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TESIS DOCTORAL

Función de PARP-1 en rutas de inestabilidad genómica e hipoxia: implicaciones en el control tumoral

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En Granada, a de del 2008

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ÍNDICE

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I. ABREVIATURAS

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ADP: adenosín difosfato [*adenosine diphosphate*]
Angptl4: angiopoyetina tipo 4 [*angiopoietin-like 4*]
ARNT: transportador nuclear del receptor de hidrocarburos aromáticos [*aryl hydrocarbon receptor nuclear translocator*]
A-T: ataxia telangiectasia [*ataxia telangiectasia*]
ATM: ataxia-telangiectasia mutada [*ataxia-telangiectasia mutated*]
ATP: adenosín 5'-trifosfato [*adenosine-5'-triphosphate*]
ATR: relacionada con A-T y RAD-3 *[A-T and RAD-3-related*]
BER: reparación por excisión de bases [*base excision repair*]
bHLH-PAS: básico hélice-giro-hélice Per-ARNT-Sim [*basic helix-loop-helix Per-ARNT-Sim*]
BRCT: porción C-terminal del gen BRCA1 [*BRCA1 C terminus*]
Ca^{2+}: ion calcio [*calcium ion*]
Ca 9: anhidrasa carbónica 9 [*carbonic anhydrase 9*]
CBP: proteína de unión a CREB [*CREB binding protein*]
Ctgf: factor de crecimiento de tejido conectivo [*connective tissue growth factor*]
DBD: dominio de unión al DNA [*DNA binding domain*]
DSB: rotura de cadena doble [*double-strand break*]
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*]
EPAS1: proteína con dominio PAS, endotelial 1 [*endothelial PAS domain protein 1*]
Fabp4: proteína de unión a ácidos grasos 4 [*fatty acid-binding protein 4*]
FAT: FRAP, ATM, TRRAP [*FRAP, ATM, TRRAP*]
FATC: FRAP, ATM, TRRAP C terminal [*FRAP, ATM, TRRAP* C terminus]
Fe: ion (ferroso/férrico) [*ferroso/ferricum ion*]
FIH-1: factor inhibitorio de HIF [*factor inhibiting HIF*]
GLUT-1: transportador de glucosa 1 [*glucose transporter 1*]

HEAT: huntingtin, factor de elongación 3, subunidad A de la proteína fosfatasa 2A y TOR1 [*Huntingtin, Elongation factor 3, A subunit of protein phosphatase 2A and TOR1*]

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

HR: recombinación homóloga [*homologous recombination*]

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

H_2O_2: peróxido de hidrógeno [*hydrogen peroxide*]

iNOS: óxido nítrico sintetasa inducible [*inducible nitric oxide syinthase*]

IR: radiación ionizante [*ionizing radiation*]

LDHA: lactato deshidrogenasa A [*lactate dehydrogenase A*]

MEF: fibroblasto embrionario murino [*mouse embryonic fibroblast*]

Mg^{2+}: ion magnesio [*magnesium ion*]

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

MRN: complejo Mre11, Rad50, Nbs1 [*Mre11, Rad50, Nbs1 complex*]

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

mTOR: diana mamífera de la rapamicina [*mammalian target of rapamycin*]

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

NCS: neocarcinostatina [*neocarzinostatin*]

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

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

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

O_2: oxígeno molecular [*molecular oxygen*]

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*]

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

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

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

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

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

siRNA: RNA pequeño de interferencia [*small interfering RNA*]

SSB: rotura de cadena sencilla [*single-strand break*]

SMC1: mantenimiento estructural de los cromosomas 1 [*structural maintenance of chromosomes 1*]

TAD: dominio de transactivación [*transactivation domain*]

VEGF: factor de crecimiento endotelial vascular [*vascular endothelial growth factor*]

II. SUMMARY

(RESUMEN)

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Poly(ADP-ribose) polymerases (PARP) constitute a family of enzymes involved in the regulation of many cellular processes such as DNA repair, gene transcription, cell cycle progression, cell death, chromatin functions and genomic stability. Among the 18 members identified so far, PARP-1 and PARP-2 are the only proteins stimulated by DNA strand breaks and implicated in the repair of DNA injury. Therefore, these molecules have been exploited as potential targets for the development of pharmacological strategies to increase the antitumor efficacy of chemotherapeutic agents, which induce DNA damage. The general aim of this research has been to place PARP-1 in the context of the tumor micro-environment studying both the response to ionising radiation, through its interaction with ATM, and its role in hypoxia.

The presence of damaged DNA in the cell activates repair mechanisms as well as signal transduction pathways leading to cell cycle arrest and programmed cell death. The protein kinase ATM is centrally involved in the cellular response to γ-irradiation (IR) and other DNA double strand breaks (DSBs)-inducing insults, whereas that poly(ADP-ribose)polymerase-1 (PARP-1) is involved mainly in base excision repair, a key pathway in the repair of DNA single strand breaks (SSBs).

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 PARP-1 deficient cells or cells treated with PARP inhibitors display a defective ATM-kinase activity and reduced γ-H2AX foci formation in response to IR.

In the absence of PARP-1 activity, spontaneous non repaired single strand breaks collapse replication forks and trigger ATM for repair of DNA double strand breaks originated. So, PARP inhibitors induce ATM activation through DSBs formation to activate repair mechanisms. In ATM-deficient cells, double

strand breaks originated can't be repaired via ATM, and as consequence there is an increased sensitivity and death cell provoked by PARP inhibitors.

On the other hand, transcriptional responses to hypoxia are primarily mediated by the hypoxia-inducible factor (HIF), a heterodimer of HIF-α and HIF-β subunits. While initial characterization of HIF mostly focused on HIF-1α, HIF-2α raised more recently as a non-redundant isoform inducing specific genes and hence performing specific functions.

In this study, we present evidence for a role of PARP-1 in the regulation of the hypoxic pathway. Furthermore, we show a specific impact of PARP-1 on the hypoxic activation of HIF-2α. By using two independent approaches (MEFs derived from *parp*-1-/- KO mice and gene silencing by siRNA) we show that PARP-1 controls HIF-2α mRNA expression as well as the hypoxic induction of the protein and HIF-2-dependent target genes. However, treatment with PARP inhibitors has no impact on HIF-2 regulation.

Therefore, we have studied two aspects of PARP-1 biology to contribute to our current knowledge of the role of PARP-1 in tumor development through genomic instability and hypoxia related-environment.

III. INTRODUCCIÓN

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1. Carcinogénesis

La hipótesis más aceptada sobre la carcinogénesis es la de la acumulación secuencial de cambios genéticos en una misma célula, que entonces adquiere características neoplásicas. Una sobreexpresión y/o mutación que aumente la función de oncogenes, o la pérdida o inactivación de genes supresores de tumores, puede directamente ofrecer ventajas de crecimiento selectivas a la célula (Bell and Ryan 2005). Estos cambios genéticos, así como alteraciones epigenéticas, desregulación transcripcional o aberraciones en modificaciones post-transduccionales, promueven el proceso de carcinogénesis (Miwa and Masutani 2007).

Así, el cáncer es un proceso constituido por varias etapas, donde cada etapa es el reflejo de alteraciones que conducen a la transformación progresiva de células normales en otras malignas derivadas de las primeras (Hanahan and Weinberg 2000). El concepto de "Iniciación/Promoción/Progresión" (Pitot and Dragan 1991) propone que, cuando una única célula se expone a un agente que altera irreversiblemente la expresión de un gen específico, se inmortaliza, sin que se le permita diferenciarse a la vez que obtiene ventajas de crecimiento, (Trosko and Ruch 1998). Pero esta célula "iniciada" no es una célula tumoral hasta que sufre otra serie de cambios genotípicos y fenotípicos. El proceso de *iniciación* elimina la capacidad de esta célula "madre" de dividirse asimétricamente para originar una célula diferenciada y una célula "madre" iniciada, de modo que cuando es estimulada para dividirse origina dos células *iniciadas*. Si estas células *iniciadas* que no llegaron a diferenciarse, no son eliminadas por las células adyacentes, proliferarán originando un conjunto de células no diferenciadas en el tejido, en lo que vendría a llamarse la fase de *promoción* (Trosko 2001). Finalmente, si una de estas células adquiere cambios genéticos o epigenéticos adicionales, tal que pueda invadir y metastatizar otros tejidos, alcanza el estado maligno o de *progresión* (Pitot, Goldsworthy et al. 1981) (Figura III 1).

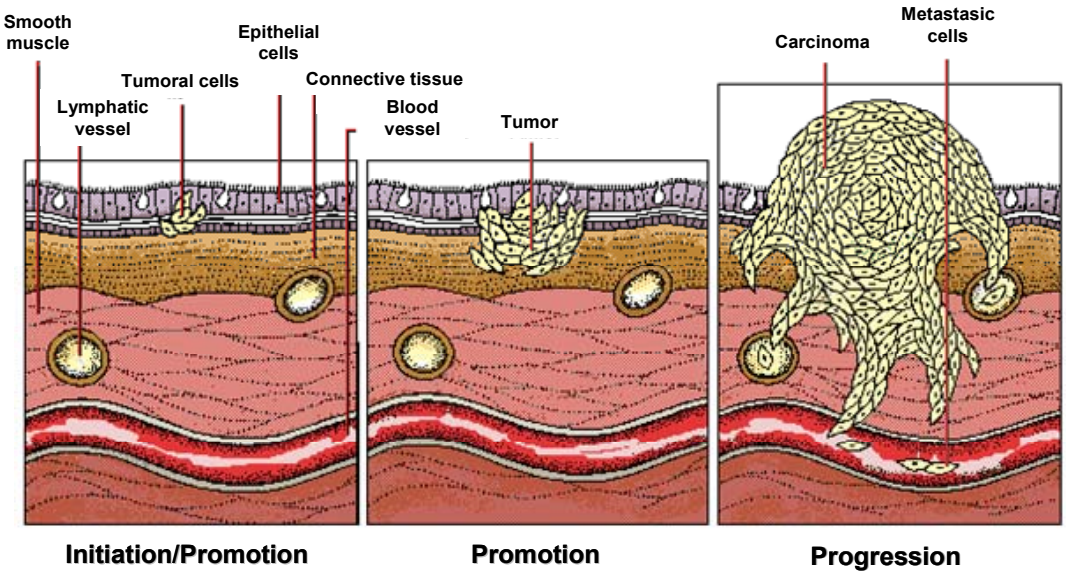

Figura III 1. Proceso de carcinogénesis basado en el modelo de "Iniciación/Promoción/Progresión"

Para evitar la iniciación tumoral, la célula responde de manera eficaz a daños en el DNA, ya sean de origen endógeno (procesos metabólicos celulares) o de origen exógeno (factores medioambientales) a través de la coordinación e integración de puntos de control y mecanismos de reparación (Hakem 2008). Sin embargo, la inestabilidad genómica responsable del crecimiento tumoral puede derivarse además de mutaciones en genes implicados en los propios procesos de reparación del DNA y de segregación cromosómica, afectando a la tasa de mutación de otros genes.

Una característica común de los procesos tumorales en lo que respecta a señalización intracelular, es la capacidad de la célula de crecer y dividirse más allá de sus límites normales. Esto es por sí mismo complejo e implica la proliferación de la célula en ausencia de señales que promuevan el crecimiento, la presencia de señales inhibitorias del crecimiento, y la capacidad de acoplar los procesos de división y crecimiento celular (Bell and Ryan 2005).

Se ha propuesto que la carcinogénesis supone la adquisición de seis alteraciones fundamentales en la fisiología celular que van a determinar la

transformación maligna: proliferación autosuficiente, insensibilidad a señales inhibidoras del crecimiento, evasión de la muerte celular programada, potencial replicativo ilimitado, angiogénesis sostenida, e invasión de tejidos y metástasis (Hanahan and Weinberg 2000).

Sin embargo, mientras todas estas características adquiridas por las células son necesarias para el proceso de carcinogénesis, posteriormente se ha planteado que no son suficientes. Un nuevo concepto propone que el desarrollo tumoral es el resultado de procesos que implican tanto a las células cancerosas como a células no cancerosas (Rakoff-Nahoum 2006). De hecho, los tumores están constituidos por múltiples tipos celulares, como fibroblastos y células epiteliales, células del sistema inmune innato y adaptativo, y células que forman la vasculatura sanguínea y linfática, así como tipos celulares mesenquimales especializados, únicos de cada microambiente tisular. Mientras la homeostasis tisular se mantiene por la colaboración de estos diversos tipos celulares, el desarrollo del cáncer aumenta cuando las células con alteraciones genéticas prescinden de la capacidad cooperativa a favor de su propia supervivencia (de Visser, Eichten et al. 2006) (Figura III 2).

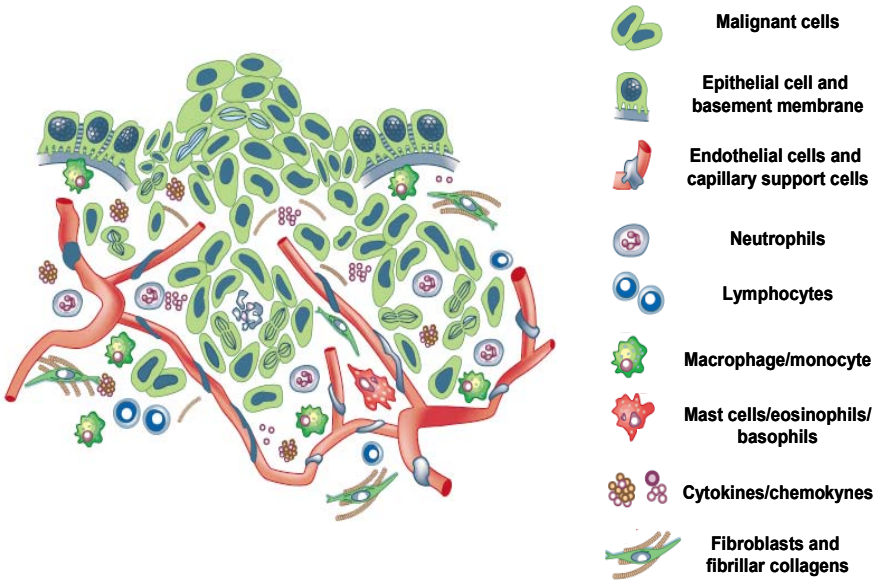

Figura III 2. Composición celular de un carcinoma invasivo. La angiogénesis asociada con neoplasia origina una organización vascular caótica donde las células neoplásicas interaccionan con otros tipos celulares (destacando la presencia del sistema inmunitario), y una remodelada matriz extracelular. Las células neoplásicas producen toda una serie de citoquinas y quimioquinas mitogénicas y/o quimioatrayentes de granulocitos, mastocitos, monocitos/macrófagos, fibroblastos y células endoteliales. Además, los fibroblastos y las células inflamatorias infiltradas, una vez activadas, secretan enzimas proteolíticas, citoquinas y quimioquinas, que son mitogénicas para las células neoplásicas, así como para células endoteliales implicadas en neoangiogénesis. Estos factores potencian el crecimiento tumoral, estimulan la angiogénesis, inducen migración y maduración de fibroblastos, y favorecen la metástasis a través de los nuevos vasos. (*Adaptado de (Coussens and Werb 2002))*

Se ha tratado de aclarar el complejo papel del sistema inmune en el desarrollo del cáncer, que implica interacciones recíprocas entre las células alteradas genéticamente, las células del sistema inmune adaptativo e innato, sus mediadores solubles y los componentes estructurales del microambiente neoplásico. Cada etapa de la carcinogénesis es regulada por el sistema inmune; pero mientras que la completa activación de las células inmunes adaptativas en el tumor puede suponer la erradicación de células malignas, la activación crónica de células inmunes innatas en lugares de crecimiento tumoral podría aumentar el mismo (de Visser and Coussens 2006).

Dentro de este mismo contexto, se ha establecido una relación funcional entre inflamación y cáncer (Coussens and Werb 2002). De hecho, muchos tumores se originan en sitios de infección, irritación crónica e inflamación. Aunque está claro que la proliferación de las células por sí sola no origina cáncer, una proliferación celular sostenida en una ambiente rico en células inflamatorias, factores de crecimiento y agentes promotores del daño en el DNA, potencian y/o promueven el riesgo neoplásico. El microambiente tumoral, donde las células inflamatorias son importantes, es un participante fundamental en el proceso neoplásico, promoviendo la proliferación, supervivencia y migración de las células tumorales.

De gran relevancia en el proceso de carcinogénesis, también relacionado con el microambiente tumoral, es la reducción en la tensión de oxígeno tisular que tiene lugar durante el crecimiento tumoral y que puede a su vez regular la proliferación de la célula cancerosa. Esta baja disponibilidad de oxígeno o hipoxia, es común de tumores sólidos localmente avanzados, que se han asociado con menor respuesta terapéutica y progresión maligna, o lo que es lo mismo, probabilidad alta de recurrencia, expansión local y metástasis. Los efectos de la hipoxia sobre la progresión maligna se deben a una serie de cambios genómicos y proteómicos que activan procesos de angiogénesis, metabolismo anaerobio y otros, para permitir a las células tumorales sobrevivir o eludir el entorno deficiente en oxígeno (Vaupel 2004) y que serán tratados en detalle en el punto 6 de esta introducción.

Además, trabajos recientes describen las conexiones moleculares entre factores de transcripción regulados por oxígeno y rutas conocidas de control de la función de células madre, sugiriendo un nuevo mecanismo por el que tales factores de transcripción inducidos por hipoxia quizá conduzcan al crecimiento tumoral mediante la expansión de células iniciadoras del tumor o "células madre" cancerosas (Keith and Simon 2007).

2. PARP-1

2.1. Definición y estructura

Poli(ADP-ribosa) polimerasa (PARP)-1 es la isoforma fundadora y más estudiada de la superfamilia PARP. PARP-1 es una enzima nuclear multifuncional de 113 kDa, altamente conservada y constituida, básicamente, por tres dominios (Kim, Zhang et al. 2005) (Figura III 3):

- Dominio de unión al DNA (DBD): N-terminal, con dos dedos de zinc (FI y FII), seguido de una señal de localización nuclear (NLS).
- Dominio de automodificación: central, con un motivo BRCT (BRCA1 C terminus) para interacciones proteína-proteína.
- Dominio catalítico: C-terminal, contiene el sitio activo.

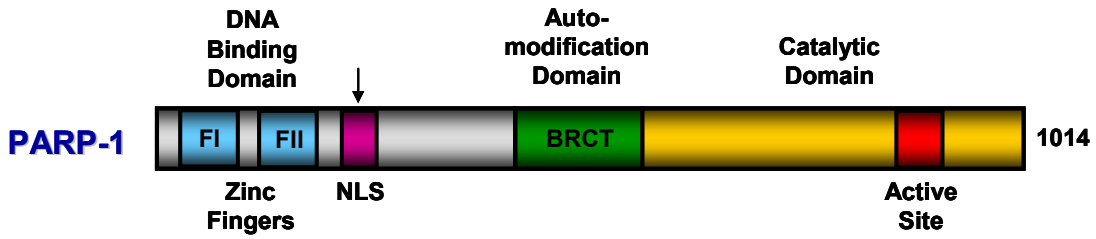

Figura III 3. Organización estructural y funcional de PARP-1. El sitio de corte por caspasa 3 y caspasa 7 está indicado mediante una flecha. *(Adaptado de(Kim, Zhang et al. 2005))*

La actividad enzimática de PARP-1 consiste en la síntesis de un polímero aniónico de ADP-ribosa (PAR) de tamaño variable, lineal o ramificado, a partir de NAD^+. La unión covalente de PAR tiene lugar sobre el grupo γ-carboxilo de residuos principalmente de glutámico de proteínas aceptoras,

normalmente relacionadas funcionalmente con el DNA (heteromodificación), o de la propia PARP-1 (automodificación) (Schreiber, Dantzer et al. 2006). De hecho, el principal aceptor *in vivo* de PAR es PARP-1, cuyo dominio de automodificación contiene varios residuos de glutámico (Kim, Zhang et al. 2005). Dianas de la actividad enzimática de PARP-1 son entre otras, histonas, proteínas de reparación del DNA o factores relacionados con la transcripción que interaccionan con PARP-1 (Huletsky, de Murcia et al. 1989; D'Amours, Desnoyers et al. 1999; Kraus and Lis 2003) (Figura III 4). Debido a la alta carga negativa del polímero, esta modificación post-transduccional altera las propiedades físico-químicas de los aceptores, tales como su afinidad de unión al DNA, o por ejemplo, su función de interacción con otras proteínas (Rouleau, Aubin et al. 2004). Además, las interacciones proteína-proteína entre PARP-1 y un cada vez más numeroso conjunto de proteínas, puede influir sobre importantes procesos celulares (Ame, Spenlehauer et al. 2004). A su vez, el polímero puede también interaccionar selectivamente con una serie de proteínas implicadas en la respuesta celular al daño en el DNA y en el metabolismo del DNA, alterando sus propiedades funcionales. Dichas proteínas contienen un motivo consenso de unión a PAR que frecuentemente se solapa con un dominio funcional, como un dominio de unión al DNA o a proteínas (Pleschke, Kleczkowska et al. 2000).

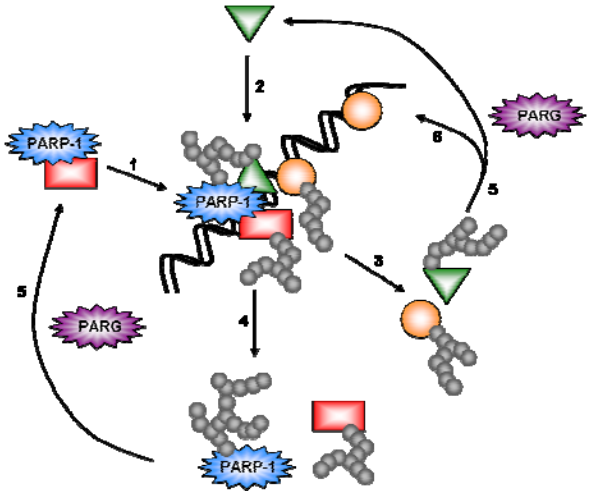

Figura III 4. PARP-1 y DNA: varios modos de actuación. *(1)* Varias proteínas (rectángulos rojos) interaccionan físicamente con PARP-1 independientemente de su automodificación y pueden ser reclutadas al DNA junto con PARP-1. Después de la unión al DNA, PARP-1 se activa y se poli(ADP-ribosil)a. *(2)* Muchas proteínas (triángulos verdes) interaccionan preferiblemente con PARP-1 automodificada y son reclutadas al DNA después de la activación de PARP-1. Las proteínas con las que PARP-1 interacciona y las proteínas de unión al DNA cercanas (círculos naranja) son también poli(ADP-ribosil)adas. *(3)* Esta modificación puede desencadenar la salida del DNA y romper los complejos de proteínas. Si las proteínas poli(ADP-ribosil)adas son enzimas que modifican el DNA o factores de transcripción, pueden ser inactivados. Si las proteínas son histonas, pueden remodelar la estructura de la cromatina. *(4)* La propia PARP-1 es inactivada por automodificación y posterior disociación del DNA. *(5)* PARG (presentada a continuación) elimina de PARP-1 y de otras proteínas el polímero sintetizado, generando así polímeros libres y ADP-ribosa. Varias proteínas pueden unirse a los mismos para evitar unirse al DNA. *(6)* Las proteínas separadas de los polímeros gracias a PARG pueden volver a unirse al DNA y llevar a cabo de nuevo su actividad. *(Adaptado de (Petermann, Keil et al. 2005))*

2.2. Expresión y activación

No se encuentran en la literatura muchos trabajos acerca de los mecanismos moleculares que regulan la transcripción del gen PARP-1 debido a que la regulación de PARP-1 se lleva a cabo principalmente a nivel de la activación enzimática de PARP-1 preexistente, más que a nivel de expresión del gen. No obstante, hay descritos casos como la activación de linfocitos humanos o la regeneración del hígado de rata, en los que la expresión de PARP-1 varía (McNerney, Tavasolli et al. 1989; Cesarone, Scarabelli et al. 1990; Liu, Ying et al. 2000). La expresión de PARP-1 parece estar relacionada con la proliferación celular, de forma que el mRNA de PARP-1 es más abundante durante la fase G1 del ciclo celular (Menegazzi, Gerosa et al. 1988; Thibodeau, Gradwohl et al. 1989; Bhatia, Kirkland et al. 1995). Pero en general, PARP-1 se expresa constitutivamente a altos niveles y su mRNA está presente en todos los tejidos, aunque a niveles variables (Meyer-Ficca, Meyer et al. 2005). Se han clonado el promotor humano (Yokoyama, Kawamoto et al. 1990), el promotor de rata (Potvin, Thibodeau et al. 1992) y el promotor de ratón de PARP-1 (Pacini, Quattrone et al. 1999), y los tres comparten motivos estructurales parecidos típicos de genes constitutivos, en los que falta una caja consenso TATA funcional, poseen un alto contenido en residuos GC y una secuencia iniciadora consenso (Inr) que se solapa con el sitio de inicio de la transcripción. Además, se ha visto que el promotor humano tiene sitios de unión para los factores de transcripción Sp1, AP-2 (Yokoyama, Kawamoto et al. 1990), YY1 (Oei, Griesenbeck et al. 1997), y Ets (Soldatenkov, Albor et al. 1999), y que un complejo formado por la proteína de adenovirus E1A y pRb disminuye la expresión del promotor murino (Pacini, Quattrone et al. 1999). La actividad del promotor proximal de PARP-1 de rata depende principal, pero no completamente, del reconocimiento de cinco sitios de unión ricos en GC por los factores de transcripción Sp1 y Sp3 (Potvin, Roy et al. 1993; Bergeron, Leclerc et al. 1997). Por otro lado, otros factores de transcripción, como los que pertenecen a la familia de factores de transcripción NFI (Nuclear Factor I) (Laniel, Bergeron et al. 1997), reprimen la actividad del promotor de PARP-1 al competir con Sp1 por la disponibilidad de un elemento del promotor con sitios

diana solapantes para ambos factores de transcripción (Laniel, Bergeron et al. 1997; Laniel, Poirier et al. 2001; Zaniolo, Gingras et al. 2006). Por tanto, en base a los efectos sinérgicos u opuestos de los factores de transcripción que se unen para, respectivamente, activar o reprimir la transcripción, la combinación de elementos reguladores del promotor de PARP-1 supone una forma eficiente de modular su expresión en diferentes contextos celulares (Zaniolo, Desnoyers et al. 2007).

La actividad enzimática basal de PARP-1 es muy baja, pero es estimulada drásticamente por una variedad de activadores alostéricos, como DNA dañado, algunas estructuras de DNA no dañado, nucleosomas y proteínas con las que interacciona (D'Amours, Desnoyers et al. 1999; Oei and Shi 2001; Kun, Kirsten et al. 2002; Kim, Mauro et al. 2004). De hecho, PARP-1 se une a diversas estructuras del DNA, incluyendo roturas de cadena sencilla y cadena doble, entrecruzamientos, formas en cruz, superenrollamientos, así como a algunas secuencias específicas de doble cadena (Kim, Zhang et al. 2005).

Sin embargo, inicialmente, se asumió que la actividad de PARP se regulaba principalmente por su capacidad para reconocer a través de sus dedos de zinc cadenas rotas de DNA (Virag and Szabo 2002). Oxidantes endógenos y radicales libres, así como radiación ionizante (IR) y compuestos genotóxicos son inductores clásicos de roturas del DNA (Szabo, Zingarelli et al. 1996). Por tanto, se han identificado como activadores endógenos de PARP-1, condiciones que pueden producir radicales reactivos y oxidantes dentro de la célula, como hipoxia-reoxigenación (Gilad, Zingarelli et al. 1997), alta concentración de glucosa extracelular (Garcia Soriano, Virag et al. 2001; Pacher, Liaudet et al. 2002; Du, Matsumura et al. 2003; Pacher and Szabo 2005), Ca^{2+} (Yakovlev, Wang et al. 2000), y angiotensina II (Szabo, Pacher et al. 2004; Pillai, Gupta et al. 2006). PARP-1 igualmente puede activarse en respuesta a roturas del DNA localizadas y transitorias, originadas por procesos biológicos como reparación del DNA, replicación, recombinación y reordenamiento génico. Además, se ha demostrado la regulación alostérica de la autopoli(ADP-ribosil)ación por Mg^{2+}, Ca^{2+}, poliaminas, ATP, y las histonas H1

y H3 (Kun, Kirsten et al. 2004). La actividad de PARP-1 puede también ser modulada por varios factores endógenos, como varias quinasas (por modificación post-transduccional), purinas y metabolitos de cafeína; y varios antibióticos del grupo de las tetraciclinas (potentes inhibidores de PARP-1) (Szabo, Pacher et al. 2006). Así, el grado de activación de PARP por daños en el DNA puede ser regulado por otros factores.

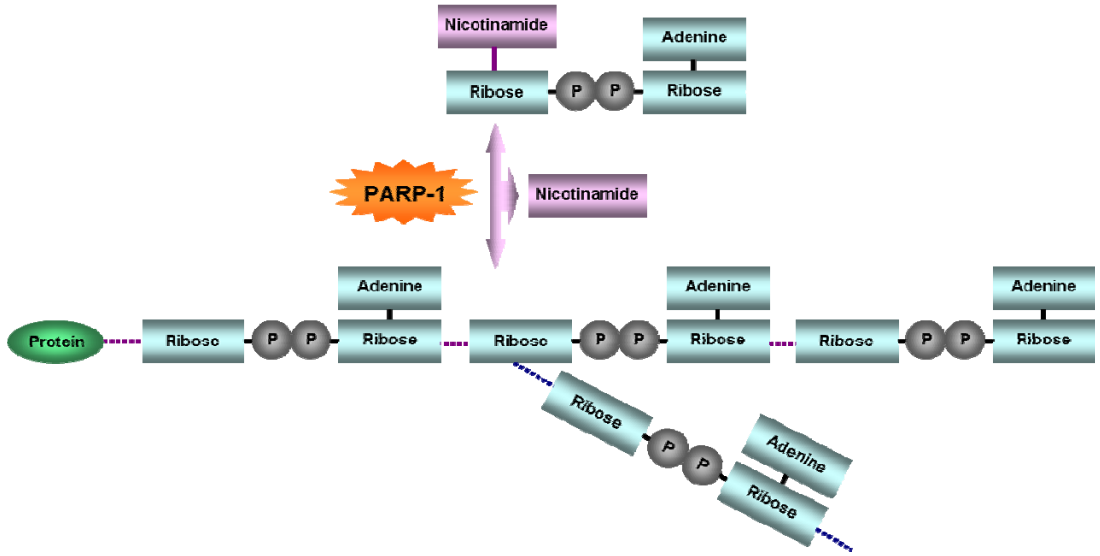

Figura III 5. Reacción de poli(ADP-ribosil)ación catalizada por PARP-1. PARP-1 rompe las moléculas de NAD^+ en nicotinamida y ADP-ribosa, y utiliza esta última para unirla covalentemente a proteínas aceptoras (reacción de iniciación) o a otras unidades de ADP-ribosa ya unidas (reacción de elongación). *(Adaptado de (Nguewa, Fuertes et al. 2005))*

Una vez activada, PARP-1 rápidamente cataliza de manera secuencial la transferencia de unidades de ADP-ribosa desde la forma oxidada del dinucleótido nicotinamida adenina (NAD^+), por ruptura del enlace glucosídico entre nicotinamida y ribosa, hasta residuos principalmente de glutámico de proteínas aceptoras o de la propia PARP-1, como ya se comentó previamente. El resultado es la formación de un enlace éster entre el aceptor y el residuo de ADP-ribosa (reacción de iniciación) (Diefenbach and Burkle 2005). Seguidamente, PARP-1 cataliza una reacción de elongación y ramificación a partir de unidades de ADP-ribosa adicionales, con nuevas uniones ribosil-ribosil, dando origen a polímeros de longitud de cadena de hasta 200 unidades de ADP-ribosil y varios puntos de ramificación (Alvarez-Gonzalez and Jacobson 1987) (Figura III 5). Por tanto, la reacción catalizada sería: $NAD^+ + X \rightarrow$ X-1'-ribosa-5'-ADP + nicotinamida, resultando en la formación de polímeros lineales de homo-ADP-ribosa unidos a proteína (Brown and Marala 2002); donde X es

una proteína que va a ser modificada (iniciación), o el grupo 2'-hidroxilo de la ribosa unida a adenina (elongación de cadena) o la ribosa no perteneciente al grupo ADP (ramificación) (Nguewa, Fuertes et al. 2005). La proporción ramificación/elongación es aproximadamente del 2% (Keith, Desgres et al. 1990).

La poli(ADP-ribosil)ación es un proceso dinámico, tal y como indica la corta vida media *in vivo* del polímero (inferior a 1 minuto) (Whitacre, Hashimoto et al. 1995), ya que como otras modificaciones post-transduccionales de proteínas, la poli(ADP-ribosil)ación es un proceso reversible. La ruptura del PAR la lleva a cabo la enzima poli(ADP-ribosa) glicohidrolasa (PARG), enzima con actividades exo- y endoglucosidasa, que hidroxila el enlace glucosídico entre unidades de ADP-ribosa terminales (en el caso de la actividad exoglucosidasa) e internos (en el caso de la actividad endoglucosidasa) (Brochu, Duchaine et al. 1994; Davidovic, Vodenicharov et al. 2001). PARG rompe los enlaces ribosa-ribosa tanto de las porciones lineales como ramificadas del homopolímero, liberando grandes fragmentos de oligo(ADP-ribosa) o ADP-ribosa monomérica. Además, se ha descrito que una ADP-ribosil protein liasa elimina la porción proximal del monómero ADP-ribosa unido a la proteína aceptora (Davidovic, Vodenicharov et al. 2001). El valor de la *Km* de PARG es mucho más bajo para los polímeros de $(ADP\text{-}ribosa)_n$ de mayor tamaño que para los más pequeños (Hatakeyama, Nemoto et al. 1986), por lo que la enzima cataboliza los fragmentos más grandes en primer lugar. Entonces, PARG cambia a modo exoglucosídico y elimina unidades de ADP-ribosa una a una. Aparte, la alta actividad específica de PARG compensa la poca abundancia de la enzima.

Como se expuso anteriormente, PARP-1, como aceptor principal de su propia actividad enzimática, se automodifica en una reacción de autopoli(ADP-ribosil)ación intermolecular. Debido a que el dominio de automodificación también media las interacciones proteína-proteína, dicha reacción de autopoli(ADP-ribosil)ación altera las propiedades de interacción de PARP-1 con otras proteínas, del mismo modo que la presencia del polímero cargado negativamente, hace que PARP-1 se libere de su sustrato

(Meyer-Ficca, Meyer et al. 2005). Así, el resultado de esta automodificación es la inhibición de PARP-1 (Nguewa, Fuertes et al. 2005), por lo que la degradación del polímero por PARG será fundamental a su vez, para la reactivación de PARP-1.

2.3. La superfamilia PARP

Se conoce por "firma PARP" a una secuencia altamente conservada entre especies del dominio catalítico de PARP-1 (100% conservada entre vertebrados). La "firma PARP" se ha utilizado para buscar homólogos humanos de PARP y de este modo se han identificado nuevas PARP putativas hasta incrementar el número de los miembros de la familia PARP a 17 (Ame, Spenlehauer et al. 2004). Sin embargo, la conservación en la "firma PARP" varía significativamente entre los miembros de la familia PARP (Schreiber, Dantzer et al. 2006). De hecho, algunos miembros de la familia PARP no tienen actividad enzimática PARP intrínseca (Ame, Spenlehauer et al. 2004). Por otro lado, es posible encontrar actividad enzimática propia de las enzimas PARP en todos los organismos multicelulares, incluyendo plantas y en algunos eucariotas unicelulares inferiores, pero está ausente en procariotas y levaduras (Burkle 2005).

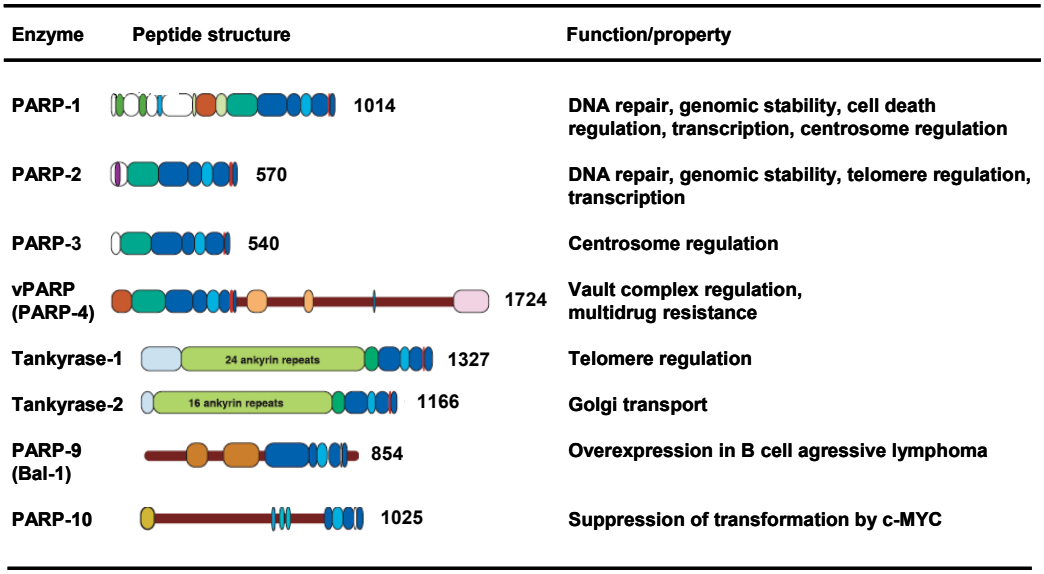

Figura III 6. Funciones/propiedades de las proteínas de la familia PARP relacionadas con carcinogénesis. El número de aminoácidos de cada proteína se indica al lado del correspondiente diagrama esquemático para cada una de ellas. *(Adaptado de (Miwa and Masutani 2007))*

Se piensa que los miembros de la familia PARP podrían tener funciones celulares divergentes, en base a que fuera del dominio de homología, contienen dominios únicos que las distinguen unas de otras, por lo que puede establecerse una clasificación de los miembros de la familia PARP de acuerdo con sus dominios funcionales putativos (Schreiber, Dantzer et al. 2006). Además, las múltiples localizaciones subcelulares de las proteínas PARP hacen que sea improbable una función común entre ellas (Nguewa, Fuertes et al. 2005). Por ello, la translocación de PARG entre citoplasma y núcleo, así como la presencia de diferentes isoformas de PARG generadas por *splicing* diferencial distribuidas por compartimentos celulares diferentes de la célula, aseguran el recambio dinámico de las unidades de ADP-ribosa (Bonicalzi, Vodenicharov et al. 2003; Ohashi, Kanai et al. 2003). En la figura III 6, a modo de resumen, se muestran la estructura y función sólo de los miembros de la familia PARP relacionados con carcinogénesis.

2.4. Función

Los dominios y actividades de PARP-1 sugieren papeles importantes en varias funciones nucleares.

2.4.1. Reparación del DNA y mantenimiento de la integridad genómica

PARP-1 es un sensor molecular de daños en el DNA, de modo que su actividad catalítica se estimula más de 500 veces por unión a roturas del DNA, 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).

En respuesta a daños en el DNA, 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 DNA dañado a

través de su doble dedo de zinc del dominio de unión al DNA (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 DNA, modificándolas por poli(ADP-ribosil)ación y/o reclutándolas al DNA dañado. (Okano, Lan et al. 2003; Lan, Nakajima et al. 2004). 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 DNA, 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, así como regulará su propia actividad (Figura III 7). Ya se ha comentado que la autopoli(ADP-ribosil)ación modifica las propiedades de unión de PARP-1 con proteínas, y que la presencia de cargas negativas promueve la liberación de PARP-1 del DNA, inactivándose.

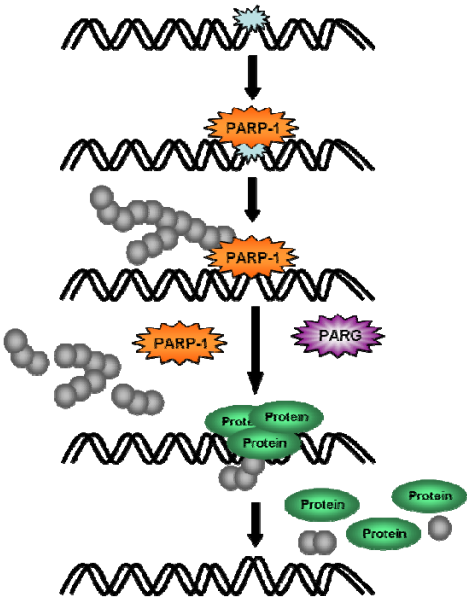

Figura III 7. Mecanismo de reparación del daño en el DNA por PARP-1. PARP-1 detecta el daño y rápidamente se une al DNA, catalizando la poli(ADP-ribosil)ación de ella misma, principalmente. Entonces PARP-1 se libera del DNA y permite el acceso de proteínas de reparación que son reclutadas, y en ocasiones modificadas, por el PAR. PARG se encarga de degradar el polímero para volver a la situación inicial. *(Adaptado de (Herceg and Wang 2001))*

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 DNA; 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 DNA. Además, si estas roturas persisten, pueden convertirse en roturas de cadena doble (Horton, Watson et al. 2008).

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 DNA dañado y por tanto, para el proceso de SSBR/BER (Masson, Niedergang et al. 1998; Caldecott 2003; Okano, Lan et al. 2003).

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 DNA, 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, Harrigan 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, Dejsuphong 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, Harrigan et al. 2003). También, PARP-1 está implicado, junto con Ligasa III, en una ruta alternativa de NHEJ de más baja fidelidad (Wang, Wu et al. 2006). Sin embargo, se ha descrito que PARP-1 promueve la HR, antagonizando con NHEJ (Saberi, Hochegger 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).

En el punto V A de esta memoria se discutirá la interacción de PARP-1 con otro importante sensor de daños en el DNA, ATM, y también la conexión entre la reparación de SSBs dependiente de PARP-1 y la reparación de DSBs, dependiente de HR (Bryant, Schultz et al. 2005; Farmer, McCabe et al. 2005).

El DNA dañado que no se repara apropiadamente puede dar lugar a inestabilidad génomica, predisponiendo al organismo a cáncer (Hakem 2008) (Figura III 8). Ya se ha comentado la contribución de los cambios genéticos en el desarrollo tumoral. Así, PARP-1, como factor de reparación del DNA, está estrechamente ligado al proceso de carcinogénesis.

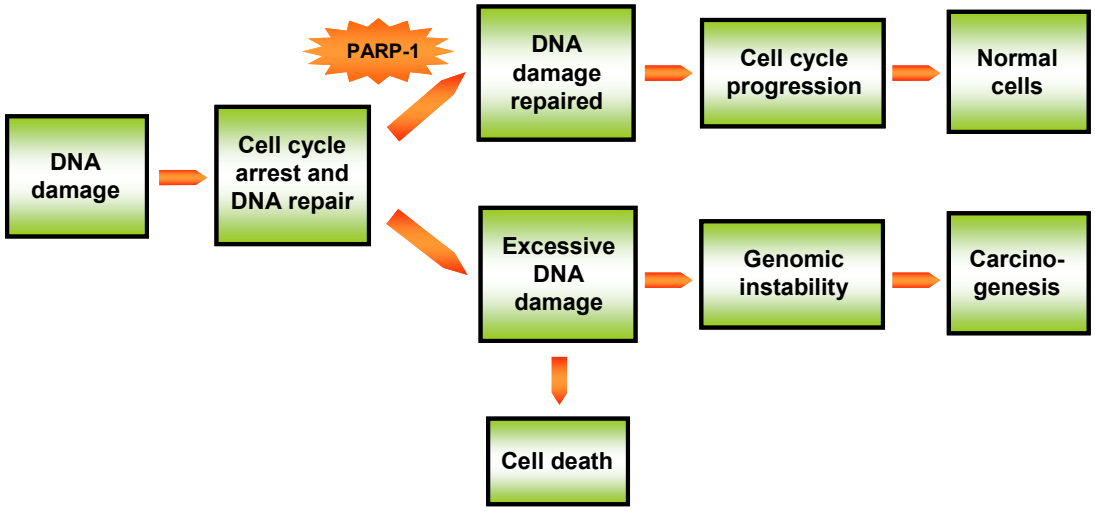

Figura III 8. Correlación entre daños en el DNA y carcinogénesis. *(Adaptado de (Bernstein, Bernstein et al. 2002))*

2.4.2. Muerte celular

Con niveles leves o moderados de daño en el DNA, PARP-1 actúa como un factor de supervivencia implicado en la detección del daño del DNA y en su reparación, sin el riesgo de arrastrar genes mutados. Por el contrario, con grandes niveles de daño en el DNA, PARP-1 promueve mecanismos de muerte

celular (Burkle 2001). Así, la intensidad del estímulo genotóxico, determinará el destino celular (Jagtap and Szabo 2005) (Figura III 9).

