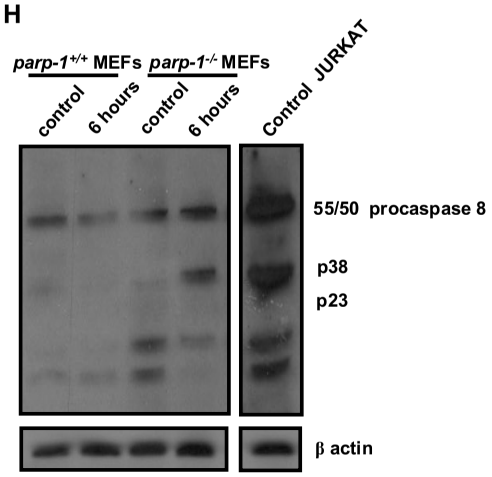


Figure 5


A
parp-1+/+ MEFs
parp-1-/- MEFs
PJ34 10μM
siRNA PARP-1
% Cell Death (PI)
control
HANK 8 hours
B
parp-1+/+ MEFs
parp-1+/+ MEFs 3MA 2mM
parp-1-/- MEFs
parp-1-/- MEFs 3MA 2mM
MTT % Survival
Starvation (hours)
C
Untreated
siRNA ATG7
siATG7 + PJ34 10μM
Cell Death (%)
Starvation (hours)
Control
SIMA
siATG7
ATG7
α tubulin


D
MEFs Bax-/- / Bak-/- GFP-LC3
% LC3 punctated cells
Untreated
3MA 2 mM
DPQ 40μM
control
30 min.
60 min.
120 min.
Rapa 100 nM
Starvation
MEFs Bax-/- / Bak-/- GFP-LC3
Starvation
LC3 I
LC3 II
α - tubulin
E
MEFs Bax-/- / Bak-/- GFP-LC3
% Cell Death
control
2 hours
4 hours
6 hours
8 hours
Starvation
F
MEFs Bax-/- / Bak-/- GFP-LC3
Untreated
siRNA PARP-1
% Cell Death)
control
4 hours
6 hours
8 hours
Starvation
MEFs Bax-/- / Bak-/- GFP-LC3
control
SIMA
siRNA PARP-1
PARP-1
α - tubulin


G
parp-1+/+ 3T3 MEFs
parp-1-/- 3T3 MEFs
Caspase 3/7 activation
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
**
**
0.0
2.5
5.0
7.5
10.0
Starvation (Hours)


H
parp-1+/+ MEFs
parp-1-/- MEFs
control
6 hours
control
6 hours
Control JURKAT
55/50 procaspase 8
p38
p23
β actin

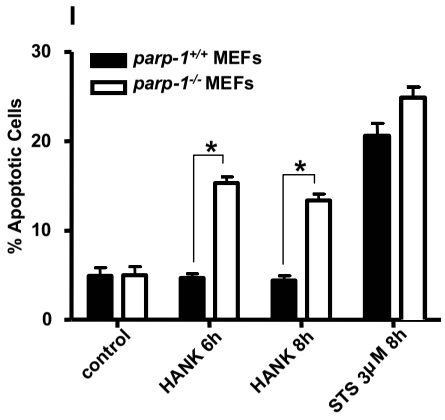
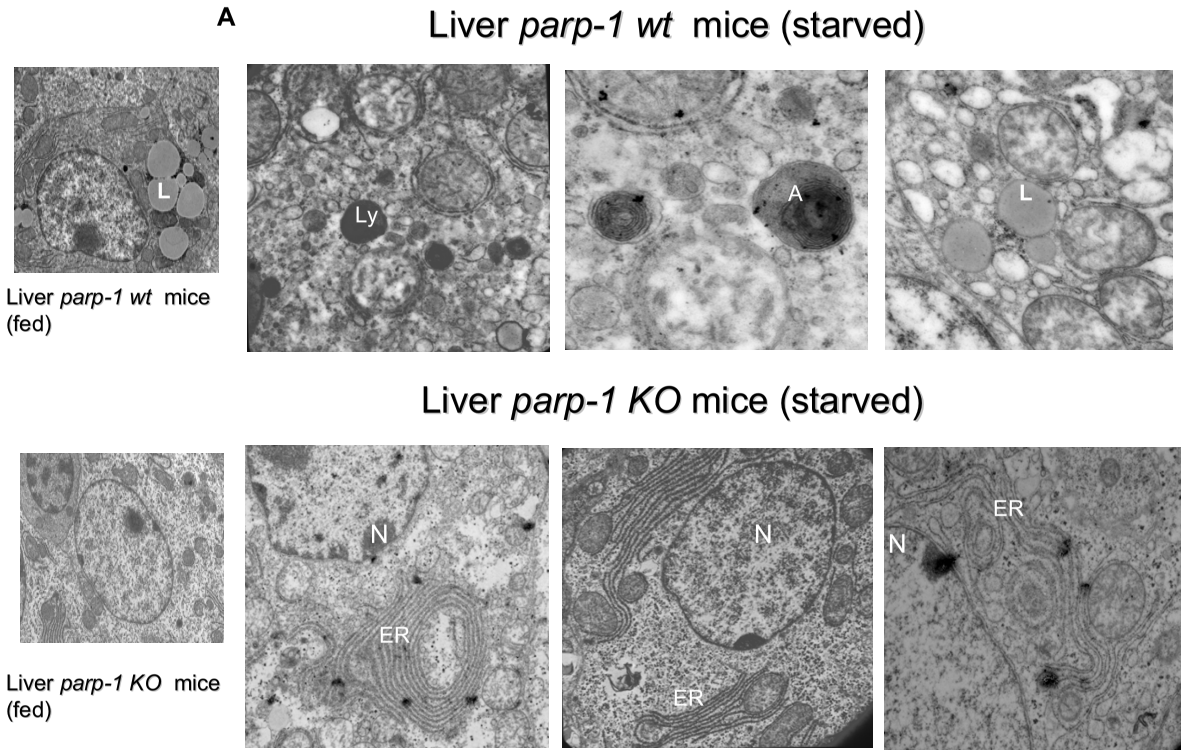

I
parp-1+/+ MEFs
parp-1-/- MEFs
% Apoptotic Cells
30
20
10
0
*
*
control
HANK 6h
HANK 8h
STS 3μM 8h

Figure 6


A
Liver parp-1 wt mice (starved)
L
Ly
A
L
Liver parp-1 wt mice (fed)
Liver parp-1 KO mice (starved)
N
ER
N
ER
N
ER
Liver parp-1 KO mice (fed)

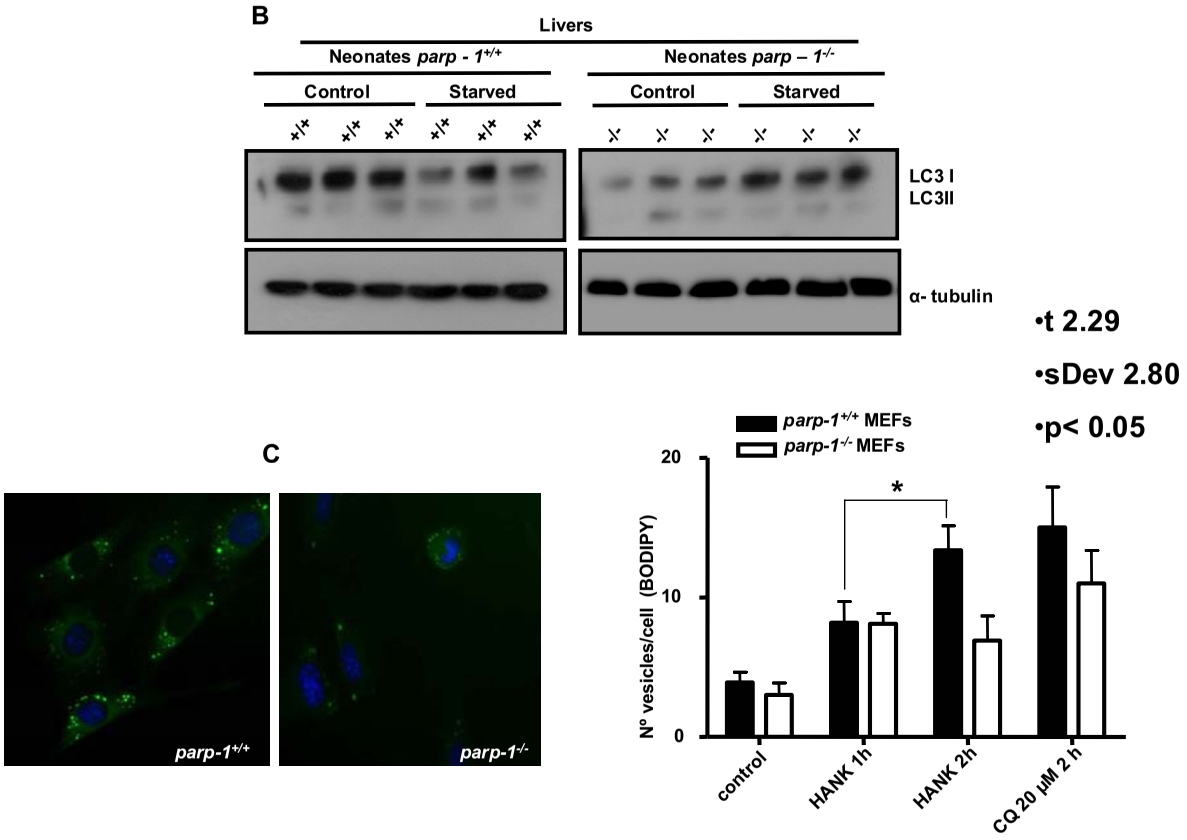

B
Livers
Neonates parp - 1+/+
Neonates parp – 1-/-
Control
Starved
Control
Starved
+/+
-/-
LC3 I
LC3II
α- tubulin
•t 2.29
•sDev 2.80
•p< 0.05
C
parp-1+/+
parp-1-/-
parp-1+/+ MEFs
parp-1-/- MEFs
Nº vesicles/cell (BODIPY)
20
10
0
*
control
HANK 1h
HANK 2h
CQ 20 µM 2 h

Figure 7

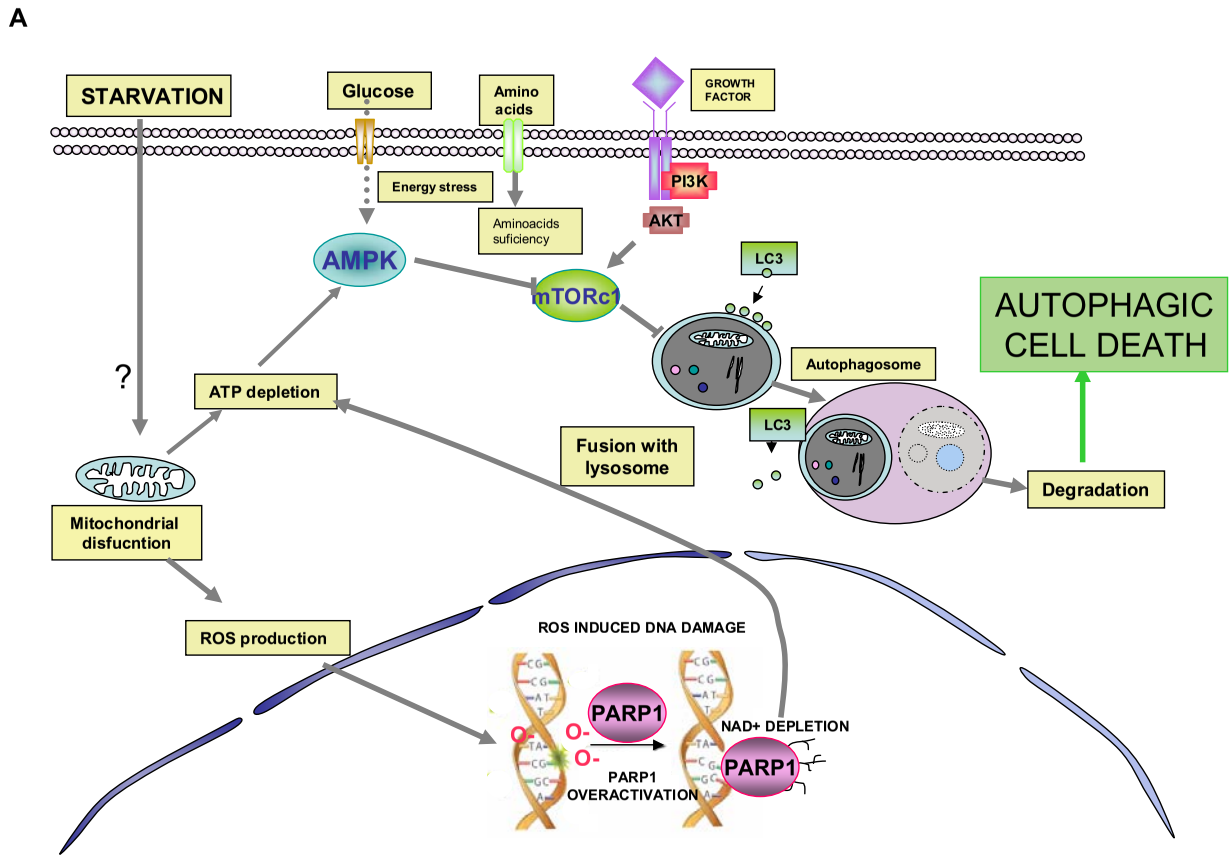
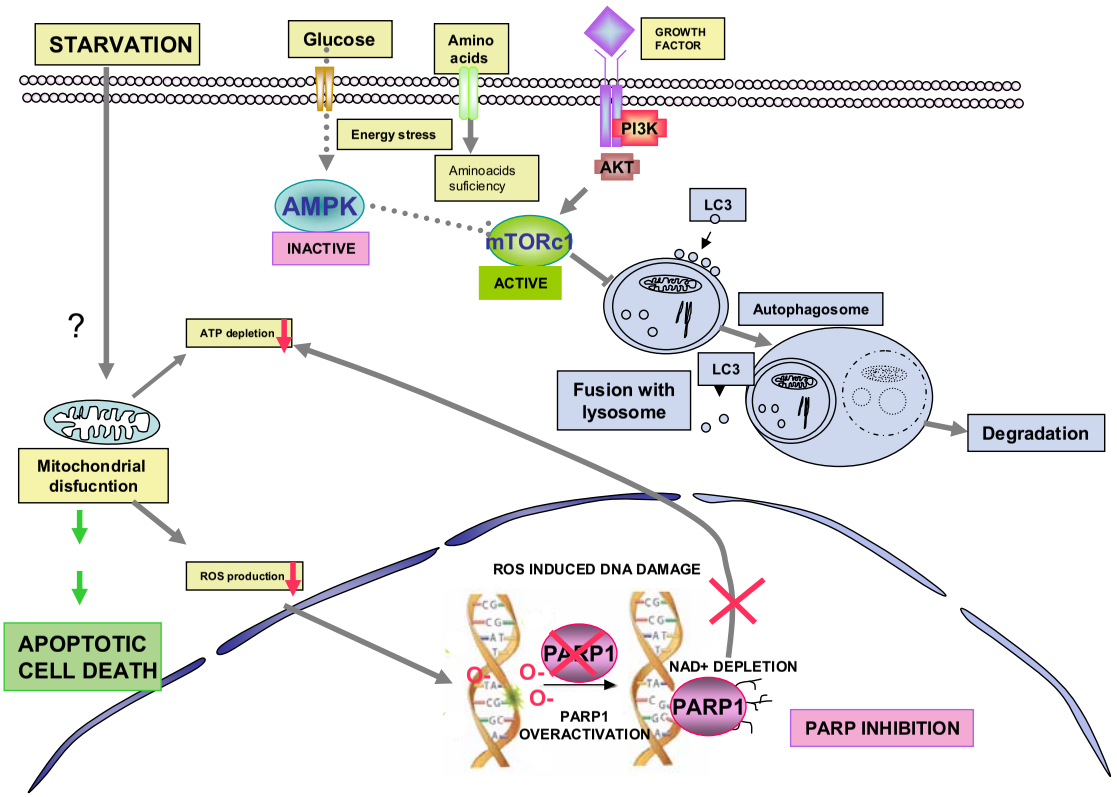