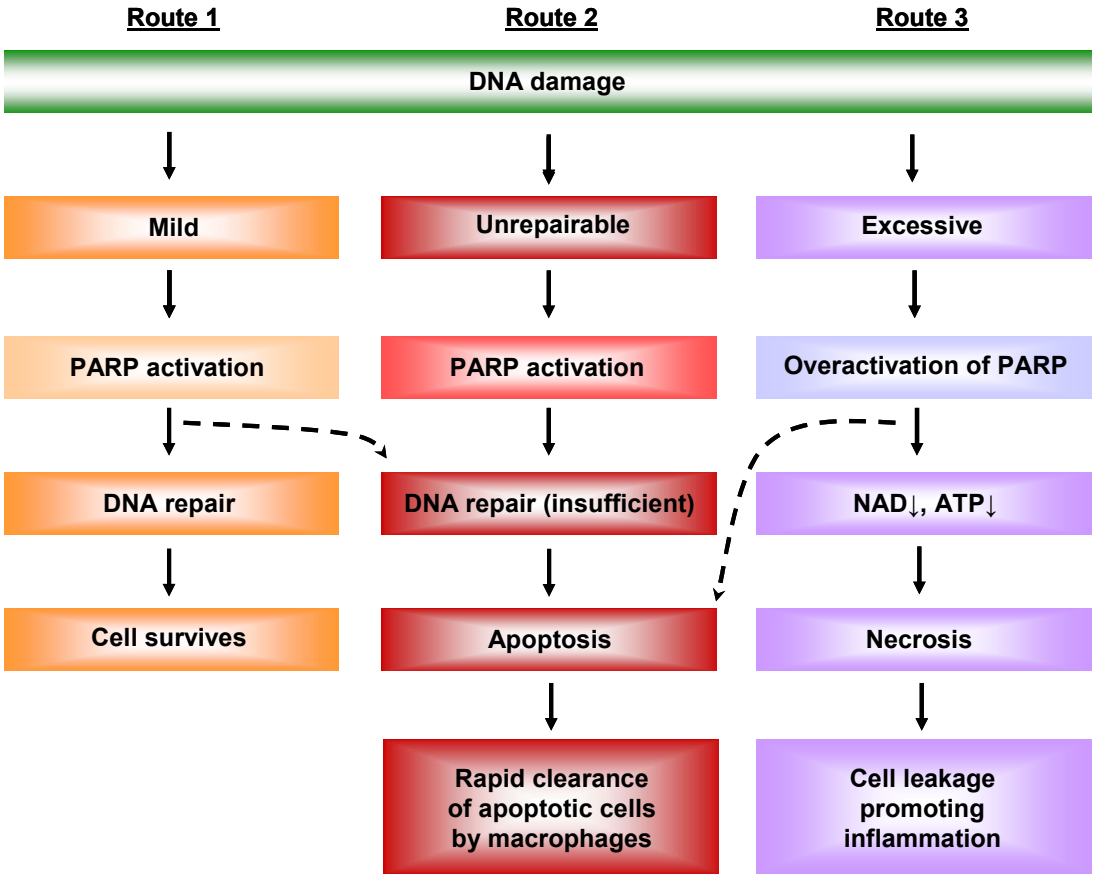

Figura III 9. La intensidad del estímulo de daño del DNA determina el destino celular: supervivencia, apoptosis o necrosis. Dependiendo de la intensidad del estímulo, PARP-1 regula tres rutas diferentes. En el caso de daño leve en el DNA, la poli(ADP-ribosil)ación facilita la reparación del DNA y por tanto, la supervivencia (ruta 1). Estímulos genotóxicos más intensos activan la apoptosis para eliminar células con DNA dañado (ruta 2). Un daño en el DNA 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 (Jagtap and Szabo 2005). 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 activadad inducida por el daño en el DNA (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; Ivana Scovassi and Diederich 2004). La ruptura proteolítica de PARP-1 impide su sobreactivación en respuesta a la fragmentación del DNA 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 (Herceg and Wang 1999; Aikin, Rosenberg et al. 2004). Además, previniendo intentos inútiles de reparación del DNA, la ruptura de PARP-1 quizá ayude a las células a entrar en apoptosis (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, Wang et al. 2002; Yu, Andrabi et al. 2006). 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). Ya en el núcleo, AIF induce condensación de la cromatina y fragmentación del DNA (Susin, Lorenzo et al. 1999). Como las caspasas no están implicadas en este proceso, PARP-1 está intacto para ser activado por el DNA fragmentado, lo que origina una masiva síntesis de polímero, caída de los niveles de NAD^+ y ATP, y por tanto, muerte celular.

El destino celular tras el daño en el DNA, 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 DNA induce apoptosis, con las caspasas 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 DNA leve reparable, así como aquellas fuertemente dañadas, serían eliminadas por apoptosis (Figura III 9). La inhibición de PARP en estas últimas, que normalmente morirían por necrosis, conservaría los niveles de ATP necesarios para la apoptosis (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 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.

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

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 DNA se mantiene (Schreiber, Dantzer et al. 2006; Aguilar-Quesada, Munoz-Gamez et al. 2007).

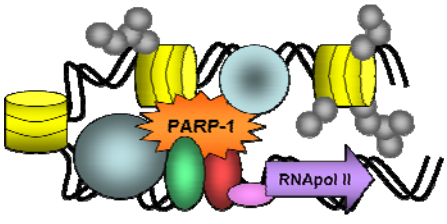

Figura III 10. Mecanismos de regulación de la transcripción por PARP-1. Se han propuesto dos modos de regulación de la transcripción por PARP-1. Primero, por modificación de las histonas (en amarillo) y otros factores asociados a cromatina, modulando la estructura de la misma; y segundo, como cofactor transcripcional, de forma dependiente o independiente de su actividad enzimática. *(Adaptado de (Kraus and Lis 2003))*

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, Dignam 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 III 10): 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).

Modificación de la estructura de la cromatina

PARP-1 afecta a la estructura de la cromatina al poli(ADP-ribosil)ar directamente al núcleo de histonas y proteínas asociadas a cromatina, de manera que favorece la disociación de los nucleosomas y la descondesación de la cromatina (Oei, Griesenbeck et al. 1998; D'Amours, Desnoyers et al. 1999; Kraus and Lis 2003; Rouleau, Aubin et al. 2004). Incluso se ha sugerido que el polímero polianiónico, bien libre o unido a proteínas como la propia PARP-1, proporciona una matriz apropiada para las histonas liberadas de los nucleosomas desestabilizados (Mathis and Althaus 1987; Realini and Althaus 1992), o incluso separa proteínas básicas como las histonas, del DNA (Mathis and Althaus 1987; Wesierska-Gadek and Sauermann 1988; Panzeter, Realini et al. 1992).

Así, en *D. melanogaster*, PARP-1 se encuentra próxima a regiones de cromatina transcripcionalmente reprimida, pero distintas de los dominios heterocromáticos altamente condensados unidos a histona H1. En respuesta a un estímulo externo, ambiental o propio del desarrollo, PARP-1 se activa, modifica a las histonas, que se separan de la cromatina, permitiendo la activación transcripcional (Tulin and Spradling 2003). Además, PARP-1 se automodifica para disociarse del DNA. Curiosamente, en este mismo modelo, se ha observado que PARP-1 promueve la formación de estructuras de cromatina más compactas en regiones de heterocromatina, pero no de eucromatina (Tulin, Stewart et al. 2002).

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. Activadores transcripcionales de unión al DNA podrían reclutar enzimas sintetizadoras de NAD^+ necesario para PARP-1. Este proceso explica la aparente contradicción en la modulación de la cromatina por PARP-1, siendo en principio independiente de la modificación de histonas, pero actuando los nucleosomas como potentes activadores de la actividad enzimática de PARP-1 (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 DNA genómico (Zardo, Reale et al. 2003). Dada la importante contribución de la metilación del DNA 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 DNA, podría suponer otra manera de que PARP-1 modulara la estructura de la cromatina (Kim, Zhang et al. 2005).

Control 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 (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 (Whitacre, Hashimoto et al. 1995; Oei, Griesenbeck et al. 1997; Le Page, Sanceau et al. 1998; Butler and Ordahl 1999; Kannan, Yu et al. 1999; Oliver, Menissier-de Murcia et al. 1999; Anderson, Scoggin et al. 2000; Cervellera and Sala 2000; Ha, Juluri et al. 2001; Ha, Hester et al. 2002; Kiefmann, Heckel et al. 2004; Zingarelli, Hake et al. 2004; Martin-Oliva, Aguilar-Quesada et al. 2006; Zaniolo, Desnoyers et al. 2007), 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 positivo (Guermah, Malik et al. 1998), esencial para la actividad de factores de transcripción como NF-κB, Sp1 y Oct-1. Otro ejemplo es el efecto regulador de PARP-1 sobre el *Mediador* (complejo multiproteico reclutado a algunas secuencias potenciadoras por proteínas activadoras). En ausencia de síntesis de polímero, PARP-1 regula el cambio del estado inactivo a activo del *Mediador* (Pavri, Lewis et al. 2005).

PARP-1 tiene diferentes efectos sobre los factores de transcripción (estimulación frente a inhibición) en función de la presencia de otros cofactores y el contexto del promotor, del tipo celular y el estado proliferativo de la célula, la concentración de NAD^+ y la presencia de roturas de la cadena del DNA, siendo el mecanismo de acción también distinto (D'Amours, Desnoyers et al. 1999; Hassa and Hottiger 2002; Kraus and Lis 2003; Aguilar-Quesada, Munoz-Gamez et al. 2007). En presencia de NAD^+, el silenciamiento por parte de PARP1 de la transcripción implica la poli(ADP-ribosil)ación de factores de transcripción específicos como p53 y fos (Amstad, Krupitza et al. 1992; Wesierska-Gadek and Schmid 2001), lo que previene su unión a las secuencias consenso de DNA respectivas y la formación de complejos de transcripción activos, teniendo PARP-1 en este caso un efecto negativo sobre la transcripción (Rawling and Alvarez-Gonzalez 1997; Oei, Griesenbeck et al. 1998; Butler and Ordahl 1999; Simbulan-Rosenthal, Rosenthal et al. 2001; Soldatenkov, Chasovskikh et al. 2002). Sin embargo, en respuesta al daño en

el DNA, la actividad catalítica de PARP-1 se dispara y la automodificación de PARP-1 previene 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, Stelzer et al. 1997) y numerosos factores de transcripción como TEF-1, AP-2, p53, NF-κB, B-Myb, Oct 1 y YY1 (Oei, Griesenbeck et al. 1997; Nie, Sakamoto et al. 1998; Butler and Ordahl 1999; Kannan, Yu et al. 1999; Oliver, Menissier-de Murcia et al. 1999; Cervellera and Sala 2000; Hassa, Buerki et al. 2003; Wesierska-Gadek, Wojciechowski 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, Naidu 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 DNA, 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 DNA ni la actividad enzimática de PARP-1 sean necesarias para la completa activación de NF-κB en respuesta a varios estímulos (Hassa, Covic et al. 2001). Además, la transactivación dependiente de NF-κB de promotores en la 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, Buerki 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 DNA, CTCF (CCCTC-binding factor) (Yu, Ginjala et al. 2004).

El punto V B de la tesis se centrará en la función de PARP-1 como regulador de la expresión génica, enmarcado dentro de la respuesta a hipoxia y de la actividad transcripcional del factor inducido por hipoxia HIF.

2.4.4. Otras funciones

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

- *Replicación del DNA*: PARP-1 interacciona con un complejo multiproteico que contiene importantes proteínas de replicación del DNA, muchas de las cuales son poli(ADP-ribosil)adas (Simbulan-Rosenthal, 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 DNA (Dantzer, Nasheuer 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 apropiada segregación de los cromosomas durante la división celular (Smith 2001; Ame, Spenlehauer et al. 2004). Por ejemplo, PARP-1 se localiza en los centrómeros (Saxena, Saffery et al. 2002) y en los centrosomas (Augustin, Spenlehauer et al. 2003), hecho que une los mecanismos de supervivencia al daño en el DNA con el punto de control de la fidelidad mitótica (Schreiber, Dantzer et al. 2006).
- C*ontrol del ciclo celular*: PARP-1 está implicado en la regulación de los puntos de control del ciclo celular tras el daño en el DNA 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).

3. PARP-1 y carcinogénesis

La función normal de PARP-1 es necesaria para prevenir la carcinogénesis inducida por daños en el DNA. Sin embargo, la naturaleza del daño del DNA determina la contribución específica de PARP-1 en la prevención del cáncer (Kim, Zhang et al. 2005).

En algunos modelos tumorales inducidos químicamente, se ha descrito que la ausencia de PARP-1 contribuye a la activación defectuosa de factores de transcripción claves en el desarrollo tumoral (Martin-Oliva, O'Valle et al. 2004; Martin-Oliva, Aguilar-Quesada et al. 2006). En concreto, en esta tesis se va a discutir el efecto de PARP-1 sobre la respuesta a hipoxia mediada por HIF, principal regulador transcripcional de la adaptación a estrés hipóxico en tumores.

Ya se ha comentado la relación existente entre PARP-1 e inflamación a través del excesivo daño en el DNA, sobreactivación de PARP-1 y muerte celular por necrosis. A esta situación, se le debe sumar la función coactivadora de PARP-1 sobre NF-κB y AP-1, que resulta en la síntesis de mediadores pro-inflamatorios (Hassa, Buerki et al. 2003; Hassa, Haenni et al. 2005). Entre ellos, la óxido nítrico sintasa inducible (iNOS) produce óxido nítrico y los derivados peroxinitritos y radicales hidroxilo altamente reactivos, que causan a su vez gran cantidad de daños en el DNA. En ausencia de PARP-1, todos estos factores se encuentran disminuidos, lo que podría emplearse como estrategia antitumoral.

Inhibidores de PARP en la terapia del cáncer

La alta citotoxicidad de los inhibidores de PARP (actualmente no hay disponibles inhibidores selectivos de PARP-1) en células proliferativas tratadas con agentes genotóxicos, probablemente por inhibición de la reparación del

DNA, promete una eficaz actividad antitumoral en terapia combinada con quimioterapia y radioterapia (Schreiber, Dantzer et al. 2006). De hecho, los inhibidores de PARP aumentan la actividad antiproliferativa del agente metilante del DNA, temozolomida, de manera que el tratamiento combinado de ambos se encuentra en evaluación clínica (Calabrese, Almassy et al. 2004; Curtin 2005).

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). Estas células acumulan SSBs por inhibición de PARP, que durante la replicación del DNA se convierten en DSBs. Debido al defecto en HR, las DSBs no pueden repararse, desencadenando muerte celular. Este tratamiento, por tanto difiere de terapias previas en que no son necesarios agentes genotóxicos adicionales para causar muerte celular, por lo que se espera que haya pocos efectos secundarios, especialmente debido a que las células wild-type muestran muy baja toxicidad a la inhibición de PARP (Bryant and Helleday 2004).

Por otro lado, se ha descrito que los inhibidores de PARP tienen efectos antiangiogénicos (Rajesh, Mukhopadhyay et al. 2006; Rajesh, Mukhopadhyay et al. 2006; Tentori, Lacal et al. 2007), aunque no se conoce aún con exactitud el mecanismo responsable. Sin embargo, de una manera u otra, los inhibidores de PARP se plantean como una estrategia terapéutica muy prometedora en oncología.

Dos factores estrechamente relacionados con iniciación, y progresión tumoral, respectivamente, son la inestabilidad genómica y la hipoxia. A lo largo de esta tesis, vamos a tratar de demostrar la implicación de PARP-1 en el control tumoral a través de la regulación de las respuestas dependientes de ATM (en reparación del DNA) y de HIF (en hipoxia).

4. ATM (Ataxia-Telangiectasia (A-T) Mutated) e inestabilidad genómica

4.1. Organización de la respuesta a DSBs

La rotura de cadena doble del DNA (DSB) constituye una de las formas más agresivas de daño del DNA, precisamente por afectar a ambas cadenas (Cann and Hicks 2007). Las DSBs se originan como subproductos de procesos endógenos, con aproximadamente 50 DSBs por célula humana en cada fase S del ciclo celular (Vilenchik and Knudson 2003). Las DSBs se forman de manera específica durante el reordenamiento de los genes de las inmunoglobulinas en las células B y de los genes del receptor de la célula T en las células T, y también durante la recombinación meiótica en células germinales (Hoeijmakers 2001; Khanna and Jackson 2001). En cuanto a factores exógenos, la exposición de las células a IR (radiación ionizante: rayos X y radiación γ) origina toda una variedad de daños en el DNA, incluyendo DSBs (Ward 1985; Hoeijmakers 2001; Khanna and Jackson 2001). Además, el daño en el DNA producido durante la fase S puede colapsar la horquilla de replicación del DNA, originando a su vez DSBs (Paulsen and Cimprich 2007).

Las DSBs son potencialmente letales para la célula y por tanto, deben ser reconocidas rápidamente para su reparación (Agarwal, Tafel et al. 2006). Las DSBs no reparadas correctamente pueden originar daño genómico del tipo delecciones, inversiones, translocaciones, fusión de cromosomas, y pérdida de cromosomas (Tsukamoto and Ikeda 1998; Karlsson, Deb-Basu et al. 2003; Jonnalagadda, Matsuguchi et al. 2005), que a su vez puede desencadenar muerte celular o transformación tumoral. Ya se han citado previamente los dos mecanismos fundamentales de reparación de DSBs: HR (recombinación homóloga) y NHEJ (unión de extremos no homólogos). Pero para que las roturas sean reparadas por uno de estos mecanismos, debe producirse un proceso de detección, reconocimiento y señalización del daño no sólo a la maquinaria de reparación, sino también a los procesos de control del ciclo celular y de la transcripción génica (Zhou and Elledge 2000).

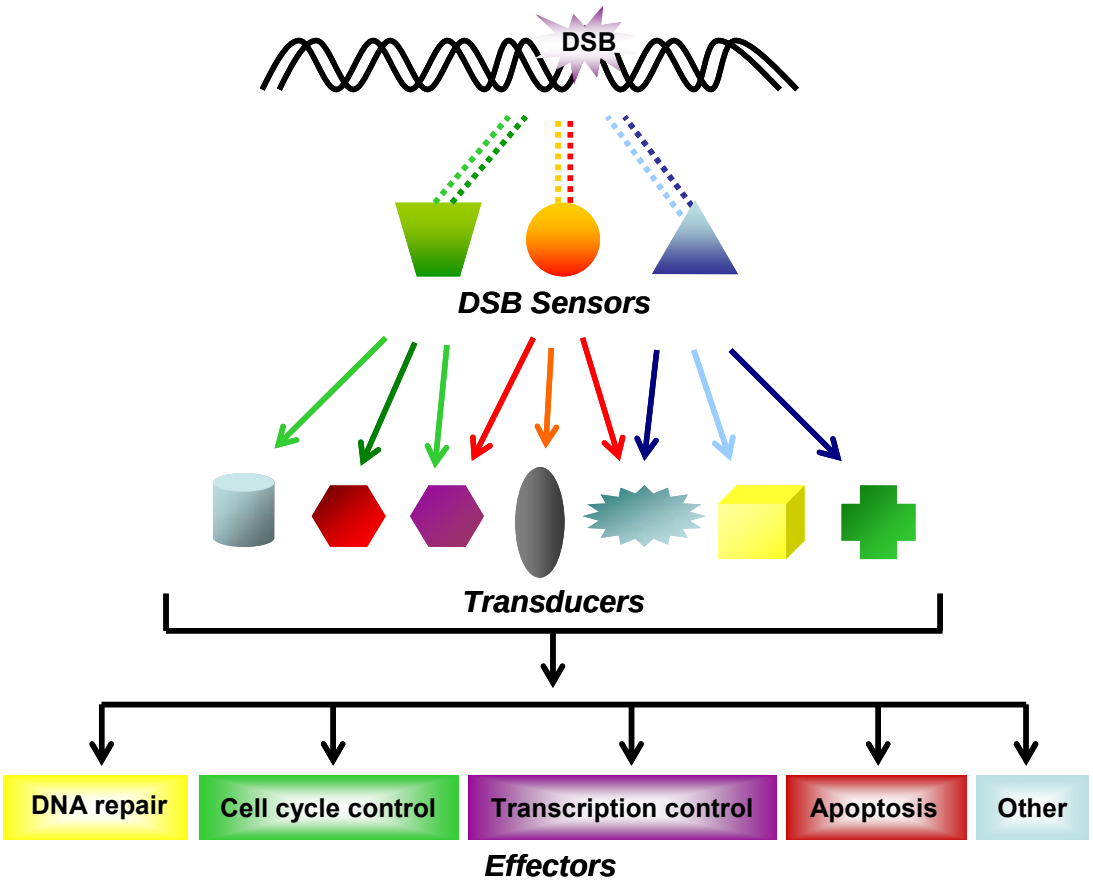

Figura III 11. Representación esquemática de la respuesta celular a DSBs del DNA. Múltiples proteínas *sensoras* reconocen físicamente el daño en el DNA, y entonces activan a gran cantidad de proteínas *transductoras* que amplifican y diversifican la señal del daño en el DNA a través de una serie de *efectores* que regulan varios aspectos de la función celular. *(Adaptado de (Jackson 2002))*

Al menos en algunos casos, estos sistemas de detección pueden entenderse como cascadas de transducción de señales clásicas en las que una *señal*, el daño en el DNA, es detectada por un *sensor* (proteína de unión al daño en el DNA), que entonces dispara la activación de un sistema *transductor* que amplifica y diversifica la señal por modificación de una serie de *efectores* de la respuesta al daño en el DNA (Figura III 11). Por supuesto, tales sistemas necesitan ser extremadamente sensibles y selectivos, y deben activarse rápida y eficazmente por un bajo número de DSBs (incluso una), así como permanecer inactivos bajo otras condiciones (Jackson 2002).

Una de las respuestas celulares a DSBs es activar y/ó inducir los niveles de proteínas de reparación del DNA, las cuales son entonces físicamente reclutadas al sitio de la lesión del DNA para iniciar su reparación

(Jackson 2002). Así, los sitios de reparación de DSBs están marcados por la agregación de múltiples proteínas de reparación en los llamados *foci*. Estos *foci* de reparación son sitios de síntesis de DNA no programada capaces de conectar varias DSBs (Lisby, Mortensen et al. 2003). Se trata de estructuras muy dinámicas que pueden ensamblarse y desensamblarse en minutos, sin la presencia de complejos proteicos preformados (Essers, Houtsmuller et al. 2002; Lisby, Mortensen et al. 2003). La organización en *foci* de la reparación de DSBs favorece una alta concentración local de proteínas de reparación en la lesión, posiblemente necesaria para ciertos pasos bioquímicos durante el proceso de reparación. Además, en algunas ocasiones los dos extremos de una DSB se separan y estos *foci* permiten acercarlos de nuevo para la correcta unión (Lisby, Antunez de Mayolo et al. 2003). Por otra parte, la localización de múltiples lesiones del DNA en unos pocos *foci* podría facilitar la coordinación de los procesos de reparación del DNA con la progresión del ciclo celular (Zink, Mayr et al. 2002; Lukas, Falck et al. 2003).

El mayor avance en el estudio del reconocimiento y la señalización de DSBs del DNA se ha conseguido a partir del estudio de una serie de enfermedades genéticas humanas raras que conllevan una alta sensibilidad a agentes que originan roturas en el DNA (Lukas, Bohr et al. 2006). En nuestro caso, por ejemplo, hemos utilizado un modelo celular derivado del síndrome de inestabilidad genómica Ataxia-Telangiectasia (A-T) para el estudio de la señalización mediada por la proteína mutada en A-T, ATM (A-T mutated).

4.2. Ataxia-Telangiectasia (A-T)

Ataxia-Telangiectasia (A-T) es un desorden genético raro autosómico recesivo que se caracteriza por neurodegeneración progresiva (debido a la pérdida de células de Purkinje en el cerebelo y malfuncionamiento de otras células neuronales) que se manifiesta con ataxia, telangiectasia ocular y cutánea, inestabilidad genómica, un alto riesgo de cáncer, e inmunodeficiencia. Además, los pacientes de A-T son muy sensibles a la radioterapia. La esperanza de vida de los pacientes afectados por esta enfermedad es de 15-20 años y no existen terapias específicas. Sobre la hipótesis de que algunas de

las disfunciones neurológicas observadas en pacientes con A-T se deben a estrés oxidativo, precisamente se ha llevado a cabo un ensayo clínico con la combinación de un antioxidante y un inhibidor de PARP, para controlar algunos de los efectos secundarios de dicho estrés oxidativo (Lavin, Gueven et al. 2007). Como resultado, se observó que ambos fármacos no producían efectos adversos de manera significativa, y que dos marcadores de estrés oxidativo mejoraban en estos paciantes de A-T tratados con esta terapia combinada.

La proteína ATM (ataxia-telangiectasia mutated) fue identificada como el producto del gen mutado (perdido o inactivado) en A-T. ATM es un componente crucial de la cascada de señalización de DSBs del DNA y las severas patologías asociadas con A-T se atribuyen fundamentalmente, si no enteramente, al defecto en los procesos de reconocimiento y reparación de DSBs, que supone la acumulación de una gran cantidad de DNA no reparado (Shiloh 2003).

4.3. La familia PIKK

ATM pertenece a una familia conservada de proteínas, la mayoría con actividad quinasa serina/treonina (Abraham 2001; Durocher and Jackson 2001; Khanna, Lavin et al. 2001; Shiloh 2001; Shiloh and Kastan 2001). Todas ellas contienen un dominio con motivos típicos de las fosfatidilinositol 3-quinasas (PI3K), por lo que se denominaron proteínas quinasas tipo PI3K (PIKKs), y en este dominio llamado entonces PI3K, es donde se encuentra el sitio catalítico de las proteínas quinasa activas de la familia PIKK (Shiloh 2003).

Otros miembros de esta familia implicados en la respuesta al daño en el DNA son ATR (ATM- and Rad3-related), DNA-PK (DNA-dependent protein kinase) y ATX (Abraham 2001; Durocher and Jackson 2001; Shiloh 2001; Shiloh and Kastan 2001; Sesto, Navarro et al. 2002). Mientras que ATM y DNA-PK responden fundamentalmente a DSBs, ATR y ATX responden tanto a DSBs como a daños de bases por luz ultravioleta (UV), y ATR además, a la parada de la maquinaria de replicación y a hipoxia

(Abraham 2001; Shiloh 2001; Hammond, Denko et al. 2002; Heffernan, Simpson et al. 2002; Denko, Wernke-Dollries et al. 2003; Hammond and Giaccia 2004). Es más, en la respuesta a DSBs, ATM interviene durante la fase inicial, que dura 1-2 horas, mientras que ATR se le une más tarde y mantiene el estado fosforilado de determinados sustratos (Shiloh 2003). Así, ATR comparte varios sustratos con ATM (Abraham 2001). Incluso se ha descrito que ATM, junto con el complejo MRN (introducido a continuación), pueden activar a ATR después de DSBs en las fases S y G_2 del ciclo celular (Cuadrado, Martinez-Pastor et al. 2006; Jazayeri, Falck et al. 2006; Myers and Cortez 2006). Por otro lado, otra quinasa perteneciente a la familia PIKK que no está implicada en la respuesta al daño en el DNA es mTOR (o FRAP) (Shiloh 2003).

4.4. ATM: estructura, localización y activación

ATM es una quinasa de gran peso molecular (350 kDa) que fosforila a sus sustratos sobre residuos de serina o treonina seguidos por glutamina (motivo “SQ/TQ”) (Kim, Lim et al. 1999; O'Neill, Dwyer et al. 2000). ATM presenta dos dominios FAT (nombre derivado de los miembros de la familia PIKK FRAP, ATM y TRRAP), uno denominado simplemente FAT, que contiene un residuo de serina (la serina en posición 1981) que es autofosforilado durante la activación de ATM; y otro localizado en el extremo C-terminal y que se denomina por tanto, FATC (Shiloh 2003). También en la parte C-terminal de la proteína se encuentra el dominio PI3K (con los sitios de unión al sustrato y a ATP), bien conservado entre diferentes especies (Lavin, Scott et al. 2004), aunque aparte, ATM cuenta con un segundo sitio N-terminal de unión a sustratos. Se ha sugerido que el significado funcional de los dominios FAT es el establecimiento de una configuración que asegure la correcta función del dominio PI3K (Bosotti, Isacchi et al. 2000). ATM también cuenta con múltiples repeticiones HEAT (*h*untingtin, *e*longation factor 3, *A* subunit of protein phosphatase 2A and *T*OR1) , un motivo incompleto de cremallera de leucina y una región rica en prolinas, que pueden favorecer la interacción con otras proteínas (Groves and Barford 1999; Perry and Kleckner 2003). No menos importante es la presencia de un dominio de asociación a cromatina en el extremo N-terminal de ATM, que puede estar conduciendo a ATM a sitios de

DNA dañado (Young, Jonnalagadda et al. 2005). Por otra parte, se han identificado en la secuencia de ATM dos sitios de corte por caspasa 3 (Smith, d'Adda di Fagagna et al. 1999) (Figura III 12).

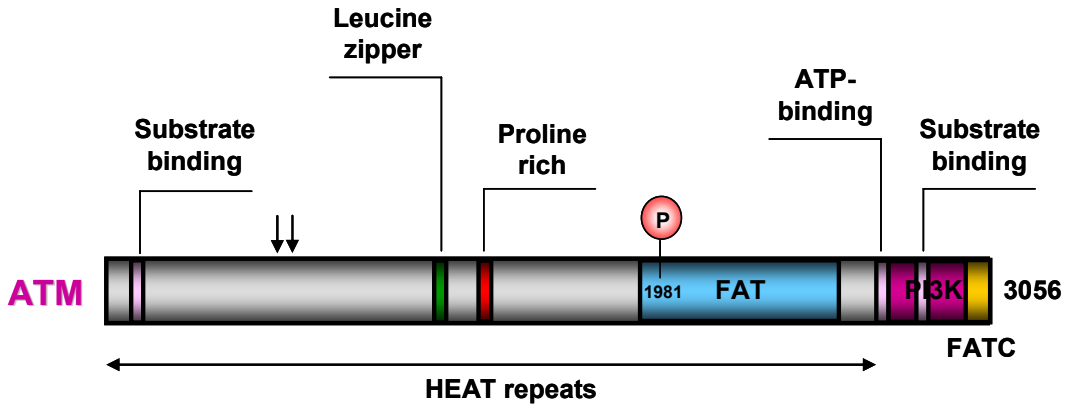

Figura III 12. Representación esquemática de la estructura de ATM, indicando los diferentes dominios y motivos descritos. Los dos sitios de corte por caspasa 3 son señalados mediante flechas. *(Adaptado de (Lavin, Scott et al. 2004))*

En células en proliferación, ATM se localiza fundamentalmente en el núcleo, de acuerdo con su función en el reconocimiento y señalización del daño del DNA. Sin embargo, incluso en estas células, aproximadamente el 10% de la proteína es extranuclear, presente en vesículas citoplasmáticas identificadas como peroxisomas y endosomas (Jain, Maltepe et al. 1998; Watters, Kedar et al. 1999). Así, el estrés oxidativo asociado con pérdida de ATM puede deberse en parte a un defecto en la función peroxisomal (Lavin, Scott et al. 2004). Es importante anotar, por su implicación en neurodegeneración asociada con A-T, que en células de Purkinje humanas, la proteína ATM está presente en el citoplasma, mientras que no se detecta en el núcleo (Oka and Takashima 1998). Así, el potencial estrés oxidativo derivado de la pérdida de ATM, tendrá una contribución muy importante en este tipo celular.

Inmediatamente después de la formación de DSBs, ATM responde con un rápido aumento en su actividad quinasa (pero no en su cantidad) (Banin, Moyal et al. 1998; Canman, Lim et al. 1998), lo que permite la fosforilación de sus sustratos en cuestión de minutos. La activación de ATM requiere la formación de monómeros catalíticamente activos de la proteína a partir de dímeros o multímeros inactivos presentes normalmente en la célula

(Bakkenist and Kastan 2003). En la configuración dimérica (ó multimérica), el dominio PI3K de cada monómero se encuentra bloqueado por el dominio FAT de otro. Sin embargo, la formación de DSBs provoca la auto- o trans-fosforilación del residuo serina 1981 del dominio FAT, y la posterior disociación de los complejos inactivos de ATM en monómeros catalíticamente activos listos para la fosforilación de sustratos.

Se han propuesto un par de modelos de reconocimiento de DSBs relacionados con ATM (Cann and Hicks 2007). Uno de ellos postula que los cambios provocados por las DSBs en la estructura de la cromatina son suficientes para activar a ATM, en cuyo caso ATM se comportaría como un sensor del daño en el DNA (Bakkenist and Kastan 2003). Este modelo se basa en la gran cantidad de moléculas de ATM activadas tras la formación de sólo unas pocas DSBs en el genoma. Así, la señal para la activación de ATM serían tales alteraciones en la cromatina y no el contacto directo de ATM con el DNA dañado. Sin embargo, se ha descrito que tras el daño, una proporción nuclear de ATM es reclutada a las DSBs (Andegeko, Moyal et al. 2001).

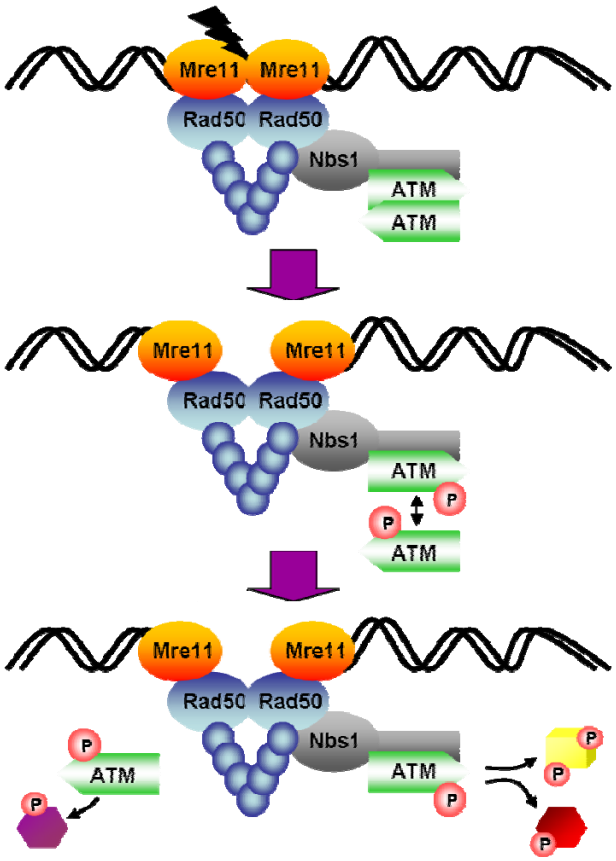

Figura III 13. El complejo MRN y activación de ATM. La presencia de DSBs en el DNA lleva a la apertura de la cromatina de forma dependiente de MRN y ATM. MRN actúa como un sensor del daño, se une a los extremos rotos y recluta a ATM. ATM forma entonces monómeros activos y fosforila múltiples sustratos en la proximidad de las DSBs o en el nucleoplasma. Es importante anotar que este modelo se aplica a células humanas, ya que en células murinas no se han obtenido los mismos datos. *(Adaptado de (Abraham and Tibbetts 2005))*

En el otro modelo de reconocimiento de DSBs, es el complejo MRN (formado por Mre11, Rad50 y Nbs1) el que detecta las DSBs por

unión a los extremos rotos, y una vez unido, recluta y promueve la activación de ATM y consecuente fosforilación de sustratos (Lee and Paull 2005; Paull and Lee 2005) (Figura III 13). De hecho, se ha identificado dentro del extremo C-terminal de Nbs1 un motivo de interacción con ATM, necesario para la retención de ATM en sitios de daño del DNA y para la activación de puntos de control (Falck, Coates et al. 2005). Sin embargo, esta interacción no es necesaria para la autofosforilación de ATM. Pero independientemente de cuál sea la proteína que físicamente reconoce las DSBs, parece ser una mutua promoción de actividad entre el complejo MRN y ATM (Cann and Hicks 2007). ATM fosforila a Nbs1 (Gatei, Young et al. 2000; Lim, Kim et al. 2000), y el complejo MRN aumenta la actividad quinasa de ATM (Lee and Paull 2005).

4.5. Sustratos de ATM

La activación de ATM por DSBs inicia rutas de transducción de señales que promueven procesos de reparación del DNA, puntos de control del ciclo celular y apoptosis (Cann and Hicks 2007). ATM responde rápidamente a DSBs fosforilando numerosos sustratos, de forma que activa o reprime su actividad, afectando a procesos específicos en los que estas proteínas están implicadas (Shiloh 2003). Estos sustratos pueden fosforilarse bien en sitios de daño del DNA o en el nucleoplasma (Abraham and Tibbetts 2005). Algunos de los sustratos de ATM implicados en uno o varios de los anteriores procesos se enumeran a continuación:

- **Reparación del DNA:** H2AX (una variante de la histona H2A) (Rogakou, Pilch et al. 1998; Burma, Chen et al. 2001), 53BP1 (Anderson, Henderson et al. 2001), MDC1 (Goldberg, Stucki et al. 2003), BRCA1 (Xu, Kim et al. 2001; Xu, O'Donnell et al. 2002), Nbs1 (Gatei, Young et al. 2000; Lim, Kim et al. 2000) o c-Abl (Baskaran, Wood et al. 1997).

- **Control del ciclo celular:** p53 (Banin, Moyal et al. 1998; Canman, Lim et al. 1998; Andegeko, Moyal et al. 2001), CHK2 (Bartek, Falck et al. 2001; McGowan 2002), MDM2 (Khosravi, Maya et al. 1999; Maya, Balass et al. 2001), BRCA1 (El-Deiry 2002; Jasin 2002; Venkitaraman 2002; Yarden, Pardo-Reoyo et al. 2002), Nbs1 (Gatei, Young et al. 2000; Lim, Kim et al. 2000; Wu, Ranganathan et al. 2000; Zhao, Weng et al. 2000), SMC1 (Jeong, Bae et al. 2002; Yazdi, Wang et al. 2002), FANCD2 (Taniguchi, Garcia-Higuera et al. 2002), CHK1 (Shiloh 2003), RAD17 (Bao, Tibbetts et al. 2001; Post, Weng et al. 2001) o RAD9 (Chen, Lin et al. 2001).
- **Apoptosis** (cuando la reparación del daño no es factible)**:** p53 (Bree, Neary et al. 2004) o MDM2 (Khosravi, Maya et al. 1999; Maya, Balass et al. 2001).

Muchas de las respuestas al daño en el DNA terminan modulando la expresión génica. En el caso de la señalización dependiente de ATM, el ejemplo es evidente con p53. Además, otro factor de transcripción con un papel central en el control del ciclo celular es E2F1, que es fosforilado y estabilizado por ATM (Aragones, Jones et al. 2001).

En numerosas ocasiones, se requiere la fosforilación de un sustrato por ATM para la fosforilación de otro, y muchas de estas proteínas con frecuencia se encuentran físicamente cerca, lo que permite una gran rapidez en la respuesta. Finalmente, cuando se tiene una visión de conjunto de las rutas de señalización dirigidas por ATM, se llega a la conclusión de que el mecanismo de amplificación de la señal del daño en el DNA, y el reclutamiento de factores para su procesamiento, son procesos cíclicos y no una serie de pasos con una jerarquía lineal (Shiloh 2003). Así, por ejemplo, H2AX fosforilada rápidamente forma *foci* en las DSBs, proceso esencial para el reclutamiento a su vez del complejo MRN, Rad51 o BRCA1 (Celeste, Petersen et al. 2002).

4.6. Activadores e inactivadores de ATM

Debido a la gran cantidad de sustratos de ATM, es posible que diferentes cofactores sean requeridos para la función de ATM sobre un determinado sustrato. Otra posibilidad es que los cofactores sean necesarios para la activación de ATM por un determinado estímulo (Chen, Su et al. 2007).

Dos fosfatasas, la proteína fosfatasa 2A (PP2A) y la proteína fosfatasa 5 (PP5), de la familia proteína fosfatasa serina/treonina, afectan a la actividad de ATM *in vivo* (Ali, Zhang et al. 2004; Goodarzi, Jonnalagadda et al. 2004). PP2A regula la autofosforilación de ATM, por lo que se piensa que quizá en células humanas, defosforile constitutivamente a ATM (Chen, Su et al. 2007). Por otro lado, PP5 puede estar regulando la actividad quinasa de ATM defosforilando a la propia ATM, al complejo MRN o a otros factores implicados en la activación de ATM. Debido a que la actividad fosfatasa de PP5 es necesaria para la autofosforilación y activación de ATM, se plantean tres posibilidades: que ATM sea fosforilada sobre sitios de no autofosforilación en células no tratadas y tras el daño en el DNA sea defosforilada por PP5; que PP5 actúe junto con otros factores proteicos todavía por identificar para activar ATM mediante defosforilación de estos factores desconocidos; o que PP5 defosforile a PP2A y como resultado, se active ATM. Además, otra fosfatasa, Wip1, regula negativamente la actividad quinasa de ATM (Shreeram, Demidov et al. 2006).

53BP1 (p53-binding protein 1) es un coactivador de ATM que colocaliza con H2AX fosforilada en DSBs del DNA (Schultz, Chehab et al. 2000; Anderson, Henderson et al. 2001; Rappold, Iwabuchi et al. 2001; Xia, Morales et al. 2001; Shang, Bodero et al. 2003), y cuya ausencia da lugar a defectos en las paradas en fase S y G_2-M después de IR, y a disminución de la autofosforilación de ATM y de la fosforilación de sus sustratos (DiTullio, Mochan et al. 2002; Wang, Matsuoka et al. 2002).

También BRCA1 es necesario para la localización de ATM activada en DSBs del DNA y puede modular la actividad quinasa de ATM hacia varios de sus sustratos (Foray, Marot et al. 2003; Fabbro, Savage et al. 2004; Kitagawa, Bakkenist et al. 2004). Otro coactivador potencial de ATM es p18, necesario para la correcta autofosforilación y activación de ATM (Park, Kang et al. 2005).

Es importante destacar que se ha descrito la activación de ATM de manera independiente de Nbs1, en ausencia de roturas del DNA, y a través de un nuevo factor, ATMIN (ATM interacting protein) (Kanu and Behrens 2007). Estos resultados apoyan los que describían la autofosforilación de ATM en la serina 1981 por agentes que modifican la cromatina, en ausencia de roturas del DNA (Bakkenist and Kastan 2003).

Las histona acetiltransferasas son también posibles cofactores para la activación de ATM. ATM es acetilada por el complejo Tip60 tras daños en el DNA, siendo la acetilación necesaria para la autofosforilación y activación de ATM (Sun, Jiang et al. 2005; Sun, Jiang et al. 2006). La histona acetiltransferasa hMOF también afecta a la autofosforilación y activación de ATM (Gupta, Sharma et al. 2005). Aunque tanto Tip60 como hMOF acetilan histonas, lo que puede alterar la estructura de la cromatina y afectar a ATM indirectamente, Tip60 acetila directamente a ATM. Así, la acetilación de histonas y de ATM van a ser importantes en la regulación de ATM *in vivo* (Chen, Su et al. 2007).

En el punto V A de esta memoria se mostrarán resultados del efecto de PARP-1 sobre la activación de ATM, y se enmarcarán dentro del conocimiento actual de dicha interacción.

5. HIF (Hypoxia-Inducible Factor) e hipoxia

Los organismos responden rápidamente a cambios en la disponibilidad de oxígeno, y esta respuesta, disparada por muchos sensores de oxígeno localizados a diferentes niveles en el organismo, activa rutas de señalización

que culminan en el control de la expresión génica (Benizri, Ginouves et al. 2008).

Cuando la demanda de oxígeno (O_2) por parte de la célula supera la disponibilidad del mismo, se produce una situación de *hipoxia*. Esta situación es normal durante el desarrollo embrionario, o situaciones de gran altitud, anemia o cicatrización de heridas. Debido a que el O_2 es la principal fuente de energía metabólica para las células eucarióticas, a lo largo de la evolución han desarrollado la capacidad de responder a hipoxia. Así, la hipoxia tiene un gran efecto sobre el transcriptoma celular, un efecto que variará en función del tipo celular y del estado específico de la célula. Se ha demostrado que son varios los factores de transcripción que responden a hipoxia (Cummins and Taylor 2005), como NF-κB, AP-1, p53 o Sp1/Sp3. De hecho, Sp1/Sp3 o AP-1 son algunos de los factores de transcripción que regulan la expresión de VEGF (vascular endothelial growth factor), muy importante en el proceso de adaptación a hipoxia a través de la formación de nuevos vasos sanguíneos (angiogénesis) (Josko and Mazurek 2004; Pages and Pouyssegur 2005). Sin embargo, la homeostasis de O_2 (que incluye no sólo la expresión de VEGF, sino de muchos otros genes) es principalmente controlada por HIF (Hypoxia-Inducible Factor), tanto durante el desarrollo embrionario como en la vida postnatal, en procesos fisiológicos y patofisiológicos (Semenza 1998).

5.1. Composición y estructura

HIF es un factor de transcripción heterodimérico constituido por una subunidad α- y una subunidad β-, ambas pertenecientes a la gran familia de proteínas bHLH-PAS (basic helix-loop-helix Per-ARNT-Sim) (Brahimi-Horn and Pouyssegur 2005), con dos dominios característicos en la parte N-terminal (Zagorska and Dulak 2004) (Figura III 14):

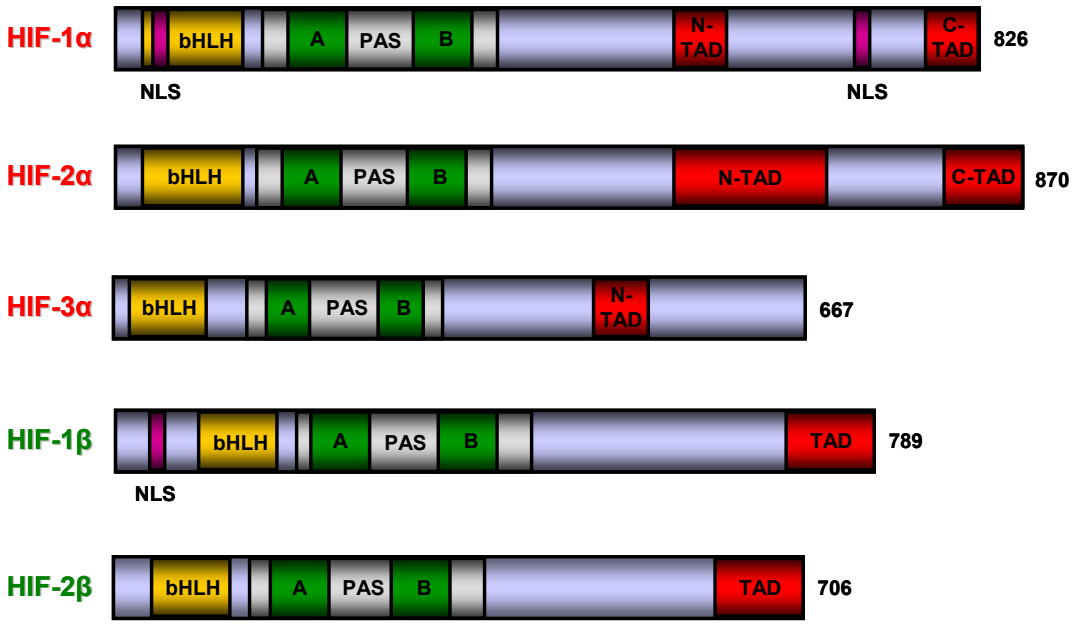

Figura III 14. Diagrama esquemático de la estructura de las proteínas bHLH-PAS implicadas en la respuesta a hipoxia. El número de aminoácidos de cada proteína se indica a la derecha de su representación esquématica (*Adaptado de (Brahimi-Horn and Pouyssegur 2005)*)

- Dominio bHLH (basic-helix-loop-helix): común para un gran número de factores de transcripción, es necesario para la dimerización de la proteína y para la unión al DNA.
- Dominio PAS: denominado así como acrónimo de los primeros miembros conocidos de la familia (Per-ARNT-Sim) (Crews 1998), contiene dos unidades homólogas, A y B, y está implicado en interacción con proteínas.

Se han descrito tres isoformas de la subunidad α- y dos de la subunidad β- de HIF implicadas en la respuesta a hipoxia y todas ellas comparten los dominios anteriores (Brahimi-Horn and Pouyssegur 2005):

- **HIF-1α** posee dos dominios de transactivación (responsables de la regulación de la actividad de HIF) en su parte C-terminal, denominados N-TAD (N-transactivation domain) y C-TAD (C-transactivation domain), entre los que se encuentra un dominio inhibitorio transcripcional. Se han identificado dos señales de localización nuclear (NLS), una N-terminal asociada con el dominio bHLH, y otra C-terminal, que parece ser la única funcional. Un dominio conocido como el dominio de degradación dependiente del oxígeno (ODDD), en el centro de la proteína, es fundamental para determinar la vida media de la proteína.
- **HIF-2α** (también conocida como EPAS1) tiene un 48% de identidad total con HIF-1α, mayoritariamente en el extremo N-terminal y en una pequeña región C-terminal. También cuenta con dos dominios funcionales de transactivación inducibles por hipoxia intercalados por secuencias inhibitorias, aunque el N-TAD de HIF-2α es más largo que el de HIF-1α y contiene una región rica en prolinas al final del dominio. Como HIF-1α, presenta un ODDD que regula la estabilidad de la proteína en función del O_2.
- **HIF-3α**, a pesar de compartir los dominios comunes bHLH y PAS N-terminales, presenta una estructura más divergente. No tiene el dominio C-TAD, y además, se ha visto que uno de sus productos de splicing reprime la expresión génica inducible por hipoxia dependiente de HIF. Como es característico de las subunidades HIF-α, cuenta con un dominio ODDD.
- **HIF-1β** y **HIF-2β** (también conocidas como ARNT y ARNT2, respectivamente) tienen una estructura similar con un único dominio de transactivación. Además, en el caso de HIF-1β, se ha descrito un NLS en su parte N-terminal.

5.2. Regulación de HIF

El peso de la regulación de la actividad de HIF recae sobre la subunidad HIF-α (siendo constitutiva la subunidad HIF-β) y se lleva a cabo a múltiples niveles como la estabilización de la proteína, la heterodimerización o la

activación transcripcional. Varios de estos procesos son regulados de manera independiente por el O_2, aunque la etapa más crucial es la regulación de la degradación proteosómica de HIF-α dependiente del O_2 (Berra, Ginouves et al. 2006). Sin embargo, también se han descrito cambios en la expresión del mRNA de HIF-α (Wang, Jiang et al. 1995; Wiener, Booth et al. 1996; Yu, Frid et al. 1998; Semenza 2000; Heidbreder, Frohlich et al. 2003; Naranjo-Suarez, Castellanos et al. 2003; Shimba, Wada et al. 2004; Agarwal, Tafel et al. 2006; Frede, Berchner-Pfannschmidt et al. 2007; Audebert, Salles et al. 2008).

5.2.1. Regulación transcripcional

La expresión del gen HIF-1α se debe principalmente al factor de transcripción Sp1, aunque también se han encontrado sitios de unión en su región promotora para AP-1, AP-2, NFI o NF-κB (Dery, Michaud et al. 2005). De hecho, un trabajo reciente ha puesto de manifiesto la regulación del gen HIF-1α por NF-κB (van Uden, Kenneth et al. 2008). En cuanto a la transcripción de HIF-2α, se ha descrito que Sp1 y Sp3 están implicados en la regulación transcripcional de la expresión de HIF-2α durante la adipogénesis (Wada, Shimba et al. 2006), y que la NADP(H) oxidasa Nox4 es esencial para la expresión de HIF-2α en células tumorales renales (Maranchie and Zhan 2005).

Poco después del descubrimiento de HIF-1α, fue aceptado que no existía regulación a nivel transcripcional por hipoxia, tal y como indicaban experimentos en células de hepatoma expuestas a hipoxia aguda (Wang and Semenza 1993). Sin embargo, en experimentos *in vivo* desarrollados en animales en condiciones de hipoxia prolongada o intermitente, se observaron variaciones en los niveles de mRNA de HIF-1α como consecuencia de un aumento en su transcripción (Semenza 2000). De hecho, un trabajo en células musculares lisas de arterias pulmonares (PASMC), ha descrito un mecanismo mediado por la ruta PI3K/AKT y por NF-κB que explica el aumento del mRNA de HIF-1α por hipoxia (Belaiba, Bonello et al. 2007). También, se ha observado que el mRNA de HIF-3α aumenta en hipoxia en diferentes órganos de rata (Heidbreder, Frohlich et al. 2003) y en un modelo de células epiteliales pulmonares (Li, Wang et al. 2006). Curiosamente, en este trabajo realizado en

rata no se observó aumento ni en la transcripción de HIF-1α ni en la de HIF-2α tras hipoxia, por lo que la variación de los niveles de mRNA de las subunidades HIF-α por hipoxia se encuentra en continuo debate. Por otro lado, sucesivas investigaciones han demostrado que varias sustancias son capaces de modular el mRNA de HIF-α. (Frede, Berchner-Pfannschmidt et al. 2007). Así, el lipopolisacárido (LPS) induce la expresión génica del gen HIF-1α via NADP(H) oxidasa y Sp1 (Oh, Lee et al. 2008). Incluso se ha descrito la regulación del mRNA de HIF-1α por la diana transcripcional del propio HIF, VEGF, efecto que es mediado por el anión superóxido (Deudero, Caramelo et al. 2008). También el mRNA de HIF-2α, como hemos detallado, es regulado independientemente del O_2 (Wada, Shimba et al. 2006), y también por factores de crecimiento (Naranjo-Suarez, Castellanos et al. 2003).

Tanto el mRNA de HIF-1α como el de HIF-2α se expresan en una gran cantidad de tejidos, aunque la expresión de HIF-2α es más alta en tejidos de mayor vascularización (Ema, Taya et al. 1997; Tian, McKnight et al. 1997). Una diferencia destacable es la presencia de HIF-1α y ausencia de HIF-2α en leucocitos (Tian, McKnight et al. 1997). En un principio, la hibridación del mRNA de HIF-2α *in situ* en el embrión murino mostró la presencia de HIF-2α principalmente en células endoteliales vasculares (Tian, McKnight et al. 1997; Jain, Maltepe et al. 1998), al contrario que HIF-1α. Sin embargo, posteriormente, se ha observado que HIF-2α también se expresa en otros tipos celulares como fibroblastos renales, hepatocitos, células gliales, células epiteliales del lumen intestinal, células intersticiales pancreáticas, células derivadas de la cresta neural, miocitos cardiacos, y neumocitos de tipo II pulmonares (Compernolle, Brusselmans et al. 2002; Rosenberger, Mandriota et al. 2002; Wiesener, Jurgensen et al. 2003). HIF-3α se expresa en timo adulto, cerebro, corazón y riñón (Gu, Moran et al. 1998). HIF-1β también se expresa en una amplia variedad de tejidos mientras que HIF-2β predomina en el sistema nervioso central del ratón en desarrollo (Jain, Maltepe et al. 1998).

5.2.2. Regulación traduccional

La traducción de la subunidad HIF-α es estimulada por la quinasa mTOR en respuesta a varios estímulos, en condiciones de normoxia. La ruta de las PI3K/AKT y su diana, mTOR, incrementan los niveles de la proteína HIF-1α (Jiang, Jiang et al. 2001; Hudson, Liu et al. 2002) a nivel de traducción a través de la fosforilación de la proteína de unión a la caperuza del mRNA, eIF4E (eukaryotic initiation factor 4E). Por otra parte, durante la hipoxia, la traducción de la proteína HIF-1α se mantiene gracias a la presencia de un sitio IRES (internal ribosomal entry site) en la región no traducida 5' (5'-UTR) del gen HIF-1α (Lang, Kappel et al. 2002). También se ha descrito que las proteínas de unión al RNA, HuR y PTB, se unen al mRNA de HIF-1α para aumentar su traducción tras el tratamiento con el mimético de hipoxia $CoCl_2$ (Galban, Kuwano et al. 2008). Además, en diferentes tipos celulares (Stiehl, Jelkmann et al. 2002; Zhou, Fandrey et al. 2003; BelAiba, Djordjevic et al. 2004; Frede, Freitag et al. 2005; Bert, Grepin et al. 2006) se ha observado un aumento de la traducción del mRNA de HIF-1α por mediadores inflamatorios, aunque las rutas de señalización implicadas dependen del tipo celular y del estímulo (Frede, Berchner-Pfannschmidt et al. 2007). En el caso de HIF-2α, también se ha descrito otro mecanismo de regulación a nivel de traducción. Dependiendo de la disponibilidad de hierro en la célula, la unión de proteínas reguladoras del hierro a un elemento conservado de respuesta al hierro (IRE) en la región 5'-UTR del mRNA de HIF-2α, controla su traducción (Sanchez, Galy et al. 2007).

5.2.3. Regulación de HIF-α dependiente del O_2

La regulación por el O_2 de la subunidad HIF-α comprende tanto la regulación de su estabilidad como la de su actividad, mediante un mecanismo de hidroxilación de residuos de prolina (Pro) o asparagina (Asn) totalmente dependiente de la presencia de O_2 (Ivan, Kondo et al. 2001; Jaakkola, Mole et al. 2001; Lando, Peet et al. 2002).

El dominio de degradación dependiente del O_2 (ODDD) está implicado en la regulación por el O_2 de la degradación proteosómica de HIF-α, que constituye la etapa limitante en la regulación de HIF-α, y en su consecuente activación (Salceda and Caro 1997; Huang, Gu et al. 1998). En células bien oxigenadas, los niveles de HIF-α son muy bajos, ya que la proteína es rápidamente degradada, con una vida media de menos de 5 minutos en presencia de un 21% de O_2 (Huang, Gu et al. 1998). Por el contrario, una bajada en la disponibilidad de O_2 acumula HIF-α al disminuir su degradación por el proteosoma. La ubiquitinización y degradación proteosómica de HIF-α requiere a pVHL, producto del gen supresor de tumores von Hippel-Lindau (Maxwell, Wiesener et al. 1999), el cual funciona como una ubiquitina E3 ligasa (Kaelin 2002; Kim and Kaelin 2003) y como componente de reconocimiento de HIF-α por un complejo multiproteico. Para que pVHL se una a HIF-α y este se degrade, deben hidroxilarse dos residuos de prolina conservados dentro del ODDD (Ivan, Kondo et al. 2001; Jaakkola, Mole et al. 2001). De hecho, la estabilización de HIF-α se debe a la ruptura de la interacción pVHL/HIF-α en condiciones hipóxicas (Figura III 15).

Las enzimas que catalizan esta reacción de hidroxilación son las PHDs (HIF prolyl hydroxylases o prolyl hydroxylase domain-containing proteins) (Bruick and McKnight 2001; Epstein, Gleadle et al. 2001). Las PHDs pertenecen a la superfamilia de las dioxigenasas dependientes de Fe(II) y 2-oxoglutarato. Al necesitar también el O_2 como cosustrato proporcionan la base molecular para su función como sensores de O_2 (Hirsila, Koivunen et al. 2003). Las PHDs también utilizan como cofactores ácido cítrico, para activar el O_2 y como molde para la ordenada unión de reactivos, y ascorbato, necesario para la máxima actividad de las enzimas, al reactivarlas por reducción del Fe(III) a Fe(II) (Berra, Ginouves et al. 2006). Se conocen tres isoformas de las PHDs: PHD1 (nuclear), PHD2 (principalmente citoplasmática) y PHD3 (nuclear y citoplasmática), las tres expresadas en todos los tejidos, aunque a diferentes niveles (Lieb, Menzies et al. 2002; Cioffi, Liu et al. 2003; Metzen, Berchner-Pfannschmidt et al. 2003). Aunque la contribución de cada PHD a la regulación de HIF-α depende de su abundancia relativa (Appelhoff, Tian et al. 2004), se ha

descrito que PHD2 es la enzima responsable de los bajos niveles de HIF-1α en normoxia, mientras que PHD1 y PHD3 contribuyen a la regulación de HIF-1α sólo en el caso de hipoxia crónica (Berra, Benizri et al. 2003). Por otro lado, la expresión y la actividad de las PHDs son reguladas de manera precisa tanto a nivel transcripcional como a nivel post-transcripcional (Semenza 2003; Berra, Ginouves et al. 2006). De hecho, uno de los mecanismos de regulación de la expresión de PHDs es dependiente de HIF e hipoxia, de manera que la expresión de PHD2 y de PHD3 es transcripcionalmente inducida por hipoxia, lo que promueve un proceso de retroalimentación negativa que permite la degradación de HIF-α tras largos períodos de hipoxia (Berra, Richard et al. 2001; Epstein, Gleadle et al. 2001; Berra, Benizri et al. 2003). Pero mientras que la inducción hipóxica de PHD3 depende de HIF-1α y HIF-2α, HIF-2α no afecta a PHD2 (del Peso, Castellanos et al. 2003; Aprelikova, Chandramouli et al. 2004).

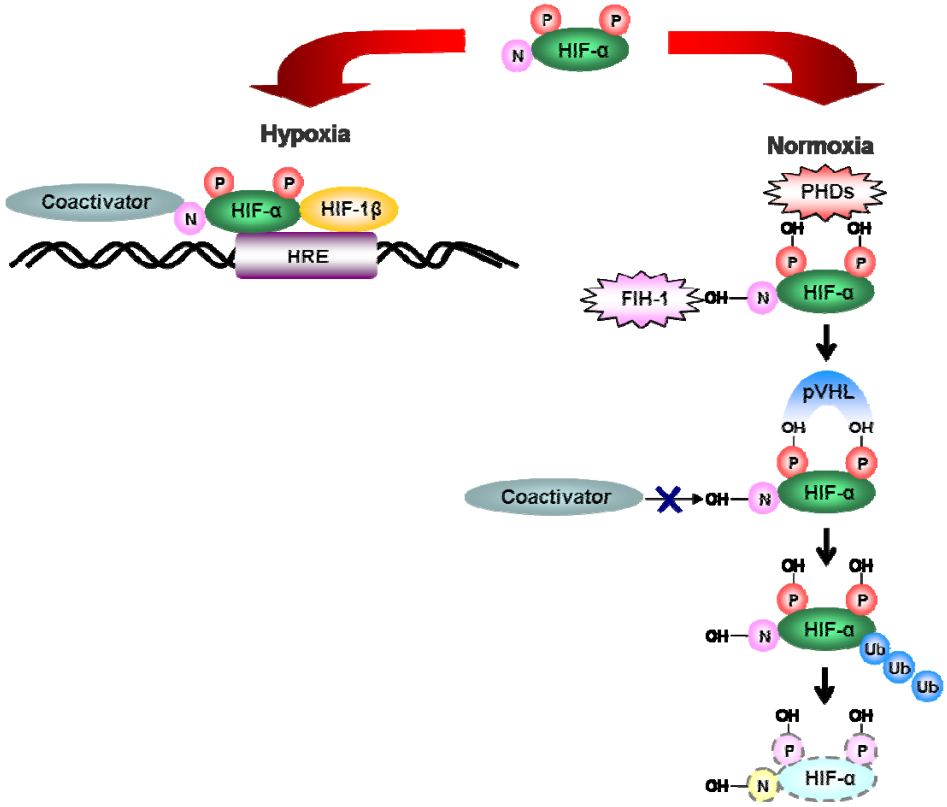

Figura III 15. Regulación de HIF-α por el O_2. En normoxia, la hidroxilación de dos residuos de prolina por las PHDs promueve la asociación de HIF-α con pVHL y la destrucción de HIF-α via ubiquitina/proteosoma, mientras que la hidroxilación de un residuo de asparagina por FIH-1 bloquea la asociación con coactivadores. En hipoxia, estos procesos quedan suprimidos, permitiendo a las subunidades HIF-α escapar de la proteolisis, dimerizar con HIF-1β, reclutar coactivadores, y activar la transcripción via HREs. N, asparagina; P, prolina; OH, grupo hidroxilo; Ub, ubiquitina. *(Adaptado de (Ratcliffe 2007))*

En una situación de hipoxia, la reacción de prolil hidroxilación de HIF-α por parte de las PHDs disminuye debido a la falta de O_2, lo que permite la estabilización de la subunidad HIF-α. HIF-α entonces forma un complejo en el núcleo con HIF-β y se unen a unas secuencias específicas del DNA, los elementos de respuesta a hipoxia (HRE), para activar la transcripción génica (Dery, Michaud et al. 2005).

A este nivel, la hidroxilación de un residuo específico de asparagina del dominio C-TAD de HIF-α por FIH-1 (factor inhibiting HIF), impide la unión del coactivador p300/CBP, y así, la actividad transcripcional de la proteína (Mahon, Hirota et al. 2001; Lando, Peet et al. 2002). FIH-1 no afecta entonces a la estabilidad de HIF-α, pero modula su transactivación. La hidroxilación por FIH-1 también depende de la disponibilidad de O_2, demostrando la estricta regulación de HIF en presencia de O_2. En base a este mecanismo de regulación y a la presencia de dos TADs, N-TAD y C-TAD, se ha propuesto un modelo de expresión génica modulado por la disminución gradual de O_2 (Dayan, Roux et al. 2006). Estudios de la *Km* de los dos sensores de O_2, PHDs y FIH-1, indican que las PHDs requieren niveles mayores de O_2 que FIH-1 para su actividad (Koivunen, Hirsila et al. 2004). Así, cuando las células dispongan de suficiente O_2, tanto las PHDs como FIH-1 estarán activos, por lo que sólo habrá una mínima cantidad basal de HIF-α inactivo. Conforme disminuye la disponibilidad de O_2, HIF-α se estabiliza por inactivación de las PHDs, pero FIH-1 mantiene su bloqueo sobre C-TAD. De forma que si la expresión de ciertos genes sólo depende de la transactivación de N-TAD, se activarán bajo esos niveles de O_2 (*bnip3* o *eno1*, por ejemplo), y cuando la cantidad de O_2 disponible siga disminuyendo, los genes cuya expresión es dirigida por C-TAD, además o no de N-TAD, se inducirán (como *ldha* o *cited2*).

5.2.4. Otros mecanismos de regulación

Se han descrito otros mecanismos que pueden contribuir o modular la regulación dependiente del O_2 de HIF-α. Otra modificación post-transduccional de HIF-α, la acetilación, regula la afinidad de la interacción pVHL/HIF-α

(Jeong, Bae et al. 2002). Por su parte, las especies reactivas de oxígeno pueden aumentar la expresión génica dependiente de HIF al regular tanto los niveles de HIF-α como su actividad transcripcional (Kietzmann and Gorlach 2005). A su vez, el CO y el ácido fosfatídico afectan a la regulación de HIF (Goldberg, Dunning et al. 1988; Aragones, Jones et al. 2001).

Otros estudios describen la inducción de HIF en condiciones de disponibilidad de O_2, en respuesta a estímulos como factores de crecimiento, citoquinas, hormonas o contactos célula-célula. Varios grupos han demostrado que señales como Ilgf o insulina (Zelzer, Levy et al. 1998; Feldser, Agani et al. 1999), interleuquina-1 (Thornton, Lane et al. 2000), factor de necrosis tumoral (Sandau, Zhou et al. 2001), angiotensina (Richard, Berra et al. 2000), o EGF/heregulina (Zhong, Chiles et al. 2000; Laughner, Taghavi et al. 2001), llevan a la estabilización de la proteína HIF-α. Aunque a simple vista, existe mucha diversidad entre los anteriores factores, es probable que la mayoría estabilicen HIF-α vía rutas de quinasas comunes activadas por receptores específicos de célula, como las rutas PI3K/AKT o MAPK. Además, la comunicación entre diferentes rutas permitiría una regulación precisa de HIF, específica del tipo celular y para un determinado gen (Powis and Kirkpatrick 2004).

En conclusión, la regulación de HIF es un mecanismo complejo y otros factores aparte del O_2 pueden contribuir a la actividad de este factor de transcripción.

5.3. Funciones biológicas de HIF

En condiciones de hipoxia, las subunidades HIF-α y HIF-β dimerizan dentro del núcleo, y dicho dímero, HIF, se une a los elementos de respuesta a hipoxia (HRE) dentro de las regiones promotoras o activadoras de los genes diana. Entonces, HIF recluta coactivadores y forma un complejo de iniciación de la transcripción para la síntesis de mRNA, que en último término resulta en la biosíntesis de proteínas que llevan a cabo la respuesta a hipoxia. La estructura del HRE, la metilación de un residuo de citosina del HRE, o la

presencia de factores de transcripción adicionales pueden además influir en la respuesta de HIF. De hecho, la eficiente activación génica con frecuencia requiere el reclutamiento de factores transcripcionales adicionales que no son dependientes de hipoxia, y que pueden amplificar la respuesta hipóxica en determinadas condiciones, variar la respuesta a hipoxia entre diferentes tejidos y aumentar la inducción de los genes diana de HIF (Zagorska and Dulak 2004).

Los HREs contienen la secuencia de reconocimiento 5'-[A/G]CGTG-3', que en la mayoría de los casos es 5'-ACGTG-3' (Wenger and Gassmann 1997). Además, los factores de transcripción HIF muestran preferencia por bases específicas en la proximidad de la anterior secuencia lo que ha llevado a la descripción de una secuencia consenso HRE de mayor tamaño (Wenger and Gassmann 1997).

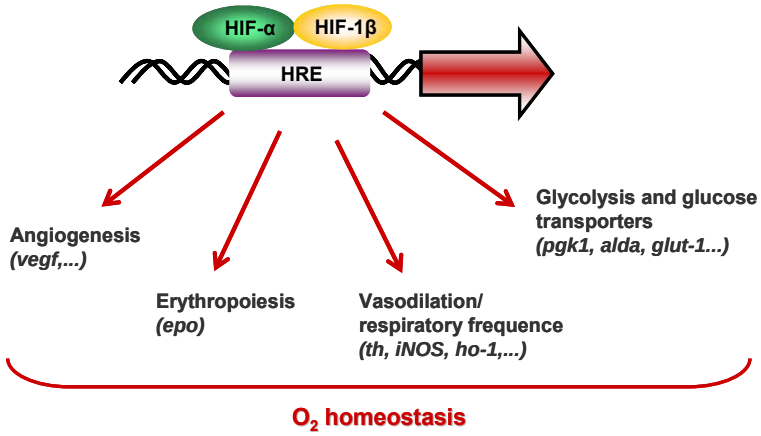

Figura III 16. Procesos de homeostasis de O_2 en los que están implicados genes regulados por HIF. *(Adaptado de (Berra, Ginouves et al. 2006))*

HIF regula la transcripción de muchos genes implicados en las respuestas celulares y sistémicas a hipoxia, con la finalidad de mantener la homeostasis de O_2 (Figura III 16). Las consecuencias de la activación de HIF varían significativamente de un tipo celular a otro, lo que no es sorprendente dado que las diferentes células y órganos *in vivo* necesitan activar diferentes mecanismos adaptativos para hacer frente a cambios en el aporte de O_2 (Maxwell 2005). Los genes diana de HIF están implicados en procesos de eritropoyesis, angiogénesis, transporte de glucosa y glucolisis, entre otros. El incremento en el aporte de glucosa (por ejemplo, gracias al transportador de glucosa GLUT-1) y en la expresión de enzimas glucolíticas (como PGK1 o ALDA) aumenta la capacidad de las células para generar ATP por glucolisis, lo

que puede compensar el fallo en el transporte de electrones mitocondrial debido a hipoxia. El aumento de la señalización angiogénica (a través de VEGF fundamentalmente) promueve un mayor aporte vascular, y la eritropoyesis (gracias a EPO) aporta las células rojas sanguíneas necesarias para un aumento en el transporte de O_2. Otros mecanismos adaptativos disparados por hipoxia son la vasodilatación (procesos en los que interviene la iNOS y la hemo oxigenasa HO1) y la regulación de la frecuencia respiratoria (regulada por TH) (Benizri, Ginouves et al. 2008). Otras dianas de HIF son las anhidrasas carbónicas Ca 9 y Ca 12, importantes para compensar el aumento local en la generación de iones hidrógeno. Además, la activación de HIF también es importante para decidir el destino celular en hipoxia: proliferación o apoptosis (por ejemplo, a través de BNIP3). Otros genes interesantes regulados por HIF son CXCR4 y su ligando, el factor 1 derivado de célula estromal, que proporcionan una vía por la que células madre multipotentes circulantes pueden ser guiadas a sus nichos donde es necesaria la reparación de tejidos en respuesta a daños (Ceradini, Kulkarni et al. 2004).

Curiosamente, muchas enzimas que utilizan O_2 como cosustrato son dianas de HIF (al igual que PHD2 o PHD3), quizá porque el aumento en su expresión proporciona una forma efectiva de mantener la velocidad de la reacción a concentraciones más bajas de O_2 (Maxwell 2005).

En el contexto de la carcinogénesis, la hipoxia puede inducir cambios en el proteoma de las células tumorales que conducen a una parada del crecimiento o muerte celular. Pero alternativamente, la hipoxia puede inducir cambios proteómicos que permiten a las células tumorales adaptarse o superar su situación de bajada de O_2 y sobrevivir o escapar del ambiente hostil (Vaupel 2004).

5.4. HIF-1α frente a HIF-2α

La expresión de HIF-2α en modelos murinos deficientes en HIF-1α no disminuye la letalidad embrionaria (Covello, Kehler et al. 2006). Esto viene a decir que, a pesar de la gran similitud de secuencia, HIF-1α y HIF-2α tienen

funciones distintas e independientes, esenciales, al menos, para el desarrollo embrionario.

La diferencia en cuanto a función de ambas subunidades puede explicarse por la regulación de la expresión de un conjunto de genes diferente en cada caso, aunque también compartan genes comunes. HIF-1α es el responsable de la expresión de genes de enzimas glucolíticas, mientras que HIF-2α fundamentalmente regula la expresión de TGFα (transforming growth factor-α), lisil oxidasa, Oct-4 y ciclina D1 (Augustin, Spenlehauer et al. 2003; Baba, Hirai et al. 2003; Gunaratnam, Morley et al. 2003; Wang, Davis et al. 2005; Covello, Kehler et al. 2006; Erler, Bennewith et al. 2006).

Por tanto, la eliminación de los genes HIF-1α o HIF-2α da lugar a efectos sustancialmente distintos: el ratón knockout de HIF-1α muere con defectos neuronales durante el desarrollo embrionario, por fallo cardíaco y vascular (Carmeliet, Dor et al. 1998; Iyer, Kotch et al. 1998; Ryan, Lo et al. 1998); mientras que el ratón knockout de HIF-2α presenta diferentes fenotipos en función de la cepa, con letalidad embrionaria por bradicardia y defectos vasculares, letalidad perinatal por fallo en la maduración de los pulmones, y letalidad embrionaria y postnatal causada por fallo multiorgánico y disfunción mitocondrial (Tian, Hammer et al. 1998; Peng, Zhang et al. 2000; Compernolle, Brusselmans et al. 2002; Scortegagna, Ding et al. 2003).

Los resultados anteriores en cuanto a genes diana y fenotipos knockout sugieren que HIF-1α y HIF-2α podrían promover también un fenotipo distinto en los tumores que los expresen (Gordan and Simon 2007).

En base a estas claras diferencias entre la función de HIF-1α y HIF-2α, muchos trabajos tratan de explicar el mecanismo responsable de las mismas, proponiendo interacciones con diferentes proteínas o diferentes modificaciones post-transduccionales (Lando, Pongratz et al. 2000; Elvert, Kappel et al. 2003; Bracken, Whitelaw et al. 2005; Agarwal, Tafel et al. 2006; Aprelikova, Wood et al. 2006; Bracken, Fedele et al. 2006; Covello, Kehler et al. 2006). Esta tesis, de hecho, en el punto V B, estudiará el papel que PARP-1 puede estar

jugando en la regulación de HIF-1α y HIF-2α a distintos niveles. Como adelanto, nuestros datos indican que el efecto de PARP-1 sobre ambas subunidades difiere tanto en el modo de regulación como en la proporción.

6. HIF y cáncer

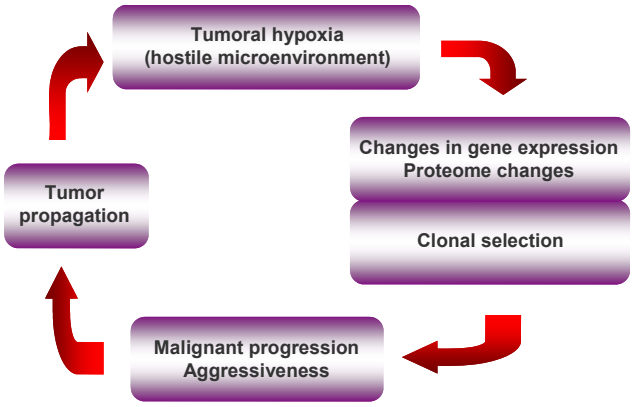

Figura III 17. Hipoxia tumoral y progresión maligna. Algunas células tumorales, ante una situación de hipoxia, responden con cambios proteómicos adaptativos para favorecer su supervivencia. Así, tendrán ventajas selectivas con respecto a células no adaptadas y proliferarán a mayor velocidad, convirtiéndose en el clon dominante. Estas células, gracias a las capacidades adquiridas, serán más agresivas y favorecerán la propagación del tumor. El crecimiento del tumor agravará de nuevo la situación de hipoxia, estableciéndose un círculo de progresión maligna e hipoxia. *(Adaptado de (Vaupel 2004))*

El crecimiento tumoral requiere la presencia de una red vascular local que aporte tanto O_2 como nutrientes a las células tumorales. Sin embargo, la masa tumoral en proliferación se desarrolla más rápidamente que la vasculatura, por lo que las células tumorales rápidamente se encuentran con un ambiente deficiente en O_2, hipóxico (Folkman, Hahnfeldt et al. 2000; Vaupel 2004; Brahimi-Horn, Chiche et al. 2007). La respuesta a hipoxia de las células tumorales favorecerá el crecimiento tumoral, que a su vez generará una nueva situación de hipoxia. De hecho, se ha sugerido que la hipoxia ejerce una fuerte presión selectiva sobre las células malignas (Graeber, Osmanian et al. 1996; Hockel and Vaupel 2001; Hockel and Vaupel 2001; Vaupel, Briest et al. 2002) (Figura III 17).

6.1. Expresión de HIF en cáncer

El aumento en la expresión de HIF-α (y en la actividad de HIF) puede producirse por pérdida de supresores de tumores como pVHL, por activación de oncogenes, o por un aumento de la actividad de las rutas de señalización PI3K y MAPK, de forma que la sobreexpresión de HIF-α generalmente promueve carcinogénesis (Maynard and Ohh 2007). En efecto, tanto HIF-1α

como HIF-2α se expresan de manera elevada en varios tumores y líneas celulares tumorales (Talks, Turley et al. 2000; Hopfl, Ogunshola et al. 2004).

Niveles altos de HIF-1α se han correlacionado con progresión tumoral y pobre prognosis en pacientes con tumor cerebral, esofágico, de mama, de estómago, carcinoma pulmonar de células no pequeñas (NSCLC), fibrosarcoma, carcinoma colorectal (CRC), de próstata, de ovario, uterino y cervical (Zhong, De Marzo et al. 1999; Birner, Schindl et al. 2000; Talks, Turley et al. 2000; Birner, Gatterbauer et al. 2001; Birner, Schindl et al. 2001; Koukourakis, Giatromanolaki et al. 2001; Schindl, Schoppmann et al. 2002; Sivridis, Giatromanolaki et al. 2002; Bos, van der Groep et al. 2003; Takahashi, Tanaka et al. 2003; Yoshimura, Dhar et al. 2004; Detwiller, Fernando et al. 2005; Generali, Berruti et al. 2006; Liu, Yu et al. 2006; Nakamura, Martin et al. 2006; Winter, Shah et al. 2006).

La sobreexpresión de HIF-2α se ha correlacionado con progresión tumoral y pobre prognosis en pacientes con tumor cerebral, de mama, NSCLC, carcinoma de célula escamosa de cuello y cabeza (HN-SCC), CRC, y carcinoma de célula clara renal (CCRC) y hemangioblastomas asociados a pVHL (Flamme, Krieg et al. 1998; Giatromanolaki, Koukourakis et al. 2001; Harris 2002; Koukourakis, Giatromanolaki et al. 2002; Yoshimura, Dhar et al. 2004; Raval, Lau et al. 2005; Holmquist-Mengelbier, Fredlund et al. 2006; Liu, Yu et al. 2006; Winter, Shah et al. 2006). Curiosamente, un ejemplo más de divergencia entre las subunidades HIF-1α y HIF-2α, se observa en CCRCs asociados a pVHL, en el que HIF-2α, pero no HIF-1α, promueve el crecimiento tumoral (Kondo, Klco et al. 2002; Maranchie, Vasselli et al. 2002; Kondo, Kim et al. 2003; Raval, Lau et al. 2005). Además, estos resultados vienen apoyados por otro estudio independiente en el que se demuestra que es HIF-2α el que preferencialmente está implicado en el desarrollo neoplásico (Covello, Simon et al. 2005).

6.2. Genes regulados por HIF en el desarrollo y en la progresión del cáncer

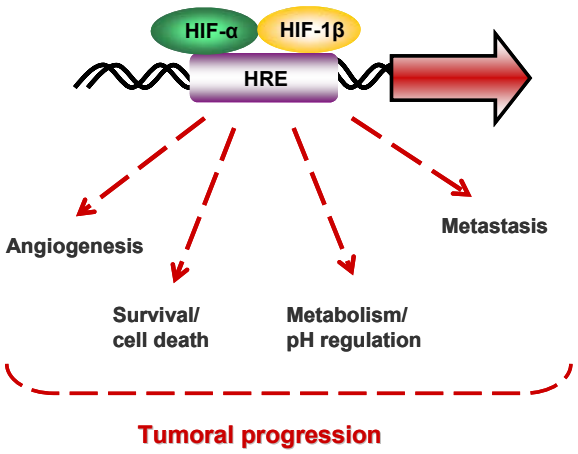

Figura III 18. Mecanismos dependientes de HIF que aprovechan las células tumorales para su crecimiento y desarrollo. *(Adaptado de (Berra, Ginouves et al. 2006))*

HIF se encuentra normalmente activado en cáncer y la transcripción de sus genes diana favorece el crecimiento y la supervivencia de las células tumorales (Maynard and Ohh 2007). Muchos de los genes regulados por HIF-1 y HIF-2 permiten a la célula adaptarse a las condiciones hipóxicas para promover la supervivencia y desarrollo del fenotipo tumoral, a través de los siguientes mecanismos (Brahimi-Horn, Chiche et al. 2007) (Figura III 18):

- *Angiogénesis:* mediada por VEGF y angiopoyetina-2 fundamentalmente, restablece un ambiente rico en O_2 y nutrientes para el mantenimiento del crecimiento tumoral (Ferrara and Kerbel 2005). Sin embargo, los nuevos vasos formados son con frecuencia irregulares y menos eficientes.
- *Supervivencia o muerte celular:* mientras que la hipoxia desencadena unos mecanismos que permite a las células tumorales seguir proliferando, si dicha hipoxia es bastante severa, puede también causar muerte celular, como refleja la zona necrótica central de muchos tumores. El desarrollo espacio-temporal del tumor se acompaña de niveles muy variables de hipoxia, poniéndose de manifiesto multitud de respuestas de las células tumorales.
- *Metabolismo:* las células tumorales se caracterizan por la conversión citoplasmática del piruvato a lactato, previniendo su entrada en la fosforilación oxidativa mitocondrial. La expresión de genes dependientes de HIF favorece el conocido como fenotipo glucolítico, caracterizado por un aumento de la glucolisis y de los transportadores de glucosa que compensa la reducción de la producción de ATP por formación de lactato. Además, la hipoxia suprime mTOR para ahorrar el consumo

energético derivado de la síntesis de proteínas y permitir la adaptación celular y la supervivencia.

- *Regulación del pH:* una de las consecuencias del fenotipo glucolítico es la disminución en el pH extracelular o acidosis (Swietach, Vaughan-Jones et al. 2007), aumentada además por la pobre vasculatura. Sin embargo, el pH intracelular de las células tumorales se mantiene en unos valores relativamente normales, necesario para el crecimiento celular, gracias a la expresión y activación mediada por HIF de transportadores de membrana, intercambiadores, bombas y ectoenzimas implicadas en la homeostasis del pH.
- *Metástasis:* la activación de HIF se asocia con pérdida de E-caderina, un supresor de la invasión y metástasis (Sullivan and Graham 2007). Además, las células que sobreviven a la acidosis no sólo desarrollan una ventaja de crecimiento sino que también se vuelven más agresivas e invasivas (Vaupel 2004; Walenta and Mueller-Klieser 2004).

En vista de los puntos anteriores, el factor de transcripción HIF representa una diana importante en la terapia del cáncer, y la búsqueda de una estrategia para regular su expresión y actividad es fundamental para hacer frente a los procesos de carcinogénesis.

IV. JUSTIFICACIÓN Y OBJETIVOS

JUSTIFICACIÓN Y OBJETIVOS

Las células de organismos superiores han desarrollado mecanismos específicos que permiten el reconocimiento y la reparación de daños en el DNA. Las roturas en la cadena del DNA generadas directamente por agentes genotóxicos (radicales de oxígeno, radiaciones ionizantes, agentes alquilantes) o indirectamente por la incisión enzimática de bases del DNA, inducen la síntesis de poli(ADP-ribosa) por el enzima PARP-1. PARP-1 es una proteína nuclear que se une al DNA cuando detecta la presencia de roturas, y se activa para catalizar la síntesis de un polímero utilizando NAD^+ como sustrato. Este polímero es transferido a distintos aceptores nucleares implicados en la arquitectura de la cromatina (histonas H1, H2B, lamina B, etc.) o en el metabolismo del DNA (topoisomerasas I y II, proteínas de reparación), y a la propia PARP-1, que resulta inactivada.

Las líneas de investigación actuales de nuestro grupo se centran, por un lado, en el estudio del papel de PARP-1 en la integridad del genoma en células tumorales y en el estudio de la susceptibilidad al desarrollo tumoral de los ratones PARP-1 knockout. En nuestro laboratorio hemos demostrado, por un lado, que PARP-1 es necesaria para la respuesta celular óptima a radiación ionizante a través de la fosforilación de p53 (Valenzuela et al, 2002) y, por otra parte, (utilizando análisis de expresión génica masiva o cDNA chip array, en un modelo de carcinogénesis dérmica) 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, Aguilar-Quesada et al. 2006). Uno de los genes cuya expresión disminuye fuertemente en ausencia/inhibición de PARP en dicho modelo es el de HIF-1α (hypoxia-inducible factor-1α).

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:

En el microambiente tumoral la elevada inestabilidad genómica, y el entorno hipóxico son dos factores determinantes de la agresividad tumoral y de la respuesta al tratamiento antineoplásico. PARP-1 puede jugar un papel modulador de la respuesta de los tumores a estas situaciones de estrés, y su ausencia podría mejorar el control de la progresión tumoral.

Para comprobar esta hipótesis se ha estudiado la relación de PARP-1 con dos factores fundamentales implicados en las citadas señales de estrés: ATM y HIF; y hemos abordado los siguientes OBJETIVOS:

A. OBJETIVO GLOBAL: Estudio de la interacción entre ATM y PARP-1 en respuesta al daño en el DNA y sensibilización de células deficientes en ATM mediante inhibición de PARP

Objetivos concretos:

A1. Estudiar la interacción física entre ATM y PARP-1 en respuesta a daños en el DNA.

A2. Estudiar si ATM es modificada por PAR en respuesta a IR, y las consecuencias funcionales de dicha poli(ADP-ribosil)ación sobre la actividad de ATM.

A3. Estudiar si la ausencia de actividad de PARP (mediante inhibidores de esta proteína) activa a ATM.

A4. Estudiar la integridad de la reparación de DSBs en células deficientes en ATM tras el tratamiento con IR y/o inhibidores de PARP, y su viabilidad en tales condiciones.

B. OBJETIVO GLOBAL: Efecto específico de PARP-1 sobre la respuesta a hipoxia mediada por HIF

Objetivos concretos:

B1. Estudiar la implicación de PARP-1 en la respuesta a hipoxia.

B1. Estudiar el papel de PARP-1 en la regulación de HIF.

B2. Estudiar el efecto de PARP-1 sobre la expresión y actividad de las subunidades HIF-α en respuesta a hipoxia.

B3. Estudiar el mecanismo responsable de la regulación establecida por PARP-1 de las subunidades HIF-α.

V. RESULTADOS

A. INTERACCIÓN ENTRE ATM Y PARP-1 EN RESPUESTA AL DAÑO EN EL DNA Y SENSIBILIZACIÓN DE CÉLULAS DEFICIENTES EN ATM MEDIANTE INHIBICIÓN DE PARP

RESUMEN

ATM y PARP-1 son dos de los más importantes factores en la respuesta de la célula al daño en el DNA. PARP-1 y ATM reconocen y se unen a roturas de cadena sencilla y/ó doble del DNA en respuesta a diferentes estímulos. En el siguiente trabajo, vemos que ATM y PARP-1 forman un complejo molecular *in vivo* en células no dañadas y que esta asociación aumenta después de radiación-γ. ATM además es modificada por PARP-1 durante el daño en el DNA. Hemos analizado también el efecto de la ausencia de PARP-1 o su inhibición sobre la actividad quinasa de ATM y hemos encontrado que mientras que las células deficientes en PARP-1 presentan una actividad quinasa de ATM disminuida y una reducción en la formación de *foci* de γ-H2AX en respuesta a radiación-γ, la inhibición de PARP por sí sola es capaz de aumentar la actividad quinasa de ATM. La inhibición de PARP produjo acumulación de *foci* de γ-H2AX de forma dependiente de ATM, y originó roturas de cadena doble del DNA, en mayor medida en ausencia de ATM. Como consecuencia, las células deficientes en ATM mostraron una mayor sensibilidad a la inhibición de PARP. En resumen, nuestros resultados muestran que mientras PARP-1 es necesaria en la respuesta de ATM a radiación-γ, la inhibición de PARP produce roturas de cadena doble del DNA que son reparadas a través de una ruta dependiente de ATM, y por tanto, capaces de activar a ATM.