A
STARVATION
Glucose
Amino acids
GROWTH FACTOR
Energy stress
PI3K
AKT
Aminoacids suficiency
AMPK
mTORc1
LC3
AUTOPHAGIC CELL DEATH
?
ATP depletion
Autophagosome
LC3
Fusion with lysosome
Degradation
Mitochondrial disfucntion
ROS production
ROS INDUCED DNA DAMAGE
PARP1
NAD+ DEPLETION
PARP1
PARP1 OVERACTIVATION

Figure 7

B



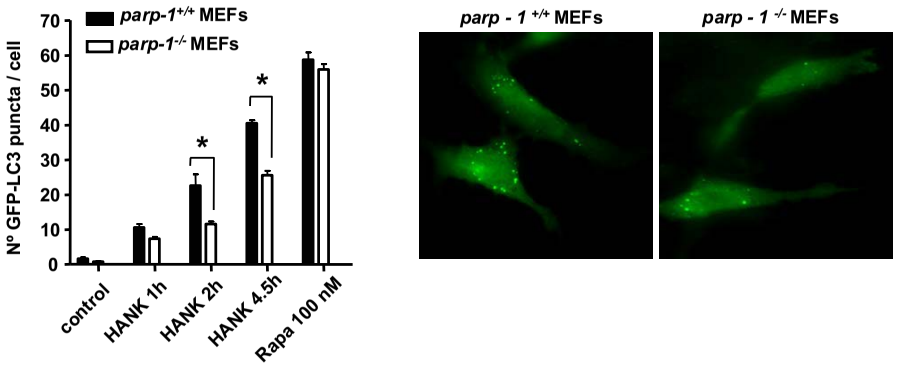


Figure S1. *parp-1*$^{+/+}$ MEFs 3T3 and *parp-1*$^{-/-}$ transfected transiently with GFP-LC3. 24 hours after the transfection, were starved with HANK solution during 1, 2 and 4,5 hours and the number of vesicles positive to GFP were counted under fluorescence microscopy (LEFT PANEL). Rapamycin 100 nM 4 hours was used as positive control of induction of GFP-LC3-vesicles in both cell lines. The images shown the differences in the number of vesicles at 2 hours of starvation (RIGHT PANEL). Approximately 250 cells were counted in a fluorescent microscope in 3 independent experiments. * p <0.01 as compared to starved *parp-1*$^{+/+}$ MEFs 3T3.

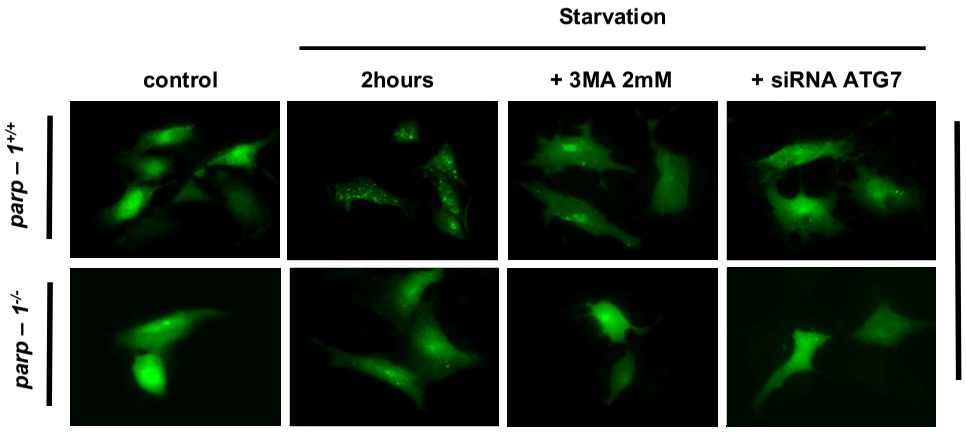


FIGURE S2: Effect of the chemical inhibition (3MA) and of ATG7 silencing on autophagy during starvation in *parp-1*$^{+/+}$ *and parp-1*+/+ MEFs 3T3

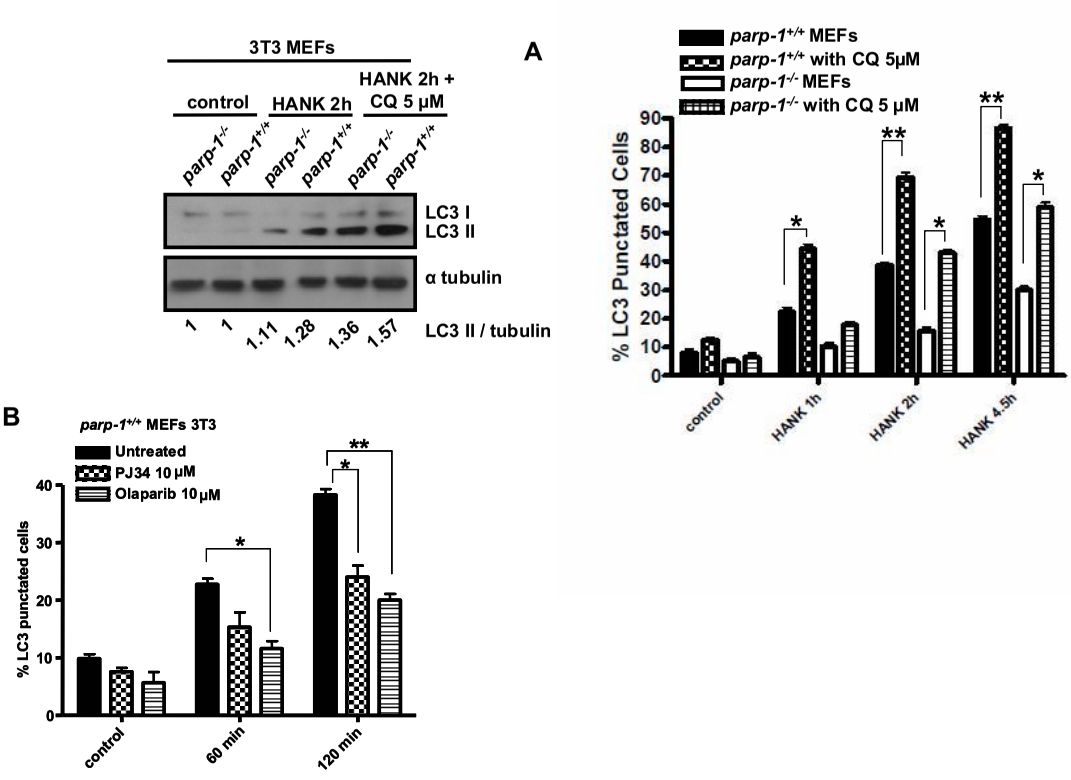


Figure S3: (A) Lysosome fusion with autophagosomes is not affected in *parp-1* -/- cells. Treatment with chloroquine to inhibit lysosome fusion results in a similar accumulation of LC3 vesicles in *parp-1* +/+ and parp-1 -/- . **(B)** Delay of starvation-induced autophagy after treatment with two different inhibitors of PARP, PJ34 (10 mM) or Olaparib (10 mM).

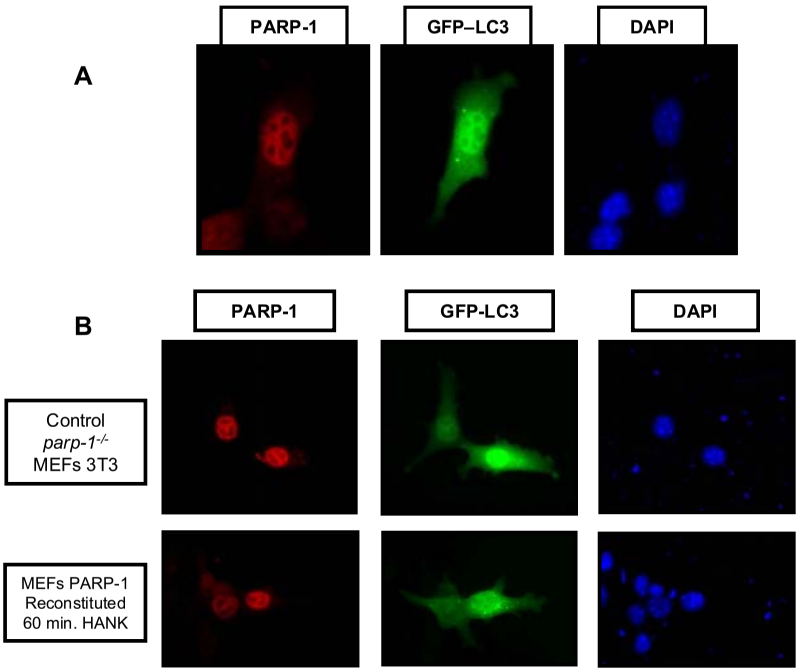


Figure S4. Reconstituted expression of PARP-1 in *parp-1-/-* MEFs 3T3 restored the autophagy levels

A. *parp-1*$^{-/-}$ MEFs 3T3 co-transfected with GFP-LC3 and pBC-PARP-1. PARP-1 located in nucleus and GFP-LC3 homogeneously distributed in cytosol and nucleus.

B. *parp-1*$^{-/-}$ MEFs 3T3 with PARP-1 restored, recovered the response to starvation and the number of vesicles at 60 minutes of treatment with HANK solution , increased.

Similar results were obtained in 4 independent reconstitution assays by inmunofluorescence.

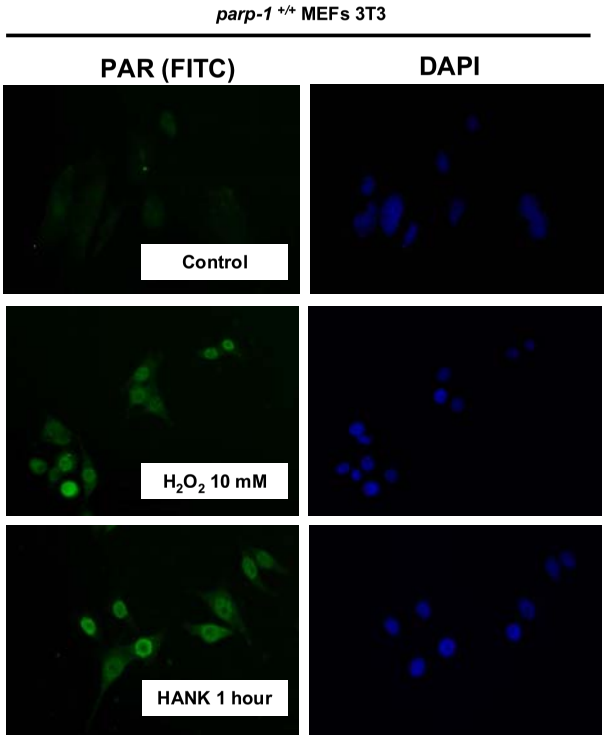


Figure S5. PAR polymer production confirms PARP-1 over-activation during starvation Immunofluorescence of polymer of poly(ADP-ribose) in *parp-1*$^{+/+}$ MEFs 3T3 confirms PARP-1 activation during nutrient deprivation. H2O2 10 mM act as positive control of activation of PARP-1. This signal is similar to PAR signal obtained during the HANK solution treatment. This results was confirmed in 4 independent immunofluorescence assays.