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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

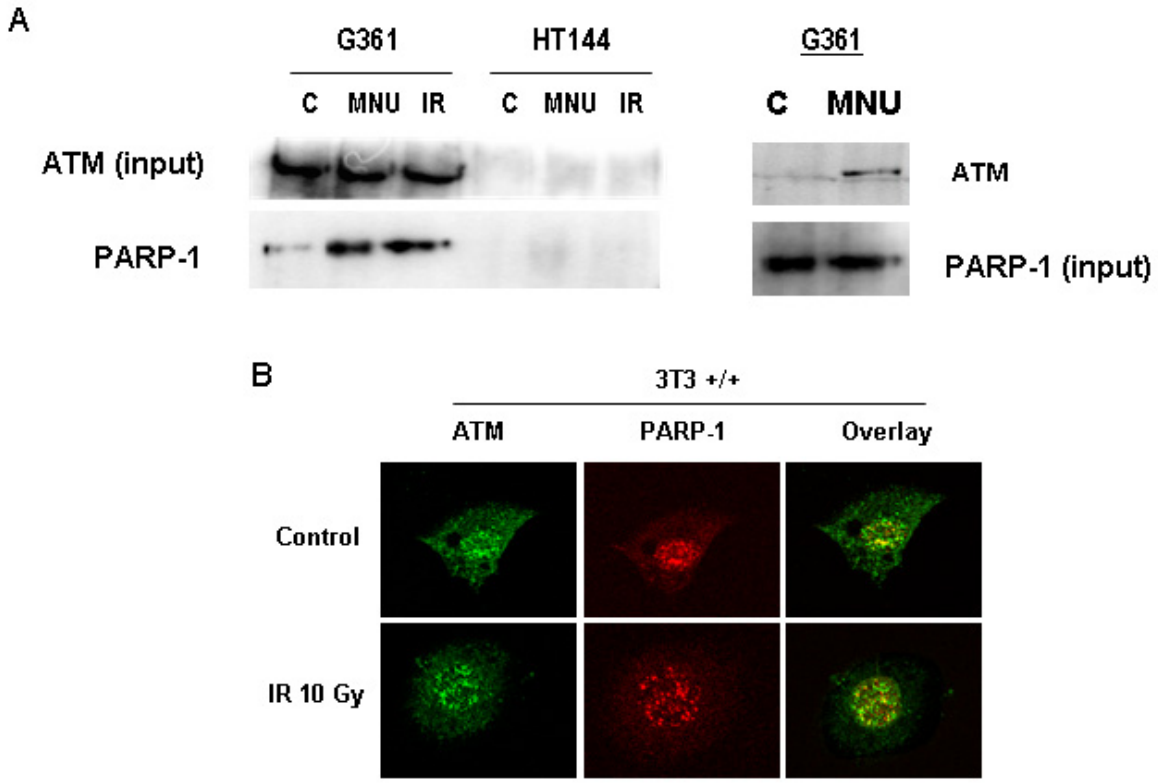

Figure 1
ATM form a complex with PARP-1 in DNA that is much more evident after treatment with DNA damaging agents. A: ATM was immunoprecipitated in human melanoma cell lines G361 and HT144 (ATM deficient) as explained under methods and the presence of PARP-1 was tested by immunoblot analysis. Cells were 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

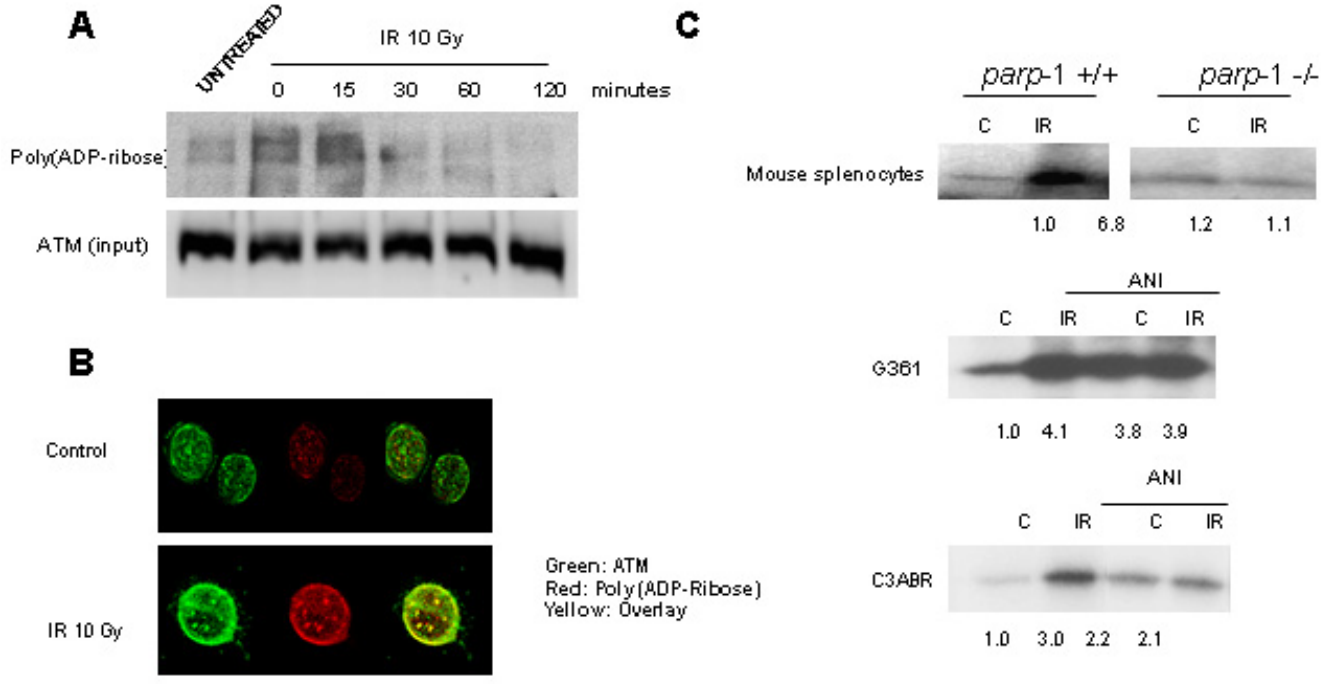

Figure 2
ATM is modified by poly(ADP)ribose. A: ATM was immunoprecipitated in G361 cells and the presence of poly(ADP ribose) was tested by immunoblot analysis in a time course, reaching a maximum after 15 min of IR (10 Gy). Equal loading was normalized by the input of ATM. B: Double indirect immunofluorescence 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

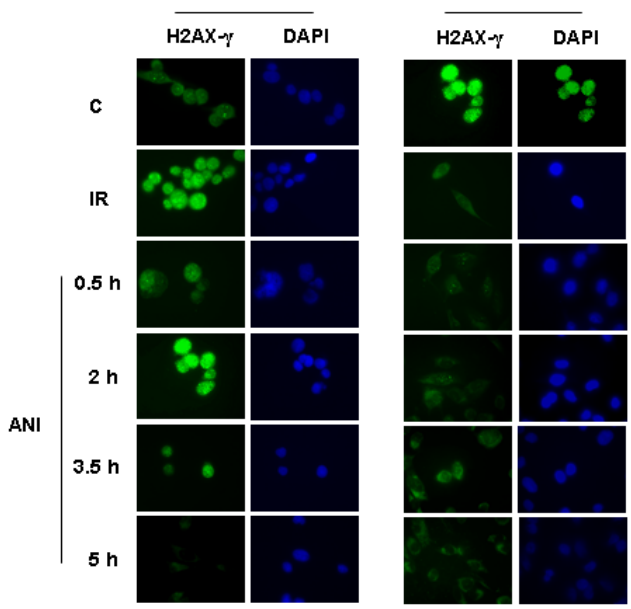

Figure 3
ATM is activated by PARP inhibitors. Immunostaining for γ-H2AX in G361 (ATM wild type) and HT144 (ATM deficient) cells exposed to γ-irradiation (positive control) or ANI (time course after drug exposure). H2AX (green signal) is phosphorilated by ATM after ANI treatment in absence of γ-irradiation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

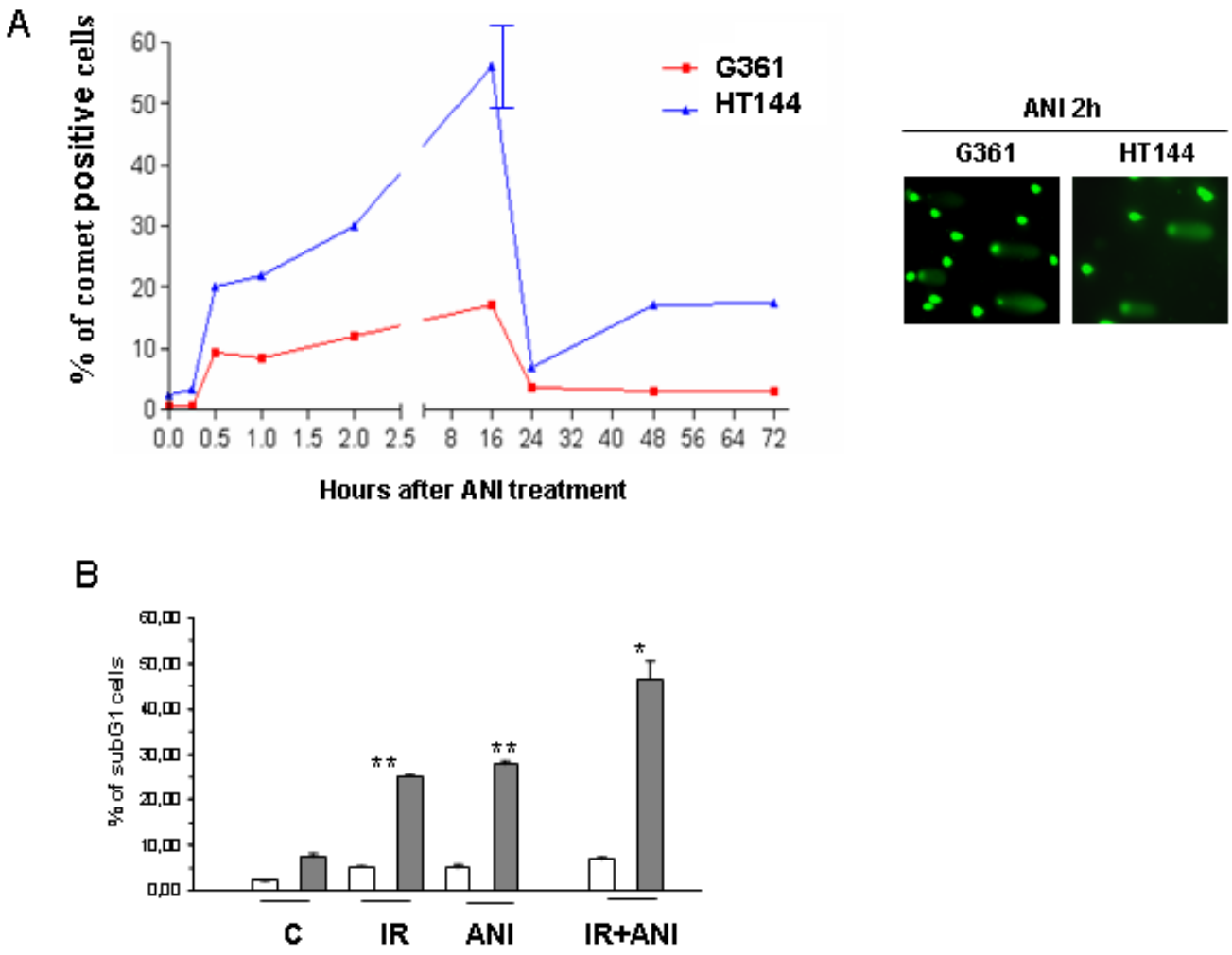

Figure 4

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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CONCLUSIONES

A1. PARP-1 y ATM interaccionan físicamente, y su asociación aumenta tras el tratamiento con radiación ionizante.

A2. ATM es modificada por poli(ADP-ribosil)ación. La activación de ATM en respuesta a IR es menor en células deficientes en PARP-1 o células tratadas con inhibidores de PARP.

A3. La inhibición de PARP por sí misma activa a ATM, como demuestra la fosforilación de su sustrato H2AX.

A4. La inhibición de PARP origina roturas de cadena doble del DNA que son reparadas por una ruta que implica a ATM, de forma que las células deficientes en ATM son mucho más sensibles a los inhibidores de PARP que las células parentales.

SUPPLEMENTARY UNPUBLISHED RESULTS *A*

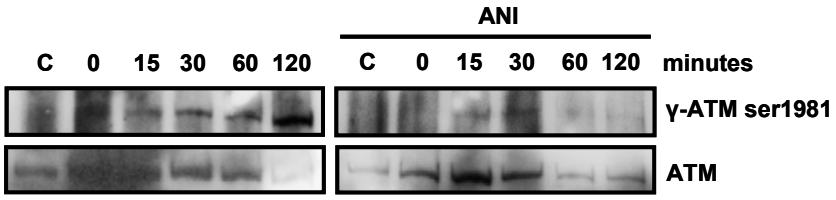

Figure A1. Autophosphorylation of ATM on serine 1981 is delayed after inhibition of PARP. ATM-proficient cells G361 were pre-treated with 10μM ANI for 1 h prior to DNA damage induction with γ-irradiation (10 Gy) and compared with ATM-proficient cells G361 exposed only to IR. ATM was immunoprecipitated in a time course after IR and its level of phosphorylation was tested by immunoblot analysis using an anti-ATM pS1981 polyclonal antibody (Abcam Ltd, Cambridge, UK) at a dilution of 1:500. Equal loading was normalized by the input of ATM.

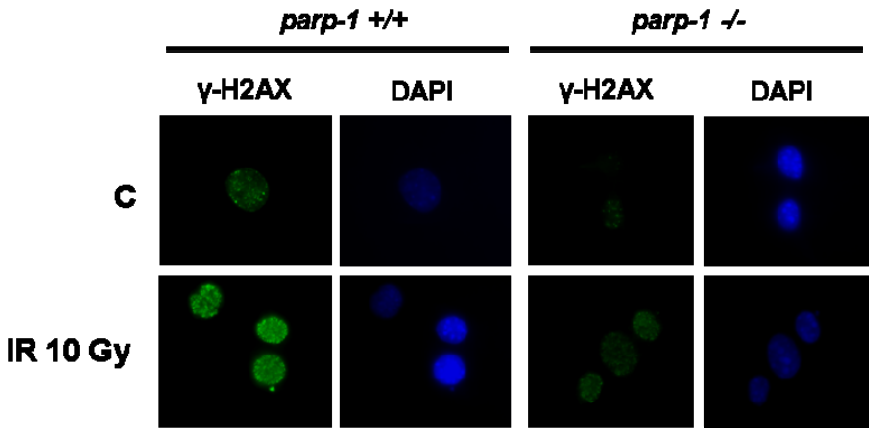

Figure A2. PARP-1 is needed for optimal activation of ATM in response to ionizing irradiation. Immunostaining for γ-H2AX in *parp-1* +/+ and *parp-1* -/- cells exposed to 10 Gy of γ-irradiation for 2 h was achieved as described in Methods. ATM activity (as measured by γ-H2AX) increases significatively after γ-irradiation in wild-type PARP-1 cells, but not in knockout PARP-1 cells. DAPI is shown in blue and both views are in coincidence.

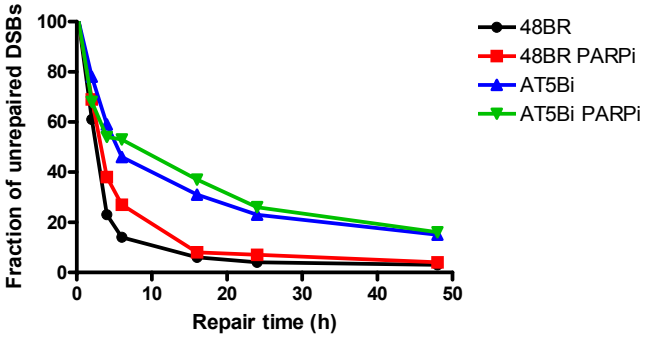

Figure A3. Kinetics of DSB rejoining in confluence-arrested primary human fibroblasts after inhibition of PARP and X-ray treatment. PARP inhibitor delays DSB rejoining primarily in wild-type cells (48BR) but also in ATM-deficient cells (AT5Bi). 48BR and AT5Bi cells were pre-treated with 10μM KU0058684 PARP inhibitor (PARPi) for 1 h prior to DNA damage induction with X-rays (1 Gy) and compared with untreated cells exposed only to X-rays. γ-H2AX foci disappearance was measured in cells using an anti-H2AX pS139 monoclonal antibody (Upstate) at a dilution of 1:800.

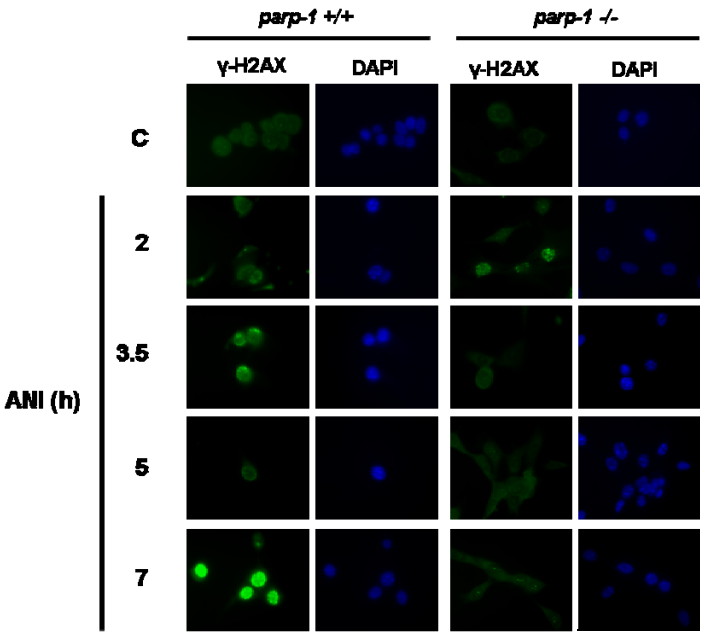

Figure A4. Inhibition of PARP activates ATM while PARP-1 deficient cells display a limited ATM activation. Immunostaining for γ-H2AX in *parp-1* +/+ and *parp-1* -/- cells treated with ANI (time course after drug exposure) was performed as described in Methods. DAPI is shown in blue and both views are in coincidence.

B. EFECTO ESPECÍFICO DE PARP-1 SOBRE LA RESPUESTA A HIPOXIA MEDIADA POR HIF

RESUMEN

Las respuestas transcripcionales a hipoxia son fundamentalmente dirigidas por el factor de transcripción HIF, un heterodímero formado por las subunidades HIF-α y HIF-β. Mientras que la caracterización inicial de HIF se centró principalmente sobre HIF-1α, recientemente, HIF-2α ha surgido como una isoforma no redundante que induce la expresión de genes específicos y, por lo tanto, desarrolla funciones específicas. PARP-1, por otra parte, ha sido implicada en el control de la transcripción génica al modular la estructura de la cromatina y al formar parte de complejos de unión a activadores/promotores específicos de genes. En este estudio, los resultados indican que PARP-1 está implicada en la regulación de la ruta de respuesta a hipoxia. Además, observamos un efecto específico de PARP-1 sobre la activación hipóxica de HIF-2α. Utilizando dos aproximaciones independientes (MEFs derivados de ratones *parp-1* knockout y silenciamiento génico de PARP-1 mediante siRNA), vemos que PARP-1 controla la expresión del mRNA de HIF-2α, así como la inducción hipóxica de la proteína y los genes diana dependientes de HIF-2. Sin embargo, el tratamiento con inhibidores de PARP no tiene efecto sobre la regulación de HIF-2. En resumen, estos resultados sugieren que PARP-1 está implicado en la fina regulación de la respuesta hipóxica.

Specific impact of PARP-1 on HIF-mediated response to hypoxia

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Running Title: PARP-1 differentially regulates HIF-1α and HIF-2α

Abstract

Transcriptional responses to hypoxia are primarily mediated by the hypoxia-inducible factor (HIF), a heterodimer of HIF-α and HIF-β subunits. While initial characterization of HIF mostly focused on HIF-1α, HIF-2α raised more recently as a non-redundant isoform inducing specific genes and hence performing specific functions. Poly (ADP-ribose) polymerase-1 (PARP-1) is a nuclear protein, which has been recently involved in the control of gene transcription by modulating chromatin structure and acting as part of gene-specific enhancer/promoter-binding complexes. In this study, we present evidence for a role of PARP-1 in the regulation of the hypoxic pathway. Furthermore, we show a specific impact of PARP-1 on the hypoxic activation of HIF-2α. By using two independent approaches (MEFs derived from *parp*-1-/- KO mice and gene silencing by siRNA) we show that PARP-1 controls HIF-2α mRNA expression as well as the hypoxic induction of the protein and HIF-2-dependent target genes. However, treatment with PARP inhibitors has no impact on HIF-2 regulation. Altogether, these results suggest that PARP-1 is involved in the fine tuning of the hypoxic response.

Introduction

Because oxygen (O_2) is absolutely required for invertebrate and vertebrate life, the ability to recognize changes in O_2 availability is an essential feature of mammalian survival. When O_2 availability decreases (hypoxia), adaptative mechanisms are switched on by increasing the transcription of specific genes that contribute to restore O_2 homeostasis [1, 2]. These hypoxia-induced genes are involved in glucose transport, glycolysis, erythropoiesis, angiogenesis, vasodilation, and respiratory rate. Furthermore, these genes cooperate to minimize the effects caused by O_2 deprivation by decreasing O_2 consumption and/or increasing O_2 availability at cellular, tissue and systemic levels [3-5]. These adaptative mechanisms play a central role in the normal physiology such as during embryonic development but they are also related to several pathological situations like cancer [3]. Indeed, tumor hypoxia and activation of the hypoxic pathway have been associated with aggressive and malignant phenotypes across a range of cancers [6].

The up-regulation of many hypoxia-regulated genes is mediated by a ubiquitously expressed transcription factor called hypoxia-inducible factor (HIF), a heterodimer of an α- and a β-subunit [7]. Both subunits belong to the superfamily of basic helix-loop-helix/Per-Arnt-Sim (bHLH-PAS) transcription factors. HIF binds to *cis*-acting hypoxia response elements (HREs) located within the target genes [8].

The HIF complex is regulated by O_2 availability through the abundance and activity of the HIF-α subunits [9]. Whereas HIF-β, also known as ARNT [aryl hydrocarbon receptor nuclear translocator] remains largely unaffected by changes in O_2 levels, the HIF-α subunit is rapidly degraded and transcriptionally repressed in the presence of O_2, as well as strongly accumulated and transcriptionally activated following exposure to hypoxic conditions.

To date, three HIF-α isoforms, encoded by different genes, have been described, with the best characterized being HIF-1α and HIF-2α (also known as endothelial PAS domain protein 1 [EPAS 1], HIF-1-like factor [HLF], and HIF-1-related factor [HRF]). Although HIF-1α is ubiquitously expressed, HIF-2α transcripts are enriched in selected tissues and cell types [10-14]. HIF-1α and HIF-2α share a 48% overall identity, underscored by their shared domain architecture, ability to heterodimerize with ARNT, HREs binding, and activation mechanisms. Despite these similarities, targeted disruption of the HIF-1α or HIF-2α gene in mice results in strikingly different phenotypes, demonstrating non-redundancy of their functions during embryonic development [15-22]. Furthermore, targeted inactivation of HIF-1α and HIF-2α in embryonic stem cells is associated with different patterns of response to hypoxia and low glucose stress [23]. Thus, it is possible that HIF-1α and HIF-2α regulate the expression of overlapping but non-identical target genes with different contribution of these factors in mediating hypoxic adaptation.

Under normoxic conditions (21% O_2), HIF-α subunits are constitutively transcribed translated, and degraded by the proteasome. Indeed, the oxygen-dependent

degradation domain (ODDD) within the HIF-α subunit is rapidly recognized by the product of the von-Hippel Lindau tumor suppressor gene (pVHL) [24]. pVHL is a component of a protein-ubiquitin ligase complex that targets the HIF-α subunit for degradation by the proteasome 26S [25-27]. pVHL recognition of HIF-α is dependent on hydroxylation of specific proline residues within the ODDD [28, 29]. The prolyl hydroxylation is carried out by a family of prolyl hydroxylase domain-containing proteins (PHDs) [30, 31]. Three different PHDs (PHD1, PHD2 and PHD3) have been characterized in mammals [32]. Because these enzymes bind O_2 directly and use it as a co-factor, they act as critical O_2 sensors. Under hypoxic conditions, hydroxylation is blocked, resulting in increased HIF-α stability [32] . HIF-α translocate into the nucleus and heterodimerize with the HIF-β subunit and thus form the active HIF complex. Besides prolyl hydroxylation, HIF-1α is hydroxylated on an asparagine residue within its carboxy-terminal transactivation domain by factor inhibiting HIF (FIH-1; [33]). FIH-1, which is subjected to the same regulatory mechanisms as the PHDs, suppresses HIF-1 transcriptional activity under normoxic conditions by blocking its association with the coactivator p300/CBP, a critical component of the HIF transcriptional complex.

PARP-1 (poly (ADP-ribose) polymerase-1) is an abundant nuclear enzyme that uses NAD^+ as a substrate to catalyse the covalent attachment of ADP-ribose units on nuclear acceptor proteins, or on PARP-1 itself. The resulting polymer of ADP-ribose (PAR) alters the functional properties of the different target proteins. Indeed, this posttranscriptional covalent modification has consequences in a number of biological functions (reviewed in [34]). Initially, it was assumed that PARP-1

regulation and activation primarily occurs as a consequence of DNA damage, but recent studies have provided evidence that PARP-1 activity can also be modulated by several endogenous factors, including various kinases, purines and caffeine metabolites [35]. Otherwise, in some circumstances, PARP-1 catalytic activity is not required, as it is the case for the regulation of some transcription factors where PARP-1 acts as a component of the enhancer/promoter regulatory complexes [36].

In contrast to a growing body of evidence demonstrating clear differences between HIF-1 and HIF-2 function, few mechanisms are known to account for this specificity. New efforts are now on their way to identify the molecular basis of the functional differences between HIF-1α and HIF-2α. In the present work, we studied the relationship between PARP-1 and HIF-1α and HIF-2α subunits expression upon hypoxia, and we found that depletion of PARP-1, but not the inhibition of its activity, dramatically decreased HIF-2α at the mRNA, protein levels, and consequently the transcription of HIF2-dependent genes, whereas HIF-1α is mostly not affected. Our results suggest that PARP-1 exerts an exquisite and specific regulatory effect on HIF-2α that could be exploited to specifically target HIF-2α to counteract tumor development.

Results

HIF-dependent response is impaired in parp-1 -/- MEFs

In a previous work, we found that wild-type mice treated with PARP inhibitors and *parp-1* -/- mice showed decreased level of HIF-1α after treatment with DMBA plus TPA in a model of skin carcinogenesis. Consequently, HIF activity was decreased in MEFs derived from the KO mice [37]. Based on these results, we decided to further evaluate the impact of PARP-1 on HIF activity upon hypoxia.

To test whether PARP-1 influences HIF activity, we used two already known HIF-dependent genes as reporter: PHD2 and PHD3 [38, 39]. Indeed, the expression of PHD2 and PHD3 has been shown to be transcriptionally induced by hypoxia, and hence promote a negative feedback loop [31, 40]. The expression of both PHDs was determined by Quantitative RT-PCR in immortalized MEFs *parp-1* +/+ and *parp-1* -/- upon hypoxia (1% O_2) for 16 hours. As shown in Figure 1, hypoxia-induced PHD3 mRNA levels are drastically decreased in *parp-1* -/- cells, whereas PHD2 mRNA levels exhibited only a modest decrease. PHD3 and PHD2 mRNA levels were also quantified in another independent clone with similar results (data not shown). These results suggested that PARP-1 might impact on HIF response in a specific manner.

HIF-2-dependent gene expression is specifically impaired in parp1 -/- MEFs

Interestingly, it has been described that silencing of HIF-1α or HIF-2α results in decreased hypoxia-dependent induction of PHD3 expression, whereas PHD2 is

not affected by HIF-2α invalidation [38, 41]. Hence, we hypothesized that PARP-1 might mostly impact on HIF-2 activity. However, since PHD2 and PHD3 participate in the modulation of the HIF pathway, alterations in their expression could mask the real impact of PARP-1 on HIF regulation. To obviate the masked effect and to further validate our hypothesis, we look for the expression of two additional HIF-dependent genes, which expression is differentially regulated by HIF-1 or HIF-2. Indeed, in studies designed to identify unique HIF-1 and HIF-2 target genes, it has been shown that HIF-1 exclusively induces the hypoxic transcription of glycolytic genes such as phosphoglycerate kinase 1 (Pgk1), aldolase A (Alda) and lactate dehydrogenase A (LDHA), whereas HIF-1 and HIF-2 both activate the expression of vascular endothelial growth factor (VEGF) or glucose transporter 1 (GLUT-1) [42-45]. Hence, GLUT-1 (HIF-1 and HIF-2 target) and LDHA (HIF-1 target) mRNA levels were compared in immortalized MEFs *parp-1 +/+* and *parp-1 -/-* after 16 hours of hypoxia 1%. Interestingly, we observed that the absence of PARP-1 reduces the hypoxic induction of GLUT-1 whereas it has almost no impact on LDHA induction (Figure 2). GLUT-1 and LDHA mRNA levels were also quantified in another independent clone with similar results (data not shown).

HIF-2α expression is reduced in parp-1 -/- MEFs

We next evaluated by Western-blot HIF-1α and HIF-2α protein accumulation after 16 hours of 1% hypoxia in immortalized MEFs *parp-1 +/+* and *parp-1 -/-* (Figure 3a). The hypoxic induction of HIF-2α was greatly impaired in *parp-1 -/-* MEFs while

a relatively small change in HIF-1α level was observed between *parp-1 +/+* and *parp-1 -/-* cells.

HIF-2α accumulation upon hypoxia in MEFs was in contradiction with a previous report showing that endogenous HIF-2α protein in these cells is expressed at constant levels regardless of oxygenation and is primarily localized into the cytoplasm [46]. In view of this discrepancy, we checked the subcellular localization of HIF-1α and HIF-2α in our immortalized MEFs *parp-1 +/+* and *parp-1 -/-* after hypoxic incubation (1% O_2) by immunofluorescence microscopy. Indirect immunostaining of HIF-1α confirmed our results by WB regarding the induction of the protein and shows nuclear HIF-1α accumulation when cells were treated with hypoxia (Figure 3b). More interestingly, results shown in Figure 3b clearly demonstrate HIF-2α nuclear immunostaining after incubation at 1% O_2 only in immortalized *parp-1 +/+* MEFs. These results support the hypothesis that PARP1 acts as a modulator of HIF-2α expression rather than a transcriptional regulator of HIF-2 activity.

Then, we aimed to investigate if the observed impact on HIF-2α protein was due to a PARP-1-mediated effect on HIF-2α mRNA expression. As in the case of the protein, HIF-2α mRNA levels measured by Quantitative RT-PCR dramatically decreased in *parp-1 -/-* MEFs compared to *parp-1 +/+* MEFs either in normoxia or hypoxia (Figure 3c and data not shown). HIF-2α mRNA levels were also quantified

in another independent clone with similar results (data not shown). Furthermore, HIF-1α mRNA levels were also affected even if the impact was lower.

Silencing of PARP-1 reduces HIF-2–dependent transcriptional activity

To ascertain that the effect observed in immortalized MEFs is specific of PARP-1 ablation, we proceeded to invalidate PARP-1 by siRNA. We transiently transfected the VHL-defective renal carcinoma cell line 786-O, that exclusively express HIF-2α and not HIF-1α [24], with a chemically synthesized PARP-1 siRNA that properly decreased PARP-1 mRNA levels (Figure 4a). As a control, we used an irrelevant siRNA (SIMA) which, as expected, had no impact on PARP-1 mRNA (Figure 4a).

When we determined PHD3 and PHD2 mRNA levels following transient transfection with the PARP-1 siRNA, the 786-O cells displayed an important decrease in PHD3, but not in PHD2 (Figure 4b), as we observed previously in immortalized MEFs (Figure 1). Furthermore, constitutive HIF-2α protein accumulation in 786-O and HIF-2α mRNA expression were decreased after knockdown of PARP-1 by siRNA (Figure 4c and d, respectively).

Inhibition of PARP-1 catalytic activity has no impact on HIF-2

To investigate whether PARP-1 enzymatic activity was important for HIF-2-dependent response, we studied the effect of the PARP inhibitor, DPQ. Efficacy of the inhibitor raised 87% of inhibition of poly(ADP-ribose) formation for immortalized wild-type MEFs. As shown in Figure 5a, neither PHD2 nor PHD3 hypoxia-induced

mRNA were significantly affected by DPQ compared to the DMSO-treated control cells. When we examined HIF-1α and HIF-2α protein accumulation, we couldn't reveal any significant difference between cells treated with DMSO or DPQ (Figure 5b). However, DPQ treatment increases HIF-2α mRNA levels whereas it has no impact on HIF-1α mRNA (figure 5C). Thus, inhibition of PARP-1 catalytic activity, in contrast to PARP-1 knockout or silencing, appears to have no impact on HIF-dependent response.

Discussion

Our previous work in a model of skin carcinogenesis [37] reported that PARP-1 knockout mice and mice treated with PARP inhibitors showed decreased level of HIF-1α after DMBA plus TPA treatment. In this paper, we show a differential effect of PARP-1 on HIF-1α and HIF-2α upon hypoxia. Indeed, hypoxia-induced HIF-2α accumulation and transcriptional activity appear to be strongly down-regulated in PARP-1 deficient cells. However, HIF-1α expression does not appear to be affected by PARP-1 in response to hypoxic stress. Thus, we postulate that PARP-1 effect on HIF-1α during the treatment of mice with DMBA plus TPA (where PARP-1 itself and its activity are fundamental) takes place through a different pathway from what occurs under hypoxia.

HIF-1α is ubiquitously expressed and has been suggested to play a primary role in hypoxic responses. HIF-2α is also widely expressed, but its transcripts are

enriched in specific cell types, such as vascular endothelial cells, kidney fibroblasts, hepatocytes, glial cells, interstitial cells of the pancreas, epithelial cells of the intestinal lumen, neural crest cell derivatives, and lung type II pneumocytes [10-14]. In contrast to the restricted expression observed in embryonic and adult tissues, HIF-2α is detected in many human tumors, including those associated with VHL disease (renal clear cell carcinomas and hemangioblastomas) as well as tumors not associated with VHL disease, such as breast, head and neck squamous cell carcinoma, and non-small cell lung cancers [47].

There has been a long-term interest in distinguishing the roles of HIF-1α and HIF-2α on gene transcription, and a growing number of physiological and mechanistic differences between HIF-1α and HIF-2α have being reported. Hence, it seems well accepted that HIF-1α and HIF-2α play complementary rather than redundant functions. As an exemple, the NF-κB essential modulator (NEMO) has been shown to exclusively interact with HIF-2α and to promote its transcriptional activity by enhancing binding to CBP/p300 [48]. The redox factor Ref-1 enhances the HIF-2α DNA binding capacity by reducing HIF-2α and not HIF-1α based on a single amino acid difference between HIF-1α and HIF-2α within the N-terminal DNA-binding basic regions [49]. Similarly, a single amino acid substitution between HIF-1α and HIF-2α contributes to the intrinsically higher FIH-1-mediated asparaginyl hydroxylation of HIF-1α and hence, lower HIF-1α activity detected in several cell lines [50]. Furthermore, the specific impact of HIF-1α in NBS1 repression and hence DNA repair has been shown to be mediated by the phosphorylation status of

a threonine residue within the PAS-B domain; in contrast to HIF-1α, this residue is phoshorylated on HIF-2α and this phosphorylation impairs NBS repression [51].

In the present study, we show a differential effect of PARP-1 on HIF-1α and HIF-2α mRNA expression, and consequently protein and transcriptional activity, supporting PARP-1 protein (but not its catalytic activity) as a new element in the transcriptional regulation of HIF-2α. In line with these results, a recent report by Elser et al found that tumor cells stably depleted of PARP-1 express reduced levels of Glut-1 [52].

In this work, we have used MEFs derived from wild-type and *parp-1-/-* knockout mice. Previously, it was reported that in MEFs, HIF-2α is transcriptionally inactive and retained in the cytoplasm [46]. In addition, the authors showed that endogenous HIF-2α escapes from O_2-dependent protein degradation and accumulates in immortalized MEFs under normoxia, whereas HIF-1α is subject to tight regulation by O_2 levels. In contrast to this previous report, our results clearly show that the induction of both isoforms is dependent on O_2 availability. Moreover, when we checked the subcellular localization of HIF-1α and HIF-2α in our wild-type MEFs under hypoxia, we obtained nuclear HIF-1α and HIF-2α accumulation compared with normoxia. On the contrary, we could not find any nuclear HIF-2α after hypoxic stimuli in *parp-1 -/-* MEFs due to PARP-1 absence. These apparent discrepancies observed in wild-type MEFs for HIF-2α between both reports might be due to differences on the inmortalization method or on the specifity of the antibody used in both studies. To avoid any non-specific staining, prior to use it for

the immunofluorescence we have validated our antibody using specific siRNAs against HIF-2α (data not shown).

Why PARP-1 has a specific effect on the regulation of HIF-2 alpha is still unknown. The role of PARP-1 in transcriptional regulation is an important aspect of the biological function of this protein (reviewed in [36]) and PARP-1 can achieve an enhancer/promoter binding cofactor activity that can act in conjunction with other transcription-related factors [53]. In this context, one interesting possibility concerning the specificity of the PARP-1's effect on HIF-2α comes from the fact that PARP-1 associates with the transcription factor Sp1. Indeed, Sp1 has been shown to promote HIF-2α transcription during adypocyte differentiation and the expression of Sp1 is reduced in *parp-1 -/-* cells [54]. Co-immunoprecipitation assays revealed that PARP-1 physically interacts with Sp1 in a DNA-independent manner, but not with Sp3 in *parp-1 +/+* cells [54].

Furthermore, PARP-1 promotes activator-dependent transcription by interacting with an increasing number of transcription factors, including AP-2, B-Myb, YY-1, Oct-1, NF-kB, and p53 [55-61]. In agreement with our results this transcription regulatory function requires nuclear PARP-1, but it is not dependent on its enzymatic activity. Computer analysis of the 5'-flanking region of the HIF-2α gene has revealed several putative binding sites for transcription factors as YY-1, AP-1, AP-2 or E2F-1. Hence, it is possible that the interaction of PARP-1 with any of the

previous transcription factors would be responsible, at least partially, of the impact of PARP-1 on HIF-2α expression.

Another possibility is that PARP-1 might contribute to HIF-2α expression through regulation of NAD(P)H oxidases since the silencing of the NAD(P)H oxidase Nox4 downregulates HIF2-α mRNA expression and hence its transcriptional activity [62].

Our results clearly demonstrate that knock-down and/or silencing of PARP-1 impacts on HIF-2 whereas inhibition of the catalytic activity has no effect. It remains intriguing to understand mechanistically how this duality between PARP activity and PARP (the protein itself) may affect differentially the hypoxic response. Nevertheless, the same duality has been shown for the effect of PARP-1 on the activity of different transcription factors, including NF-kB [63].

Nonetheless, there are still many open questions about the specific function of PARP-1 in HIF-2α expression and function. Both HIF-1α and HIF-2α are expressed at high levels in a variety of human tumors and tumor cell lines, although the relative contribution of each protein to tumor initiation and progression is not clear yet. For reasons that remain unclear, in neoplastic epithelial cells of CCRCs, the normal predominance of HIF-1α expression in non-neoplastic renal tubules is altered strikingly in favour of HIF-2α expression [64]. Furthermore, genetic manipulation in CCRC cells indicates that activation of HIF-2α but not HIF-1α promotes tumor growth [44, 65-67]. Hemangioblastomas, highly vascularized

tumors of the central nervous system, are the most frequent manifestation of the autosomal dominantly inherited von Hippel-Lindau (VHL) disease and they present up-regulation of HIF-2α [68], which is in addition an important factor determining neuroblastoma aggressiveness [69]. In view of the previous examples, HIF-2α could therefore represent an important target in cancer therapy, and it would be very interesting to rely on a tool to specifically target HIF-2α and to counteract tumor development. Based on our results PARP-1 appears as a good candidate even if further studies should be necessary to elucidate the molecular mechanism underlying the specific impact of PARP-1 on HIF-2α.

Materials and methods

Cell culture

Immortalized mouse embryonic fibroblast cells (MEFs), derived from both wild-type and knock-out PARP-1 mice [70], were grown in Dulbecco's modified Eagle's medium (DMEM) High Glucose (4.5g/l) supplemented with 10% inactivated fetal bovine serum (FBS) and antibiotics (penicillin G, 50 U/ml; and streptomycin, 50 µg/ml) (Gibco) at 37 °C in a humidified 5% CO2 atmosphere. 786-O cells were grown in the same conditions but with 7.5% inactivated FBS. Hypoxic conditions (1% O_2) were produced by incubation of cells in a sealed "Bug-Box" anaerobic workstation (Ruskin Technologies, Jouan).

Reagents and antibodies

PARP inhibitor, 3, 4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone (DPQ), was purchased from Alexis Biochemicals. The anti-HIF-1α (antiserum 2087) has been previously described [71]; anti-HIF-2α was purchased from Novus Biologicals; anti-PARP (Ab-2) from Oncogene; and anti-β-actin was from Sigma. For indirect immunofluorescence, anti-HIF-1α from BETHYL was used.

Quantitative RT-PCR

Immediately after treatments, cells were washed with PBS and harvested for total RNA extraction with RNeasy Mini Kit from Qiagen. Total RNA was quantified, and integrity was tested by gel electrophoresis. 500 ng of total RNA from each sample was retro-transcribed to cDNA (MultiScribe Reverse Transcriptase; Applied Biosystems) and amplified with the qPCR MasterMix Plus for SYBR Green I (Eurogentec) in a one step reaction. PCR amplifications were carried out in a 7300 Real Time PCR System (Applied Biosystems), and data were analyzed with 7300 System software. For each sample, duplicate determinations were made, and the gene copy number was normalized for the amount of Arbp in MEFs and Rplp in 786-O cells.

For HIF-2α, 2000 ng of total RNA was used in a reverse transcription reaction to synthesize cDNA using the iScript cDNA Synthesis kit (Bio-Rad) according to the manufacturer's protocol. Quantitative PCR analysis was done using iQ SYBR Green Supermix and the iCycler iQ detection system (Bio-Rad) according to the manufacturer's protocol.

Reverse Transcription PCR

cDNAs were synthesized from 500 ng of total RNA isolated with RNeasy Mini Kit (Qiagen) by using Omniscript RT Kit (Qiagen) together with oligo(dT)15 primer and recombinant RNasin ribonuclease inhibitor (Promega). PCRs were performed with Taq PCR Master Mix Kit from Qiagen and the following primers: human PARP-1 sense (5'-CAGCGGAAGCTGGAGGAGTG-3') and antisense (5'-GATTTCTTCTTCGCCACTTC-3'); and Rplp sense (5'-CAGATTGGCTACCCAACTGTT-3') and antisense (5'-GGCCAGGACTCGTTTGTACC-3'). PCR product was resolved and visualized in agarose gel.

Western Blotting

Cells were lysed and sonicated in Laemmli buffer after PBS wash. The protein concentration was determined using the Lowry assay and 40 μg for HIF-1α and PARP-1, or 60 μg for HIF-2α of whole-cell extracts were resolved by SDS-PAGE (7.5%). Next, proteins were transferred onto a PVDF (Millipore) membrane for HIF-1α and PARP-1, or nitrocellulose (Amersham Biosciences) for HIF-2α. The same membranes were reprobed with anti-β-actin. Immunoreactive bands were visualized with the ECL system (Amersham Biosciences).

siRNA Preparation and Transient Transfection

The 21-nucleotide RNAs were chemically synthesized and annealed by Eurogentec. The siRNA targeting PARP-1 (GenBank accession No. NP_001609)

correspond to the coding region 421-441 relative to the start codon. The siRNA used as an irrelevant control (SIMA) has been previously described [39]. Transfection was carried out using Dharmafect transfection reagent (Dharmacon) according to the manufacturer's instructions. Cells were analysed 48 hours after transfection.

Immunofluorescence analysis

Cells were plated on coverslips the day before of treatment with hypoxia for 16 hours. Medium was removed and cells were fixed in 3% paraformaldehyde 2% sucrose PBS and permeabilized with 0.2% triton in PBS. Primary antibody incubations were achieved in PBS 2% BSA at a dilution of 1:200 for HIF-1α and 1:250 for HIF-2α. The secondary antibody used in this study was FITC-conjugated goat anti-rabbit IgG (Sigma) in PBS 2% BSA at a dilution of 1:400 for HIF-1α and 1:600 for HIF-2α. 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 Laboratories), coverslipped and stored in the dark at 4ºC.

PARP assay

PARP activity in whole-cell extracts was measured with Universal Colorimetric PARP Assay Kit (Trevigen) according to the manufacturer's recommendations.

Figures

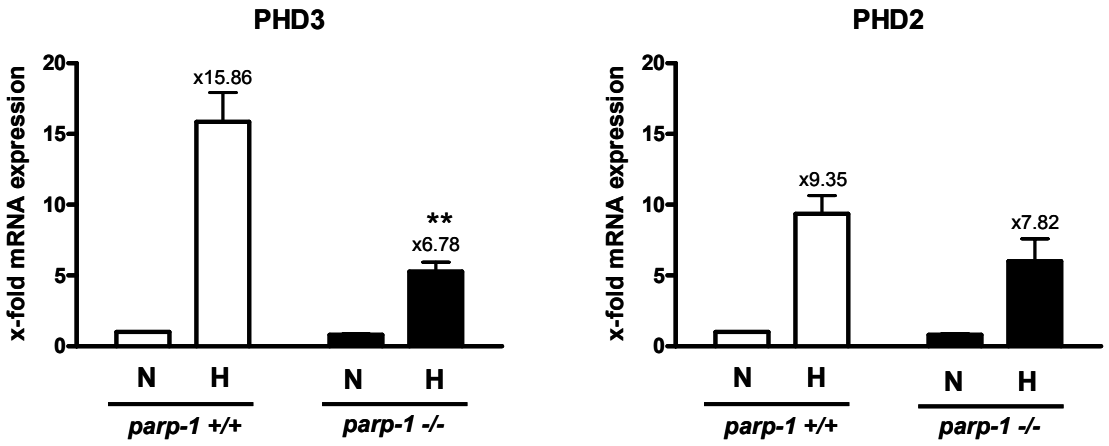

Figure 1. HIF-dependent response is impaired in *parp-1 -/-* immortalized MEFs. *parp-1 +/+* and *parp-1 -/-* immortalized MEFs were not treated (N, normoxia) or incubated upon hypoxia 1% (H) for 16 hours. PHD3 (left panel) and PHD2 (right panel) mRNA levels were determined by Quantitative RT-PCR. The level of mRNA in each sample was normalized by the level of Arbp mRNA and expressed as fold of the normoxia (untreated) value in *parp-1 +/+* cells, which was assigned with the value of 1. Columns represent mean of five independent experiments done in duplicate and the numeric value represents mean of hypoxic induction in each cell line. (**) means $p < 0.01$ with respect to the hypoxic induction in wild-type cells.

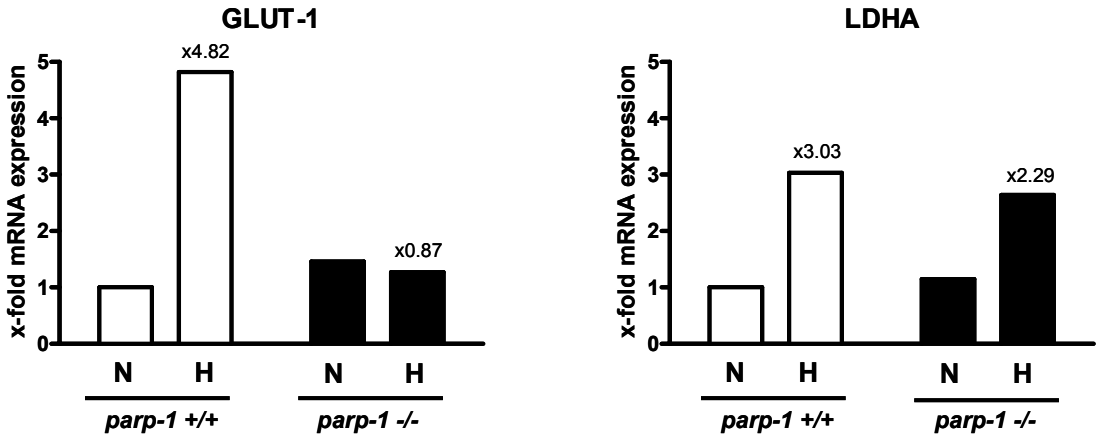

Figure 2. HIF-2-dependent gene expression is specifically impaired in *parp1 -/-* immortalized MEFs. *parp-1 +/+* and *parp1 -/-* immortalized MEFs were treated as in Figure 1 and GLUT-1 (left panel) and LDHA (right panel) mRNA levels were determined by Quantitative RT-PCR. The level of mRNA in each sample was normalized by the level of Arbp mRNA and expressed as fold of the normoxia (untreated) value in wild-type cells, which was assigned with the value of 1. Numeric value represents hypoxic induction in each cell line.

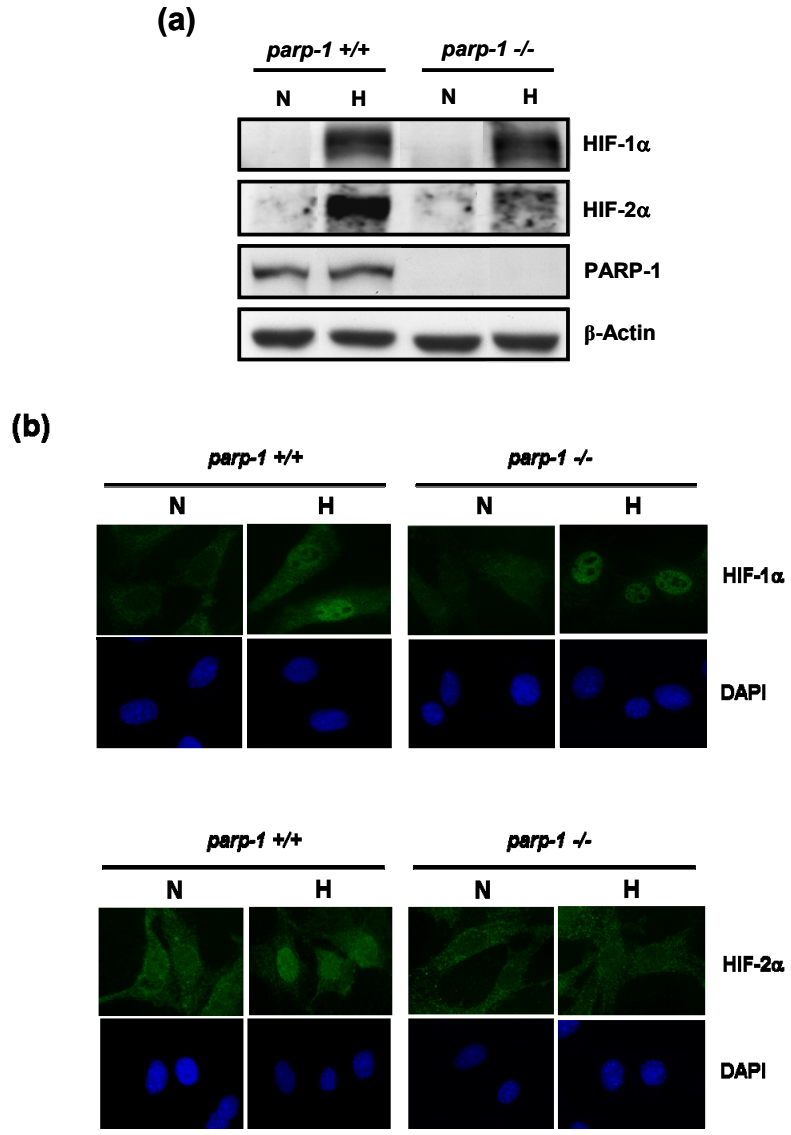
(a)
parp-1 +/+
parp-1 -/-
N
H
N
H
HIF-1α
HIF-2α
PARP-1
β-Actin
(b)
parp-1 +/+
parp-1 -/-
N
H
N
H
HIF-1α
DAPI
parp-1 +/+
parp-1 -/-
N
H
N
H
HIF-2α
DAPI
(c)
HIF-1α
x-fold mRNA expression
1.00
0.75
0.50
0.25
0.00
***
x0.69
parp-1 +/+
parp-1 -/-
HIF-2α
x-fold mRNA expression
1.00
0.75
0.50
0.25
0.00
***
x0.16
parp-1 +/+
parp-1 -/-

Figure 3. HIF-2α expression is decreased in PARP-1 deficient immortalized MEFs. (a-b) HIF-2α protein accumulation by hypoxia is greatly reduced in *parp-1 -/-* immortalized MEFs. **(a)** Western Blots of whole-cell extracts of *parp-1 +/+* and *parp1 -/-* immortalized MEFs treated as previously described were performed in order to look for HIF-1α and HIF-2α. PARP-1 was used as an internal control to confirm the specific character of cells and β-actin to check for protein loading. **(b)** Immunofluorescence staining of *parp-1 +/+* and *parp1 -/-* immortalized MEFs was achieved after 16 hours without treatment (N, normoxia) or incubated upon hypoxia (H, 1% O_2) for HIF-1α and HIF-2α. A representative image from 3 independent experiments is shown. **(c)** HIF-2α mRNA is greatly down-regulated in *parp1 -/-* immortalized MEFs. HIF-1α (left panel) and HIF-2α (right panel) mRNA levels were determined by Quantitative RT-PCR. The level of mRNA in each sample was normalized by the level of Arbp mRNA and expressed as fold of the value in wild-type cells, which was assigned with the value of 1. Columns represent mean of at least five independent experiments done in duplicate. Numeric value represents mean of results obtained. (***) means $p < 0.001$ with respect to wild-type cells.

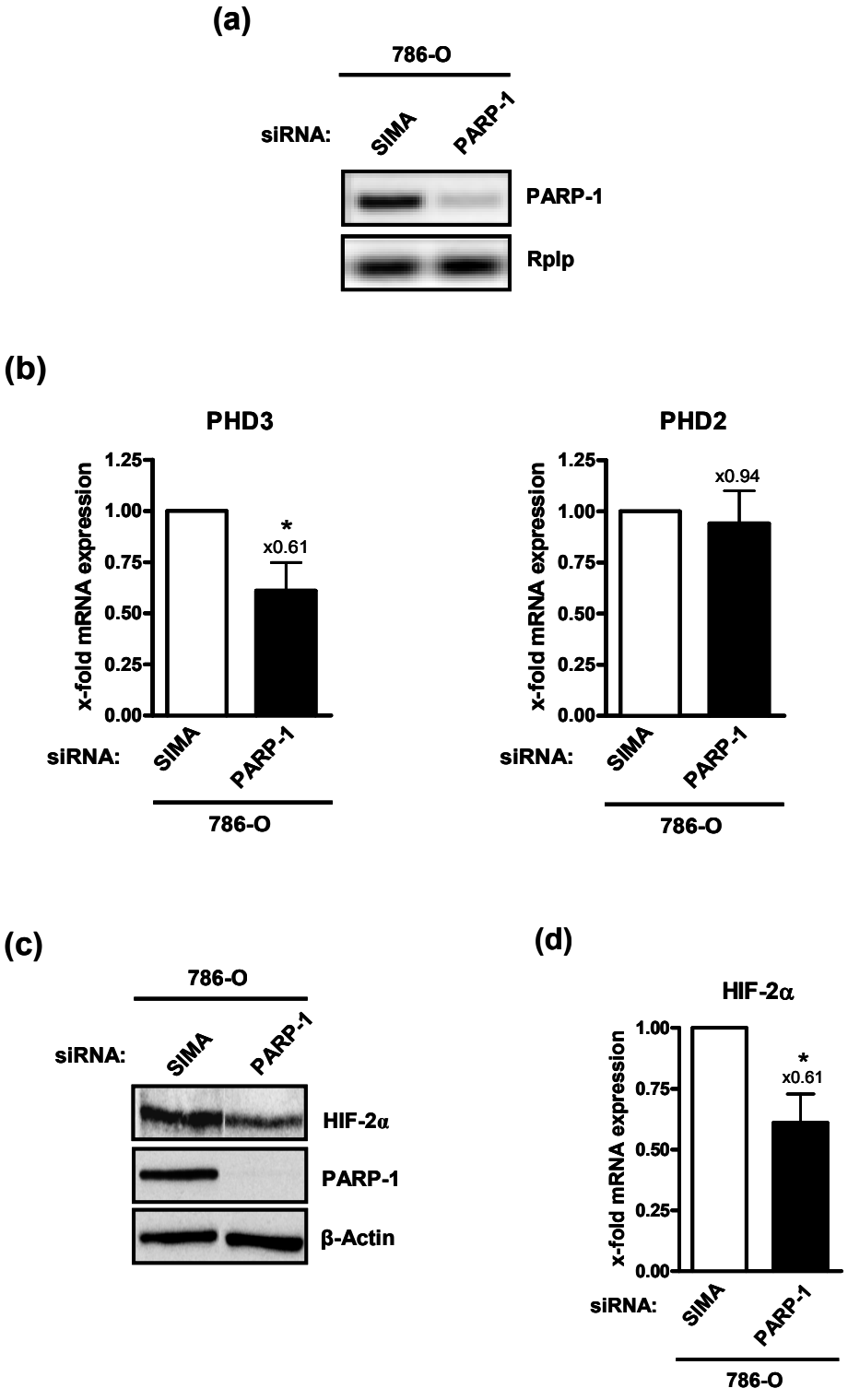
(a)
786-O
siRNA:
SIMA
PARP-1
PARP-1
Rplp
(b)
PHD3
x-fold mRNA expression
1.25
1.00
0.75
0.50
0.25
0.00
*
x0.61
siRNA:
SIMA
PARP-1
786-O
PHD2
x-fold mRNA expression
1.25
1.00
0.75
0.50
0.25
0.00
x0.94
siRNA:
SIMA
PARP-1
786-O
(c)
786-O
siRNA:
SIMA
PARP-1
HIF-2α
PARP-1
β-Actin
(d)
HIF-2α
x-fold mRNA expression
1.00
0.75
0.50
0.25
0.00
*
x0.61
siRNA:
SIMA
PARP-1
786-O

Figure 4. *parp-1* gene silencing reduces HIF-2 transcriptional activity and HIF-2α protein and mRNA levels. (a) PARP-1 siRNA validation. 786-O cells were transiently transfected with an irrelevant siRNA (SIMA) or PARP-1 siRNA, and PARP-1 mRNA levels were determined by Reverse Transcription PCR as described in Materials and methods. Rplp mRNA was used to check for equal loading sample. **(b)** PHD3 expression is decreased after PARP-1 knockdown with siRNA. 786-O cells were transiently transfected with an irrelevant siRNA (SIMA) or PARP-1 siRNA, and PHD3 (left panel) and PHD2 (right panel) mRNA levels were determined by Quantitative RT-PCR. The level of mRNA in each sample was normalized by the level of Rplp mRNA and expressed as fold of the value in SIMA transfected cells, which was assigned with the value of 1. Columns represent mean of three independent experiments done in duplicate and the numeric value represents mean of results obtained. (*) means $p < 0.05$ with respect to SIMA transfected cells. **(c)** HIF-2α protein accumulation is reduced in 786-O cell line by siPARP-1. Western Blots of whole-cell extracts of 786-O cells transiently transfected with an irrelevant siRNA (SIMA) or PARP-1 siRNA were achieved after the same experimental conditions described above in (b). Immunoblotting was performed against HIF-2α. PARP-1 was used to check that PARP-1 has been effectively silenced, and β-actin to check for protein loading. **(d)** HIF-2α mRNA is down-regulated after PARP-1 ablation with siRNA. Experimental conditions were the same as described above. HIF-2α mRNA was determined by Quantitative RT-PCR and the level of mRNA in each sample was normalized by the level of Rplp mRNA and expressed as fold of the value in SIMA transfected cells, which was assigned with the value of 1. Results represent the average of three independent experiments and numeric value represents mean of results obtained. (*) means $p < 0.05$ with respect to SIMA transfected cells.

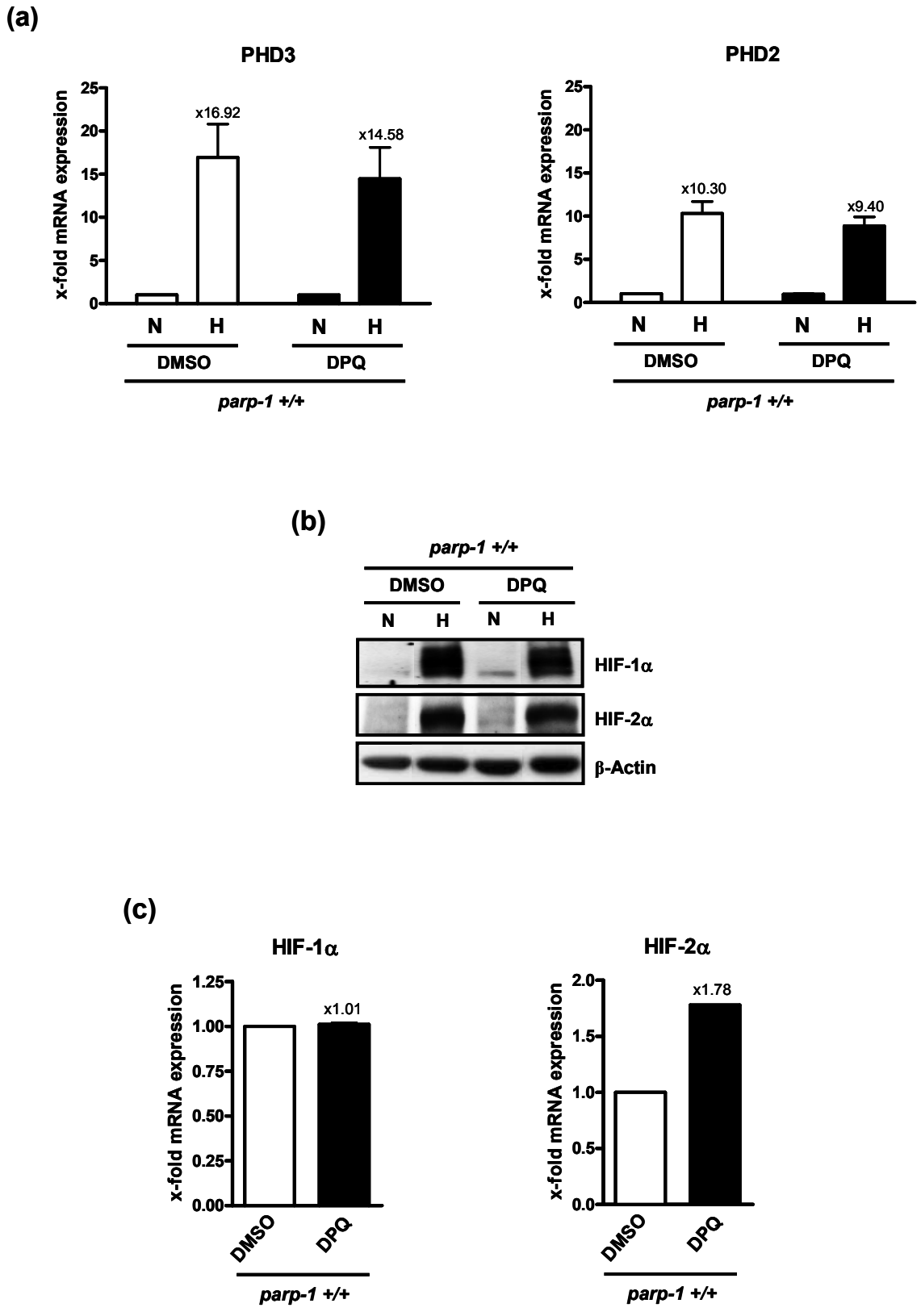
(a)
PHD3
x-fold mRNA expression
25
20
15
10
5
0
x16.92
x14.58
N
H
N
H
DMSO
DPQ
parp-1 +/+
PHD2
x-fold mRNA expression
25
20
15
10
5
0
x10.30
x9.40
N
H
N
H
DMSO
DPQ
parp-1 +/+
(b)
parp-1 +/+
DMSO
DPQ
N
H
N
H
HIF-1α
HIF-2α
β-Actin
(c)
HIF-1α
x-fold mRNA expression
1.25
1.00
0.75
0.50
0.25
0.00
x1.01
DMSO
DPQ
parp-1 +/+
HIF-2α
x-fold mRNA expression
2.0
1.5
1.0
0.5
0.0
x1.78
DMSO
DPQ
parp-1 +/+

Figure 5. Inhibition of PARP catalytic activity doesn't reduce HIF-2 transcriptional activity, neither HIF-2α protein and mRNA levels. (a) Hypoxia-induced PHD3 expression is not affected by the PARP inhibitor DPQ. *parp-1 +/+* immortalized MEFs were not treated (N, normoxia) or incubated upon hypoxia (H, 1%) for 16 hours in presence of DMSO or DPQ after 2 hours of pre-incubation. PHD3 (left panel) and PHD2 (right panel) mRNA levels were determined by Quantitative RT-PCR. The level of mRNA in each sample was normalized by the level of Arbp mRNA, and expressed as fold of the normoxia (untreated) value in cells treated with DMSO, which was assigned with the value of 1. Columns represent mean of two independent experiments done in duplicate. Numeric value represents mean of hypoxic induction in each condition. **(b)** HIF-2α protein induction by hypoxia is not affected by the PARP inhibitor DPQ. Western Blots of whole-cell extracts of *parp-1 +/+* immortalized MEFs were achieved after the same experimental conditions described above in (a). Immunoblotting was performed against HIF-1α and HIF-2α. β-actin was used to check for protein loading. **(c)** HIF-2α mRNA is not down-regulated by the PARP inhibitor DPQ. Experimental conditions were the same as described above. HIF-1α and HIF-2α mRNA was determined in *parp-1 +/+* immortalized MEFs cells by Quantitative RT-PCR. The level of mRNA in each sample was normalized by the level of Arbp mRNA, and expressed as fold of the value in cells treated with DMSO, which was assigned with the value of 1. Numeric value represents mean of results obtained.

Acknowledgements

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CONCLUSIONES

B1. Las células deficientes en PARP-1 presentan una activación defectuosa de HIF, debido especialmente al efecto sobre la expresión de mRNA de la subunidad HIF-2α. Por tanto, la ausencia de PARP-1 afecta también a la expresión de la proteína HIF-2α, a su translocación nuclear y a los genes dependientes de HIF-2.

B2. El silenciamiento de PARP-1 mediante siRNA en células tumorales tiene el mismo efecto que la eliminación genética de PARP-1 sobre la expresión de HIF-2α y la activación de HIF-2.

B3. La inhibición de la actividad enzimática de PARP-1 no afecta a los niveles de expresión de HIF-2α.

En función de los anteriores puntos, PARP-1 tiene un papel activo en la respuesta celular a la hipoxia tanto en células normales como tumorales, y su papel regulador podría tener importantes repercusiones en el diseño de nuevas estrategias encaminadas a minimizar la expresión de HIF en células tumorales.

SUPPLEMENTARY UNPUBLISHED RESULTS *B*

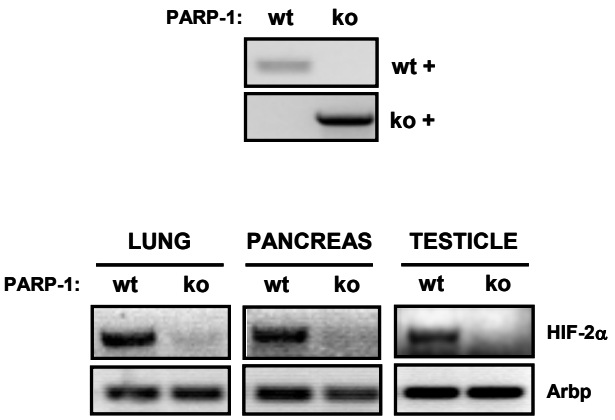

Figure B1. Preliminary results indicate that HIF-2α mRNA is down-regulated in several PARP-1 knockout murine tissues. Wild-type (wt) and knockout (ko) PARP-1 mice were genotyped for PARP-1 (upper panel) and HIF-2α mRNA levels were determined by Reverse Transcription PCR (lower panel) in different normoxic tissues: lung, pancreas and testicle. The level of mRNA in each sample was normalized by the level of *Arbp* mRNA.

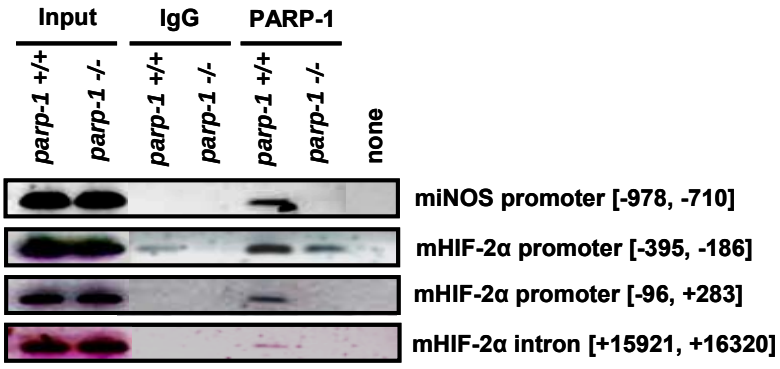

Figure B2. Binding of PARP-1 to HIF-2α gene regulatory sequences. For ChIP (Chromatin Immunoprecipitation) analysis, chromatin from wild-type (+/+) and PARP-1 knock-out (-/-) MEFs was fixed with formaldehyde and immunoprecipitated initially with control IgGs (IgG) and then overnight with polyclonal anti-PARP-1 antibody (PARP-1, Alexis Biochemicals), following the protocol described by (Pescador, Cuevas et al. 2005). After DNA purification, the presence of the selected potential regulatory sequences (evolutionary conserved regions) was assessed by PCR amplification of the region -395 to -186 (mHIF-2α promoter [-395, -186]) and -96 to +283 (mHIF-2α promoter [-96, +283]) within the murine HIF-2α promoter, and +15921 to +16320 (mHIF-2α intron [+15921, +16320]) of an intron of murine HIF-2α gene. As positive control, we used primers framing the region -978 to -710 of the murine iNOS promoter (miNOS promoter [-978, -710]). Input corresponds to PCR amplified products from sample of fragmented genomic DNA before immunoprecipitation. None, PCR products amplification without template.

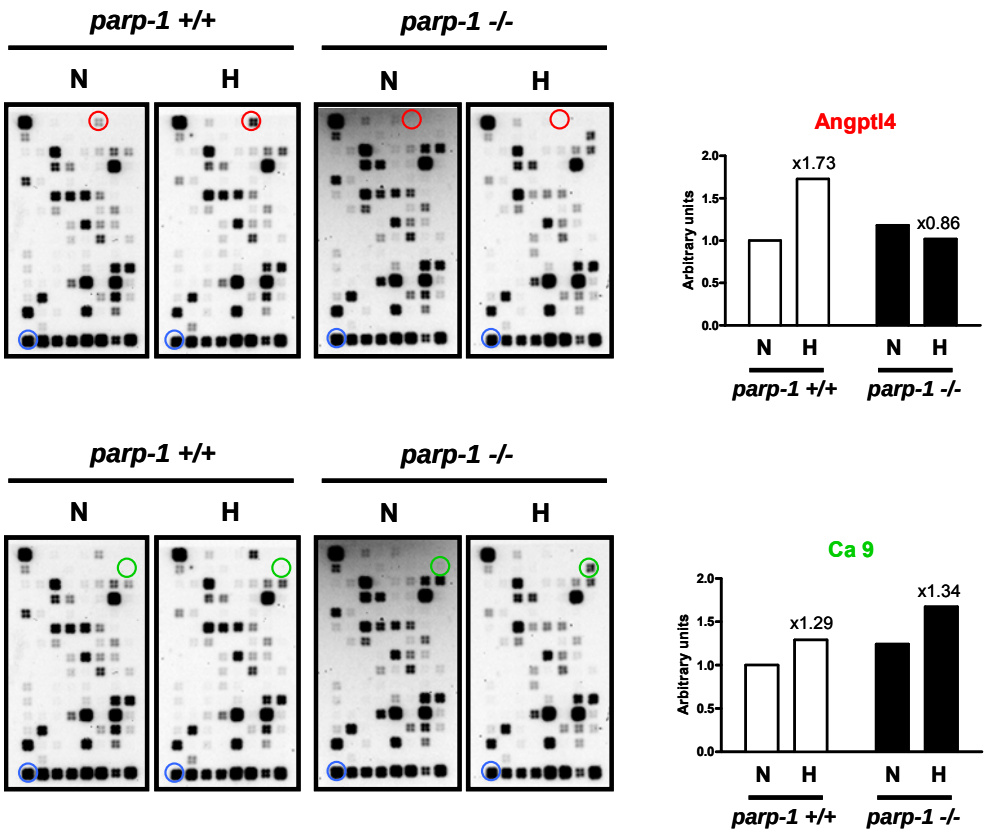

Figure B3. Hypoxic response is affected in *parp-1 -/-* MEFs. *parp-1* +/+ and *parp-1 -/-* immortalized MEFs were not treated (N, normoxia) or incubated upon hypoxia 1% (H) for 16 hours. Immediately after treatments, cells were harvested for total RNA extraction with RNeasy Mini Kit from Qiagen. Total RNA was quantified, integrity was tested by gel electrophoresis, and was used for Oligo GEArray expression analysis (SuperArray Bioscience Corporation) according to the manufacturer's instructions. Differences for two genes, *Angptl4* (*angiopoietin-like 4*, upper panel) and *Ca 9* (*carbonic anhydrase 9*, lower panel), were analysed by densitometry and represented graphically. The signal for each gene (red or green circles, respectively) was normalized by the signal for the *Rps27a* housekeeping gene (blue circles) and expressed as fold of the normoxia (untreated) value in *parp-1* +/+ cells, which was assigned with the value of 1. Numeric value represents hypoxic induction in each cell line.

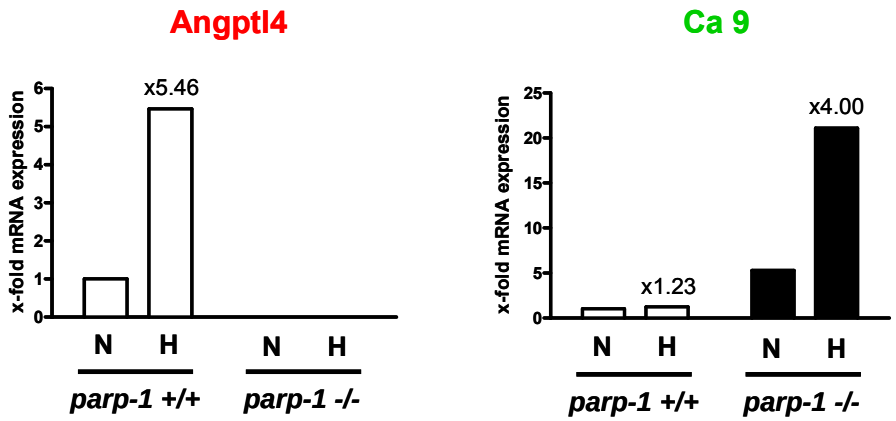

Figure B4. Hypoxia-related gene expression of *Angptl4* and *Ca 9* is affected in *parp-1 -/-* MEFs. *Angptl4* (left panel) and *Ca 9* (right panel) mRNA levels were determined by Quantitative RT-PCR from total RNA used in Oligo GEArray system. The level of mRNA in each sample was normalized by the level of *Arbp* mRNA and expressed as fold of the normoxia (untreated) value in wild-type cells, which was assigned with the value of 1. Numeric value represents hypoxic induction in each cell line.

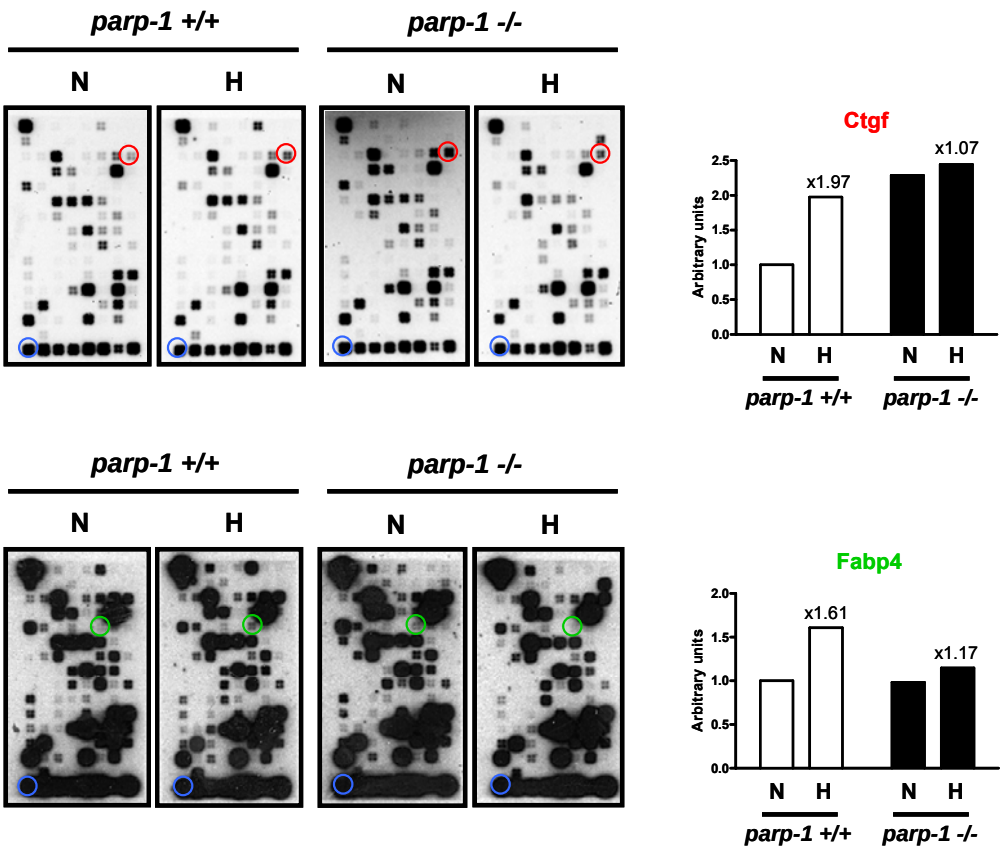

Figure B5. Hypoxic-response of *Ctgf* and *Fabp4* is affected in *parp-1 -/-* MEFs. Differences obtained by Oligo GEArray system in two genes, *Ctgf* (*connective tissue growth factor*, upper panel) and *Fabp4* (*fatty acid-binding protein 4*, lower panel) for *parp-1* +/+ and *parp-1* -/- immortalized MEFs not treated (N, normoxia) or incubated upon hypoxia 1% (H) for 16 hours, were analysed by densitometry and represented graphically. The signal for each gene (red or green circles, respectively) was normalized by the signal for the *Rps27a* housekeeping gene (blue circles) and expressed as fold of the normoxia (untreated) value in *parp-1* +/+ cells, which was assigned with the value of 1. Numeric value represents hypoxic induction in each cell line.

VI. DISCUSIÓN

DISCUSIÓN

PARP-1 está implicada en numerosas funciones celulares que han sido enumeradas y comentadas previamente en la introducción. En esta tesis, nos hemos centrado en dos de ellas, si cabe, las principales: la reparación del DNA y la transcripción génica. En concreto, durante la primera parte hemos estudiado la regulación que PARP-1 ejerce sobre un componente clave del reconocimiento y de la señalización de DSBs, *ATM*. La reparación del DNA juega un papel fundamental en el mantenimiento de la integridad genómica, previniendo los procesos de carcinogénesis, por lo que el estudio de la interacción de PARP-1 y ATM es clave para profundizar en el conocimiento de los mecanismos básicos implicados en la transformación celular hacia la neoplasia.

Otro elemento de gran interés en el desarrollo de un tumor es el status del factor de transcripción *HIF*, ya que la hipoxia tumoral determina en gran medida la evolución del proceso de carcinogénesis. Durante la segunda parte de la tesis hemos querido introducirnos en los mecanismos de regulación de HIF a través de PARP-1 como un posible regulador transcripcional.

En resumen, en esta tesis hemos avanzado en dos aspectos de la biología de PARP-1, orientando el trabajo hacia un mejor entendimiento del *desarrollo tumoral*, basándonos en los cambios en el microambiente tumoral relacionados con la inestabilidad en el genoma y la hipoxia.

A. Interacción entre ATM y PARP-1 en respuesta al daño en el DNA y sensibilización de células deficientes en ATM mediante inhibición de PARP

Los mecanismos de respuesta al daño en el DNA son esenciales para el mantenimiento de la integridad genómica. Las células de mamíferos han desarrollado una intrincada red de señalización basada en la detección de lesiones en el DNA por proteínas sensoras y en la transducción de esta señal hasta proteínas efectoras que detienen de manera transitoria la progresión del

ciclo celular y permiten la reparación del DNA. Una cuestión importante con respecto a la función de PARP-1 ha sido si la poli(ADP-ribosil)ación modula la señalización inmediata del daño en el DNA (Haince, Kozlov et al. 2007).

La implicación de PARP-1 en la ruta de reparación por excisión de bases (BER) se ha establecido en ratones con inactivación génica de PARP-1. El tratamiento de ratones *parp-1 -/-* con agentes que disparan la ruta BER, como agentes alquilantes o γ-irradiación, desencadena una extrema sensibilidad y una alta inestabilidad genómica (de Murcia, Niedergang et al. 1997). PARP-1 también se ha asociado con recombinación homóloga (HR), ya que la ausencia de PARP-1 supone un aumento en el intercambio de cromátidas hermanas y en la formación de micronúcleos (de Murcia, Niedergang et al. 1997; Wang, Stingl et al. 1997). Sin embargo, parece que PARP-1 no es necesaria para el propio mecanismo de HR debido a que los *foci* de Rad51 se forman normalmente en células deficientes en PARP-1 y los daños de cadena doble (DSBs) del DNA (inducidos por I-SceI) son reparados en células en las que se ha inhibido PARP (Schultz, Lopez et al. 2003). Además, se ha descrito que PARP-1 interviene, junto con el complejo XRCC1/DNA ligasa III, en una ruta de NHEJ independiente de DNA-PK/Artemis/XRCC4/ligasa IV que puede estar funcionando como una ruta alternativa de reserva de NHEJ (Audebert, Salles et al. 2004). Por otro lado, PARP-1 puede estar actuando directamente sobre el propio mecanismo de NHEJ a través de DNA-PK (Ruscetti, Lehnert et al. 1998). Por tanto, parece evidente que PARP-1 está relacionada no sólo con la reparación de SSBs sino también con la de DSBs (Benjamin and Gill 1980; Weinfeld, Chaudhry et al. 1997).

A pesar de su función en reparación del DNA, PARP-1 no es esencial para la supervivencia celular o la prevención de la tumorigénesis, ya que los ratones PARP-1 knockout son viables, fértiles y no desarrollan tumores (Wang, Auer et al. 1995; de Murcia, Niedergang et al. 1997; Masutani, Nozaki et al. 1999; Masutani, Suzuki et al. 1999; Conde, Mark et al. 2001). Sin embargo, la doble mutación en el ratón de PARP-1 y ATM origina un alto nivel de muerte celular y letalidad embrionaria. Como el período de embriogénesis es uno de los más sensibles al daño en el DNA por la gran tasa de proliferación y la falta

de puntos de control del ciclo celular (Snow and Bennett 1978; Heyer, MacAuley et al. 2000), estos resultados sugieren que PARP-1 y ATM actúan sinérgicamente en rutas que monitorizan o reparan el daño en el DNA durante el desarrollo murino (Menisser-de Murcia, Mark et al. 2001). Es decir, el fenotipo sinérgico de los dobles mutantes PARP-1/ATM lleva a pensar en una relación funcional entre ambas proteínas. De acuerdo con lo anteriormente expuesto, la línea celular HT144, deficiente en ATM, es muy sensible al inhibidor de PARP, ANI (Figura 4B). Otros trabajos también han descrito esta hipersensibilidad de las células A-T a un inhibidor de PARP (McCabe, Turner et al. 2006), y que células deficientes en PARP-1 son hipersensibles a un inhibidor específico de ATM (Bryant and Helleday 2006).

Tanto PARP-1 como ATM intervienen en respuesta a roturas de cadena del DNA, resultando en la inducción de una red de señalización responsable de la recuperación del DNA y de la supervivencia celular. Aparte del efecto sinérgico en ratones doble mutantes, PARP-1 y ATM comparten una serie de similitudes que sugieren que ambas proteínas podrían formar parte de un mismo mecanismo de señalización relacionado con las lesiones en el DNA: PARP-1 y ATM son proteínas nucleares de múltiples dominios con el mismo patrón de expresión y numerosos sustratos celulares, y sus actividades son inducidas tras el daño en el DNA (Schreiber, Dantzer et al. 2006).

Además, la actividad de ATM *in vitro* aumenta en presencia del polímero (PAR) (Goodarzi and Lees-Miller 2004), por lo que el PAR podría influir sobre la cascada de fosforilación iniciada por ATM en respuesta a DSBs. Sin embargo, este mismo trabajo observa que la inhibición de PARP no tiene un efecto significativo sobre los niveles de autofosforilación de ATM o de fosforilación de sustratos dependiente de ATM, inducidos por IR. Por el contrario, nosotros hemos visto que la ausencia de polímero disminuye la autofosforilación de ATM (Figura A1) en respuesta a IR, mientras que otro estudio independiente apoya nuestros resultados al mostrar que la inhibición de PARP reduce la fosforilación dependiente de ATM de p53, H2AX y SMC1 después del daño en el DNA producido por IR (Haince, Kozlov et al. 2007). Por otro lado, se ha descrito que el tratamiento con NCS (neocarcinostatina, compuesto que introduce DSBs en

el DNA) aumenta significativamente los niveles de fosforilación de H2AX y p53 en MEFs *parp-1 -/-* (Watanabe, Fukazawa et al. 2004). Pero mientras que la IR produce múltiples lesiones en proporción a la dosis de radiación, entre ellas, daño oxidativo de bases, SSBs y DSBs (Noel, Giocanti et al. 2003), NCS actúa introduciendo DSBs de manera limpia en el DNA. Por lo que es posible que las proteínas activadas tras el tratamiento con IR, así como el mecanismo de regulación establecido no sea totalmente extrapolable con el caso de la NCS. Otra posibilidad es que la activación de ATM sea una consecuencia del daño no reparado y acumulado en ausencia de PARP-1 tras NCS y no un efecto directo de PARP-1 sobre la activación de ATM. Como parte del mismo estudio, PARP-1 inhibe la actividad quinasa de ATM *in vitro* de forma dependiente del DNA (Watanabe, Fukazawa et al. 2004), aunque este efecto no depende de la poli(ADP-ribosil)ación ya que el sustrato de PARP-1, NAD^+, no fue incluido en el ensayo. Por tanto, surge la duda de si la inhibición de ATM por PARP-1 se debe a un efecto competitivo de ambas proteínas por el DNA, y no a una relación funcional directa entre ellas; de hecho, la inhibición de la actividad de ATM por PARP-1 en presencia del DNA aumenta con la cantidad de PARP-1. Nuestro ensayo quinasa de ATM *in vitro*, sin embargo, muestra que la actividad de ATM procedente de células knockout para PARP-1 o de células tratadas con inhibidores de PARP no aumenta tras IR con respecto a su control (Figura 2C). Relacionado directamente con esta observación, los MEFs derivados de ratones knockout para PARP-1 tratados con IR, presentan unos niveles inferiores de fosforilación de H2AX que los MEFs derivados de ratones *wild-type* (Figura A2). En vista de todos estos datos, no podemos obviar el hecho de que el estudio del efecto del PAR y de la presencia de PARP-1 sobre la activación de ATM ha originado resultados contradictorios, por lo que es necesario un análisis más profundo que determine el motivo de dicha discrepancia.

Otros resultados a favor de la interacción funcional de ATM con PARP-1, es la presencia del polímero en DSBs marcadas con H2AX fosforilada (Tartier, Spenlehauer et al. 2003; Haince, Kozlov et al. 2007), aunque también uno de estos trabajos muestra que la distribución de estos foci de γ-H2AX no es afectada por un inhibidor de PARP. Es más, en este trabajo, la fosforilación de

H2AX inducida por irradiación es independiente del estado de PARP-1 (Tartier, Spenlehauer et al. 2003). Por otro lado, se ha descrito que PARP-1 interacciona directamente con γ-H2AX en respuesta a IR (Du, Gu et al. 2006). Una vez más, estos resultados de no fácil interpretación nos hacen pensar que existe una relación funcional y una probable regulación de la activación de ATM por PARP-1 en unas condiciones concretas, pero que existen variables, bien sea la magnitud del daño en el DNA, la cinética de respuesta al mismo, la presencia de otras proteínas, que impiden obtener una visión clara de la situación. Además, debemos tener en cuenta que PARP-1 no es el único miembro de la familia PARP cuya actividad es estimulada en respuesta a roturas del DNA, sino también PARP-2, aunque PARP-1 es la principal enzima responsable de la síntesis de polímero dependiente de daño en el DNA (Schreiber, Dantzer et al. 2006).

De manera recíproca, se ha llevado a cabo el estudio del efecto de ATM sobre la actividad de PARP (Schreiber, Dantzer et al. 2006). En este caso, la poli(ADP-ribosil)ación no es alterada por la ausencia de la proteína ATM ya que la actividad de PARP estimulada por irradiación o H_2O_2 es normal en células A-T y en tejidos de ratones deficientes en ATM. Independientemente, se ha descrito la activación sostenida de PARP-1 en células A-T debido a la más alta acumulación de daños en el DNA (Marecki and McCord 2002).