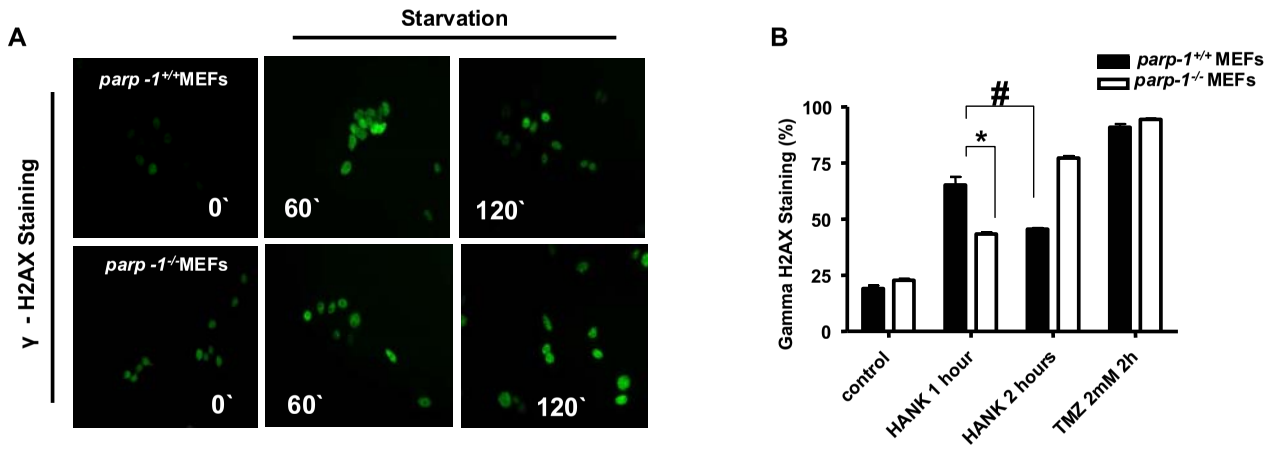


Figure 6 γ - H2AX Staining during starvation in *parp-1*+/+ MEFs 3T3 and *parp-1*- A. FIRST PANEL: Foci of γ-H2AX histone in *parp-1*$^{+/+}$ MEFs 3T3 and *parp-1*$^{-/-}$ at different times of nutrient deprivation with HANK Solution. The intensity at 120 minutes is higher in *parp-1*$^{-/-}$ MEFs 3T3. Similar results was obtained in 3 independent assays.

B : Number of cells *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ with the typical staining for Gamma H2AX histone during starvation. The percentage of cells *parp-1*$^{-/-}$ at 2 hours of starvation confirm the result obtained by immunofluorescence. Temozolomide 2mM 2 hours was used as positive staining of H2AX foci (DNA damage).

* p <0.01 and #p <0.01 as compared to starved *parp-1*$^{+/+}$ MEFs 3T3

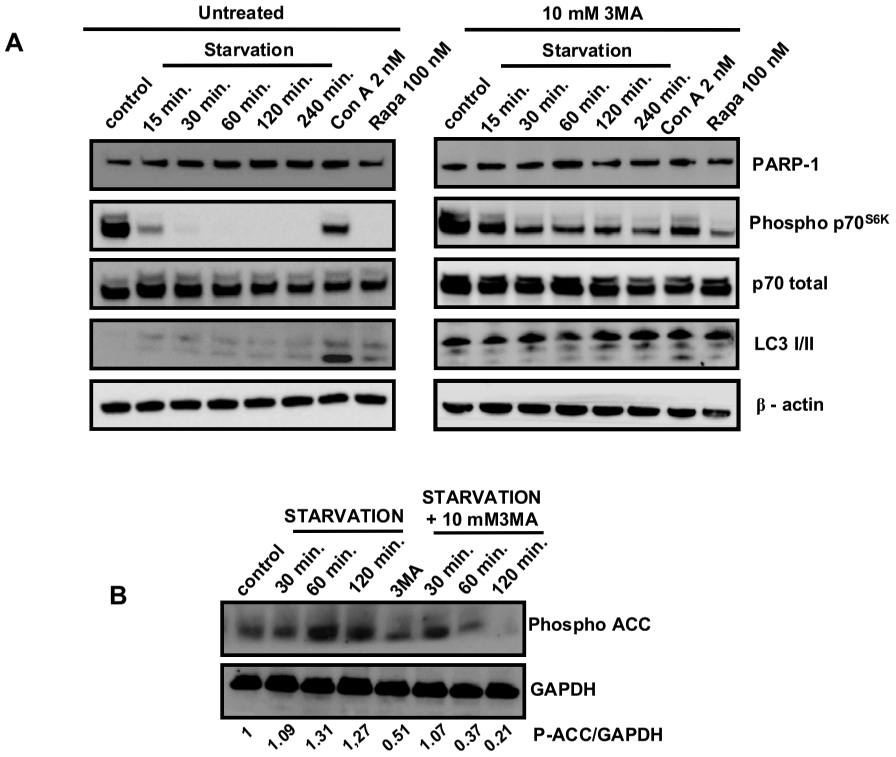


Figure S7. MCF7 cells stably transfected with GFP-LC3 were starved in the absence or presence of 10 mM 3MA . **A** Treatment with 10 mM 3MA strongly delays mTOR activation while LC3 II was not detected. **B,** an important reduction in AMPK activation 60 and 120 min after treatment with 3MA as measured by the levels of ACC posphorylation . The ratio P-ACC/GAPDH is shown in the bottom of the figure.

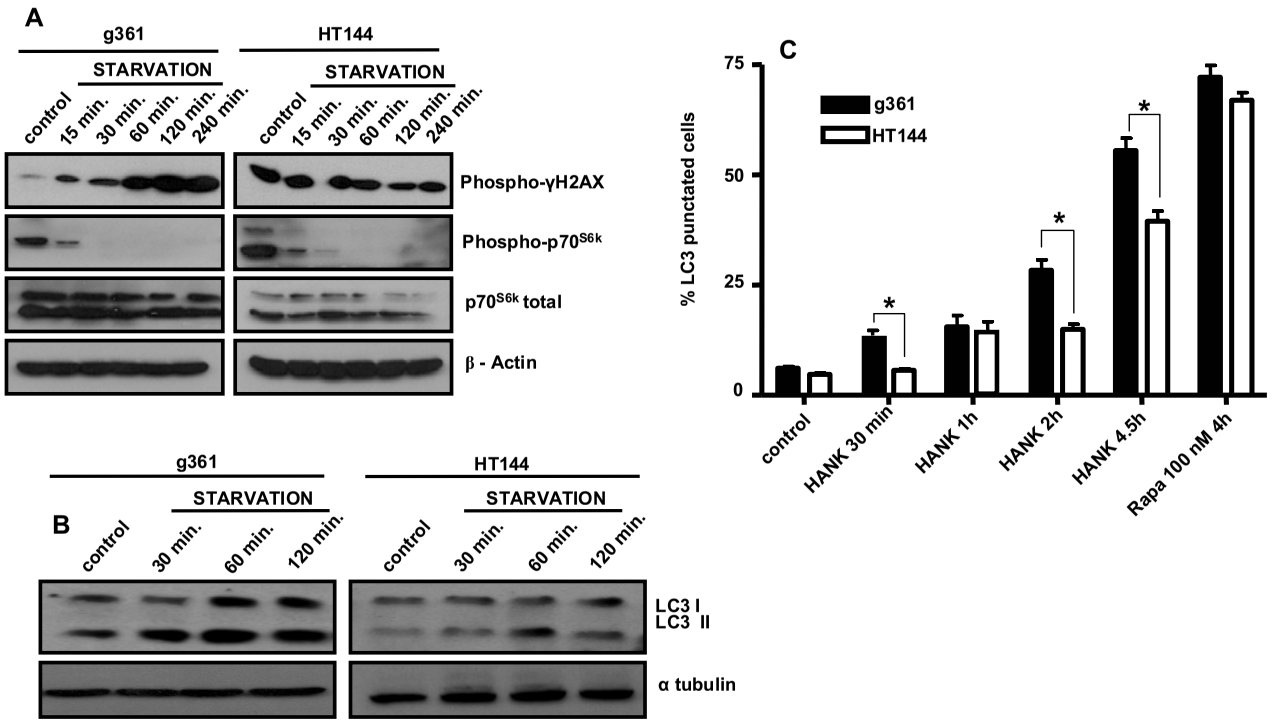


Figure S8 Delay of autophagy in a deficient DNA repair model

(A) Effect of DNA damage on mTOR pathway: g361 and HT144 (atm mutant) cells were starved for 15, 30, 60, 120 and 240 minutes and the levels of mTOR inhibition were measured as loss of phospho-$p70^{s6k}$. Different levels of phospho- γH2AX histone correlates with a delay of mTOR pathway inhibition in HT144 cells. β–actin was used as loading control. Similar results were obtained in three independent experiments.

(B) Western Blot analysis of the endogenous LC3 translocation in HT144 cell during starvation: g361 and HT144 cells were starved at different times and the levels of autophagy was measured as LC3-II translocation. Similar results were obtained in two independent experiments.

(C) g361 and HT144 were transfected with GFP-LC3, 24 hours after, were starved with HANK solution for 30, 60, 120 and 240 minutes. Percentages of LC3 translocation are shown. Rapamycin 100 nM during 4 hours was used as positive control of autophagosomes accumulation.
In C figure, 250 cells were counted in a Zeiss fluorescent microscope in both cell lines, in 3 independent experiments. * $p < 0.01$, as compared to starved g361 cells.

Targeting PARP-1 in breast cancer: the paradigm of a DNA repair-based therapeutic strategy

Chapter: DNA damage and repair. BOOK: Cancer Biomarkers

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Abstract

Poly(ADP-ribose) polymerases (PARPs) are defined as cell signalling enzymes that catalyze the transfer of ADP-ribose units from NAD(+) to a number of acceptor proteins. PARP-1, the best characterized member of the PARP family, that presently includes eighteen members, is an abundant nuclear enzyme implicated in cellular responses to DNA injury provoked by genotoxic stress. PARP is involved in DNA repair and transcriptional regulation, and is now recognized as a key regulator of cell survival and cell death as well as a master component of a number of transcription factors involved in tumor development and inflammation. PARP-1 is essential to the repair of DNA single-strand breaks via the base excision repair pathway. Inhibitors of PARP-1 have been shown to enhance the cytotoxic effects of ionizing radiation and DNA damaging chemotherapy agents, such as the methylating agents and topoisomerase I inhibitors. Recent in vitro and in vivo evidence suggests that PARP inhibitors could be used not only as chemo/radiotherapy sensitizers, but as single agents to selectively kill cancers defective in DNA repair, specifically cancers with mutations in the breast cancer associated (BRCA) 1 and 2 genes and other mutations affecting the cell's efficiency to cope with homologous recombination. The aim of the present chapter is to overview the emerging data in the literature on the mechanistic insights of poly(ADP-ribose) polymerase-1 (PARP-1) in the pathway of homologous recombination and the consequences for the therapeutic intervention in familiar and refractory breast cancer.

INTRODUCTION

PAR polymerases (PARPs) enzymes are an important family of proteins that regulates the integrity and correctness of transcription of DNA in both vegetal and animal cells, through the transfer of units of ADP-ribose to protein acceptors. The addition of polymers of ADP-ribose is an important post-translational modification, because the highly negative charge of ADP-monomer, changes the activities and function of these nuclear acceptors. PARPs

family is defined as cell signaling enzymes that are key sensors of DNA damage (1). PARP-1 (poly (ADP-ribose) polymerase-1) is the most important member of this family, which includes others 16 members of different proteins, related with DNA metabolism: PARP-2, PARP-3, PARP-4 (Vault-PARP), PARP-5 (Tankyrases 1 and 2), PARP-6, PARP-7 (tiPARP), PARP-8, PARP-9 (BAL-1), PARP-10, PARP-11, PARP-12, PARP-13 (ZAP), PARP-14 (CoaSt6), PARP-15 and PARP-16. All these proteins have different structural domains and functions in the acid nucleic metabolism (2).

PARP-1 play a key role as DNA damage sensor and in DNA repair provoked by genotoxic stress and maintenance of chromatin integrity(3) (1). Moreover, PARP-1 is a chromatin-associated enzyme with important functions in regulation of transcription, cell cycle, tumorigenesis and other important human diseases through ROS/NF-kB/PARP-1 pathway including type I diabetes and associated endothelial dysfunction, cardiovascular injury and heart failure, sepsis, rheumatoid arthritis and stroke (4-6).

STRUCTURE AND METABOLISM OF PARP-1

PARP-1 is an abundant nuclear enzyme with 113 KDa; it may interact with DNA and different DNA repair molecules (4). This protein has three different structural/functional domains (Figure 1): 1) A DNA-binding region or DBD domain, able to recognize DNA-strand breaks, through two zinc fingers (FI/Zn1 and FII/Zn2), that mediates in the interaction with DNA. Recently one group has discovered a new zinc finger or FIII, which is very important for DNA-dependent PARP-1 activation (5). The DBD domain contains a nuclear localization sequence (NLS) and a caspase-3 cleavage motif (DEVD); 2) A central automodification region rich in glutamic acid and containing a breast cancer associated protein C-terminal (BRCT) motif. This region allows PARPs enzymes to modify their activity by binding fragments of ADP-ribose polymer, synthesized by the PARP itself, which is able to increase their activity; and 3) A NAD-binding region with all the catalytic activities of the full-length

enzyme. This region is the most conserved domain in PARPs family. The WGR motif has recently been described as a conserved amino acid sequence (Trp, Gly, Arg) but the function is still unknown (8, 9).

PARP-1 is activated following recognition of the damaged DNA and synthesizes a branched polymer of residues formed monomers of ADP-ribose (PAR), from NAD+ (nicotinamide adenine dinucleotide) as substrate (Figure 2) (10, 11). The residues of ADP-ribose are covalently linked between them forming the various branches of the polymer, which will be covalently linked to glutamic residues of different nuclear acceptors related to the recognition and repair of damaged DNA, including nucleosomal core histones, topoisomerases I and II, High Mobility Group (HMGB) proteins or p53 (2, 12). PARP-1 can be hyperactivated after an important DNA damage induced by free radicals, reactive species of oxygen (ROS) or peroxynitrite, which produce oxidative stress and single strand-breaks in DNA and different alkylating agents that induce double strand-breaks (Figure 3). In this situation, PARP-1 first links this polymer to itself resulting in an increased activity; then the branched PAR polymer is linked to different protein acceptors, which are recruited to the DNA lesion. Both the polymer is synthesized under normal conditions, for example during DNA replication, such as DNA stress, should be degraded and recycled; PARP-1 becomes inactive activation to avoid energy collapse, which would have resulted in a significant consumption of NAD+ and ATP. The only protein capable of degrading the polymer is the poly (ADP-ribose) glicohydrolase (PARG). PARG is a protein that has different isoforms, each of which locates in a different organelle (6). When the complex PARP-1/PAR/proteins have carried out their function and the genetic material has been repaired, PARG will be responsible to hydrolyze the polymer that, first is joined PARP-1, decreasing the its activity, and then the polymer is attached to the base excision machinery, DNA repair and finally the free polymer that can play a role as a signaling molecule in nucleus. Recent studies have demonstrated that there are different mechanisms of recruitment of PARG to the area of damaged DNA, where there is a greater amount of polymer. These authors suggest different

models of recruitment: PARP-1/PAR-dependent, PAR-dependent and possibly PARP-1/PAR independent (7).

PARP-1 AND PATHOLOGICAL DISORDERS

PARP is known to have been rapidly activated in various pathophysiological conditions, and its activation is prolonged and continued. For example, direct detection of PAR accumulation has demonstrated the activation of PARP in stroke induced by middle cerebral artery occlusion and reperfusion (8), and in heart after myocardial infarction and heart transplantion (9). Also, PARP activation has been demonstrated in hemorrhagic and septic shock (10), in the lung of mice subjected to a model of acute respiratory distress syndrome and well as in diabetic animals (11).

POLY(ADP-RIBOSYL) ATION IN CANCER BIOLOGY

Poly(ADP-ribosyl)ation or PARylation is defined as the covalent modification of proteins with a negatively charged and highly branched polymer of ADP-ribose residues. This reaction occurs in a cell in many essential and physiological cellular processes such as replication of genetic material, where the mutation rate to duplicate the DNA is high, maintaining the integrity of the chromatin by interaction with different protein core histones, telomere integrity or modulation of the localisation of different transcriptional factor as p53 o KLF8, so PARP-1 and PAR are essential in a number of physiological and patho-physiological cellular processes, as programmed cell death, ischemia, inflammation, hypoxia, aging or cancer (15-17). The balance that exists in the cell to synthesize and degrade the polymer is essential in regulating a biological process, first because the activity of PARP-1 is going to consume energy and reducing power, and second by the ability to recycle the polymer through the enzyme PARG. Thus, the levels of PAR in a precise moment reflect that an essential

process, as repair or DNA replication, take place and may eventually drive the cell to cell death. As such, PARP-1 inhibitors have been discussed as potential chemotherapeutic agents, by potentiating other cytotoxic treatments. The use of these molecules is very attractive, because by sensitizing tumour cells to cytotoxic agents one might be able to give a lower dose and maintain the same relative efficacy while at the same time reducing the toxic side effects (18, 19). PARPs exhibit pleiotropic cellular functions ranging from maintenance of genomic stability and chromatin remodeling to regulation of cell death, thereby rendering PARP homologues promising targets in cancer therapy. Depending on the molecular status of a cancer cell, PARP inhibitors can (i) either be used as monotherapeutic agents following the concept of synthetic lethality or (ii) to support classical chemotherapy or radiotherapy. The rationales are the following: (i) in cancers with selective defects in homologous recombination repair, inactivation of PARPs directly causes cell death. In cancer treatment, this phenomenon can be employed to specifically target tumor cells while sparing nonmalignant tissue. (ii) PARP inhibitors can also be used to sensitize cells to cytotoxic DNA-damaging treatments, as some PARPs actively participate in genomic maintenance. Apart from that, PARP inhibitors possess antiangiogenic functions, thus opening up a further option to inhibit tumor growth. In view of the above, a number of high-potency PARP inhibitors have been developed during the last decade and are currently evaluated as cancer therapeutics in clinical trials by several leading pharmaceutical companies. In this chapter we will analyze the role of PARP-1 in double strands break repair by homologous recombination and the potential of PARP inhibition induced synthetic lethality in breast cancer.

PARP inhibitors have been used in vitro (11, 12) to inhibit vascular endothelial growth factor (VEGF)-induced proliferation, migration and tube formation in human umbilical vein endothelial cells (HUVECs) and in tumor models (13).

Recently we have characterized a novel regulatory mechanism controlling Snail1 protein expression through poly(ADP-ribosyl)ation during epithelial to mesenchymal transition (14).

EMT iPARP inhibition have effects on EMT phenotype of different tumor cells, including mesenchymal markers downregulation and epithelial markers upregulation (15). Also PARP inhibition is related to autophagy. Autophagy is a lysosome-dependent degradative pathway frequently activated in tumor cells treated with chemotherapy or radiation. PARP-1 deficient or inhibited cells display a decreased autophagic response (16). To have a more global overview, we will also review in short some aspects of the implications of PARP-1 in different non-neoplastic pathologies.

PARP in neuronal pathology

PARP-1 plays a significant role in necrotic cell death upon neuronal injury such as excitotoxicity, ischemia-reperfusion and traumatic brain injury (12). Traumatic brain injury (TBI), also known as intracranial injury, occurs when an external force traumatically injures the brain. TBI is a major cause of death and disability worldwide, especially in children and young adults. There is an involvement of nitric oxide (NO), oxidative and nitrosative stresses and PARP in the pathophysiology of TBI. In pathological conditions, an imbalance between the production and the elimination of reactive oxygen- and reactive nitrogen-containing intermediates (ROS and RNS, respectively) has been described and referred to as oxidative/nitrosative stress (13). Many of these species (e.g., peroxynitrite and hydroxyl radical) can cause DNA damage, which results in the activation of PARP (14, 15). PARP is markedly activated as early as 30 min after TBI and its activation persists for 72 h after TBI (16-18). This pattern of PARP activation is likely related to the continuing presence of peroxynitrite in the lesioned brain tissue. Chemical PARP inhibition and genetic depletion of PARP-1 gene block these neurotoxic effects. Therefore, INH2B (5-iodo-6-amino-1,2-benzopyrone) (17) and INO-1001 (19), prevent NAD depletion and improve post-TBI deficits. 3-aminobensamide (3-AB) and other derivatives induce neuroprotective effect on the neurological deficit and the brain lesion after closed head injury in mice (20) and after TBI induced by fluid percussion (18). PJ-34 [N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-2-(N,N-

dimethylamino)acetamide, HCl] and INO-1001 induce neurological recovery-promoting effects in a model of TBI caused by fluid percussion in rat (21).

Ischemic reperfused rats pretreated with PARP inhibitor PJ34 showed a near complete inhibition of microglia, macrophage activation and an 84% reduction in CA1 neuronal survival (22). Furthermore, recent studies demonstrated PARP is activated during learning and memory (23).

PARP-1 in cardiovascular diseases

PARP-1 has been reported in various models of heart failure, cardiomyopathies, and myocardial hypertrophy. Importantly, recent studies have also demonstrated overexpression of PARP-1 or increased activity in biopsies from human subjects with heart failure (24). Increased poly(ADP-ribosyl)ation, ROS formation and nitrosative stress were also reported in cardiomyocytes and endothelial cells in chronic heart failure associated with advanced aging or hypertension in rats and/or mice (13). PARP inhibition with PJ-34 or INO-1001 was associated with improved heart failure-associated decreased endothelial function and decreased myocardial hypertrophy and adverse remodeling (25). Endothelial dysfunction associated with diabetes, hypertension, heart failure and atherosclerosis is related to the local formation of reactive oxygen and nitrogen species in the vicinity of the vascular endothelium (13). The use of PARP inhibitor PJ-34 during 1 week after induction of diabetes ameliorated vascular PARP accumulation and restored normal vascular function, without altering systemic glucose levels, plasma glycated haemoglobin levels or pancreatic insulin content (26).

PJ-34 and INO-1001 blocked hyperglycemia and also ameliorated diabetes-induced albumin excretion and mesangial expansion. There some evidences that suggest that NAD a weak PARP inhibitor reverses neurological and neurovascular deficits in streptozotocin diabetic mice (27).

Poly(ADP-ribosylation) and inflammation

The relationship between inflammation and PARP activity has been widely investigated. NF-κB is a key transcription factor in the regulation of the inflammatory response and PARP has been shown to act as a co-activator in the NF-κB-mediated transcription (28). The administration of PARP inhibitors as 3-AB or new generation compounds to animals result in the attenuation of inflammation and rescued animals form many pathological signs was observed. The therapeutic effects of PARP inhibitors in various forms of heart failure, cardiomyopathies, circulatory shock, cardiovascular diseases and atherosclerosis have been described (13).

PARP-1 in HIV infection

An increasing body of evidence suggests the involvement of PARP in HIV infection. Prevention of PARP-1 nuclear localization may have greater consequences for HIV infection (29) and PARP is required for nicking of host cell DNA in order to incorporate the DNA of HIV-1 virus. Independent studies have shown that benzopyrone derivatives and nicotinamide possessed potent antiviral effects in HIV-infected cells. Another study using three different PARP inhibitory approach reported similar results (30). At present, it seems that PARP regulates HIV infection at two levels: integration and transcription but data with pharmacological inhibitors are still inconclusive.

PARP-1 in Parkinson

PARP activation has been implicated in the pathogenesis of Parkinson's disease, a degenerative disorder of the central nervous system. It results from the death of dopamine-containing cells in the substantia nigra, a region of the midbrain. ROS and RNS have been implicated in the pathogenesis of MPTP neurotoxicity (31). Direct evidence for the involvement of MPTP comes from a mouse model of Parkinson's disease. Simultaneous

treatment with five different inhibitors of PARP ameliorates the catecholamine depletion induced by MPTP (32, 33).

PARP-1 in aging

Aging is a multi-factorial process defined as time-dependent general decline in physiological function, which is associated with a progressively increasing risk of frailty, morbidity and mortality (34). A role for PARPs on longevity has been already postulated almost two decades ago (35). The oxidative stress theory of aging postulates that ROS induce a variety of macromolecular oxidative modification and accumulation of such oxidative damage, is a primary causal factor in the aging process (36). Interestingly, the enzymatic activity of PARP-1 can be strongly activated by treatment of cell with ROS such hydrogen peroxide promoting cellular demise and transcription of proinflammatory genes such as NF-κB, TNF-α, interleukin, cyclooxygenase-2 and adhesion molecules which may lead to the outcomes of aging (37). Recent results demonstrate that the relationship between PARP and SIRT (Sirtuin family of NAD dependent class III histone deacetylases) could affect not only metabolic control but also has an impact on aging (38). In fact PARP inhibition has proven beneficial in many cell culture and animal model system of acute and chronic inflammation and age-related diseases (39).