Continuando con la discusión de la relación PARP-1/ATM en respuesta a agentes genotóxicos, nosotros hemos observado una interacción física entre PARP-1 y ATM mediante experimentos de co-inmunoprecipitación (Figura 1a) y doble inmunofluorescencia indirecta (Figura 1b), siendo este complejo más evidente tras el daño en el DNA con el agente alquilante MNU o con IR. La formación de este complejo entre PARP-1 y ATM es además apoyado por otros grupos (Watanabe, Fukazawa et al. 2004; Haince, Kozlov et al. 2007). También se ha llevado a cabo el estudio de la interacción de ATM con poli(ADP)ribosa (Haince, Kozlov et al. 2007), con respecto a la cual, ya en trabajos previos se había descrito la colocalización de ATM y PAR en respuesta a irradiación (Tartier, Spenlehauer et al. 2003). En nuestro trabajo hemos mostrado cómo el polímero acompaña a ATM tras IR en experimentos de inmunoprecipitación de

ATM (Figura 2a) y en dobles inmunofluorescencias indirectas (Figura 2b). Nosotros hemos interpretado estos resultados como modificación de ATM por el polímero, ya que las condiciones de la inmunoprecipitación nos hacían pensar en una unión covalente del PAR a ATM. Sin embargo, en el trabajo al que se ha hecho referencia anteriormente (Haince, Kozlov et al. 2007), se describe la unión directa de ATM al polímero de forma no covalente. Es más, los autores identifican dos dominios putativos de unión a PAR dentro de la secuencia de ATM, uno N-terminal localizado en el dominio de asociación a cromatina de ATM, altamente conservado con respecto a la secuencia consenso; y otro en la región C-terminal, que parcialmente se solapa con el dominio PI3K de ATM. Por tanto, no descartamos esta otra interpretación de nuestros resultados, teniendo en cuenta que un motivo consenso de unión a PAR en una proteína (en este caso ATM), frecuentemente solapado con un dominio funcional, puede modular las propiedades funcionales de la misma (Schreiber, Dantzer et al. 2006). Varias proteínas de señalización o de reparación del daño en el DNA presentan motivos de unión al polímero de alta afinidad, entre otras XRCC1, DNA ligasa III, $p21^{Waf1}$ y p53 (Wesierska-Gadek, Bugajska-Schretter et al. 1996; Pleschke, Kleczkowska et al. 2000). Dos subunidades del heterotrímero DNA-PK, Ku70 y DNA-PKcs, también presentan estos motivos (Pleschke, Kleczkowska et al. 2000), y PARP-1 co-inmuprecipita con estas proteínas (Morrison, Smith et al. 1997; Ruscetti, Lehnert et al. 1998; Galande and Kohwi-Shigematsu 1999). Por otro lado, la unión específica de ATM al polímero no parece ser necesaria para la activación inicial de ATM (Haince, Kozlov et al. 2007) pero sí para el reclutamiento de ATM activada a sitios de daño en el DNA, y consecuente fosforilación de sustratos. Nuestros resultados, no obstante, sugieren que la autofosforilación de ATM tras IR también disminuye en presencia de un inhibidor de PARP (Figura A1).

Como se comentó en la introducción, la activación de ATM puede ser atribuida a una señal generada por un cambio estructural de la cromatina y no a una unión directa al DNA dañado (Canman, Lim et al. 1998; Bakkenist and Kastan 2003). Así, las moléculas de polímero unidas a la propia PARP-1 o a otras proteínas, podrían actuar como la señal de reconocimiento e iniciar la respuesta al daño en el DNA a través de la modulación de la ruta de

señalización de ATM. De acuerdo con este modelo, el dominio de unión al polímero en la región N-terminal de ATM, podría ser importante para reclutar ATM activada a las roturas del DNA (Haince, Kozlov et al. 2007). Sin embargo, no descartamos que se esté dando otro tipo de mecanismo de regulación mediado por PARP-1. Además, el hecho de que la fosforilación de sustratos de ATM disminuya significativamente en ausencia de PAR pero que todavía ocurra, sugiere que otros mecanismos redundantes podrían contribuir al reclutamiento de ATM a los sitios específicos de DNA dañado.

Para concluir con esta primera parte de la discusión ATM/PARP-1, se ha descrito que células deficientes en ATM presentan un defecto en la reparación de DSBs que se manifiesta por un 10% de roturas producidas por radiación ionizante sin reparar (Kuhne, Riballo et al. 2004; Riballo, Kuhne et al. 2004), como también hemos comprobado nosotros (Figura A3). La formación y velocidad de pérdida de *foci* de H2AX fosforilada (γ-H2AX) proporciona una técnica extraordinariamente sensible para monitorizar la formación y reparación de DSBs (Rothkamm and Lobrich 2003). Es importante tener en cuenta que estos estudios se llevan a cabo en células no replicativas, condición bajo la cual sólo las DSBs son marcadas como foci de γ-H2AX. La comparación del número de foci de H2AX-γ formados después de bajas dosis de radiación (1 mGy-2 Gy) ha sugerido que la mayoría de DSBs pueden ser monitorizadas por esta técnica. Mientras que la correlación quizá no sea una proporción exacta 1:1, está claro que el número de foci formados tras radiación ionizante (IR) es comparable al número previsto de DSBs introducidas. Así, la velocidad de pérdida de *foci* se correlaciona fuertemente con la velocidad de reparación de DSBs (Lobrich and Jeggo 2005). En base a lo anterior, cuando realizamos una cinética de reparación de DSBs, observamos que las células deficientes en ATM son defectuosas para la reparación de DSBs a partir de un tiempo aproximado de 4 horas y no en la etapa inicial post-irradiación (Figura A3). Esto indica que las células deficientes en ATM presentan un defecto concreto en la reparación de DSBs que son reparadas con una cinética lenta (Lobrich and Jeggo 2005). Por el contrario, la inhibición de PARP en células wild-type produce un defecto en la reparación a tiempos más cortos, alcanzando el mismo nivel de DSBs no reparadas que las células no tratadas con inhibidores

de PARP a tiempos largos, lo que no es sorprendente en función de la rápida formación de polímero tras IR (Haince, Kozlov et al. 2007). Aunque este hecho hace evidente una vez más la función de PARP en reparación de DSBs, por otra parte nos indica que no existe una colaboración entre PARP-1 y ATM en la reparación de DSBs, o lo que es lo mismo, no participan o señalizan para el mismo mecanismo de reparación de DSBs, lo que es apoyado por el hecho de que la inhibición de PARP sigue teniendo un efecto en células deficientes en ATM. De todas formas, este resultado no pone en entredicho el papel de PARP-1 en la activación de ATM, ampliamente discutido anteriormente.

Debido a su función en varios procesos de reparación del DNA, la inhibición de PARP-1 sensibiliza a las células a múltiples agentes que dañan el DNA, como radiación ionizante (IR) (Arundel-Suto, Scavone et al. 1991; Weltin, Holl et al. 1997; Bowman, White et al. 1998), inhibidores de la topoisomerasa I (Delaney, Wang et al. 2000; Bowman, Newell et al. 2001) o agentes alquilantes monofuncionales del DNA (Boulton, Pemberton et al. 1995; Bowman, White et al. 1998; Delaney, Wang et al. 2000; Tentori, Lacal et al. 2007). Por tanto, los inhibidores de PARP son potenciales quimioterapéuticos, cuando se utilizan en combinación con irradiación o con fármacos anticancerígenos de daño en el DNA (Curtin 2005). Dicha terapia combinada puede ser particularmente importante en el tratamiento de tumores con resistencia adquirida (Curtin, Wang et al. 2004; Cheng, Johnson et al. 2005). Pero además de su papel en terapia combinada antitumoral, los inhibidores de PARP por sí solos, son eficientes para tratar tumores deficientes en HR, como tumores defectivos en BRCA2 (Bryant, Schultz et al. 2005; Farmer, McCabe et al. 2005). Este hecho nos da pie a discutir a continuación el efecto de la inhibición de PARP-1 sobre la activación de ATM en ausencia de agentes genotóxicos (como IR), al contrario de lo discutido hasta ahora.

PARP-1 es importante para la supervivencia celular en ausencia de HR, probablemente por su función, junto con XRCC1, en la reparación de SSBs. Las SSBs espontáneas no reparadas, como en el caso de la inhibición de PARP, colapsan la horquilla de replicación formando DSBs, las cuales deben repararse por HR (Bryant and Helleday 2006). Si la maquinaria de HR es

defectuosa, estas células entran en un proceso de muerte celular. En este contexto, ATM se activa para señalizar la presencia de DSBs. Así, en células tratadas únicamente con inhibidores de PARP, se produce activación de ATM, tal y como indica la inmunofluorescencia indirecta de la fosforilación de H2AX (Figura 3). Previamente, ya habíamos observado que células tratadas con inhibidores de PARP (en ausencia de irradiación) presentaban una actividad quinasa de ATM significativamente superior en comparación con los respectivos controles no tratados (Figura 2C, paneles medio e inferior). Sin embargo, este efecto no era tan acusado en el caso de las células PARP-1 knockout (Figura 2C, panel superior), como también observamos para γ-H2AX (Figura A4). En este último caso, es de destacar que las células deficientes en PARP-1 tratadas con inhibidores de PARP no presentan un nivel tan alto de fosforilación de H2AX, lo que sugiere que el efecto que tienen los inhibidores de PARP sobre la formación de DSBs y consecuente activación de ATM depende de la presencia física de la proteína PARP-1. Una explicación puede ser que debido a la inhibición de su actividad, PARP-1 no puede automodificarse y favorecer su salida de las roturas del DNA que reconoció gracias a su dominio de unión al DNA. Esta retención de la proteína en el DNA junto con la incapacidad de reclutamiento de otras proteínas de reparación, bloquea la maquinaria de replicación asociada a la formación de DSBs (Bryant, Schultz et al. 2005).

ATR señaliza, principalmente, la parada de la horquilla de replicación (Bartek, Lukas et al. 2004), por lo que podría estar implicado en la señalización del daño del DNA originado por inhibidores de PARP. Sin embargo, la fosforilación de H2AX tras el tratamiento con inhibidores de PARP, es dependiente de ATM, ya que no se observa en células deficientes en ATM (Figura 3). Además, tras la inhibición de PARP, no se produce fosforilación de Chk1 (Bryant and Helleday 2006), la principal diana de ATR (Abraham 2001).

Por tanto, la activación de ATM como consecuencia del tratamiento con inhibidores de PARP, favorece la reparación de las DSBs originadas mediante HR e incluso una pequeña proporción, mediante NHEJ (Bryant and Helleday 2006). Como resultado, células deficientes en ATM presentan una mayor

cantidad de DSBs tras inhibición de PARP, quedando una pequeña proporción de estas DSBs sin reparar (Figura 4A). Así, la muerte celular es mayor en el caso de células deficientes en ATM tratadas con un inhibidor de PARP con respecto a células wild-type (Figura 4B). De hecho, el efecto sobre la muerte celular inducida mediante el uso de los inhibidores de PARP es comparable al producido por IR, mientras que el efecto es sinérgico cuando las células deficientes en ATM son tratadas con IR e inhibidores de PARP.

Se han encontrado mutaciones en el gen de ATM en leucemia prolinfocítica de célula T (Stilgenbauer, Schaffner et al. 1997; Rosenwald, Chuang et al. 2004), linfoma de la célula del manto (Stankovic, Weber et al. 1999; Stankovic, Hubank et al. 2004), y leucemia linfocítica crónica de célula B (Bullrich, Rasio et al. 1999; Schaffner, Stilgenbauer et al. 1999; Stankovic, Stewart et al. 2002; Austen, Powell et al. 2005). Tales casos serían candidatos ideales para una terapia con inhibidores de PARP, ya que las células con ATM mutada serían más sensibles a los inhibidores de PARP que las células con ATM, por lo que los efectos secundarios propios de los fármacos citotóxicos clásicos anticancerígenos, disminuirían (Bryant and Helleday 2006).

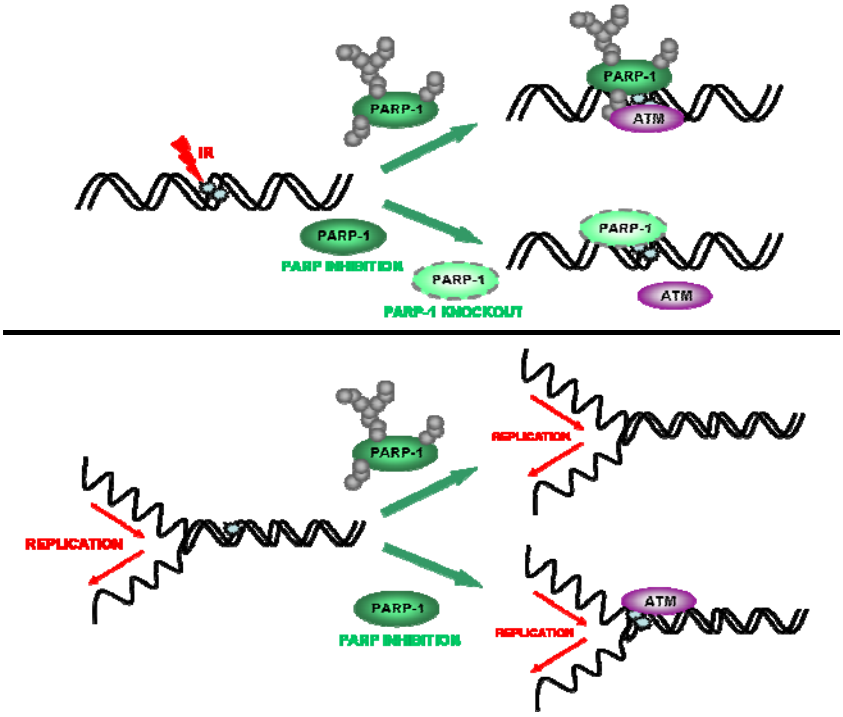

Figura VI 1. Interacción entre ATM y PARP-1 en respuesta a IR (panel superior) y activación de ATM tras inhibición de PARP (panel inferior).

En resumen, nuestro trabajo demuestra una clara asociación entre PARP-1 y ATM durante la respuesta a IR, siendo PARP-1 y su actividad necesarias para la correcta activación de ATM (Figura VI 1, panel superior). Por otro lado, y en principio como un mecanismo independiente del anterior, la inhibición de PARP da lugar a la activación de ATM como resultado de la formación de DSBs, haciendo a las células deficientes en ATM particularmente sensibles a los inhibidores de PARP (Figura VI 1, panel inferior).

B. Efecto específico de PARP-1 sobre la respuesta a hipoxia mediada por HIF

Tanto los niveles de expresión de HIF-1α como los de HIF-2α son altos en varios tumores (Talks, Turley et al. 2000; Hopfl, Ogunshola et al. 2004), lo que favorece el crecimiento y la expansión tumoral (Maynard and Ohh 2007) a través de mecanismos como la angiogénesis y la metástasis, y determina una respuesta deficiente a la quimioterapia y radioterapia. Por tanto, el estudio de la regulación de la subunidad HIF-α (y del factor de transcripción HIF) tiene un gran interés desde el punto de vista de avanzar en la búsqueda de nuevas dianas terapéuticas anti-tumorales.

La expresión de HIF-1α es ubicua y se ha sugerido que es la subunidad principal en la respuesta a hipoxia. El mRNA de HIF-2α también se expresa en una amplia variedad de tipos celulares, pero su expresión es mayor en algunos de ellos (Ema, Taya et al. 1997; Flamme, Frohlich et al. 1997; Tian, McKnight et al. 1997; Jain, Maltepe et al. 1998; Compernolle, Brusselmans et al. 2002; Rosenberger, Mandriota et al. 2002; Wiesener, Jurgensen et al. 2003). Sin embargo, en contraste a la expresión localizada en tejidos embrionarios y adultos, HIF-2α se expresa en muchos tumores humanos, incluyendo a aquellos asociados con la enfermedad de VHL (carcinomas de célula clara renal y hemangioblastomas) así como otros no relacionados con la enfermedad de VHL (carcinoma de mama, carcinoma de célula escamosa de cuello y cabeza, y carcinoma pulmonar de células no pequeñas) (Harris 2002).

Un trabajo anterior de nuestro laboratorio en un modelo de carcinogénesis dérmica (Martin-Oliva, Aguilar-Quesada et al. 2006) mostraba que los ratones knock-out para PARP-1 y los ratones wild-type tratados con inhibidores de PARP presentaban una disminución en el nivel de HIF-1α tras el tratamiento con los productos químicos DMBA y TPA. En el presente trabajo hemos observado un efecto diferente de PARP-1 sobre HIF-1α y HIF-2α tras hipoxia. Es más, la acumulación de HIF-2α y su actividad transcripcional inducidas por hipoxia disminuyen en gran medida en células deficientes en PARP-1. Sin embargo, PARP-1 no afecta a la expresión de HIF-1α en respuesta al estrés hipóxico. Varios estudios describen la regulación de HIF en condiciones de normoxia por un conjunto de estímulos como factores de crecimiento, hormonas o contactos célula-célula (Zelzer, Levy et al. 1998; Feldser, Agani et al. 1999; Richard, Berra et al. 2000; Thornton, Lane et al. 2000; Zhong, Chiles et al. 2000; Laughner, Taghavi et al. 2001; Sandau, Zhou et al. 2001; Naranjo-Suarez, Castellanos et al. 2003; Frede, Berchner-Pfannschmidt et al. 2007; Oh, Lee et al. 2008). Así, es evidente que la regulación de HIF es compleja y que otros factores aparte del O_2 pueden contribuir a la actividad de este factor de transcripción. Por tanto, pensamos que el efecto de PARP-1 sobre HIF-1α tras el tratamiento de los ratones con DMBA y TPA (donde PARP-1 y su actividad son fundamentales) tiene lugar a través de una ruta diferente de la que ocurre en hipoxia.

Desde hace algún tiempo, se está interesado en distinguir ente la función de HIF-1α y la de HIF-2α en transcripción génica, y cada vez son más numerosos los trabajos publicados acerca de diferencias fisiológicas y en cuanto al mecanismo entre ambas subunidades. Por tanto, parece que ya es bien aceptado que HIF-1α y HIF-2α llevan a cabo funciones complementarias y no totalmente solapantes. Por ejemplo, el modulador esencial de NF-κB (NEMO) interacciona exclusivamente con HIF-2α, promoviendo su actividad transcripcional al aumentar su unión con CBP/p300 (Bracken, Whitelaw et al. 2005). El factor redox Ref-1 aumenta la capacidad de unión al DNA de HIF-2α mediante una reacción de reducción de HIF-2α (pero no de HIF-1α) y este mecanismo está basado en la diferencia de un único aminoácido entre ambas subunidades dentro de las regiones básicas de unión al DNA N-terminales

(Lando, Pongratz et al. 2000). De forma similar, la sustitución de un único aminoácido entre HIF-1α y HIF-2α favorece una mayor hidroxilación por FIH-1 de la asparagina de HIF-1α, y por lo tanto, su menor actividad en varias líneas celulares (Bracken, Fedele et al. 2006). Continuando con otro ejemplo, el impacto específico de HIF-1α sobre la represión de la proteína Nbs1 (del complejo MRN) y por tanto, la reparación del DNA, es debido al estado de fosforilación de un residuo de treonina dentro del dominio PAS-B; al contrario que en HIF-1α, este residuo se encuentra fosforilado en HIF-2α, lo que impide la represión de Nbs1 por la misma (To, Sedelnikova et al. 2006).

En este contexto, nosotros hemos visto un efecto diferente de PARP-1 sobre la expresión del mRNA de HIF-1α y HIF-2α, y consecuentemente, sobre la correspondiente proteína y su actividad transcripcional, surgiendo PARP-1 (pero no su actividad catalítica) como un nuevo elemento en la regulación de la expresión de HIF-2α (Figuras 1-5 y Figura VI 2). De manera extrapolable con nuestras observaciones, un estudio reciente ha descrito que células tumorales en las que se ha eliminado de forma estable PARP-1, reducen sus niveles de expresión de GLUT-1 (Elser, Borsig et al. 2008), gen cuya expresión depende de HIF-1 y HIF-2. Independientemente, es necesario estudiar con mayor profundidad si PARP-1 actúa además a otro nivel de regulación de la actividad de HIF-2α tal y como sugieren los resultados de dicho trabajo, en el que se describe una unión directa entre HIF-2α y PARP-1 (Elser, Borsig et al. 2008).

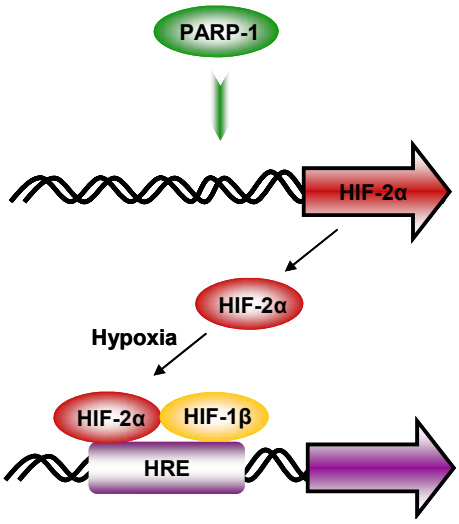

Figura VI 2. PARP-1 es fundamental para la correcta expresión de HIF-2α, que en condiciones de hipoxia se estabiliza y lleva a cabo su actividad transcripcional.

Para nuestro estudio hemos utilizado MEFs derivados de ratones wild-type y knockout para PARP-1. Previamente se ha publicado que en MEFs, HIF-2α es transcripcionalmente inactivo y se encuentra retenido en el citoplasma (Park, Dadak et al. 2003). Estos autores también mostraban que HIF-2α

endógeno escapa de la degradación proteica dependiente del O_2 y se acumula en MEFs inmortalizados en condiciones de normoxia, mientras que HIF-1α está sujeto a una regulación precisa por los niveles de O_2. Por el contrario, nuestros resultados claramente muestran que la inducción de ambas isoformas depende de la disponibilidad de O_2 (Figura 3a). Además, cuando examinamos la localización subcelular de HIF-1α y HIF-2α en nuestros MEFs wild-type tras hipoxia, obtenemos una acumulación nuclear de HIF-1α y HIF-2α con respecto a normoxia. Por el contrario, no pudimos encontrar HIF-2α en el núcleo de MEFs *parp-1* -/- después de la exposición a hipoxia debido a la ausencia de PARP-1 (Figura 3b). Estas aparentes discrepancias observadas entre ambos trabajos para HIF-2α de MEFs wild-type pueden deberse a diferencias en el método de inmortalización o a la especificidad del anticuerpo utilizado. Para evitar cualquier tinción no específica, nosotros hemos validado nuestro anticuerpo mediante siRNAs específicos de HIF-2α antes de usarlo en inmunofluorescencia.

La disminución en la inducción hipóxica de HIF-2α en ausencia de PARP-1 es, como hemos expuesto, consecuencia de la reducción en los niveles basales de mRNA. Para extrapolar el impacto de PARP-1 sobre la expresión de HIF-2α en un modelo *in vivo*, hemos analizado los niveles de mRNA de HIF-2α en diferentes tejidos de ratones *parp-1* +/+ y *parp-1* -/- en condiciones de normoxia. Resultados preliminares en pulmón, páncreas y testículo indican que en ratones *parp-1* -/- se produce una fuerte bajada del mRNA de HIF-2α (Figura B1). Sin embargo, dicha bajada no se produce ni en la misma proporción, ni en todos los tejidos analizados (datos no mostrados), y estas diferencias deben responder a un mecanismo regulador tejido-específico en el que PARP-1 está implicado.

Los ratones knock-out para HIF-2α presentan una elevada generación de especies reactivas de oxígeno (ROS) y una respuesta defectuosa al estrés oxidativo (Scortegagna, Ding et al. 2003). Los ratones knock-out para PARP-1

tiene un serio defecto en la respuesta al daño en el DNA y un aumento de la inestabilidad genómica (Shall and de Murcia 2000). Una idea interesante sería estudiar mecanismos comunes en ambos ratones knock-out para comprobar la existencia de fenotipos solapantes entre ellos.

El por qué PARP-1 tiene un efecto específico sobre la regulación de HIF-2α todavía lo desconocemos. El papel de PARP-1 en regulación transcripcional es un aspecto importante de sus funciones biológicas (revisado en (Aguilar-Quesada, Munoz-Gamez et al. 2007)), pudiendo actuar como cofactor junto con otros factores relacionados con la transcripción, uniéndose a regiones activadoras/promotoras (Kraus and Lis 2003). En esta línea, una posibilidad interesante con respecto a la especificidad del efecto de PARP-1 sobre HIF-2α se basa en la asociación de PARP-1 con el factor de transcripción Sp1. Se ha descrito que Sp1 promueve la transcripción de HIF-2α durante la diferenciación de adipocitos (Wada, Shimba et al. 2006) y a su vez, la expresión de Sp1 se encuentra disminuida en células *parp-1 -/-* (Zaniolo, Desnoyers et al. 2007). Además, ensayos de co-inmunoprecipitación en células *parp-1* +/+ han mostrado que PARP-1 interacciona físicamente con Sp1 de forma independiente del DNA (Zaniolo, Desnoyers et al. 2007).

PARP-1 promueve la transcripción dependiente de activador interaccionando con un conjunto cada vez más numeroso de factores de transcripción, como AP-2, B-Myb, YY-1, Oct-1, NF-κB, y p53 (Oei, Griesenbeck et al. 1997; Nie, Sakamoto et al. 1998; Hassa and Hottiger 1999; Kannan, Yu et al. 1999; Oliver, Menissier-de Murcia et al. 1999; Cervellera and Sala 2000; Wesierska-Gadek, Wojciechowski et al. 2003). Como en nuestro caso, esta función reguladora de la transcripción requiere a PARP-1 nuclear, pero no depende de su actividad enzimática. El análisis *in silico* de la región 5' adyacente al gen HIF-2α ha puesto de manifiesto varios sitios de unión putativos para factores de transcripción como YY-1, AP-1, AP-2 o E2F-1. Por lo tanto, también es posible que la interacción de PARP-1 con alguno de estos factores de transcripción sea responsable, al menos parcialmente, del impacto de PARP-1 sobre la expresión de HIF-2α. De hecho, mediante inmunoprecipitación de cromatina, nosotros hemos visto que PARP-1 se

encuentra unido a diferentes regiones potencialmente reguladoras del promotor y del intrón del gen HIF-2α (Figura B2).

Otra posibilidad es que PARP-1 contribuya a la expresión de HIF-2α a través de la regulación de las NAD(P)H oxidasas, ya que el silenciamiento de la NAD(P)H oxidasa Nox4 disminuye la expresión del mRNA de HIF-2α y así su actividad transcripcional (Maranchie and Zhan 2005).

Nuestros resultados claramente demuestran que la ausencia de PARP-1 afecta a HIF-2 mientras que la inhibición de su actividad catalítica no tiene efecto. Así, queda también por aclarar el mecanismo por el que la actividad de PARP y PARP (la propia proteína) actúan de manera diferente sobre la respuesta a hipoxia. No obstante, la misma dualidad se ha visto en el caso del efecto de PARP-1 sobre la actividad de distintos factores de transcripción, incluyendo NF-κB (Hassa, Covic et al. 2001). Así, todavía hay muchas preguntas sin contestar acerca de la función específica de PARP-1 en la expresión y función de HIF-2α.

No obstante, se ha descrito que los inhibidores de PARP tienen efectos antiangiogénicos (Rajesh, Mukhopadhyay et al. 2006; Rajesh, Mukhopadhyay et al. 2006), aunque los mecanismos responsables aún se desconocen. La señalización angiogénica se debe fundamentalmente a VEGF, y es posible que los inhibidores de PARP afecten a la expresión de VEGF a través de otros factores de transcripción inducidos por hipoxia relacionados con PARP-1. Además de HIF, se han identificado sitios de unión para numerosos factores de transcripción como AP-1, Sp1, NF-κB y CREB dentro del promotor de VEGF (Pages and Pouyssegur 2005) y, por ejemplo, se sabe que la activación de AP-1 es defectuosa en presencia de inhibidores de PARP (Andreone, O'Connor et al. 2003; Martin-Oliva, Aguilar-Quesada et al. 2006). Pero es más, se ha descrito que la inhibición de PARP-1 afecta a la propia actividad de HIF-1 a nivel de complejo de transcripción (Elser, Borsig et al. 2008), y todo junto, podría explicar el efecto antiangiogénico de los inhibidores de PARP.

De una forma u otra, PARP-1 afecta a la respuesta a hipoxia, tal y como además hemos obtenido por análisis de la expresión de genes relacionados con hipoxia mediante el sistema Oligo GEArray de SuperArray. Como resultado de dicho análisis, hemos visto diferencias en la inducción hipóxica de genes como *Angptl4* y *Ca9* entre MEFs inmortalizados *parp-1* +/+ y *parp-1* -/- (Figura B3), diferencias similares a las obtenidas mediante RT-PCR Cuantitativa para ese mismo RNA (Figura B4). También hemos observado variaciones en la inducción hipóxica de los genes *Ctgf* y *Fabp4* entre MEFs inmortalizados *parp-1* +/+ y *parp-1* -/- (Figura B5) y en otros genes como *Id2*, *Mycn*, *Col1a1*, *Gpx1* y *Hbb-y* (datos no mostrados). Sin embargo, las consecuencias del efecto de PARP-1 sobre la respuesta a hipoxia requieren un estudio más exhaustivo, comenzando con la implicación que cada uno de estos genes tiene sobre el fenotipo celular y cómo PARP-1 controla sus respectivas funciones. Por otro lado, no descartamos que aparte de su efecto sobre la expresión de HIF-2α, PARP-1 pueda estar actuando a otro nivel en la regulación de la respuesta a hipoxia dependiente de HIF, como el descrito por (Elser, Borsig et al. 2008), y que todo ello afecte a la expresión génica dependiente de este factor de transcripción.

Como ya se ha comentado, tanto la expresión de HIF-1α como la de HIF-2α son elevadas en una variedad de tumores humanos y de líneas celulares tumorales, aunque la contribución relativa de cada proteína a la iniciación y progresión del tumor no está del todo clara. Por razones no muy determinadas, en células epiteliales neoplásicas de carcinomas de célula clara renal (CCRCs), la mayor expresión de HIF-1α en túbulos renales no neoplásicos se altera sorprendentemente en favor de la expresión de HIF-2α (Rosenberger, Mandriota et al. 2002). Por otro lado, la manipulación genética en células CCRC indica que la activación de HIF-2α, pero no de HIF-1α, promueve el crecimiento tumoral (Kondo, Klco et al. 2002; Maranchie, Vasselli et al. 2002; Kondo, Kim et al. 2003; Raval, Lau et al. 2005). Los hemangioblastomas, tumores altamente vascularizados del sistema nervioso central, son la manifestación más frecuente de la enfermedad autosómica dominante von Hippel-Lindau (VHL), y se caracterizan por la sobreexpresión de

HIF-2α (Flamme, Krieg et al. 1998), lo que además es un factor determinante de la agresividad de los neuroblastomas (Holmquist-Mengelbier, Fredlund et al. 2006). En vista de los anteriores ejemplos, HIF-2α podría por tanto representar una valiosa diana en la terapia del cáncer, y sería muy interesante contar con una herramienta dirigida específicamente a HIF-2α que contrarrestara el desarrollo tumoral. En base a nuestros resultados, PARP-1 surge como un buen candidato a pesar de que serían necesarios más estudios para desentrañar el mecanismo molecular responsable del efecto específico de PARP-1 sobre HIF-2α.

VII. CONCLUSIONS (CONCLUSIONES)

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A1. PARP-1 and ATM interact physically, mainly in response to DNA damage by ionizing radiation.

A2. ATM is modified by poly(ADP-ribosyl)ation. PARP-1 deficient cells or cells treated with PARP inhibitors display a limited activation of ATM in response to IR.

A3. ATM kinase is activated by PARP inhibition.

A4. PARP inhibition induces DNA double strand breaks which are resolved in an ATM-dependent manner. So, ATM deficient cells are much more sensitive to PARP inhibition than ATM proficient cells.

B1. PARP-1 controls HIF-mediated response to hypoxia.

B2. HIF-2-dependent gene expression decreases in absence of PARP-1.

B3. HIF-2α mRNA expression is reduced in absence of PARP-1.

B4. Inhibition of PARP-1 catalytic activity has no impact on HIF-2.

Globally, our results contribute to place PARP-1 in two key signalling pathways involved in tumor development through its interaction with ATM and its cooperation with the HIF system and suggest new opportunities for targeting PARP-1 in antineoplasic regimes.

VIII. PERSPECTIVAS

PERSPECTIVAS

Basándonos en las propiedades y funciones de cada una de las proteínas protagonistas de este trabajo, PARP-1, ATM y HIF, así como en nuestros resultados y los de otros muchos estudios que se han ido citando en los diversos apartados, nos surgen una serie de preguntas y nos planteamos un conjunto de experimentos que nos ocuparán en el futuro:

A. Interacción PARP-1/ATM

A1. Comprobar si la modificación de la estructura de la cromatina mediada por PARP-1 puede estar afectando a la activación de ATM.

A2. Estudiar si el efecto de PARP-1 sobre la respuesta de ATM a IR es extrapolable a otros estímulos de daño en el DNA que no produzcan la misma lesión del DNA.

A3. Estudiar los niveles de estrés oxidativo en células deficientes en ATM y cómo estos pueden influir en la activación de PARP-1.

A4. Analizar la interacción PARP-1/complejo MRN, y estudiar las consecuencias de esta interacción sobre la activación y reclutamiento de ATM.

A5. Medir los niveles de fosforilación de distintos sustratos de ATM (p53, H2AX, etc.) en función del tratamiento con inhibidores de PARP y en células PARP-1 KO.

A5. Estudiar el papel de PARP-2 en la respuesta a IR mediada por ATM.

B. Papel de PARP-1 en la respuesta a hipoxia

B1. Elucidar el mecanismo de regulación de la expresión de HIF-2α por PARP-1 a través del estudio de la estabilidad del mRNA de HIF-2α y de la regulación de su promotor.

B2. Medir niveles de expresión de Nox4 y niveles de ROS en ausencia de PARP-1 en modelos de CCRCs.

B3. Estudiar en los ratones PARP-1 knockout fenotipos relacionados con la deficiencia en la activación de HIF-2 (por ejemplo, diferenciación mesénquina endotelio mediada por Oct-4, niveles de eritropoyetina).

B4. Dado que HIF-2α promueve específicamente la tumorigénesis asociada con pérdida de VHL, nos proponemos estudiar el efecto de la eliminación de PARP-1 en células deficientes en VHL sobre su capacidad tumorigénica.

B5. Generar ratones dobles deficientes en HIF-2α y PARP-1 para examinar si la ausencia de PARP-1 exacerba el fenotipo de los ratones knockout para HIF-2α.

B6. Analizar mediante estudios de TAP y proteómica los complejos asociados a HIF-1 y HIF-2 en el contexto de células PARP-1 knockout.

IX. BIBLIOGRAFÍA

BIBLIOGRAFÍA

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X. OTROS ARTÍCULOS PUBLICADOS

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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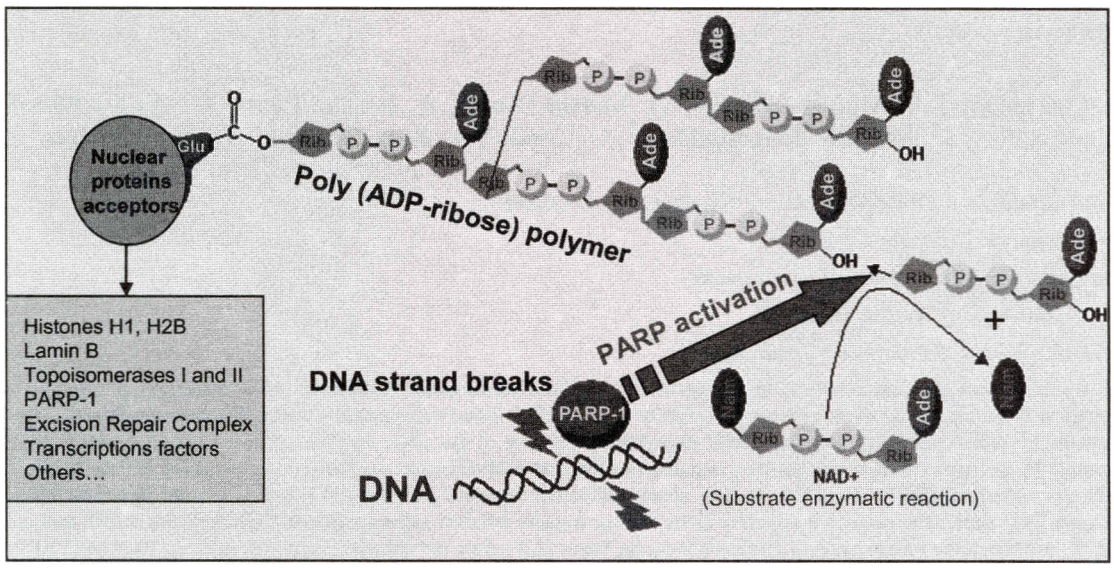

Fig. (1). DNA damage induces PARP -1 activation. A number of genotoxic agents that produces DNA strand breaks activates PARP -1 leading to poly(ADP -ribose) polymer formation from NAD+ and consequent covalent modification by poly(ADP -ribosyl)ation of different nuclear acceptor proteins.

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].

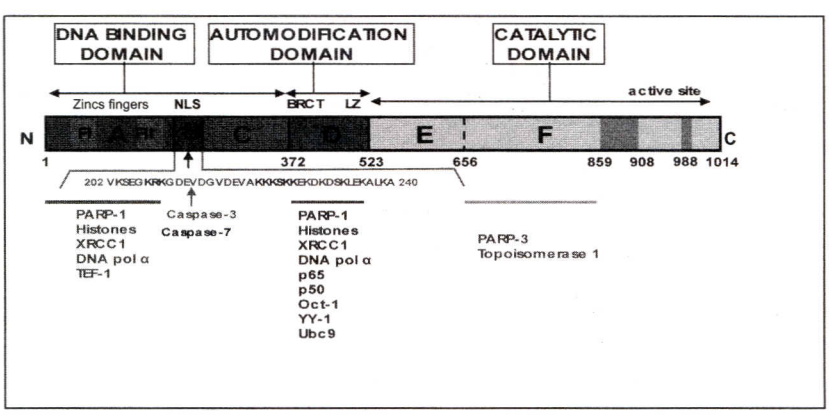

Fig. (2). Structural domains of human PARP-1. DNA binding domain (DBD) contains two zinc finger structures (FI and FII), the nuclear localization signal (NLS) and caspases -3 and -7 cleavage site; Automodification domain contains BRCT (*BRCA1* like C -terminus) motif and Leucine Zipper motif (LZ); and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

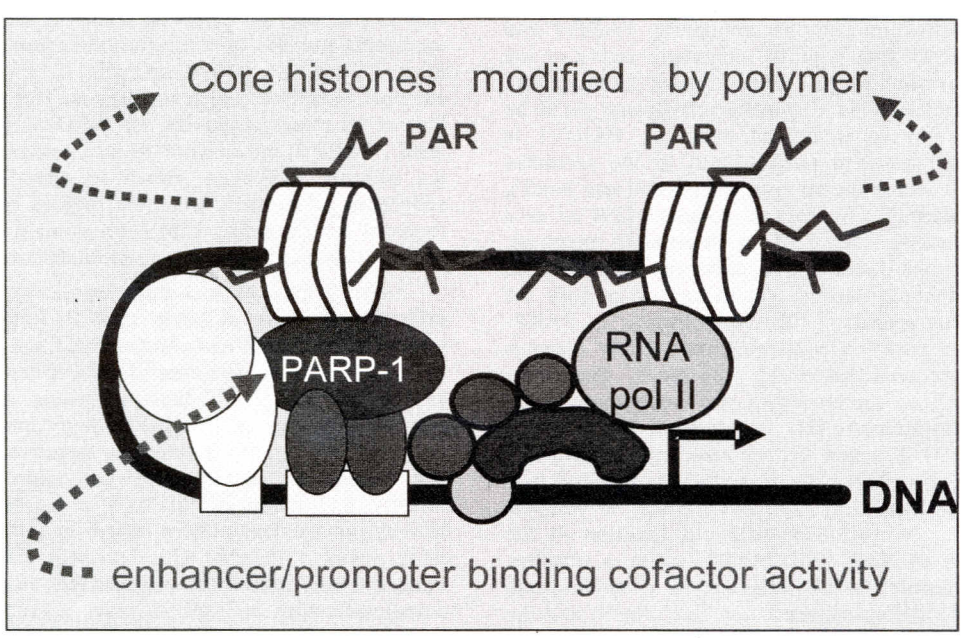

Fig. (3). Models of regulation of transcriptional activity by PARP -1. Two modes of PARP -1-dependent transcription regulation have been proposed. First, a histone -modifying by PARP -1 that can regulate chromatin structure; second, as transcriptional cofactor PARP -1 activity can enhancer the transcription 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,

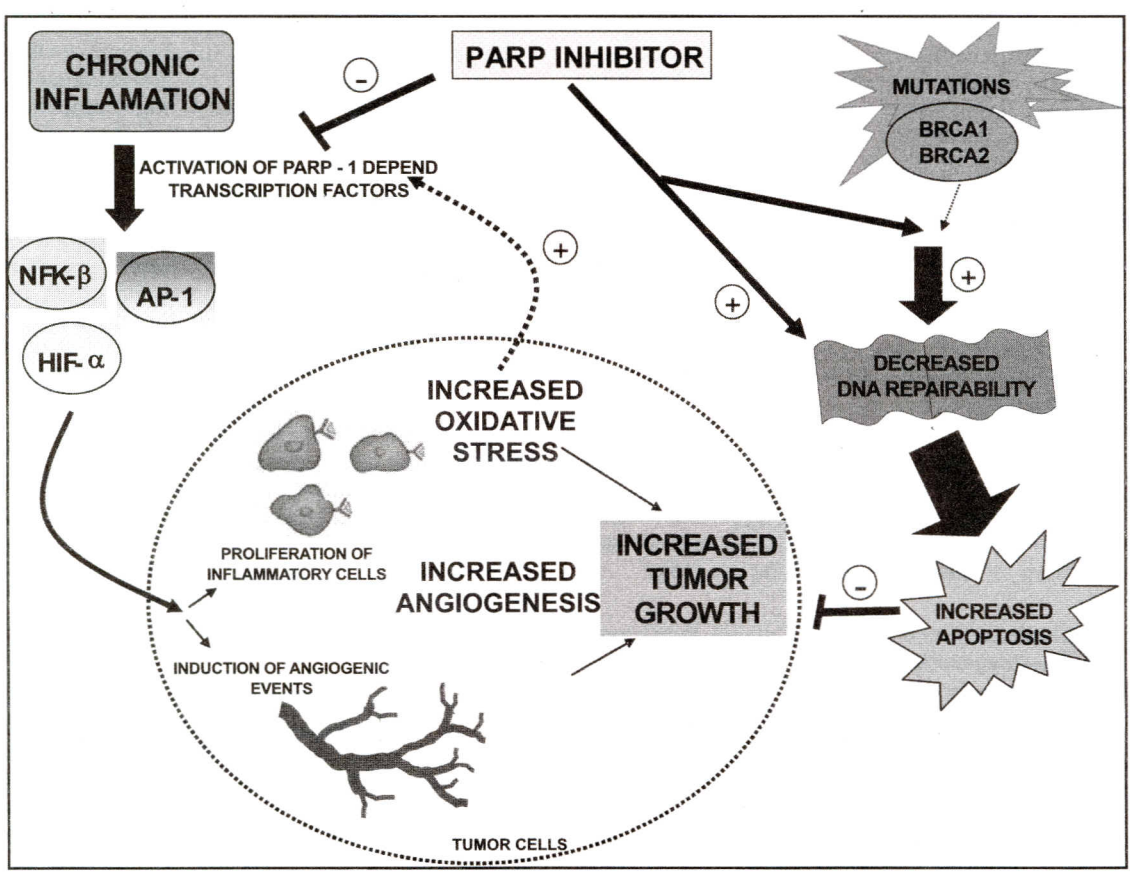

Fig. (4). Proposed model of action of PARP inhibitors in tumors. PARP inhibitors might interfere with tumor growth at different levels counteracting with the transcriptional activation of tumor-related transcription factors, angiogenesis process and in promotion of apoptosis in combination with chemotherapy, 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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Inhibition of Poly(ADP-Ribose) Polymerase Modulates Tumor-Related Gene Expression, Including Hypoxia-Inducible Factor-1 Activation, during Skin Carcinogenesis

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Abstract

Poly(ADP-ribose) polymerase (PARP)-1, an enzyme that catalyzes the attachment of ADP ribose to target proteins, acts as a component of enhancer/promoter regulatory complexes. In the present study, we show that pharmacologic inhibition of PARP-1 with 3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone (DPQ) results in a strong delay in tumor formation and in a dramatic reduction in tumor size and multiplicity during 7,12-dimethylbenz(*a*)anthracene plus 12-*O*-tetradecanoylphorbol-13-acetate–induced skin carcinogenesis. This observation was parallel with a reduction in the skin inflammatory infiltrate in DPQ-treated mice and tumor vasculogenesis. Inhibition of PARP also affected activator protein-1 (AP-1) activation but not nuclear factor-κB (NF-κB). Using cDNA expression array analysis, a substantial difference in key tumor-related gene expression was found between chemically induced mice treated or not with PARP inhibitor and also between wild-type and *parp-1* knockout mice. Most important differences were found in gene expression for *Nfkbiz, S100a9, Hif-1α*, and other genes involved in carcinogenesis and inflammation. These results were corroborated by real-time PCR. Moreover, the transcriptional activity of hypoxia-inducible factor-1α (HIF-1α) was compromised by PARP inhibition or in PARP-1–deficient cells, as measured by gene reporter assays and the expression of key target genes for HIF-1α. Tumor vasculature was also strongly inhibited in PARP-1–deficient mice and by DPQ. In summary, this study shows that inhibition of PARP on itself is able to control tumor growth, and PARP inhibition or genetic deletion of PARP-1 prevents from tumor promotion through their ability to cooperate with the activation AP-1, NF-κB, and HIF-1α. (Cancer Res 2006; 66(11): 5744-56)

Introduction

Because of the causal relationship between inflammation and tumor promotion, different proinflammatory cytokines and enzymes have been implicated in the pathophysiology of human cancer (1), and tumor microenvironment, which is largely orchestrated by inflammatory cells, is an indispensable participant in the neoplastic process, fostering proliferation, survival, and migration. Treatment with potent anti-inflammatory substances is anticipated to exert chemopreventive effect particularly in the promotion stage. Numerous intracellular signaling, including cytokines, mitogens, phorbol esters, growth factors, environmental, and ionizing radiation, converge with the activation of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), which act independently or coordinately to regulate expression of target genes. These ubiquitous eukaryotic transcription factors mediate pleiotropic effects on cellular transformation and tumor promotion.