HOMOLOGOUS RECOMBINATION

A typical diploid human cell needs to maintain about 6×10^9 base pairs in the correct sequence and chromosomal organization, a task that is usually performed nearly perfectly from one somatic cell generation to the next (44). Homologous recombination (HR) (figure 4) is a key pathway to maintain genomic integrity between generations. Recombination is required for the repair or tolerance of DNA damage and the recovery of stalled or broken replication forks (45). However, recombination is also potentially dangerous as it can lead to chromosomal rearrangements and potentially lethal intermediates (46). Defects in HR and associated processes define a number of human cancer predisposition syndromes

associated with genome instability. HR repair of DSBs involves repair using preferentially the sister chromatid, which results in error-free repair (47). HR is only active in the S and G2 phases of the cell cycle since the sister chromatid is present only after DNA replication (48). It is possible that HR could use the homologous chromosome for DSB repair when cells are in the G1 phase of the cell cycle. However, this would invariably lead to loss of heterozygosity and may also result in translocations or other gene rearrangements (23, 25-30). To avoid such a scenario, HR is not only controlled by DNA damage response signaling pathways but also by the cell cycle and relies on a high cyclin-dependent kinase activity, present only in the S, G2 and M phases of the cell cycle. HR is suppressed in mitosis, for instance by inactivating phosphorylations on BRCA2 (25). Emerging data also suggest that a subset of DSBs present in the more condensed heterochromatin act as a substrate for HR in the G2 phase of the cell cycle (26).

PARP-1 in homologous recombination

For long it has been documented that PARP-1 has a key role on DNA repair of Single Strand Breaks (SSBs). However, the involvement of PARP-1 in DNA repair of Double Strand Breaks (DSBs) is still being investigated. DSBs can be repaired by two different processes: the “error-free” mechanism of and the “error-prone” mechanism of Non-Homologous-End-Joinig (NHEJ). The current research shows that PARP-1 has a role regulating both DNA DSBs mechanism of repair. Nevertheless, this chapter will focus on the role of PARP in Homologous Recombination. There have been several evidences about the role of PARP in HR PARP inhibition stimulated HR (49). The PARP-1 KO mice developed normally but had a high recombination phenotype (50, 51); the binding of PARP to a DNA break prevented the recombining machinery from recognizing and processing DNA lesions (52); the lack of PARP lead to micronuclei formation (53). There were several lines of evidence showing that loss of PARP-1 activity caused a hyper-recombination phenotype and genomic instability. However, it wasn’t clear if PARP-1 affected only the number of lesions triggering HR or if it was involved in HR per se.

On the other hand, it has been reported that proteins involved in HR are found in nuclear foci in cells in S phase of the cell cycle, suggesting a role of HR at replication forks (54). These foci are rich in RAD51 protein and they increase their number after DNA damage (55).

In 2003, Schultz et al. further advanced in the determination of the controlling role of PARP in HR (56). This group reported that although this process was not directly affected by PARP-1, RAD51 foci increased in PARP-1 knockout MEFs or Wild Type MEFs (WT) treated with PARP inhibitors. These results suggested that PARP-1 doesn’t execute HR as such, but has a negative controlling role of lesions recognized by HR (figure 5), supported by the fact that the lack of PARP-1 generates an increase of HR phenotype in form of RAD51 foci.

In 2006, Bryan et al determined that PARP inhibition activated ATM protein, which is required for HR repair. A role of ATM in HR had previously been reported (53).This group showed that although the PARP-1 KO mice was not lethal, the PARP-1/ATM KO and the PARP-1/ku80 KO mouse were not viable, suggesting that NHEJ has a role in the survival of PARP-inhibited cells due to the involvement of ku80 in this process and second, ATM is important for cellular survival after the inhibition of PARP-1 and this survival is caused by the involvement of ATM in PARP-inhibition –induced HR repair.

The inhibition of PARP generated an increase in unsolved SSBs at the DNA replication fork that turned into DSBs generating a stalled or even a collapsed replication fork. Since it has been reported that ATM is activated by DSBs (57), it was determined that the collapsed replication fork activates this protein. Furthermore, it has been reported that ATM phosphorylates Artemis, which has a role in NHEJ (58) via activation of the NHEJ protein DNA-PK.

Thus, the increased number of SSBs following PARP inhibition collapse into DSBs at the DNA replication forks. Such collapsed replication forks need HR for their repair, which is

activated via ATM. Furthermore, an alternative pathway for the repair of the DSBs at the replication fork via NHEJ is also suggested after PARP inhibition through the ATM activation of Artemis protein.

Aguilar-Quesada et al 2007 (59) further advanced in the analyzing the relationship between PARP-1 and ATM, showing that PARP-1 interacts with ATM and ATM is modified by poly-ADP-ribosylation and, secondly, the dual effect of PARP inhibition on ATM activation. While poly-ADP-ribosylation of ATM is probably is needed for optimal ATM activation, long term exposure to PARP inhibitors result in the generation of DSBs and ATM activation. More recently, It has been shown that PARP is activated at stalled replication forks to mediate Mre11-NBS1 dependent replication restart and HR (60), (61) (figure 4). Mre11/RAD50/Nbs1 complex had previously been reported to have a role in the restart of damaged replication forks (62). Bryant et al showed that HU treatment generates stalled replication forks with short regions of SSBs that can generate the recruitment of PARP at the fork, attracting then Mre11 protein to the sites of stalling. They demonstrated that Mre11 foci and RPA foci colocalised with PAR polymer, concluding that SSBs generated after HU treatment recruited PARP to the stalled replication fork generating the activation of RPA via Mre11. Finally RPA will be replaced by RAD51, initiating HR.

Furthermore, Haince et al 2008 have documented that the recruitment of MRE11 made by PARP, is due to their physical interaction. The fact of the presence of PARP-1 in the MRE11 complex suggested the formation of a common complex involving both proteins; on the other hand, the recruitment of MRE11 by PARP can also be generated by DSBs induced by laser microirradiation, which was determined by co-inmmunostaining of PARP, ATM and γH2AX upon multiphoton laser microirradiation.

In conclusion, PARP-1 has a dual role on the regulation of HR: (i) PARP inhibition provokes genomic instability producing the accumulation of non-repaired SSBs at the replication fork that turn into DSBs activating ATM and HR; (ii) the induction of DNA damage of both simple

(HU) or double (microirradiation) strand drives to the recruitment of PARP-1 to the damage site, which forms a complex with MRE11 activating RPA and HR.

Homologous recombination and breast cancer

Homologous recombination has a dual role in eukaryotic organisms. Firstly, it is responsible for the creation of genetic variability during meiosis by directing the formation of reciprocal crossover that result in random combination of alleles and traits. Secondly, in mitotic cells, it maintains the integrity of the genome by promoting the faithful repair of DNA double-strand breaks (DSBs). In vertebrates, it therefore plays a key role in tumor avoidance (73) -Genetic polymorphisms in homologous recombination repair genes that can lead to protein haploinsufficiency and germline mutations in many of the genes that are involved in HR-mediated DNA double-strand break repair, are generally associated with various genetic disorder and increased cancer risk (63, 64).

Breast cancer is one of the major killers among malignant conditions worldwide, affecting one out of 10 women in industrialized countries, and being the leading cause of cancer-related morbidity and mortality in women. Genomic rearrangements such as translocations, deletions and duplications are extremely frequent in cancer cells and particularly, in breast cancer cells. These rearrangements are believed to result form an aberrant repair of DNA double-strand breaks (65-67). Normally, these breaks are repaired by means of the homologous recombination double-stranded DNA repair pathway, key components of which are the tumor-suppressor proteins BRCA1 and BRCA2 (68, 69).

BRCA1 and *BRCA2* mutation carriers have a cumulative lifetime tumor risk of 84% and 60-80%, respectively, for breast cancer, and 40-50% and 10-20%, respectively, for ovarian cancer. Tumors that lack *BRCA1* or *BRCA2* have an insufficiency in the repair of DNA double-strand breaks by homologous recombination and, a resultant dependence on alternative mechanisms of DNA repair, potentially error-prone. Reliance on upregulated, lower fidelity and potentially mutagenic double-strand breaks DNA repair pathway confers

cancer susceptibility through genomic instability (70). Among *BRCA1/2* gene mutations carriers, a large number of mutations have been described. While *BRCA1* operates in the maintenance of genomic integrity, chromatin remodelling, transcription regulation and cell cycle checkpoint control, the function of *BRCA2* is linked to DNA recombination and repair process (71).

After DNA damage, a number of proteins including BRCA1 are recruited to the DNA lesion where they are phosphorylated by protein kinases including ataxia telangiectasia mutated ATM protein. BRCA1 then forms a multi complex termed BRCA1-associated genomes surveillance complex [BASC] which influences the selection of the repair pathway. The presence of this BASC suggests that BRCA1 might have a key role as a coordinator of multiple processes required for the maintenance of genome integrity during the process of DNA replication. BRCA1 also participates in the regulation of the complex MRE-RAD50-NBS1 [MRN] in the initial steps of the foci formation. These foci are separate from BRCA-Rad51 foci, suggesting that BRCA1 might act in two distinct DNA double-strand breaks repair complexes. BRCA1 is also involved in other cellular functions including transcription, ubiquitination, and chromatin remodelling, among others. BRCA2 function is also important in DNA repair. Its role is to bind to the Rad51 protein, through its BRC motif and C-terminal domain, respectively. This complex has a central role in the locating and pairing of the resulting single-stranded ends with complementary sequences. The BCR motif controls the status of the Rad51 filament to govern DNA strand exchange. A 1,127-amino-acid region of BRCA2 with all eight BRC repeats was found to promote recruitment of Rad51 to ssDNA while slowing the association with dsDNA, establishing a favourable state for DNA strand exchange. Structural analysis revealed that a second structural domain within α-helix context is also essential for BRC-Rad51 interaction. In addition to its interaction with the BRC repeats, Rad51 also interacts with an unrelated C-terminal region of BRCA2, referred to as the TR2 region. This interaction serves a regulatory role in the recombinational repair. During the S and early G2 phases of the cell cycle, or after DNA damage, the C-terminal

TR2 region of BRCA2 is non-phosphorylated al serine 3291 and thus capable of interaction with Rad51, that leads to the stabilization of the filaments and as a consequence homologous recombination is active. As cells approach to mitosis, serine 3291 is phosphorylated, serving to downregulate homologous recombination, as TR2 cannot interact with and stabilize Rad51 filaments. BRCA2 has also been shown to participate in other cellular functions like cytokinesis and centrosome location. *BRCA1, BRCA2* and *Rad51* are co-expressed in development, and the three gene products also co-localize upon meiotic chromosomes during the formation of the synaptonemal complex, a structure connected with the tightly regulated events of meiotic recombination. Exposure of cells during S phase to agents that arrest DNA synthesis causes the rapid phosphorylation of BRCA1 and it coordinate relocalization, with BRCA2, Rad51 and BARD1, to sites of DNA synthesis. These observations point to a role for these protein complexes in homologous recombination during the S phase (71-76).

In cells with defective *BRCA1* and/or *BRCA2* repair of double-strand breaks occurs using alternative repair pathways, one of those routes is the base excision repair mechanism that usually repairs single-strand breaks. Inhibition of this pathway increases the number of unrepeated single-strand breaks, which leads to collapsed replication forks and produces double-strand breaks that can't be repaired. In this case, the impairment of both DNA repair pathways induces cell death. This approach follows the concept of synthetic lethality (Figure 6) applied to anti-cancer drugs. This concept refers to a biological setting in when a mutation or lack o function in one of two pathways or genes has little effect on cell survival, but the combined inhibition leads to cell death. Pre-clinical studies have shown in *BRCA1*- and *BRCA2*-defective cells, PARP-1 inhibitors have shown to be therapeutically active. Ongoing studies are also in progress to assess the combination of PARP inhibitors and taxanes in patients with both *BRCA1/2*-positive and metastatic triple-negative breast cancer (ER, PR and HER2 negative) (68, 71).

Emerging evidence indicates that in addition to the small number of germline *BRCA1* and *BRCA2* mutation-related tumors there is a wider group of sporadic cancers which manifest clinical features of *BRCA*-like functional loss either through epigenetic incativation of the *BRCA* genes or disruption of other non-redundant genes in the *BRCA*-associated homologous recombination repair pathway. These sporadic tumors display *BRCA*-like clinical properties reminiscent of hereditary cancers without *BRCA* gene loss, a phenomenon that has been described as BRCAness. Such sporadic tumors may therefore similarly benefit from the therapeutic approach of synthetic lethality with PARP inhibitors (70).

Although *BRCA1* and *BRCA2* are the most common lesions associated with genetic breast cancer, other germline mutations are also associated with familial breast cancer such as *TP53* (77), *PTEN* (78), *ATM* (79), *CHEK2* (80), *NBS1* (81), *RAD50* (82), *BRIP* (83), *PALB2* (84), *RAD51C* (63), *RAD9* (85), and other are suspected (86).

PARP inhibitors in the treatment of DNA repair deficiency-related cancers

Inhibition of PARP induces accumulation of large numbers of unrepaired SSBs, leading to the collapse of replication forks during S-phase and the consequent generation of DSBs. Single-strand breaks are usually repaired by the base excision repair pathway; therefore, inhibition of this pathway greatly increases the number of unrepaired single-strand breaks, which subsequently leads to DSBs at replication forks. Therefore, cells deficient in DNA DSB repair are highly sensitive to chemical inhibitors of PARP (31-33). In contrast, cells with intact DNA DSBs-response pathways repair damage with high fidelity and accordingly show very little sensitivity to PARP inhibitors.

Mutations in the breast cancer susceptibility genes BRCA1 and BRCA2 are responsible for the majority of hereditary breast cancers (34). Tumours in patients with heterozygous BRCA1 or BRCA2 germ-line mutations typically show somatic loss of heterozygosity at the BRCA1 or BRCA2 locus, respectively, resulting in loss of the wild-type allele (35, 36).

BRCA1 and BRCA2 encode proteins that are required for efficient homologous recombination (HR), an error-free form of DSBs repair (37, 38). Because both BRCA1 and BRCA2 deficient cells have so elevated utilization of error-prone repair pathways (87), loss of BRCA function causes genome instability (69), which renders viable cells susceptible to acquiring additional cancer initiating genetic lesions such as activation of oncogenes and inactivation of tumour suppressor genes. The tumours that arise are BRCA1 or BRCA2 deficient (BRCA1/2 -/-) whereas the remaining somatic cells still have functional BRCA proteins (BRCA1/2 +/-). BRCA1 and BRCA2-deficient cells are highly sensitive to agents that cause replication forks to stall. For example, cells with dysfunctional BRCA2 are characterized by an increased sensitivity to γ-irradiation or DNA-damaging agents (41-44) because the absences of error-free DSB repair mechanisms. PARP inhibitors are lethal in BRCA deficient cells as inhibition of PARP leads to the persistence of DNA lesions that would normally be repaired by BRCA-mediated homologous recombination. Repair of DSBs by alternative error-prone DSB repair mechanisms causes large numbers of chromatid breaks and aberrations, leading to cell cycle arrest and apoptosis. Cell lines lacking BRCA1 or BRCA2 are extremely sensitive to PARP inhibitors while the BRCA +/- and BRCA +/+ cells are relatively non-responsive to the treatment (31, 32). Indeed, *in vivo* experiments demonstrate highly efficient and selective deletion by PARP inhibition of nonneoplastic BRCA2-/- cells with no apparent deleterious effect on surrounding BRCA2-functional cells or whole animal physiology (45), and completely growth arrest of xenografted tumours derived from BRCA2-deficient cells with no effect on BRCA2-wild type tumours (31, 32). Despite these observations of selective cytotoxicity against nontumour cells with engineered BRCA2 mutations, relatively few data are available about the effect of PARP inhibitors on BRCA2-deficient mammary tumour cells. However, a recent report shows a potent growth arrest induced by PARP inhibition in BRCA2-deficient mammary cancer cell lines (46). If we exploit the fact that the PARP-1 pathway becomes essential only in homologous recombination-deficient cells, we could develop a treatment highly specific for the BRCA-deficient tumour cells (61). The aim is to generate DNA damage that, in normal circumstances, is repaired

with high fidelity but in a BRCA-dependent fashion (87). These observations suggest that PARP inhibitors might be an effective therapy for cancers arising in carriers of BRCA1 or BRCA2 mutations and have led to the assessment of these agents as monotherapy for cancer in BRCA mutation carriers (88). PARP inhibition may also be considered as a potential prophylactic treatment of non-neoplastic BRCA2-deficient cells in women heterozygous for BRCA2, as well as a primary follow-up treatment following surgery in patients in whom BRCA2 deficiency has led to tumorigenesis. It is one of the few examples where the defect causing the cancer is exploited for targeted therapy.

On the contrary, another report concluded that PARP inhibition is not a viable therapeutic approach based upon the insensitivity of one cell line treated with two weak small molecule inhibitors of PARP (89). However, the selective effect of PARP inhibition on BRCA deficiency is a property of only very potent PARP inhibitors (90), though, clinically speaking, it is not the potency of a drug that is most important but the efficacy, such as some authors point (91). Moreover, their studies indicate that the use of PARP inhibitors in mouse BRCA1 -/- mammary cancer cells seems to show only mild specificity in comparison with their controls in the xenografted nude mice. On the other hand, PARP inhibitors seem to uniformly inhibit growth of human breast cell lines regardless of the BRCA1 genotypes (91). Further observations indicate that BRCA1-/- mammary cancer cells are indeed much more resistant to PARP inhibition than BRCA1-/- nontumour cells in the allograft model. These authors propose that the non-cancerous cells are generally naïve, whereas tumour cells represent cells that have had multiple genetic mutations and that in a tumour there may be numerous fundamentally different cancer cells when classified by the type and frequency of individual genomic changes (92). Another independent study has not demonstrated either inhibition of tumour growth with the chosen PARP inhibitor, although there was reduced but detectable polymer in this samples and the lack of response may be a result of incomplete PARP inhibition (50). These observations cast doubt about the uniformity of the specific cell killing of BRCA-deficient cells upon PARP inhibition. Maybe, some specific mutations during

tumour progression due to genetic instability associated with BRCA1- and BRCA2-deficiency block the sensitivity of BRCA1/2 -/- cancer cells to PARP inhibition. In fact, it has been suggested that mutation in BRCA2 and sensitivity to therapeutics in BRCA2 mutation carriers can be suppressed by intragenic deletion (51). In the absence of functional BRCA2, mechanisms such as single-strand annealing and non-homologous end joining compensate and repair DSBs (in absence of HR), by aligning short regions of homology flanking the DSB, deleting the intervening sequence (38, 52). As consequence of this same HR deficiency, new BRCA2 proteins with deletions are capable of restore HR, leading to resistance. In summary, BRCA associated tumorigenesis may be divided into two phases regarding sensitivity to PARP inhibition. In the early stages of tumorigenesis, BRCA mutant cells are sensitive to PARP inhibition. However, in the second stage of tumorigenesis, BRCA deficient tumour cells may become insensitive to PARP inhibition due to additional mutations. Thus, the use of this strategy for therapeutic treatment for hereditary breast cancers is dependent on the continued susceptibility of BRCA mutant cells to PARP inhibitors (92).

PARP inhibitors have now entered clinical trials and initial results are promising, with frequent sustained responses in BRCA mutation carriers. Furthermore, PARP inhibitors are likely to produce few side effects, because PARP1 knockout mice are viable and healthy (53, 54) and inhibitors that are specific for PARP are relatively non-toxic and do not directly damage DNA; also, this approach is more specific than standard cytotoxic chemotherapy. Initial observations in patients with known BRCA-associated cancers, or those with a strong family history of disease suggestive of BRCA mutation, indicate that a novel, potent and orally bioavailable PARP inhibitor (93) shows low toxicity, and there are suggestions of significant antitumor activity, as assessed by radiography and by measurement of tumour biomarkers. This PARP inhibitor is being tested for efficacy as a monotherapy in BRCA-associated ovarian and breast cancer in Phase II clinical trials (56, 57). Many of the side effects are independent of the mechanism of action of these drugs and it appears that short

term inhibition of PARP is well tolerated and that PARP inhibition can be achieved at reasonable doses. Perhaps more debatable is the suggestion that the low toxicity of potent PARP inhibitors may enable their prophylactic use in women heterozygous for BRCA mutations (61). There is still no clinical experience regarding the tolerability of long term PARP inhibition. Prolonged inhibition of DNA repair could enhance the mutation rate and the potential for secondary malignancies. In fact, a recent study reveals an important role of PARP-1 in suppressing mammary tumorigenesis in mice and importantly, it suggests that long-term inhibition of PARP activity may bring increased risk for breast cancer formation (94). Nevertheless, short periods of prophylactic use may have utility but must obviously be weighed up against the actual risk a BRCA mutation carrier has of developing malignancy (95).

Very recently an interesting report has settle the basis for the resistance to PARP inhibition acquired by certain mutation on BRCA2 (61). Mutations in BRCA2 and sensitivity to therapeutics BRCA2 mutations carriers can be suppressed by intragenic deletion. Multiple mechanisms have been described for the reversion of mutations in humans, but the size and nature of the deletions they observe are uncommon. Variant of BRCA2 lacking a significant fraction of the protein are competent in mediating PARP resistance and, surprisingly, in HR. The profound sensitivity of BRCA2 deficient cells to PARP inhibitors is determined by their defect in error free repair and deletions may also arise because of this same HR deficiency.

Despite the clinical promise of PARP inhibitors in the treatment of BRCA-related cancer, extending the utility of these agents to other cancers is challenging. Sensitivity to PARP inhibition depends on homologous recombination deficiency and not on inherited BRCA1 or BRCA2 deficiency *per se*. Therefore, this approach may be more widely applicable in the treatment of other impairments of the HR pathway. The identification of novel mediators of cellular response to PARP inhibitors may highlight additional patient populations that might

benefit form this therapeutic approach. In this way, it has been used a high-throughput RNAi screen to identify new determinants of sensitivity to a PARP inhibitor (62).

Other pathways involved in HR

Loss of PARP-1 has been shown to cause an increase in Rad51 foci and sister chromatid exchanges (56) as result of an increase in the number of lesions normally repaired by HR. The sensitivity of cells deficient in proteins involved in HR to PARP inhibition suggest that treatment with PARP inhibitors maybe a useful therapeutic strategy for tumors displaying defects in the HR pathway other that the BRCAness. The observation that ATM and CHK2 depletion resulted in sensitivity to PARP inhibitors further suggest that PARP inhibition would be beneficial for a wide variety of cancers with dysfunction on genes involved in DNA damage response (33). It has been examined the effects of deficiency of several proteins involved in HR on sensitivity to PARP inhibition and it has been shown that deficiency in RAD51, RAD54, DSS1, RPA1, NBS1, ATR, ATM, CHK1, CHK2, FANCD2, FANCA or FANCC induces such a sensitivity. These results suggest that PARP inhibition might be a useful therapeutic strategy not only for the treatment of BRCA mutations but also for the treatment of a wider range of tumors bearing a variety of deficiencies in the HR pathway.

Particular attention has received the interaction between PARP and ATM. These two proteins, together with DNA-PK, are involved in responding to DNA damage to activate pathways responsible for cellular survival. A defective activation of ATM has been described in PARP-1 deficient cells; on the other hand, ATM null cells display an extraordinary sensitivity to PARP inhibitors (61), (65), suggesting that ATM and the collapse in the replication fork induced by the PARP inhibitor, function upstream of HR to repair certain types of DSBs (61). PARP-1 appears to be important for HR levels; however, it is not directly involved in this process, and the increased in HR seen in PARP-1 deficient cells is probably due to an accumulation of recombinogenic substrates in these cells. It is important to note that this treatment differs from previous therapies in that no additional genotoxic drug is

needed to cause cell death, and in this respect the it would be expected the treatment to have few side effects.

PARP inhibitors in breast and ovarian cancer

PARP catalyzes the cleavage of NAD^+ into ADP and ADP-ribose and attaches several molecules of the latter to the target protein in a process called poly(ADP-ribosyl)ation. The molecules that mimic NAD^+ block the binding of the NAD^+ to the enzyme, inhibiting PARP activity. The discovery of inhibitors of PARP was initially based on empirical, high-throughput screening, followed by optimization by chemical modifications based on structure-based design (see (96) for a review) (figures 8 and 9). Several studies have already shown the effectiveness of PARP inhibition in combination with either radiotherapy or chemotherapy in a range of human tumor mouse xenograft models (97, 98). PARP Inhibitors are being used in different Clinical Trial in Breast and Ovarian Cancer and a summary is gathered in **TABLE 1.**

AG014699 (Pf01367338) Pfizer was the first agent studied clinically in 2003 (table 1). A study evaluated 39 ovarian cancer cell lines with various molecular expressions either with single-agent AGO14699 or in combination with carboplatin, doxorubicin, gemcitabine, paclitaxel, or topotecan. The combination of AGO 146999 with carboplatin, topotecan, and doxorubicin appears to have greatest impact. The BRCA status of these cell lines was not reported. The study does suggest activity of AGO14699 in ovarian cancer and not confined to BRCA-deficient tumor or high-grade serous cancer. A phase II evaluation of single agent AG014699 in BRCA-deficient advanced breast and ovarian cancers is ongoing.

Olaparib (AZD2281, KU-0059436) AstraZeneca Olaparib is a selective and potent PARP inhibitor with a half maximal inhibitory concentration (IC_{50}) in the nanomolar range for both PARP1 and PARP2 enzymes (IC_{50} of 4.9 nM for PARP 1). Olaparib is a well-tolerated oral PARP inhibitor, which has shown promising monotherapy activity in patients with *BRCA1* or

BRCA2 mutations who have breast and ovarian cancer (99) PARP inhibitor to show activity in BRCA-related ovarian and breast cancers. It is been tested in combination with DNA damaging agents, such as topotecan, doxorubicin, carboplatin, carboplatin and paclitaxel, irinotecan, dacarbazine, and gemcitabine and cisplatin, as well as with antiangiogenesis agents (100) Two parallel open-label, multicenter, phase 2 studies of olaparib in germline *BRCA1/2* mutation carriers with advanced breast (ICEBERG1) and ovarian (ICEBERG2) cancers have provided further support for this antitumor synthetic lethal approach (Table 1)(101) .

Veliparib (ABT-888). Veliparib is an orally active PARP-1 and PARP-2 inhibitor (102). (Table 1). A phase I study of veliparib in combination with metronomic cyclophosphamide in two patients with BRCA2 ovarian cancer achieved partial response. A phase II randomized trial evaluating the role of veliparib in combination with oral cyclophosphamide in ovarian cancer patients with BRCA mutation or high-grade serous ovarian cancer will be activated in the near future. Also Veliparib in combination with TMZ was evaluated in metastatic breast cancer. Forty-one patients were treated (100)

Iniparib (BSI 201, NSC-746045; IND-71677) sanofi-aventis Iniparib, also known as BSI 201, 4-iodo-3-nitrobenzamide, is an irreversible inhibitor of PARP1 It is a prodrug with a 4-minute half-life. Iniparib is given intravenously twice a week. It is the first PARP inhibitor to show survival advantage with TNBC and has entered phase III testing. The phase I study included 23 patients with solid tumors. Six of the 23 heavily pretreated patients had stable disease for at least 2 months (up to over 9 months in 1 patient). In another study patients with solid tumors were assigned to 1 of 4 combinations of iniparib with a cytotoxic agent, topotecan, gemcitabine, TMZ, or carboplatin with taxol.