A different level of regulation in tumor progression is controlled by the response of tumor to hypoxic condition; hypoxia is a common characteristic of locally advanced solid tumors that have been associated with diminished therapeutic response and malignant progression. The transcription factor hypoxia-inducible factor-1 (HIF-1) is a major regulator of tumor cell adaptation to hypoxic stress (2). Tumor cells with proteomic and genomic changes favoring survival under hypoxic conditions will proliferate, thereby further aggravating the hypoxia. The selection and expansion of new (and more aggressive) clones, which eventually become the dominant tumor cell type, lead to the establishment of a vicious circle of hypoxia and malignant progression.

Poly(ADP-ribose) polymerase (PARP)-1 is the principal member of a family of enzymes possessing poly(ADP-ribosylation) catalytic capacity. It is a conserved nuclear protein that binds rapidly and directly to both single-strand and double-strand breaks. Both processes activate the catalytic capacity of the enzyme, which in turn modulates the activity of a wide range of nuclear proteins by covalent attachment of branching chains of ADP-ribose moieties. Organisms and cellular systems deficient in functional PARP-1 display severely impaired base excision repair and genomic instability, suggesting that the enzyme plays a primary role in the cellular response to DNA damage (3).

Increasing interest in potential clinical applications of PARP inhibition has led to the development of a wide range of new compounds, the more recently developed of which display greatly increased potency and specificity compared with the prototype PARP inhibitor, 3-aminobenzamide (3-AB; ref. 4). The understanding of the role and involvement of PARP-1 in many biological mechanisms, health, and diseases as well as its role in carcinogenesis has steadily increased in recent years. (5). In a previous report, we have shown that *parp-1* knockout (KO) mice are protected against chemically induced skin carcinogenesis (6). In

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the present study, we show that the inhibition of PARP activity prevents from tumor growth during skin carcinogenesis due to its ability to modulate the activity of key transcription factors involved in tumor promotion (such as AP-1 and HIF-1) and also to interfere with the expression of genes involved in both tumor promotion/progression and inflammation.

Materials and Methods

Mice/tumor induction experiments and cell culture conditions. Tumor induction in mice on the C57BL/6 background was done as described previously (6), except that one group was treated with 3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone (DPQ; Alexis Biochemicals, San Diego, CA), inhibitor of PARP, and applied simultaneously with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Control animals were also treated in parallel with acetone alone. Visible skin tumors were counted weekly, and the experiment was terminated at week 25. The incidence of papilloma, expressed as the percentage of animals with one or more papillomas, and its papilloma, expressed as the number of papillomas per surviving mouse, were calculated each time tumors were counted.

A short initiation/promotion protocol was used to assess for changes in NF-κB and AP-1 activation, HIF-1α, and genomic expression profiles of skin epithelial cells in the initial steps of carcinogenesis with or without DPQ inhibitor as that by Martin-Oliva et al. (6). This treatment consisted in a single dose of 7,12-dimethylbenz(*a*)anthracene (DMBA; 25 μg) and four doses of 12 μg TPA with or without 30 μg DPQ given 7 days (6) before evaluation of NF-κB and AP-1 activation, HIF-1α, and differential genes expression.

Immortalized mouse embryonic fibroblasts (3T3) from either *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ mice were cultured at 37°C (5% CO_2) in DMEM containing 10% fetal bovine serum, 0.5% gentamicin (Sigma, St. Louis, MO), and 4.5% glucose.

NF-κB, AP-1, and HIF-1α–binding activity. Gel shift assays were used to detect NF-κB, AP-1, and HIF-1α–binding activity as that by Martin-Oliva et al. (6) and Lok et al. (7).

RNA isolation. DMBA and TPA or TPA plus DPQ were applied to the skin of the backs of mice as described above for gel shift assays. Total RNA was isolated from the skin of the mice 24 hours after the last TPA or TPA plus DPQ treatment by Trizol (Life Technologies, Inc., Gaithersburg, MD) extraction method, then purified with the RNeasy kit (Qiagen, Inc., Valencia, CA), and digested with RNase-free DNase I following the manufacturer's instructions.

Construction and analysis of cDNA microarray. For all microarray studies, the mouse CNIO OncoChip was used. The mouse CNIO OncoChip is a cDNA microarray that has been designed for looking at genes involved in cancer and contains both the NIA15K and the 7.4K clone sets from the National Institute on Aging (http://lgsun.grc.nia.nih.gov/cDNA/cDNA.html) plus additional 600 clones specifically associated to cancer, angiogenesis, apoptosis, signal transduction, and stress processes. Briefly, the mouse cDNA microarray consists of 15,747 unique cDNA clones (rearrayed among 52,374 expressed sequence tags from preimplantation and periimplantation embryos, E12.5 female gonad/mesonephros, and newborn ovary) and 50% novel genes with an average insert size of 1.5 kb (8).

Target preparation. T-7-based RNA amplifications and preparations of cDNA probes were done as described previously (9, 10).

Data analysis. Fluorescence intensity measurements from each array element were compared with local background, and background subtraction was done as that by Tamames et al. (11).

Reverse transcription reaction. Amplified RNA (0.2 μg) was used in 20 μL reverse transcription reaction to synthesize cDNA using the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol. A detailed description is included in Fig. 3.

Quantitative PCR. Real-time PCR analysis was done using iQ SYBR Green Supermix and the iCycler iQ detection system (Bio-Rad Laboratories) according to the manufacturer's protocol. The sequences of primers used for these studies are shown in Supplementary Table S1. We used the 18S rRNA (12) as endogenous control gene.

Hypoxia mimicking treatment, transient transfection, and Western blot analysis. Hypoxic conditions were mimicked using the iron chelator deferrioxamine (DFO; 200 μmol/L; Sigma), a nonselective prolyl 4-hydroxylase inhibitor. Experimental conditions are explained in Fig. 4. Western blot analysis was done as described previously (13) using an anti-HIF-1α antibody (Bethyl Laboratories, Montgomery, TX).

Histologic techniques. Histologic techniques for conventional morphology and evaluation of blood vessels density were done according to previously published techniques (6).

Statistical analysis. For data shown in Fig. 1*A, i*, we have fitted the values of the average number of tumors per mouse during carcinogenesis treatment using the Mann-Whitney *U* test. Statistical analysis of other experiments used unpaired Student's *t* test.

Results

Inhibition of PARP activity delayed tumor promotion. To test the effect of the inhibition of the catalytic activity of PARP in skin tumor promotion, we treated mice with a single dose of DMBA plus TPA twice weekly with or without the PARP inhibitor DPQ for 25 weeks (see tumor induction experiments in Materials and Methods). Before analyzing the antitumor effect of DPQ, we confirmed in both skin and fibroblasts the inhibitory effect of DPQ in PARP activity, confirming the high potency and low toxicity of this inhibitor at the dose used (results not shown). Papillomas were first found in the TPA mice 8 weeks after promotion, although TPA plus DPQ mice developed tumors starting at week 10, 2 weeks later than carcinogen-treated mice. Tumor multiplicity, found after papilloma promotion, is similar to previously reported data for *parp-1* KO versus wild-type (WT) mice (6). A total of 4.6 papillomas per mouse ($n = 10$) and 1.7 papillomas per mouse ($n = 13$) in TPA and TPA plus DPQ–treated mice, respectively, developed at the end of the treatment (Fig. 1*A, i*). The differences in the number of tumor per mice obtained during the 25 weeks were statistically significant between these two groups of mice ($P < 0.05$). The percentage of tumor-bearing mice at the end of the treatment was 90% for TPA-treated mice ($n = 10$) versus 69.2% for TPA plus DPQ–treated mice ($n = 13$; Fig. 1*A, ii*). DPQ-treated mice, however, started to lose papillomas at week 25. This was due to the fact that these papillomas from DPQ-treated mice presented a fragile, tubular-like morphology (Fig. 1*A, iii*). The papilloma latent period (number of weeks to obtain at least one papilloma per mice) for TPA-treated mice was ~10.4 weeks, whereas TPA plus DPQ–treated mice was 16.6 weeks, suggesting that the time of tumor latency in TPA plus DPQ–treated mice is delayed with respect to the TPA-treated mice. Figure 1*A* (*iii*) shows the difference in the number of tumor per mice and sizes between these two groups of mice taken at the end of carcinogen treatment. In TPA plus DPQ–treated mice, the number and size of papillomas were reduced compared with TPA-treated mice (Table 1). Therefore, this decrease may be attributed to the absence of PARP activity per se.

Histologic examination shows that the epithelium of all mice treated with TPA (without DPQ) developed epithelial hyperplasia with significant increase and irregular thickness of epithelium (0.096 mm; $P < 0.0001$) and granulosum stratum (Fig. 1*B, iii*) versus control (Fig. 1*B, i*) and TPA plus DPQ–treated (Fig. 1*B, ii*) mice. In TPA plus DPQ–treated mice, epithelium is compounded by two or three layers of cells with very thin granulosum stratum, in some cases absent, and scant corneum stratum and a homogeneous thickness of a maximum of 0.02 mm at the end of treatment in the epidermis without lesion (Fig. 1*B, ii*). This homogeneous thickness is similar to control mice treated with acetone alone (0.019 mm; Fig. 1*B, i*).

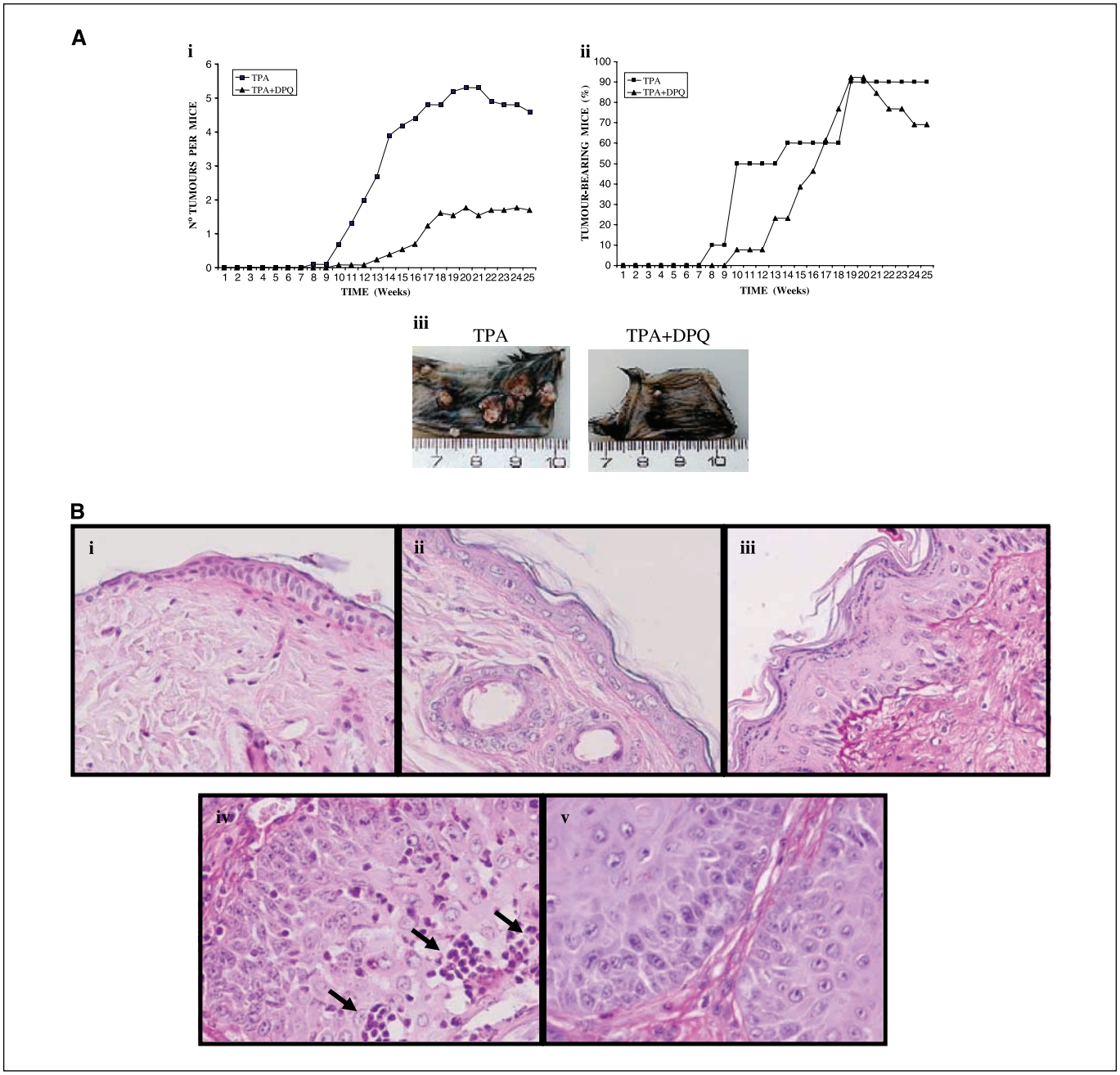
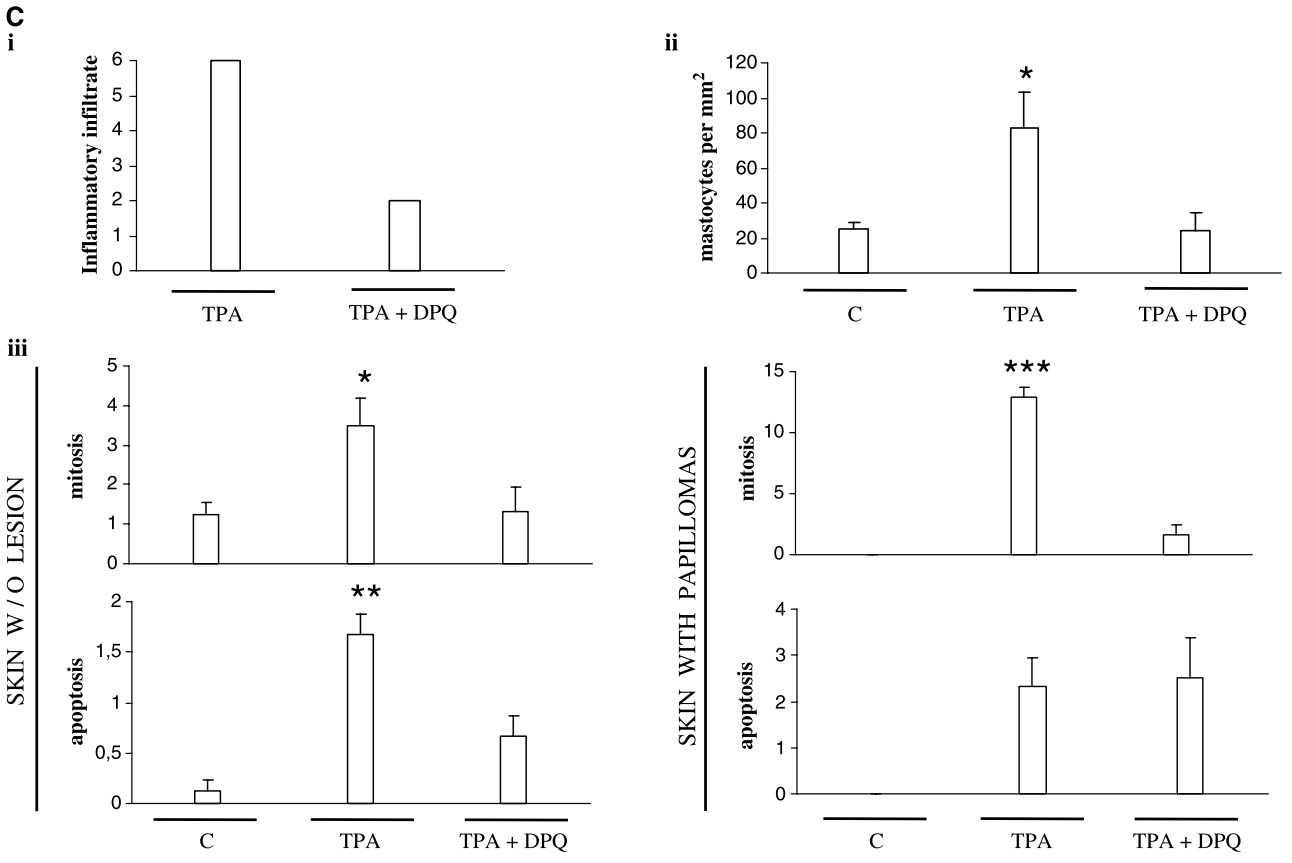


Figure 1. Skin carcinogenesis in mice treated with the PARP inhibitor DPQ. C57BL/6 mice were treated with one dose of 25 μg DMBA and then with 12 μg TPA with or without 30 μg DPQ twice weekly for 25 weeks. Tumors were counted every week. *A, i* to *iii,* tumor multiplicity, incidence, and tumor size at the end of carcinogen treatment. *i,* average number of tumors per mouse by tumor promotion in TPA-treated mice ($n = 10$; ■) and TPA + DPQ–treated mice ($n = 13$; ▲). The differences in tumor multiplicity are significant between these two groups ($P < 0.05$). *ii,* final number of tumor-bearing mice in TPA ($n = 10$; ■) and TPA + DPQ–treated mice ($n = 13$; ▲). *iii,* examples of skin papilloma size in TPA (*left*) and TPA + DPQ–treated mice (*right*) at the end of carcinogen treatment. The tumors were measured with a caliper. *B, i* to *v,* morphologic evaluation of epithelial hyperplasia and skin inflammation. For quantitative evaluation, skin longitudinal tissue sections of TPA-treated mice ($n = 6$), TPA + DPQ–treated mice ($n = 6$), and untreated control mice ($n = 2$) were stained with procedure periodic acid-Schiff (PAS). *i,* homogeneous thickness of epithelium in untreated control. This epithelium is compounded by two or three layers of cells with very thin granulosum stratum and scant corneum stratum (PAS ×400). *ii,* epithelium of TPA + DPQ–treated mice with morphology similar to nontreated control mice (PAS ×400). *iii,* irregular thickness of epithelium and granulosum stratum in mice treated with DMBA + TPA that show epithelial hyperplasia (PAS ×400). *iv* and *v,* mice treated with DMBA + TPA with evident acute and chronic inflammatory infiltrate in papilloma (PAS ×400; *iv*) or treated with TPA + DPQ (*v*), where no signs of inflammatory infiltrate were found. *C, i* to *iii,* histologic examination and diagnosis of inflammation, cell proliferation, and apoptosis in tumors and nonlesional-treated skin. *i,* number of mice with presence of inflammatory infiltrate in the papillomatous lesion. Presence of inflammatory infiltrate in papilloma was assessed by examining their presence in 10 high-power field at ×400 magnification per mouse in treated skin with a single dose of DMBA and promotion with TPA or with TPA + DPQ during 25 weeks. *ii,* number of mastocytes per mm^2 in papilloma from TPA mice and TPA + DPQ–treated mice during 25 weeks. Mastocytes were counted by examining their number in 10 high-power field at ×400 magnification per mouse in nonlesional control (*C*) mice and lesional back skin from treated mice. *iii,* increased cell turnover in skin of TPA-treated mice versus TPA + DPQ–treated and control mice. The average number of mitotic and apoptotic cells was determined by examining their number in 10 high-power field at ×600 magnification per mouse in nonlesional treated mice (*left*) and lesional back skin from treated mice (*right*). *TPA,* skin treated with DMBA plus TPA; *TPA + DPQ,* skin treated with DMBA plus TPA + DPQ. *D,* decreased angiogenesis in DPQ-treated tumors. *Left,* tumor blood vessels were stained with an antibody against lectin as described in Materials and Methods; *right,* quantitation of blood vessels per mm^2 in tumors. *Columns,* mean of at least five different mice in each case; *bars,* SE. *, $P < 0.05$, with respect to control and TPA + DPQ–treated mice; **, $P < 0.01$, with respect to control and TPA + DPQ–treated mice; ***, $P < 0.0001$, with respect to control and TPA + DPQ–treated mice.


C
i
Inflammatory infiltrate
TPA
TPA + DPQ
ii
mastocytes per mm2
C
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SKIN W / O LESION
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apoptosis
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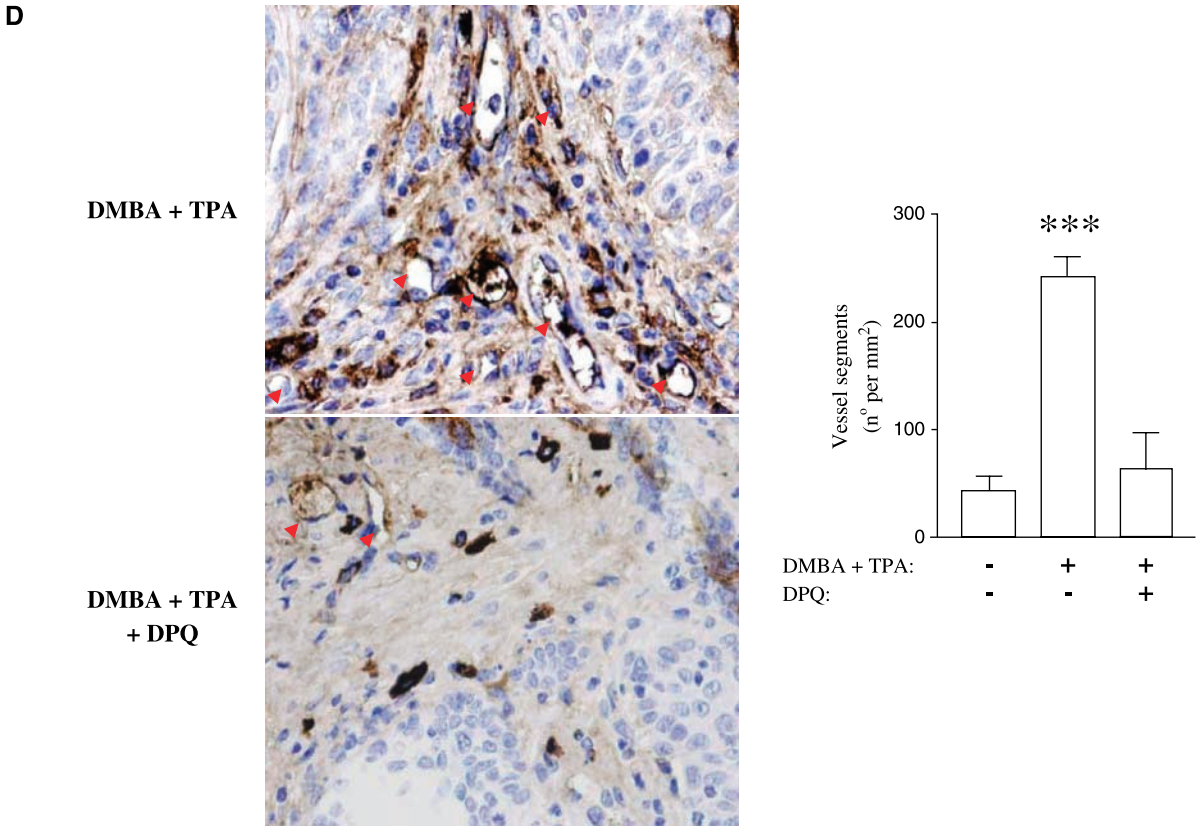
D
DMBA + TPA
DMBA + TPA
+ DPQ
Vessel segments
(n° per mm2)
DMBA + TPA:
DPQ:

Table 1. Percentage and total numbers of papillomas of different sizes in diameter at the end of carcinogen treatment

Tumors (mm)	Total no. papillomas		% Papillomas	
	TPA (*n*=10)	TPA + DPQ (*n*=13)	TPA	TPA + DPQ
>5	6	2	16.2	8.7
1-5	31	12	83.8	52.2
<1	0	9	0	39.1

The presence of acute inflammatory infiltrate [TPA-treated skin (Fig. 1*B, iv*) and TPA plus DPQ–treated skin (Fig. 1*B, v*); see also Fig. 1*C, i*] and the number of mastocytes per mm^2 in papilloma (Fig. 1*C, ii*) are significantly decreased in TPA plus DPQ–treated mice versus TPA-treated mice ($P < 0.05$). The presence of elevated infiltrates of mastocytes has been correlated with increased vascularization. Cell proliferation is also clearly increased in the papillomas and in the skin without lesion of TPA-treated mice versus TPA plus DPQ–treated mice, displaying an important increased mitosis in tumors (12.2 per high-power field versus 1.7 per high-power field, respectively; $P < 0.0001$; Fig. 1*C, iii*). A striking difference was found in the apoptotic versus mitotic rate between DMBA plus TPA–treated mice and DPQ-treated mice. This accelerated apoptotic activity in DPQ-treated mice may explain why the percentage of mice bearing tumors can increase to ~90% and then decrease to ~70% (Fig. 1*A, ii*).

To analyze tumor-associated vascularization, papillomas of varying sizes were stained with lectin Ulex europaeus biotin conjugated. The "angiogenic switch" from vascular quiescence to up-regulation of angiogenesis was observed in the early stages of skin carcinogenesis (data not shown). Quantitation of vessel density in papilloma showed an important decrease in DPQ-treated mice (Fig. 1*D*), revealing that differences in tumor vascularity may account for the decreased size and incidence after treatment with the PARP inhibitor.

Decrease of AP-1 DNA-binding activity but not NF-κB during tumor promotion after inhibition of PARP. Activation

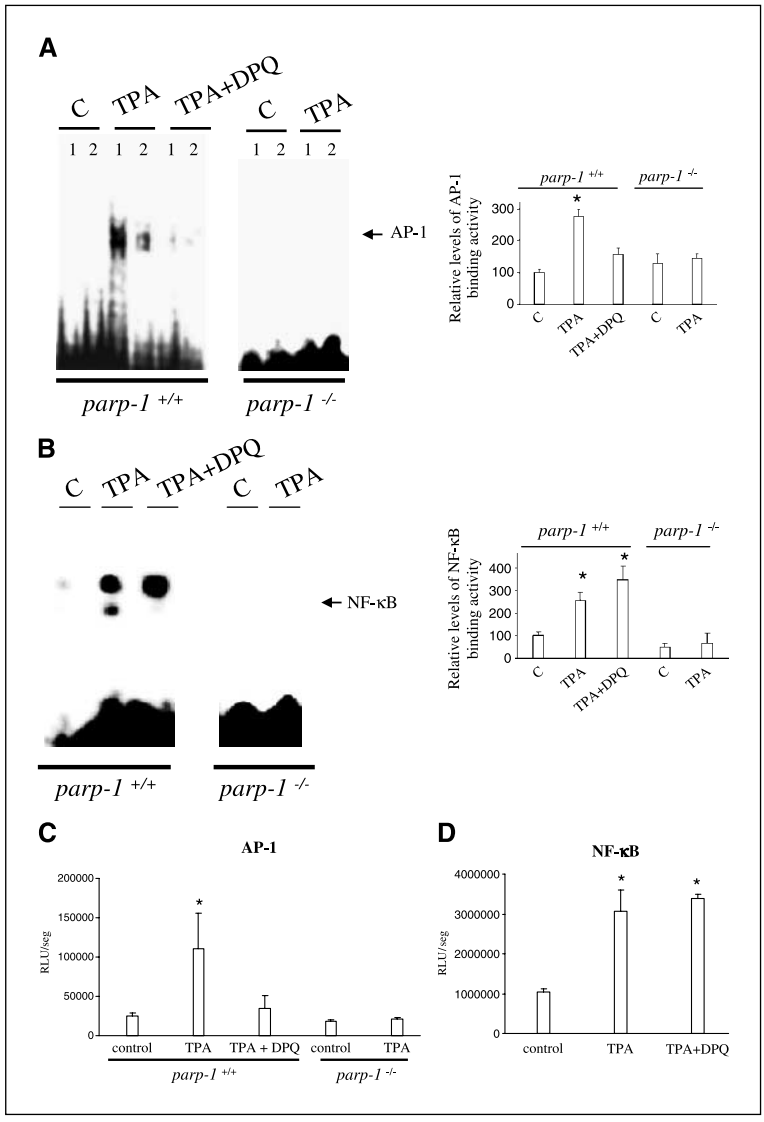

Figure 2. NF-κB and AP-1 DNA-binding activity in TPA or TPA + DPQ–treated mice during tumor promotion. *A,* band shift analysis of AP-1 activation using AP-1-binding promoter sequence in untreated (*C*) and treated skin of *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ mice with one dose of 25 μg DMBA and four applications of 12 μg TPA together with 30 μg DPQ (TPA + DPQ) or without PARP inhibitor (TPA) as described in Materials and Methods. *1 and 2,* two independent experiments in each condition. *B,* band shift analysis of NF-κB activation using κB iNOS promoter sequence in untreated (*C*) and treated skin of *parp-1*$^{+/+}$ mice with one dose of 25 μg DMBA and four applications of 12 μg TPA together with 30 μg DPQ (TPA + DPQ) or without inhibitor (TPA) as described in Materials and Methods. Representative of four independent experiments. *Right,* quantitation of four independent experiments. *, $P < 0.01$. *C* and *D,* AP-1 and NF-κB luciferase reporter assays. Fibroblasts derived from either *parp-1*$^{+/+}$ or *parp-1*$^{-/-}$ mice were transfected as specified in Materials and Methods and treated with 12 μg TPA or TPA + DPQ (30 μg) in a final volume of 3 mL culture medium for 24 hours. *, $P < 0.05$, with respect to the rest of experimental conditions.

of both AP-1 and NF-κB shows progressive elevation in human and mouse keratinocyte progression models (14). Thus, the observation that targeting AP-1 and NF-κB elevation prevents tumor promotion and progression has been extended from the mouse JB6 model to mouse and human keratinocyte progression models (15). *Parp-1* KO fibroblasts and mice deficient in PARP-1 display a decreased activation of the transcription factor NF-κB (16, 17) and AP-1 (18). To find a mechanistic explanation for the reduced and delayed papilloma formation in mice treated with DPQ, we determined activation of these transcription factors. In nuclear extracts from the epidermis of TPA-treated mice, TPA markedly induced AP-1 and NF-κB activation. However, in the skin of TPA plus DPQ–treated mice and TPA-treated *parp-1* KO mice, there was no activation of AP-1 during skin carcinogenesis (Fig. 2*A*). In contrast, there was no difference in NF-κB activation in the skin of mice treated with TPA plus PARP inhibitor DPQ (Fig. 2*B*). Our previous report suggested that *parp-1* KO mice treated with TPA display a decreased activation of the transcription factor NF-κB (6). Figure 2*A* and *B* (*right*) shows a densitometric analysis of AP-1 and NF-κB band shift assays. The effect of pharmacologic inhibition of PARP by DPQ in TPA-treated mice results in a significant reduction of the relative levels of AP-1-binding activity compared with mice treated with TPA only (Fig. 2*A*). On the other hand, DPQ treatment to TPA-treated skins does not reduce NF-κB-binding activity compared with carcinogen-treated mice (Fig. 2*B*). Results in Fig. 2*C* and *D* show that AP-1 and NF-κB activation using luciferase reporter assay in fibroblasts derived from *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ also show the deficient activation of AP-1 after deletion/inhibition of PARP-1, whereas NF-κB is not affected by the use of PARP inhibitors as has been shown by different groups (19).

Analysis of differential gene expression. We used cDNA microarrays to obtain gene expression patterns of treated skins during the first week of tumor promotion. For this purpose, we compared the expression data derived from the skin of mice treated as follows: untreated *parp-1*$^{+/+}$ versus *parp-1*$^{+/+}$ treated with carcinogens (DMBA plus TPA) with or without DPQ; untreated *parp-1*$^{-/-}$ versus *parp-1*$^{-/-}$ treated with carcinogens (DMBA plus TPA).

In this way, we were able to monitor mRNA expression of 4,615 known unique murine genes and 11,096 of unknown or hypothetical proteins. Our means of identifying differentially expressed genes were based on consistent fold change and statistical significance assessed by *t* distribution applied to the average and SE to calculate probability values in each tumor/control skin pair. By using the massive screening method, our objective was to select key cancer-related genes that were differentially expressed in mice treated with the PARP inhibitor.

The genes found differentially expressed in skin treated with carcinogens versus in normal skins belong to a variety of different categories. The up-regulated (Table 2) and the down-regulated genes (Table 2) include genes encoding for signal molecules, tumor-associated genes, molecules involved in cell adhesion, inflammatory, and immune reaction, *red-ox* regulation, different metabolic pathways, etc. Analysis of the mRNAs, which are deregulated (up-regulated or down-regulated) at least 2-fold in TPA plus DPQ–treated skin compared with TPA-treated mice, revealed the following results: 40 genes up-regulated and 29 genes down-regulated in the skin of carcinogen-treated mice; 19 genes up-regulated and 21 genes down-regulated in DPQ-treated mice; and 11 genes up-regulated and 20 genes down-regulated in *parp-1* KO mice treated with carcinogens. These results are summarized in Fig. 3*A*. Cluster analysis of all tumor samples revealed 91 genes that match the arbitrary criterion of 2-fold change in either direction with a statistical significance of $P < 0.01$ (Table 2). Of considerable interest is that, depending on the presence of the PARP inhibitor or in *parp-1* KO mice, each promoted skin sample revealed a somewhat unique expression pattern together with an impressive reduction in both the number of altered genes as well as in the up-regulation of significant genes involved in different cellular functions (Table 2).

Real-time PCR gene expression and HIF-1α protein expression. We confirmed by real-time PCR the up-regulation of some of the known genes involved in inflammation and angiogenesis (*S100a9, Hif-1α*, and *Nfkbiz*; Fig. 3*B, left*) as well as other crucial genes known to have a role in transformation of preneoplastic cells that were not reproducibly up-regulated in the microarray. We have analyzed the expression of *osteopontin* (*OPN*), *cathepsin B* (*CtsB*), *cathepsin L* (*CtsL*), *Pecam-1*, and *Igfbp3* by RT-PCR (Fig. 3*C*). The results show that expression of these genes was greatly influenced by either inhibition or genetic deletion of PARP-1 during carcinogenesis. HIF-1 transcriptional activity is dependent on increased levels of HIF-1α protein and on its heterodimerization with HIF-1β (20). To investigate whether carcinogenesis treatment influences HIF-1 activity by altering expression of HIF-1α, the levels of HIF-1α protein were determined in *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ mice exposed to the inhibitor DPQ as described in Materials and Methods. HIF-1α expression was clearly absent in DPQ-treated and *parp-1*$^{-/-}$ mice (Fig. 3*B, right*).

The RT-PCR results confirmed the array prediction (Fig. 3*B, left*), but more obvious differences were found in the expression levels of the previously described genes (Table 2). Rest of the selected genes matched the prediction of the array, and the enormous increase in the expression of *S100a9* (calgranulin B), whose overexpression has been related with progression of skin carcinogenesis, is particularly striking (21). Induction of the expression of this chemokine has been also related with the migration of neutrophils to inflammatory sites (22) that constitute a remarkable pathologic event during the process of skin carcinogenesis (Fig. 1*B*).

Regulation of HIF-1α activation by PARP-1. In view of the pivotal role of HIF-1α in carcinogenesis, we have focused particular attention to the regulation of HIF-1α by PARP inhibitors and in PARP-1-deficient cells. Cells were treated with de Fe-chelator DFO (which is an iron chelator and activator of this transcription factor) for the time indicated in Fig. 4 and in Materials and Methods. Reporter gene assays were done using a reporter plasmid containing the luciferase gene under the control of nine hypoxia-responsive elements (HRE; ref. 23). HIF-1 activation was clearly attenuated by DPQ and completely absent in *parp-1*$^{-/-}$ cells (Fig. 4*A, left*). Importantly, treatment of PARP-1-deficient cells with DPQ did not have further effect on the transcriptional activation of HIF-1, suggesting that DPQ was inhibiting specifically PARP-1 but not other PARPs. We confirmed this finding by electrophoretic mobility shift assay (EMSA) in nuclear extracts from PARP-1 WT and PARP-1-deficient cells. We determinated that DFO induced HIF-1 activation in *parp-1*$^{+/+}$ cells. Interestingly, WT cells, treated with DFO plus DPQ, and PARP-1-deficient cells showed no activation of HIF-1 during hypoxic treatment, as in Fig. 4*A* (*right*).

Protein levels for HIF-1α were also determined (Fig. 4*B*) after activation with DFO. Again, after PARP inactivation and in *parp-1*$^{-/-}$ cells, a down-regulation of HIF-1α protein expression was observed. The expression of HIF-1α target genes was determined by real-time

Table 2. Gene names in skin of DPQ untreated WT mice (WT TPA), DPQ-treated WT mice (WT TPA + DPQ), and PARP-1 KO mice (KO TPA) versus control mice during tumor promotion in two independent experiments using microarray analysis

Symbol	2-Fold deregulated gene names	Mean			Gene Ontology Biological Process (http://www.ebi.ac.uk/GOA/)
		WT TPA	WT TPA + DPQ	KO TPA	
(A) 2-Fold up-regulated genes*					
Alb1	*Albumin 1*	2			Transport
Cntn4	*Contactin 4*	2			Cell adhesion, transport
Axl	*AXL receptor tyrosine kinase*	2.46	2.14		Cell growth, protein amino acid phosphorylation, regulation of cell cycle
Bmp15	*Bone morphogenetic protein 15*	3.48	3.03	2.3	Cell growth
Ckmt1	*Creatine kinase mitochondrial 1*	2.83	2.3		Unknown
Ctla2b	*Cytotoxic T lymphocyte-associated protein 2β*	3.25			Unknown
Dbnl	*Drebrin-like*	2.14			Rac protein signal transduction, endocytosis, immune response
Degs	*Degenerative spermatocyte homolog*	2.64			Unknown
Eno1	*Enolase 1, α nonneuron*	2.14	2.14		Glucolisis
Expi	*Extracellular proteinase inhibitor*	3.25			Unknown
Ly6g6c	*Lymphocyte antigen 6 complex, locus G6C*		2.14		Unknown
Glrx1	*Glutaredoxin 1 (thioltransferase)*	7.46	5.28	3.25	Electron transport
Gltp	*Glycolipid transfer protein*	2.14			Lipid transport, transport
Gm2a	*GM2 ganglioside activator protein*	2.3			Sphingolipid metabolism
Gp49a	*Glycoprotein 49 A*	3.25		2.14	Unknown
Gpx1	*Glutathione peroxidase*	3.48			Induction of apoptosis by oxidative stress, response to oxidative stress, response to ROS
Gpx2	*Glutathione peroxidase 2*		6.96	5.28	Response to reactive oxygen species
Gsta4	*Glutathione S-transferase α4*	3.25			Unknown
Gsto1	*Glutathione S-transferase ω1*	2.64			Metabolism
Hdc	*Histidine decarboxylase*	3.25	2.64		Unknown
Hif-1a †	*Hypoxia inducible factor 1α sub unit*	2.46			Transduction
Hsd3b4	*Hydroxysteroid dehydrogenase-4, δ5-3-β*	2.14			Steroid biosynthesis, C21-steroid hormone biosynthesis
Ier3	*Immediate early response 3*	2.64		2.46	Unknown
Il18	*Interleukin-18*	3.73		3.03	Immune response
Itgb4bp	*Integrin β_4 binding protein*		2.14		Integrin-mediated signaling pathway, protein biosynthesis, translational initiation
Klf5	*Kruppel-like factor 5*	2.64			Regulation of transcription
Krt1-18	*Keratin complex 1, acidic, gene 18*		2.14		Cytoskeleton organization and biogenesis
Krt2-8	*Keratin complex 2, basic, gene 18*		2.46		C biogenesis, protein amino acid phosphorylation, response to pathogen...
Lcn2	*Lipocalin 2*	2.83	3.48	4.29	Transport
Lgals7	*Lectin, galactose binding, soluble 7*	4.29	2.46	2.3	Apoptosis
Ly6e	*Lymphocyte antigen 6 complex, locus E*	2.3			Defense response
Lyzs	*Lysozyme*	3.03			Carbohydrate metabolism, cell wall catabolism, cytolysis, defense response
Nfkbiz †	*Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, ζ*	3.03			Immune response, regulation of transcription
Map17	*Membrane-associated protein 17*	3.48	2.14	2.46	Unknown
Mglap	*Matrix γ-carboxyglutamate (gla) protein*		2.3		Regulation of bone mineralization
Npc1	*Niemann Pick type C1*	2.3			Cholesterol transport
Pold2	*Polymerase (DNA directed), δ2, regulatory subunit*	2.3			DNA replication
Ppgb	*Protective protein for β-galactosidase*	2.14			Proteolysis and peptidolysis
S100a9 †	*S100 calcium binding protein A9 (calgranulin B)*	36.76	13	10.56	Unknown
Sgk	*Serum/glucocorticoid-regulated kinase*	2.64	2.83		Apoptosis, protein amino acid phosphorylation

(*Continued on the following page*)

Table 2. Gene names in skin of DPQ untreated WT mice (WT TPA), DPQ-treated WT mice (WT TPA + DPQ), and PARP-1 KO mice (KO TPA) versus control mice during tumor promotion in two independent experiments using microarray analysis (Cont'd)

Symbol	2-Fold deregulated gene names	Mean			Gene Ontology Biological Process (http://www.ebi.ac.uk/GOA/)
		WT TPA	WT TPA + DPQ	KO TPA	
Slc34a2	*Solute carrier family 34 (sodium phosphate), member 2*	2.64			Phosphate transport, transport
Soat1	*Sterol O-acyltransferase 1*	2.14			Cholesterol transport
Tagln2	*Transgelin 2*		2.3		Muscle development
Tnc	*Tenascin C*	3.73		2.46	Unknown
Tfrc	*Transferrin receptor*	2.46			Endocytosis, iron ion homeostasis, proteolysis, and peptidolysis
Trps1	*Trichorhinophalangeal syndrome I (human)*	2			Regulation of transcription
Ucp2	*Uncoupling protein 2, mitochondrial*		2.64		Mitochondrial transport, transport
Ugcg	*UDP-glucose ceramide glucosyltransferase*	2.46			Epidermis development, glucosylceramide and glycosphingolipid biosynthesis
(B) 2-Fold down-regulated genes [‡]					
Anapc7	*Anaphase promoting complex subunit 7*			−2.64	Cell cycle, mitosis, cytokinesis, ubiquitin cycle
Arl6ip	*ADP-ribosylation factor-like 6 interacting protein 1*	−2.14			Cotranslational membrane targeting
Bcl2l	*Bcl2-like 1*	−2.3			Regulation negative of apoptosis, regulation of apoptosis, response to radiation
Calmbp1	*Calmodulin binding protein*		−2.3		Mitosis
Calm2	*Calmodulin 2*	−2			G-protein coupled receptor protein signaling pathway, cell cycle
Catns	*Catenin src*		−3.03		Cell adhesion
Cla3	*Cerebellar ataxia 3*	−2.46	−2.14		Unknown
Col1a1	*Procollagen, type I, α1*		−2.83	−3.03	Cell adhesion, phosphate transport
Col1a2	*Procollagen, type I, α2*		−3.73	−2.83	Cell adhesion, phosphate transport
Col3a1	*Procollagen, type III, α1*		−2.83	−2.3	Cell adhesion, phosphate transport
Epb4.1l4b	*Erythrocyte protein band 4.1-like 4b*	−2.3			Transport
Fasn	*Fatty acid synthase*			−2.3	Biosynthesis, fatty acid biosynthesis
Fgfr2	*Fibroblast growth factor receptor 2*	−2.46			Protein amino acid phosphorylation, regulation of cell proliferation, signal transduction
Gclc	*Glutamate-cysteine ligase, catalytic subunit*	−2.46			glutathione biosynthesis
Hbb-b1	*Hemoglobin, β adult major chain*	−2.64			Hemopoiesis, transport, oxygen transport
Hbb-y	*Hemoglobin Y, β-like embryonic c*	−2.14			Transport, oxygen transport
Hmgb1	*High mobility group box 1*	−2.46			DNA packaging, nitric oxide biosynthesis, transport
Hmgb2	*High mobility group box 2*	−2.3	−2.3		DNA packaging, regulation of transcription
Idb3	*Inhibitor of DNA binding 3*	−2.46	−2.46		Negative regulation of transcription from Pol II promoter
Igfbp5	*Insulin-like growth factor binding protein 5*		−2.14	−2.46	Regulation of cell growth, cell growth and/or maintenance
Itm2a	*Integral membrane protein 2A*			−2.83	Unknown
Stmn1	*Stathmin 1*	−4	−3.25		Intracellular signaling cascade, microtubule depolymerization, mitotic spindle assembly
Mt2	*Metallothionein 2*	−2.3	−2.3	−3.25	Nitric oxide mediated signal transduction, zinc ion homeostasis
Myef2	*Myelin basic protein expression factor 2, repressor*	−2.64			Unknown
Mylpf	*Myosin light chain, phosphorylatable, fast skeletal muscle*			−2.46	Cytoskeleton organization and biogenesis, muscle development

(*Continued on the following page*)

Table 2. Gene names in skin of DPQ untreated WT mice (WT TPA), DPQ-treated WT mice (WT TPA + DPQ), and PARP-1 KO mice (KO TPA) versus control mice during tumor promotion in two independent experiments using microarray analysis (Cont'd)

Symbol	2-Fold deregulated gene names	Mean			Gene Ontology Biological Process (http://www.ebi.ac.uk/GOA/)
		WT TPA	WT TPA + DPQ	KO TPA	
Nap1l1	*Nucleosome assembly protein 1-like 1*	−2.46	−2.14		Nucleosome assembly
Psmb5	*Proteasome (prosome, macropain) subunit, β type 5*			−2.46	Ubiquitin-dependent protein catabolism
Purb	*Purine-rich element binding protein B*	−2.14	−3.25	−3.25	Unknown
Pvrl3	*Poliovirus receptor-related 3*	−3.48	−3.25	−3.73	Cell adhesion, cell-cell adhesion
Slc4a2	*Solute carrier family 4 (anion exchanger), member 2*	−3.03			Ion transport, anion transport, transport
Smarca5	*SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin*	−2.14	−2.14		Chromatin assembly or disassembly, chromatin remodeling
Sox4	*SRY-box containing gene 4*	−2.83			Regulation of transcription
Spr	*Sepiapterin reductase*	−2.83	−2.46	−3.03	Metabolism, tetrahydrobiopterin biosynthesis
Sp1	*Trans-acting transcription factor 1*		−2.14	−2.3	Regulation of transcription, positive regulation of transcription
Tera	*Teratocarcinoma expressed, serine rich*	−2.64	−2.64	−2	Unknown
Tia1	*Cytotoxic granule-associated RNA-binding protein 1*	−2.46			Apoptosis
Tm4sf6	*Transmembrane 4 superfamily member 6*	−2.83			Unknown
Tnfrsf19	*Tumor necrosis factor receptor superfamily member 19*	−2.46	−2.83	−2.83	Unknown
Ttc3	*Tetratricopeptide repeat domain 3*	−2.14	−2.14	−2.14	Unknown
Tubb5	*Tubulin, β5*	−2.14			Microtubule-based process
Ube2s	*Ubiquitin-conjugating enzyme E2S*			−2.64	Protein modification, ubiquitin cycle
Utrn	*Utrophin*			−2.46	Signal transduction, muscle development, muscle contraction, chemotaxis
5830426I05Rik	*RIKEN cDNA 5830426I05 gene*	−2.14	−2.46	−2.3	Inner cell mass cell proliferation

*Mean gene names with fold <2 between control and treated mice are not indicated.
†Real-time PCR analysis gene expression.
‡Mean gene names with fold greater than −2 between control and treated mice are not indicated.