MK4827 MK4827 is an oral PARP-1 and 2 inhibitor with IC50 of 3.8 nM for PARP 1. Preclinical data showed single-agent antitumor activity against BRCA mutant cell lines in

culture and xenograft models. Also, MK4827 showed activity in combination with DNA damaging agents in cell cultures and xenograft models. It is currently being tested in phase 1 development as a single agent, in advanced ovarian tumors, and as combination therapy in patients with advanced solid tumors in combination with carboplatin, carboplatin with paclitaxel, and carboplatin with liposomal doxorubicin. MK4827 in Ovarian Cancer is in the phase I trial as a single agent enriched with BRCA1 or -2 mutation patients. An expansion cohort of high-grade serous ovarian cancer patients were added at the maximum toleranted dose (MTD). Responses were seen in all dose levels. The expansion cohort in high-grade serous ovarian cancer is ongoing. Once the biologically relevant dose is achieved, a phase Ib expansion in BRCA-mutant ovarian cancer patients is planned.

Currently there is a significative development in the PARP inhibitors, this agents are a promise in the cancer therapy, specifically the BRCA-associated tumorigenesis. However In the early stages of tumorigenesis, BRCA-mutant cells are sensitive to PARP inhibition. The problem is in the second stage of tumorigenesis when the cell acquired resistance to this inhibitor. Clinical disorders have been found to have the potencial to revert inherited mutation to a normal stated. A mutational event that converts BRCA2 mutation status to wild-type also converts the cells to being resistant to platinium agents and PARP inhibitor because there are a restoration of HR pathway. (100) BRCA deficient tumor cells may become insensitive to PARP inhibition owing to additional mutations. Thus, the use of this strategy for therapeutic treatment of hereditary breast cancers is dependent on the continued susceptibility of BRCA mutant cells to PARP inhibitors (92) .

The predictive Biomarker of Defective HR DNA Repair is very important in the therapeutic utility of PARP inhibitor. The identification of subgroup of patient with non.BRCA mutation-related HR repair-deficient tumor is possible with Array-Bassed Assay. The array-based strategies such gene expression profiling (103) (104), or comparative genomic hybridization (CGH) have been evaluated to define distinct BRCA1/2 – related profiled in ovarian and breast cancer. (105) (106) (101).

PERSPECTIVES

Pharmacological inhibition of key proteins involved in the response to DNA damage has emerged as an effective tool for cancer treatment, as the resistance of cancer cells to DNA-damaging agents originates from the modulation of DNA repair pathways. PARP has important pro-survival and protective functions in terms of DNA repair. A multitude of novel pharmacological inhibitors of PARP has entered clinical testing either as adjunct antitumor therapeutics or as monotherapy in familiar breast and ovarian cancer. Besides the antineoplasic action of PARP inhibitors, the evidence summarized above strongly supports a crucial role of the ROS/NF-κB/PARP pathway in mediating multiple inflammatory diseases including type I diabetes and associated endothelial dysfunction, cardiovascular injury and heart failure, sepsis, reumathoid arthritis and stroke. The information available to date supports the view that PARP over-activation during inflammatory damage is a pivotal feature of tissue damage in different inflammatory-based pathologies, and that the pharmacological inhibition of PARP may provide significant benefits in these conditions by salvaging affected tissues from necrosis, and by reducing, as well as by downregulating, the inflammatory responses. As with all new therapeutic areas, the usefulness of this target is unproven, but the potential effect of this class of agents is large. Whereas the clinical benefit of PARP inhibitors is being tested, additional new areas of research are also opening up in the preclinical front, which should eventually help in the consecution of new and more effective PARP inhibitors able to regulate the fine tuning of this pathway, including the specific inhibition of different PARP members, the association of PARP-1 in different complexes, and the activation/inactivation of poly(ADP-glycohydrolase).

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Figures

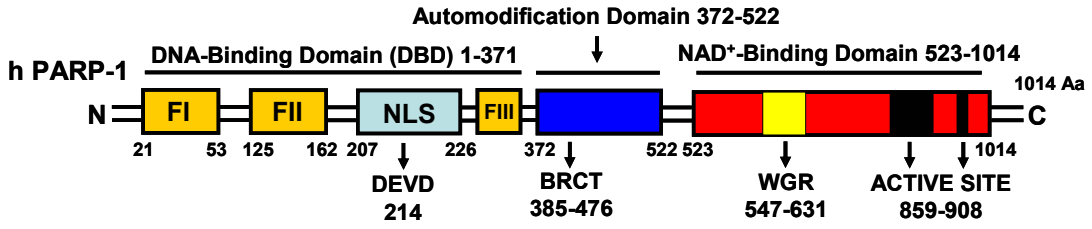

Figure 1. Structural Organization of PARP-1: Schematic representation of human PARP-1 with the different domains and active regions.

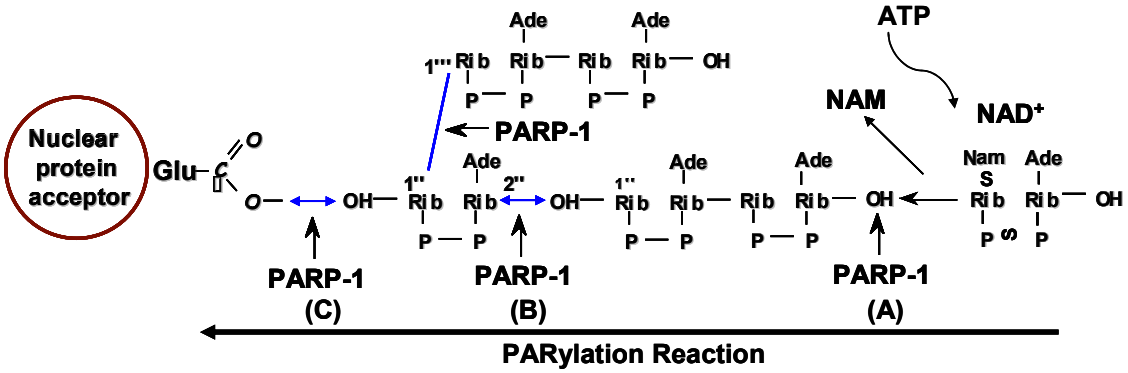

Figure 2. Poly (ADP-ribose) polymer formation: Biochemical reaction catalysed by PARP-1. (A): PARP-1 cleaves the glycosidic bond of NAD+ between Nicotinamide and ribose. (B): The different ADP - ribosyl monomers are followed and the chain is branched. (C): Covalent binding with an ester bond between glutamate residues of protein acceptor and the last ADP-ribose residue.

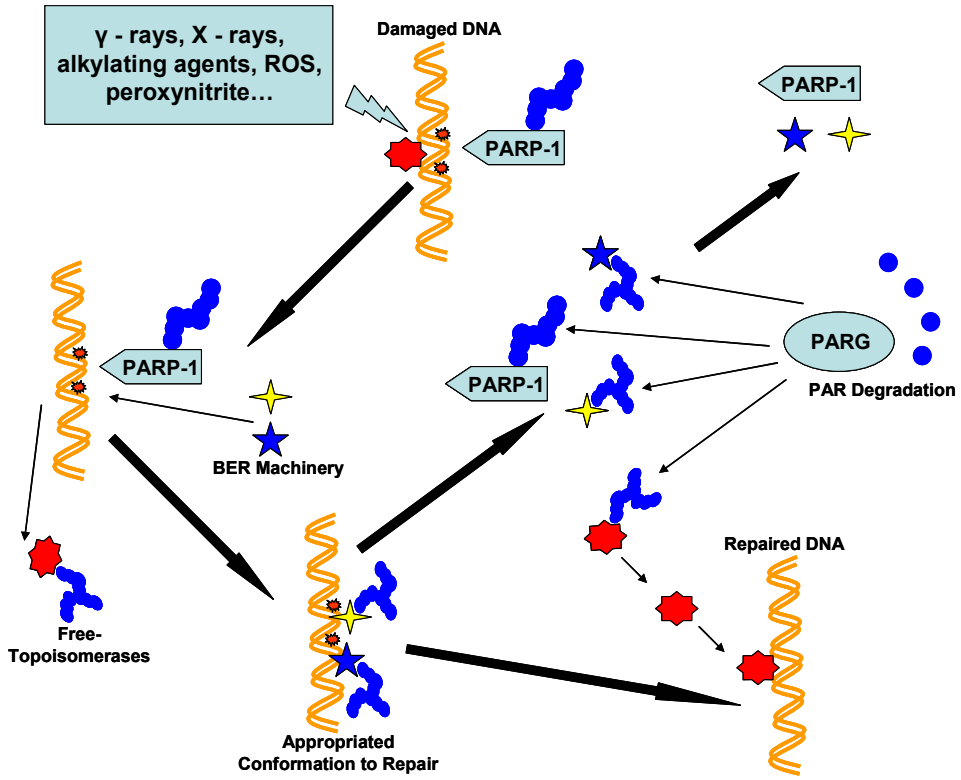

Figure 3. Metabolism of Poly(ADP - ribosyl)ation: During DNA damage, PARP-1 units PAR polymer to himself and glutamate residues of different DNA-binding proteins and protein of BER system to repair the DNA. When the genetic material is repaired the polymer attached to PARP-1 and other proteins is degraded by PARG and the system returns to the initial situation.

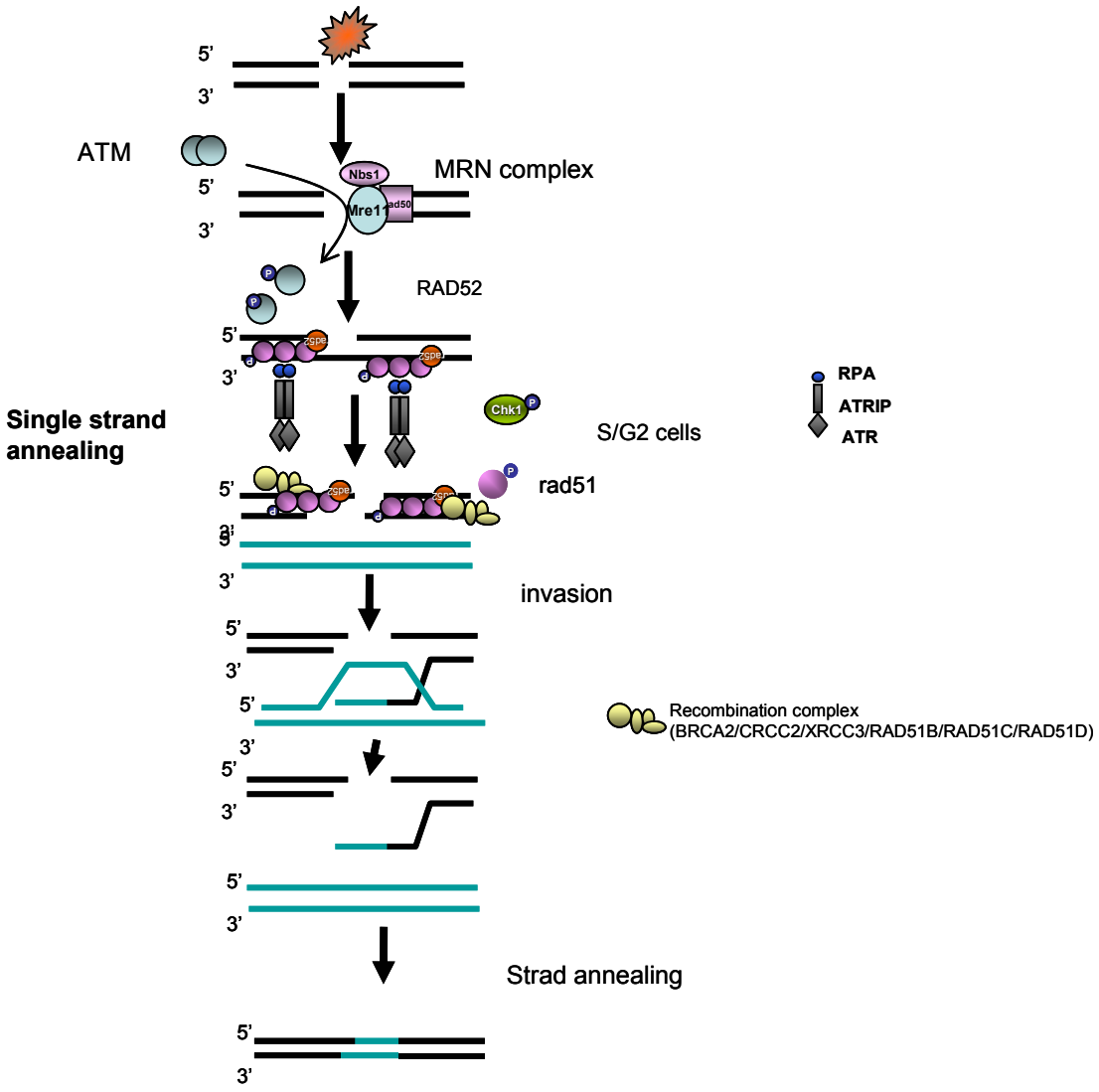

Figure 4: Homologous recombination pathway. DSBs or thoseoccurring in heterochromatic (packed) DNA activate the ATM kinase that inturn activates the full DNA damage response, allowing recruitment of endprocessing factors at the DNA ends to facilitate end joining (C). (D) Cells in the S, G2 or M phases have a high cyclin-dependent kinase activity, which trigger end resection. The exposed single stranded DNA (ssDNA) is coated with the ssDNA-binding protein RPA, which in turn triggers an ATR response that activates HR repair.

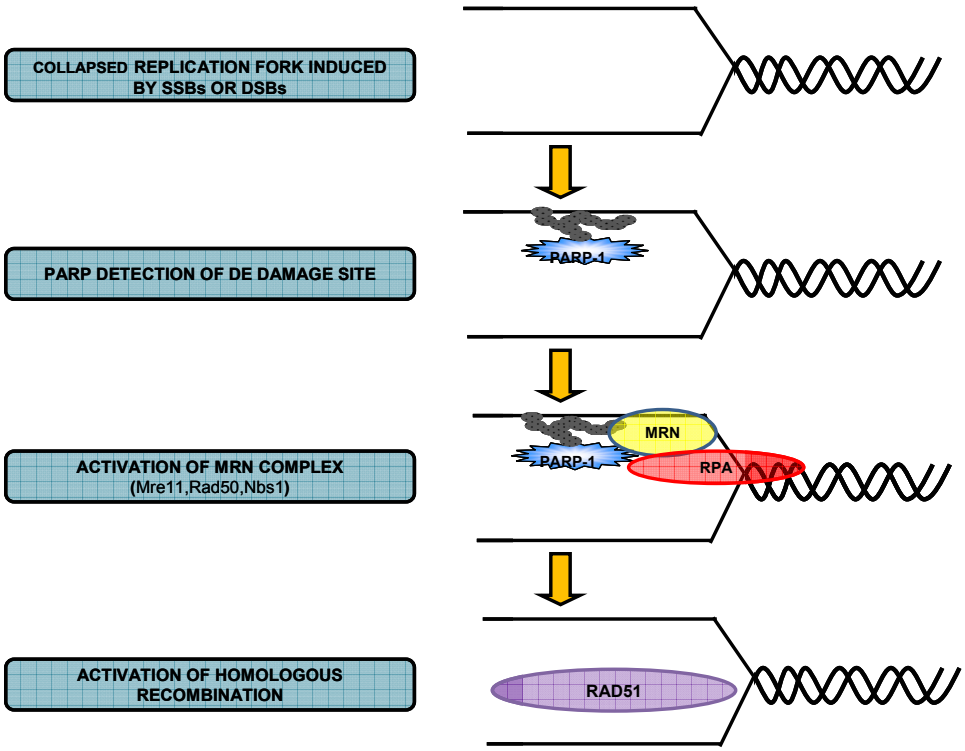

Figure 5: Role of PARP-1 in the regulation of homologous recombination pathway.

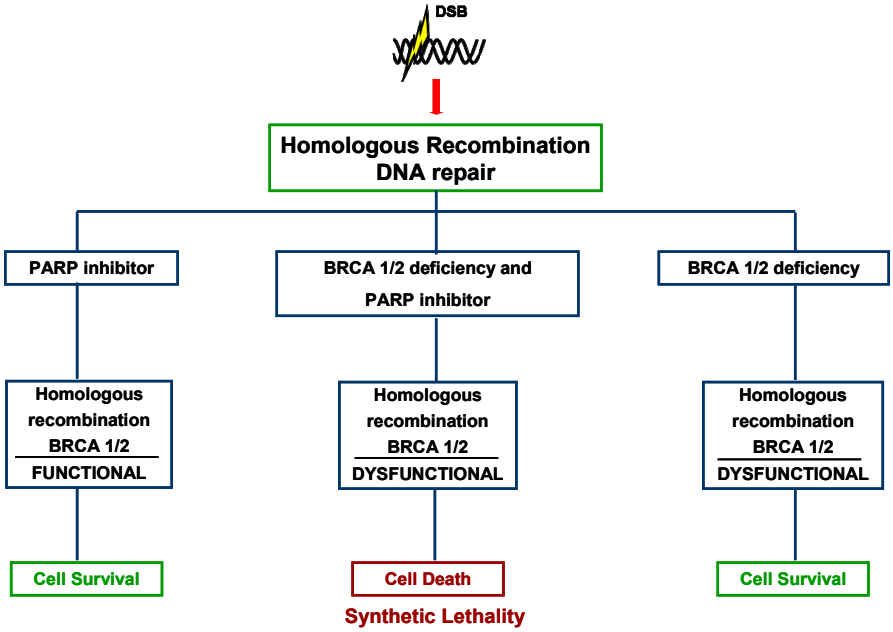

Figure 6: Inhibiton of PARP in HR deficient tumor cells selectively target mutant (tumoral cells).

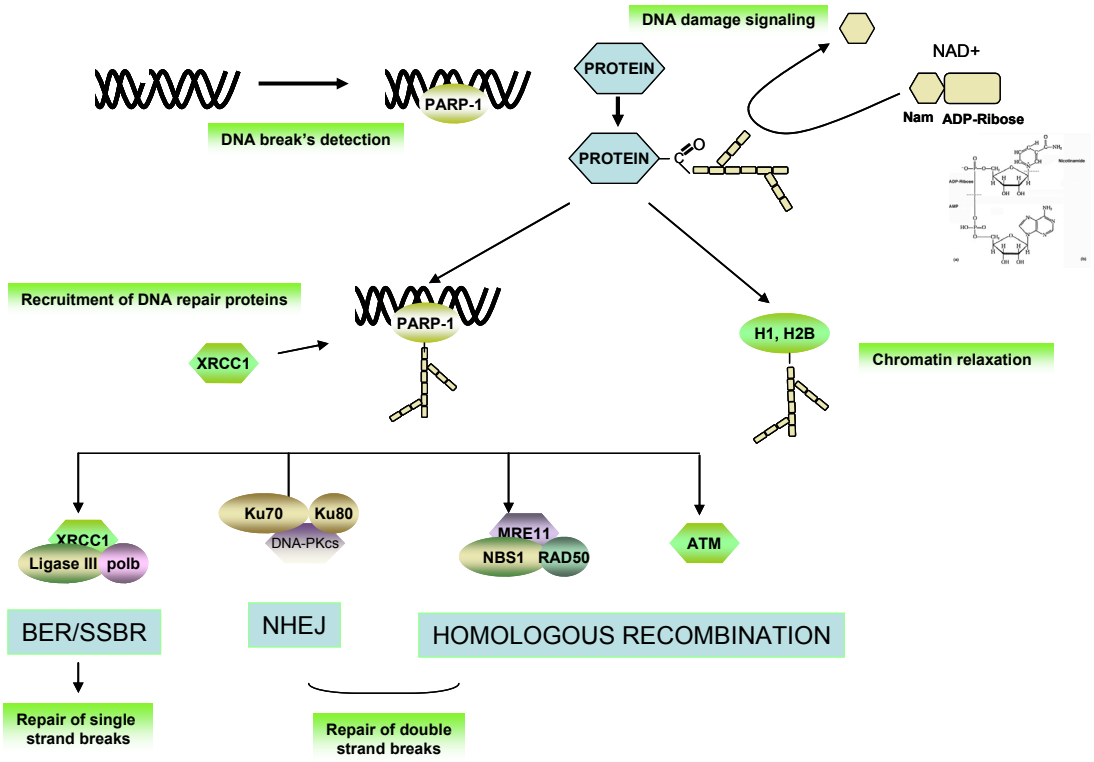

Figure 7. Multiple functions of PARP-1 in DNA damage response

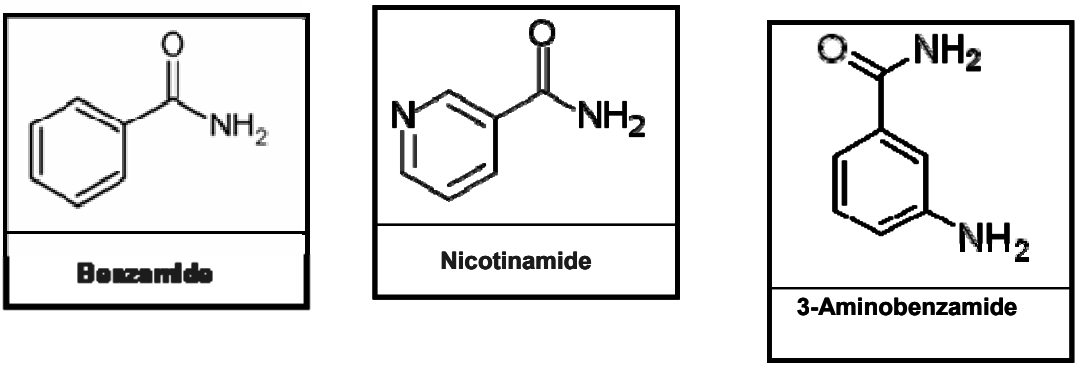

Figure 8. Inhibitors of PARP. The classical inhibitors are nicotinamide, benzamide, and substituted benzamide, in particular 3-aminobenzamide. [12]

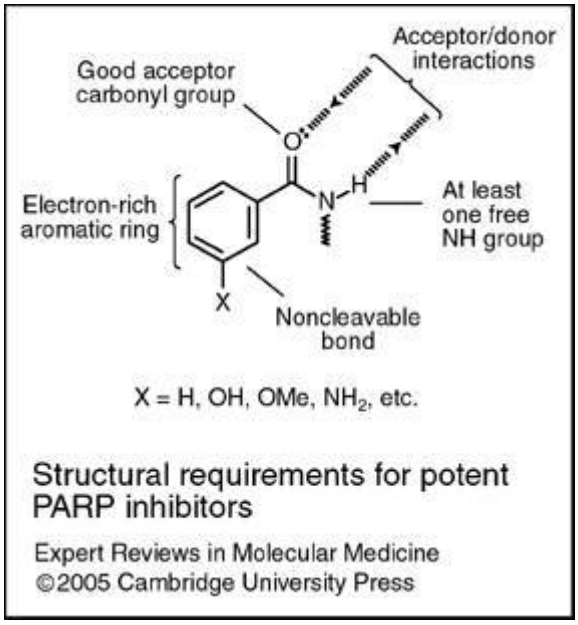

Figure 9. Structural requirements for potent PARP inhibition.
The benzamide pharmacophore is shown, illustrating the desired orientation of the carboxamide moiety for hydrogen bonding with critical residues in the NAD+-binding site of poly(ADP-ribose) polymerase 1 (PARP-1), and the desired unsaturation of the aromatic ring with a noncleavable bond at position 3. NAD+, oxidised nicotinamide adenine dinucleotide.

TABLE 1. PARP Inhibitor. Clinical Trial in Breast and Ovarian Cancer.

Agent	Company	Single/Combination therapy	Disease	Clinical status	
AG014699	Pfizer [New York, NY]	Single agent/Combination with carboplatin, doxorubicin, gemcitabine, paclitaxel, or topotecan	Ovarian cancer Breast cancer	Phase 2	Ihnen 2010 Wiel 2010(100) www.clinicaltrials.gov
Olaparip (KU59436) (AZD2281)	AstraZeneca/KuDOS [London, United Kingdom]	Single	Advanced solid tumors Women with advanced breast cancer measurable BRCA1- or BRCA2-positive advanced ovarian cancer TNBC	Phase 1 Phase 2 Phase 2 Phase 2	Fong et al 2009(68) http://clinicaltrials.gov Tutt 2010 (99) Gelmorn 2010 Dent 2010
Veliparib (ABT 888)	Abbott	With cyclophosphamide With temozolomide	ovarian cancer breast cancer metastasic breast cancer	Phase 2 Phase 2	Kummar 2010 Tan 2010 Isakoff 2010
Iniparib (BSI 201)	BiPar (Brisbane, CA, USA)	SingleAdvanced	Ovarian cancer Breast TNBC	Phase 1 complete; phase 2 ongoing Phase 3	http:www.biparsciences.com O'Shaughnessy 2010
MK4827	Merck	Single agent	Ovarian cancer	Phase 1	Sandhu 2010 http://clinicaltrials.gov
INO-1001	Inotek/Genentech [Beverly, MA]	Combination with doxorubicin	p53-deficient breast cancer	Preclinical	Mason et al 2008(107)
BSI-201 and Temozolomide	Bipar sciences	Combination with temozolomide (TMZ)	Malignant glioma	Phase I and II	http://clinicaltrials.gov
ABT-888 and Temozolomide	Massachusetts General Hospital	Combination of ABT-888 and temozolomide is a safe and effective treatment.	Metastasic Breast cancer	Phase II	http://clinicaltrials.gov
ABT-888	Warren Grant Magnuson Clinical Center	Combined cyclophosphamide in treating patients who did not respond to previous therapy.	Solid tumors, leukemia, lymphoma	Phase I	http://clinicaltrials.gov
AZD2281 and Carboplatin	Warren Grant Magnuson Clinical Center	Combination with Carboplatin.	Breast cancer or ovarian epithelial cancer	Phase I	http://clinicaltrials.gov