RT-PCR. The expression of *Igfbp3, Bnip3*, and *Vegf-A* genes was all strongly up-regulated by this drug in WT fibroblasts (Fig. 4*C* and *D*). However, after inhibition of PARP and in cells deficient in PARP-1, there was a deficient activation in the expression of these genes (Fig. 4*C* and *D*).

Discussion

Tumorigenesis is a complex multistage process, in which a series of genetic changes is thought to deregulate the cellular processes that control cell proliferation, differentiation, genome integrity, DNA repair, and induction of apoptosis. The murine model of chemically induced skin carcinogenesis is one of the best defined experimental *in vivo* models of epithelial carcinogenesis, representing an important tool for the understanding of current concepts about human neoplasia, including the multistage nature of tumor development. In this model, the development of squamous cell malignancy of the skin can be subdivided into three phases: initiation, promotion, and progression (24). Although genetic events are crucial to initiation and progression, the rate-limiting step in this model, the tumor promotion, is predominantly characterized by epigenetic events. In this context, changes in the RNA expression level, which are closely related to the amount of protein product and thus the biochemical activity, may be considered as first evidence for a gene with oncogenic potential.

PARP-1 may influence the stress/inflammation response through regulation of transcription factors and associated gene transcription. PARP-1 has been reported to either activate or repress transcription activity (25). PARP-1 influences on transcription activity may involve direct protein-protein interaction with PARP-1 or the catalytic activity of the PARP-1 enzyme, which can poly(ADP-ribosyl)ate transcription factors. Transcription factors, such as AP-2 (26), B-MYB (27), Oct-1 (28), YY-1 (29), and TEF-1 (30), have been shown to bind directly to PARP-1. On the other hand, transcription factors, such as p53 (31), fos (32), and RNA polymerases I and II (25), are poly(ADP-ribosyl)ated. NF-κB transcription activation after stress/inflammatory stimuli is reduced in *parp-1*$^{-/-}$ cells (16, 19).

The global analysis of gene expression during carcinogenesis as function of either PARP inhibition or in the skin of PARP-1-null mice revealed striking differences between the three groups analyzed (Fig. 3*A*; Table 2). Genes up-regulated in carcinogen-treated skin from WT mice (without DPQ treatment) include several tumor-associated genes in mouse and human [i.e., *AXL receptor tyrosine kinase* (*Axl*), *Enolase 1* (*Eno1*), *Hif-1α, immediate*

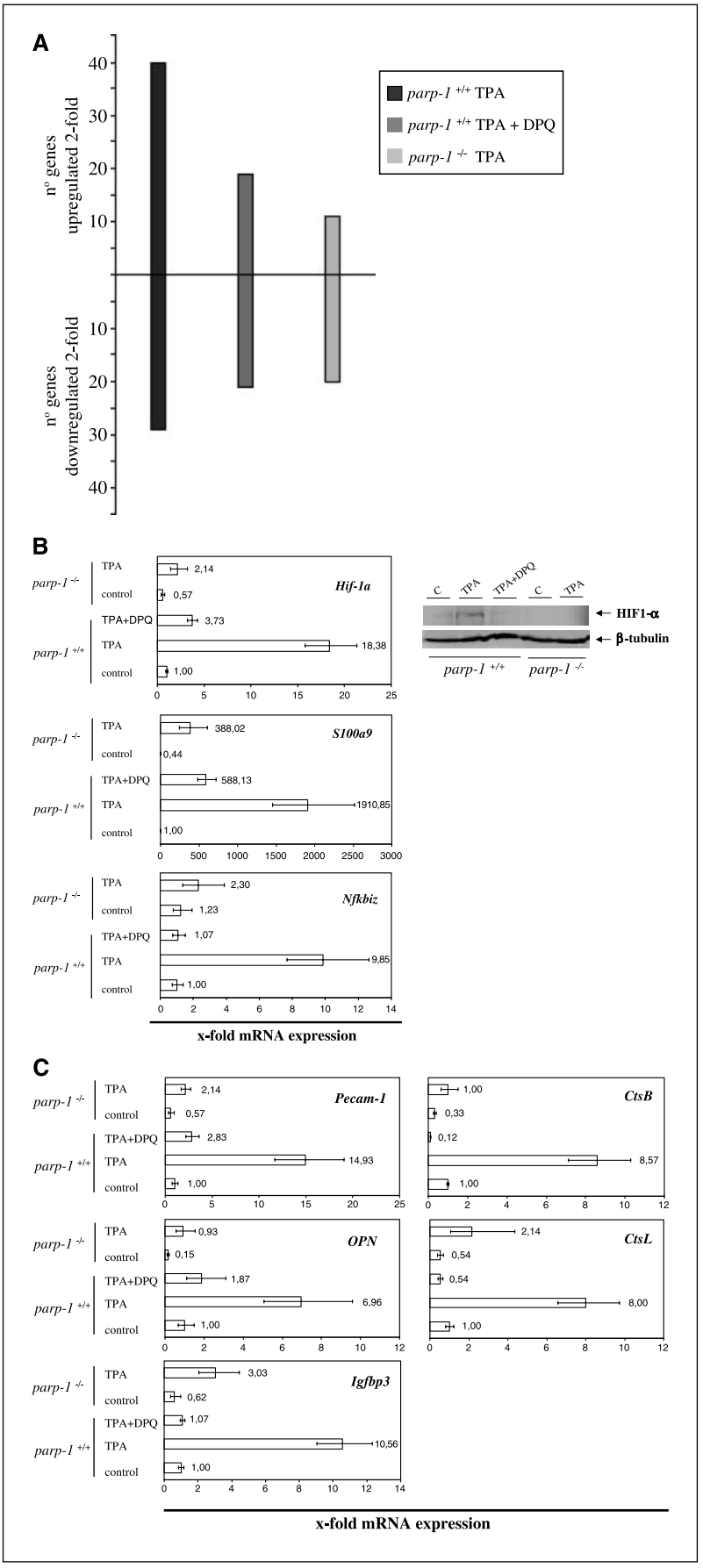

Figure 3. Numbers of genes that are deregulated (up-regulated or down-regulated) by at least 2-fold in carcinogen-treated skin versus normal skin by using cDNA microarray analysis. Twenty-four hours after the short initiation/promotion protocol, RNA was obtained from control, TPA, or TPA + DPQ–treated mice, and amplified RNA was used for hybridization of microarray slide as described in Materials and Methods. For data analysis, fluorescence intensity measurements from each array element were compared with local background, and background subtraction was done. To normalize the data, the Cy3/Cy5 ratio was adjusted using the diagnosis and normalization array data tool. In addition, spots with background-subtracted signal intensities <500 fluorescence units (sum of the two channels) were excluded from the analysis. Furthermore, bad spots or areas of the array with obvious defects were manually flagged. The Cy3/Cy5 ratios of the duplicated spots of the array were averaged. Genes were deemed to be up-regulated or down-regulated if the difference ratio was at least 2-fold. *A,* global gene expression differences between the three groups of mice used in the study: *parp-1*$^{+/+}$ TPA, *parp-1*$^{+/+}$ TPA + DPQ, and *parp-1*$^{-/-}$ TPA. *B* and *C,* differential gene expression measured by quantitative real-time PCR. DMBA and TPA or TPA + DPQ were applied to the skin of the backs of *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ mice with the short protocol as described in Materials and Methods. Total RNA was isolated from the skin of the mice 24 hours after the last TPA or TPA + DPQ treatment, and 0.2 μg RNA was used in 20 μL RT reaction to synthesize cDNA. *B,* real-time PCR of genes recently identified as possibly NF-κB regulated (*Nfkbiz* or *Mail*), AP-1 regulated (*S100a9* or *calgranulin B*), and *Hif-1α. Right,* expression of HIF-1α protein. *C,* real-time PCR of some of the known genes involved in inflammation and tumor progression: *Pecam-1, OPN, CtsB, CtsL*, and *Igfbp3*. The results were normalized to the expression of 18S rRNA for all of the samples. Real-time PCR analysis was done using iQ SYBR Green Supermix and the iCycler iQ detection system according to the manufacturer's protocol. The sequences of primers used for these studies are shown in Supplementary Table S1. In all cases, a standard curve containing at least four concentrations (represented in triplicate) of a control cDNA was constructed for both the endogenous control gene (18S rRNA) and the gene of interest. In all cases, standard curves had a coefficient of correlation >0.98. For data analysis, the cycle threshold value (C_T; arbitrary number of PCR cycles, in which all of the PCR amplification graphs is comparing, is in the linear range) was calculated in each case. A lower C_T value means more transcript, and a higher C_T value means less transcript. To normalize the endogenous control gene, the gene of interest C_T value was divided by the endogenous control gene C_T value. We used the 18S rRNA endogenous control gene because its level of expression is very stabile and so high that small fluctuations will not result in detectable fluctuations in the normalized fluorescence signal of the target gene. In each case, all samples were represented in triplicate for relative quantitative fold calculations with respect to untreated sample mice.

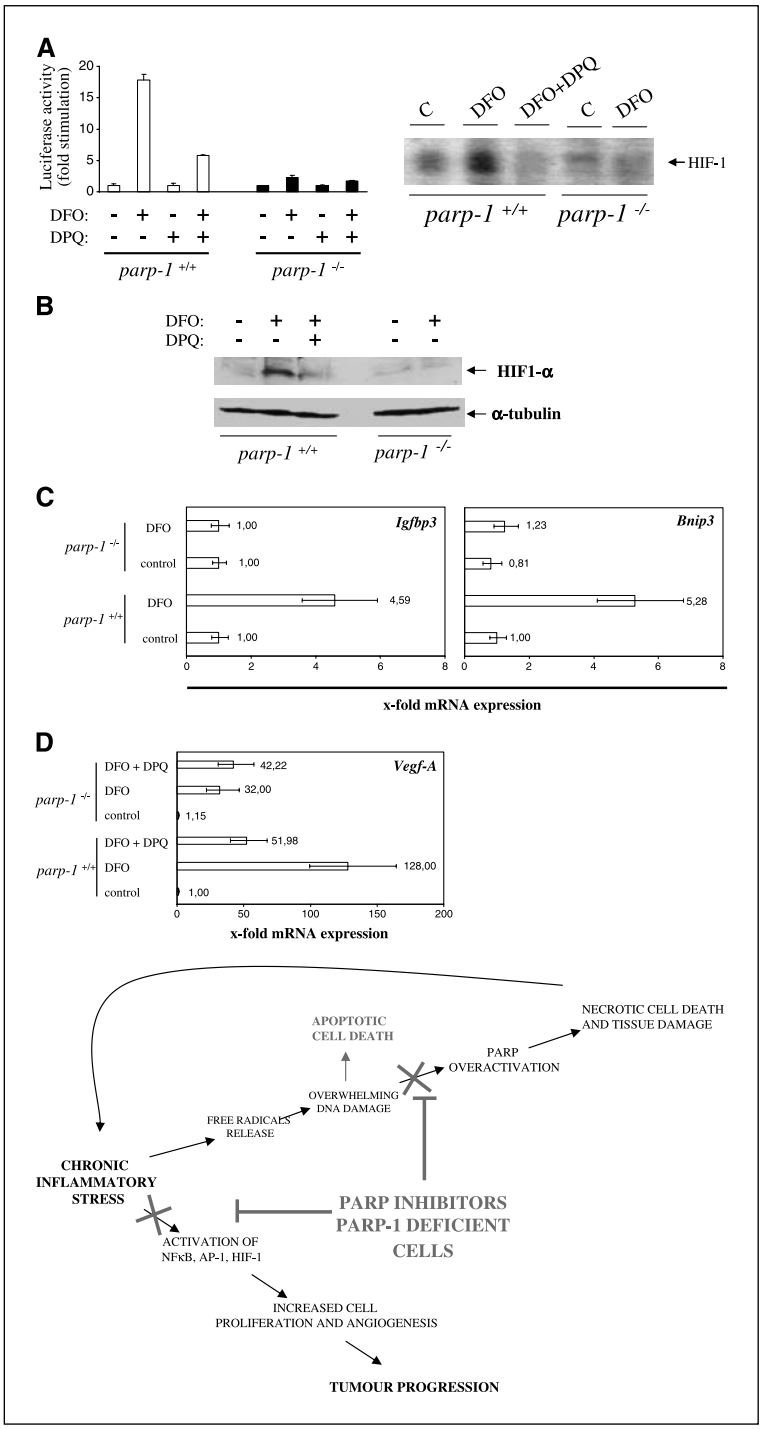

Figure 4. Defective HIF-1–dependent transcriptional activation in DPQ-treated and *parp-1*$^{-/-}$ cells. Cells were transiently cotransfected with 0.25 μg β-galactosidase reporter vector (as control of transfection) together with 1 μg of the HIF/luciferase plasmid (kind gift from Dr. Del Peso, Hospital Universitario de La Princesa, Madrid, Spain) or 1 μg of the NF-κB/luciferase plasmid or 1 μg of the AP-1/luciferase plasmid (both are kind gifts from Dr. López-Rivas, Centro Andaluz de Biologia del Desarrollo-CSIC, Sevilla, Spain) using jetPEI cationic polymer transfection reagent (Polytransfection, Illkirch, France) according to the manufacturer's instructions. *A,* 3T3 *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ were transfected with a reporter luciferase plasmid containing 9× HRE as described in Materials and Methods. Luciferase activity was measured 36 hours after DFO treatment. EMSA for HIF-1 activation. Conditions for EMSA are explained in Materials and Methods. *B,* protein levels of HIF-1α 24 hours after DFO treatment in 3T3 *parp-1*$^{+/+}$ [with or without DPQ (20 μmol/L)] and *parp-1*$^{-/-}$ cells treated with 200 μmol/L DFO. *C* to *D,* real-time RT-PCR of HIF-1-dependent genes (*Igfbp3, Bnip3*, and *Vegf-A*). mRNA levels were determined 36 hours after DFO treatment. *Columns,* mean of at least three independent experiments done in duplicate and HIF-1α protein levels are representative experiments of three. *D, bottom,* proposed model of action of PARP inhibitor in tumors, in which the inflammatory component is key for their development.

early response 3 (*Ier3; Iex-1*), *Kruppel-like factor 5* (*Klf5*), *calgranulin B* (*S100a9*), *tenascin C* (*Tnc*), *UDP-glucose ceramide glucosyltransferase* (*Ugcg*), *lectin galactose binding soluble 7* (*Lgals7*), *tranferrin receptor* (*Tfrf*), *membrane-associated protein 17* (*Map17*), *lysozyme* (*Lyzs*), *extracellular proteinase inhibitor* (*Expi*), *trichorhinophalangeal syndrome I* (*Trps1*), *Niemann Pick type C1* (*Npc1*), and *serum/glucocorticoid-regulated kinase* (*Sgk*)]; genes involved in oxidative stress, inflammation, and immune response include *glutathione peroxidase 1* (*Gpx1*), *glutathione* S-*transferase α4* (*Gsta4*) and *glutathione* S-*transferase ω1* (*Gsto1*), *interleukin-18* (*Il18*), *Nfkbiz* (also called *Mail*), *glutaredoxin 1* (*Glrx1*), *sterol* O-*acyltransferase* (*Soat-1*), *CTL-associated protein 2b* (*Ctla2b*), *glycoprotein marker of natural killer cells* (*Gp49a*), *lymphocyte antigen 6 complex* (*Ly6e*), and *drebrin-like* (*Dbnl*). All the above genes were either not up-regulated, or the expression significantly decreased with DPQ treatment or in *parp-1*$^{-/-}$ mice (Table 2). Some of the above genes are targets of NF-κB [*Nfkbiz, Iex-1, Tnc, Lyzs*, and *procollagen type I α2* (*Col1a2*); ref. 33] and AP-1 (*Tnc, procollagen type I, S100* family members, and *Pold2*; ref. 34), supporting our previous results that defective activation

of these two key transcription factors by either PARP inhibition or genetic deletion of PARP-1 results in an effective blockage of gene expression. During the promotion of skin carcinogenesis, the expression of several genes is also down-regulated. Of note are some genes that have been reported to be involved in the progression of different human and mouse tumors, which were only down-regulated or the expression significantly decreased with DPQ treatment or in *parp-1*$^{-/-}$ mice (Table 2): dermatofibrosarcoma and skin neoplasms (procollagens type I and III), *Igfbp5b, metallothionein 2* (*Mt2*), *purine-rich element binding protein B* (*Purb*), *trans*-acting transcription factor-1 (*Sp1*), and *fatty acid synthase* (*Fasn*). Other gene 2-fold down-regulated only in carcinogen-treated mice (without DPQ treatment) was the *fibroblast growth factor 2* (*Fgfr2*), whose loss of expression accompanies malignant progression of both animal and human prostate tumors (35).

One of the more important outcome in the current study is the ability of PARP-1/PAR to modulate the expression of genes involved in angiogenesis, particularly *Hif-1α, Pecam-1*, and probably *OPN*. Of particular interest is the absence of induction of *Hif-1α* after treatment with DPQ and in *parp-1*-deficient mice. This transcription factor has been largely involved in tumor progression by promoting global response to hypoxia, including new vessels formation (36). Hypoxia is an almost universal hallmark of solid tumors. Adaptation to hypoxia is critical for tumor survival and growth and is mediated in large part by transcriptional activation of genes that facilitate short-term adaptive mechanisms (e.g., increased vascular permeability, vasodilation, glucose transport, and switch to anaerobic metabolism) as well as long-term adaptive mechanisms. This coordinated homeostatic response is mediated, in large part, through the activation of the heterodimeric transcription factor HIF-1. Tumor hypoxia and overexpression of HIF-1 have been associated with resistance to certain therapies, increased risk of invasion and metastasis, and poor outcome in certain malignancies. The near universality of hypoxia in human tumors and centrality of the HIF pathway may have therapeutic utility as an antitumor strategy. Inhibition of HIF function in tumors may attenuate and contribute directly to tumor cell death through metabolic derangement. This role seems to be accomplished by the PARP inhibitor DPQ because HIF-1α is unable to accumulate after treatment with DPQ (Fig. 4*B*), and increased rates of apoptotic cells are present in the incipient tumors of DPQ-treated mice. Thus, inhibition of PARP could be of great potential interest in the design of new antiangiogenic therapies. Targeting PARP is not only a way to prevent efficient DNA repair during treatment with classic chemotherapy and radiotherapy as has been classically envisaged but also its effective targeting has more general effects on transcription of key genes involved in tumor progression. Up to date, the molecular link between the HIF-1 and PARP-1 has not been identified. What is clear from our results is that stabilization of HIF-1α differs drastically between *wt* and *parp-1*-deficient cells and also in the presence of the PARP inhibitor DPQ. PARP-1 has been reported repeatedly as a transcriptional cofactor of several transcription factor, including NF-κB and AP-1. As a hypothesis, we are working in the possible involvement of CPB/p300 protein as a link between PARP-1 and HIF-1α. CBP binding to HIF-1α is a key step for HIF-1-dependent transcriptional activation, and PARP-1 is a substrate for CBP (CBP-dependent PARP-1 acetylation is necessary for PARP-1 acting as a cofactor of NF-κB activation).

Different groups have proven the benefits of inhibiting PARP-1 in the potentiation of classic antineoplasic treatments, either radiotherapy or chemotherapy (4, 37, 38). In this study, we show that the PARP inhibitor DPQ on itself has antitumor activity. The effect of DPQ on tumor multiplicity, tumor incidence, and tumor size (Fig. 1*A*) clearly indicates that the sole inhibition of PARP is able to slow down tumor growth.

As we have previously shown, genetic deletion of PARP-1 completely prevented TPA-induced cell proliferation, with a clear effect in tumor latency, incidence, and multiplicity (6). Although it is now clear that proliferation of cells alone does not cause cancer, sustained cell proliferation in an environment rich in inflammatory cells and DNA damage-promoting agents (as free radicals derived reactive oxygen and nitrogen species during the inflammatory response do) potentiates and/or promotes neoplastic risk. Our hypothesis is that inhibition or genetic elimination of PARP-1 interferes with the promotion of tumors of epithelial origin, in which inflammatory processes play a critical role in this step (39). The implications of DNA repair inhibitors for anticancer therapy are well recognized (40), and the role of PARP-1 in DNA damage repair has been extensively characterized. First-generation PARP-1 inhibitors, such as 3-AB, lacked the potency, specificity, and pharmacologic properties required for detailed preclinical evaluation of their ability to increase the sensitivity of tumors to anticancer chemotherapy and radiotherapy. New highly potent PARP inhibitors have shown their specificity and *in vivo* activity to enhance chemotherapy and radiotherapy of human cancer (40). More recently, two different groups provided strong evidences in the sense that monotherapy with PARP inhibitors was effective in eliminating 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 (41, 42).

It is now evident that inflammation has powerful effect on tumor development. As summarized in the proposed model (Fig. 4*D, bottom*), PARP inhibitors may affect tumor progression by interfering with the activation of key transcription factors involved in cell proliferation, angiogenesis, and inflammation. Early in the neoplastic process, proinflammatory factors and cells are powerful tumor promoters, producing an attractive environment for tumor growth, facilitating sustained DNA damage, and promoting angiogenesis. Thus, effective inhibition of PARP might have a clear benefit in antitumor therapy by facilitating a reduced oxidative status (thus minimizing reactive oxygen and nitrogen species release and the DNA damage) and minimizing the activation of proinflammatory and proangiogenic factors.

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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, 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. 9999: 1–13, 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

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HIF-1α subunit level. In normoxic conditions, and with the presence of iron, HIF-1α is 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[Q1] et al., 2003]. 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[Q2] et al., 2003]. 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[Q3]) and then blocked. Polyclonal antibodies to HIF-1α (1/1000, Bethyl Lab, Inc.[Q4]) and Mn-SOD (1/8000, StressGen Biotechnologies[Q5]) 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.[Q6]) 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 ± SD 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).

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 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.

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

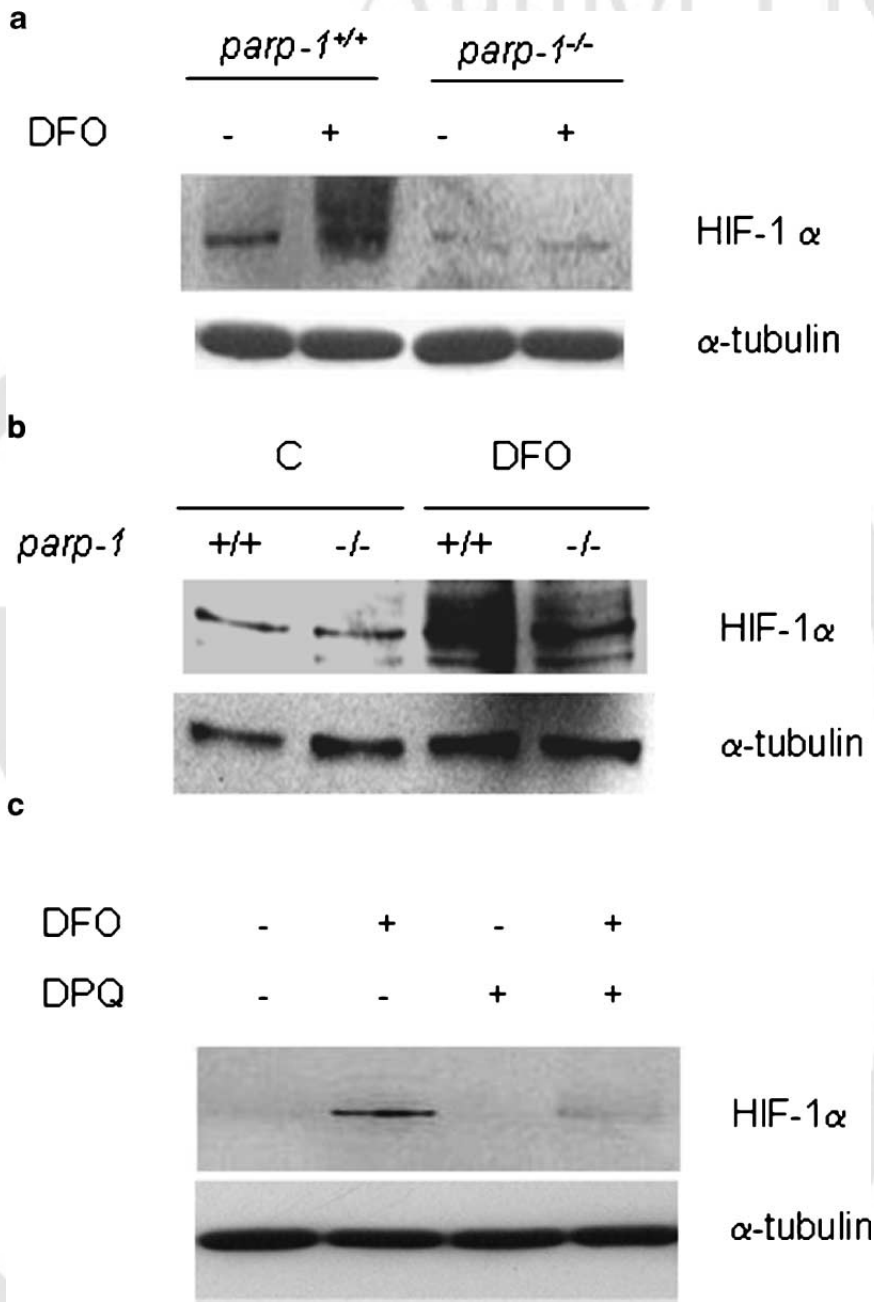

Fig. 1. Western blot analysis of the effect of PARP-1 on DFO-mediated HIF-1α stabilization after 24 h of treatment. Representative immunoblot of HIF-1α expression in control and DFO-treated wild-type and *parp-1*$^{-/-}$ immortalised **(a)** or primary **(b)** MEFs. **(c)** Representative Western blot 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.

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

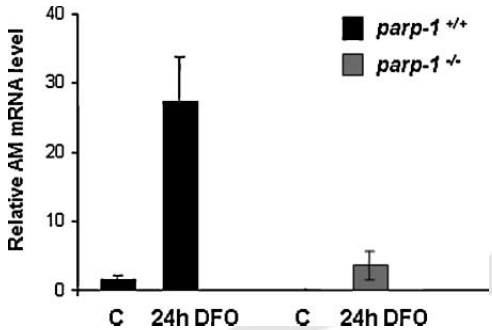

Fig. 2. Effect of DFO treatment on AM mRNA expression in *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ cells. The results are expressed as mRNA expression relative to control wild-type cells after normalization against beta-actin. Each sample was analysed in triplicate. The mean ± SD of three RNA extracts for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

(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 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.

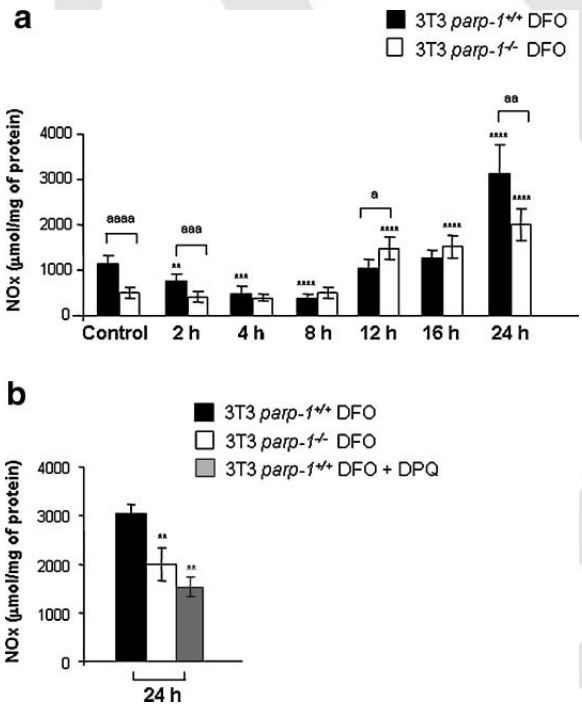

Fig. 3. **a**: Effect of DFO treatment on NOx levels (µmol/mg of protein) in *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ cells. **b**: NOx levels in *parp-1*$^{+/+}$, *parp-1*$^{-/-}$ and PARP inhibited cells (DPQ) after 24 h of DFO treatment. Values represent the mean ± SD from three independent experiments.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

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 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

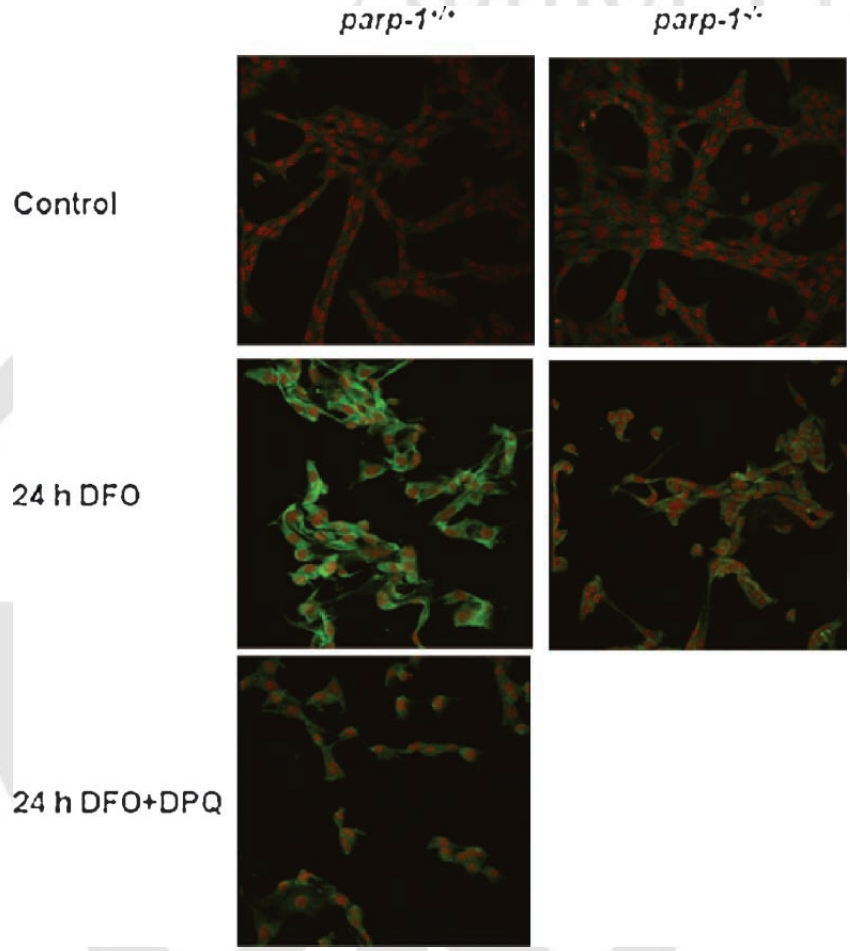

Fig. 4. Confocal immunofluorescence of iNOS in *parp-1*$^{+/+}$, DPQ-treated, and *parp-1*$^{-/-}$ cells after 24 h of DFO exposure. Control cells of the two different genotypes are also shown.

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 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).

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

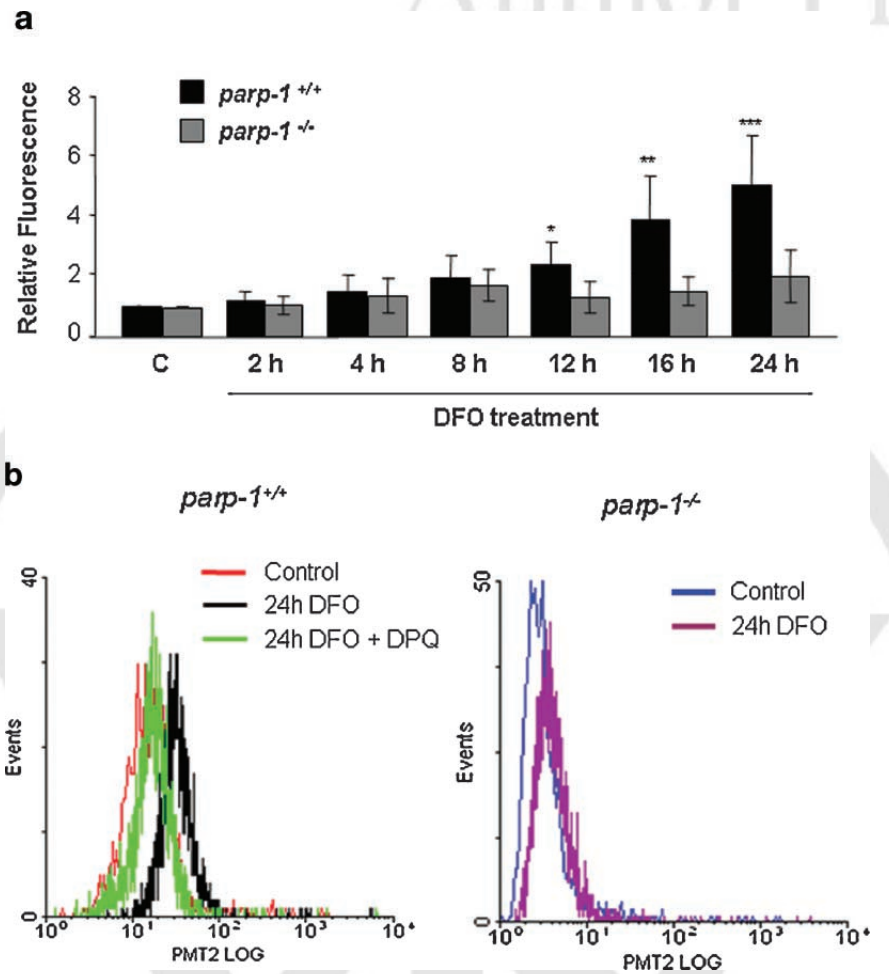

Fig. 5. **a**: Time course of ROS generation in DFO-treated cells. DCF fluorescence as a measurement of ROS production was determined by flow cytometry. Mean values of the log fluorescence for *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ cells at different times after DFO treatment. Results were normalized 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.

COLOR

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 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,

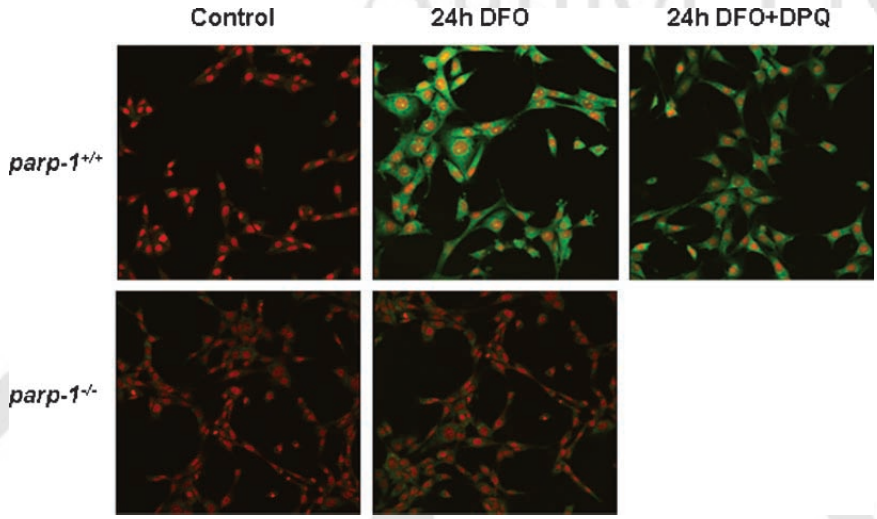

Fig. 6. Confocal immunofluorescence of DCF fluorescence in *parp-1*$^{+/+}$ cells, with and without DPQ, and in *parp-1*$^{-/-}$ cells after 24 h of DFO treatment. Photographs of cells without treatment are also shown.

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 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.

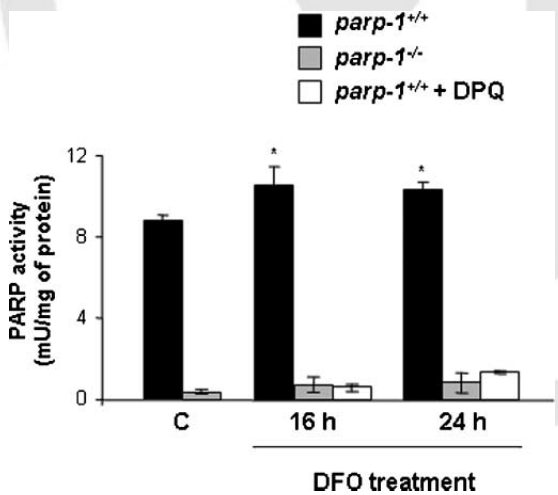

Fig. 7. PARP activity in control, 16 and 24 h DFO-treated *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ cells. Data obtained in DPQ+DFO (16 and 24 h) *parp-1*$^{+/+}$ cells are also included. Values represent mean ± SD from three independent experiments. Unit definition: 1 U PARP incorporates 100 pmol poly(ADP)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

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

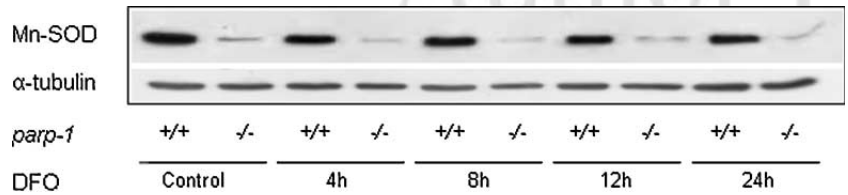

Fig. 8. Analysis of Mn-SOD expression in control and DFO-treated *parp-1*$^{+/+}$ and *parp-1*$^{-/-}$ cells. A representative immunoblot is shown. α-tubulin immunodetection was also included as a protein-loading control.

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

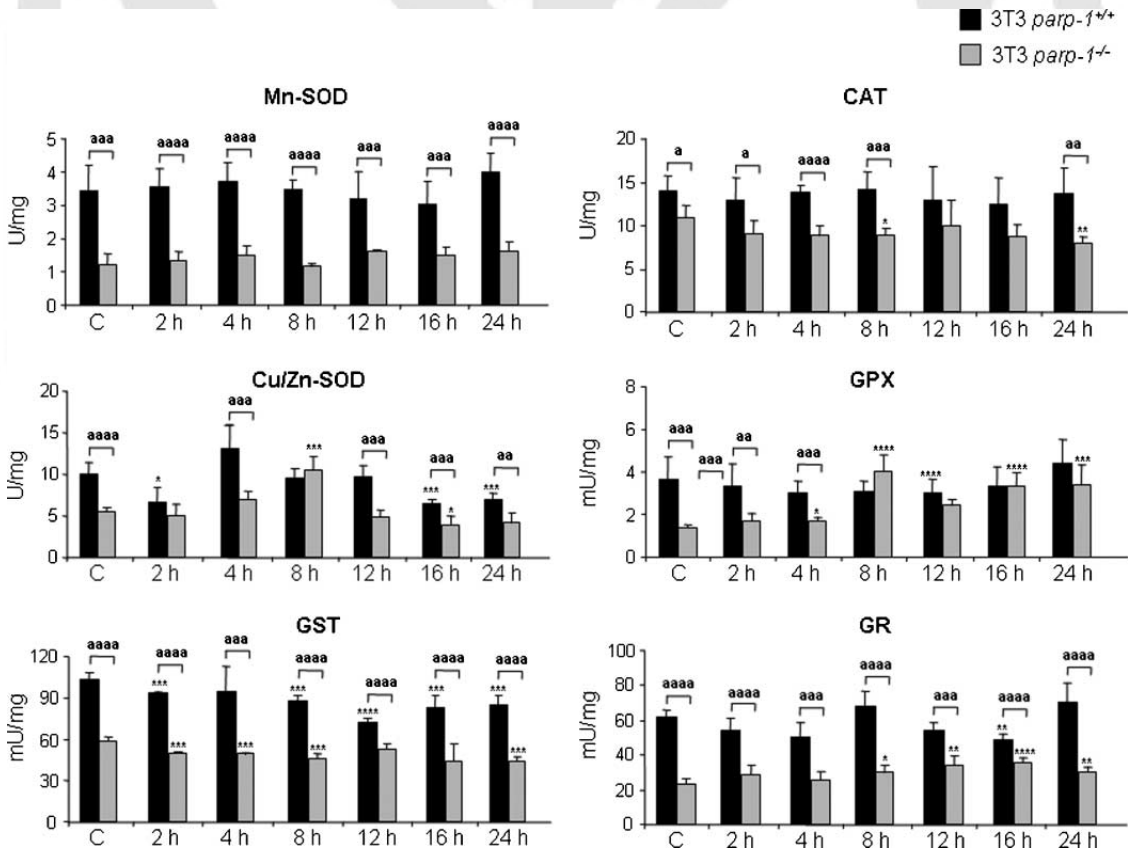

Fig. 9. Effect of DFO treatment on Mn-SOD, Cu/Zn-SOD, GST, CAT, GPX and GR activity. Data are expressed as mean ± SD for n = 4 per group in U/mg or mU/mg of protein. One SOD unit is defined as the amount of enzyme that inhibits the rate of cytochrome *c* reduction by 50%. One unit of GST activity is 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 $^{aaaa}p < 0.001$.

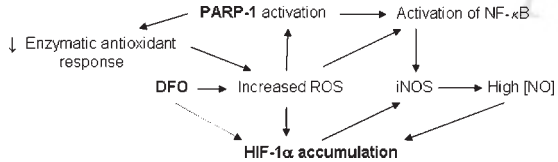

Fig. 10. Proposed model of action of PARP-1 in DFO-induced HIF-1α accumulation. DFO induces HIF-1α accumulation directly (. . .) or indirectly (—). PARP-1 plays a pivotal role, through its effect on ROS and NO production, in the indirect pathway. For details, please see the text.

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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PARP inhibition sensitizes p53-deficient breast cancer cells to doxorubicin-induced apoptosis

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p53 deficiency confers resistance to doxo (doxorubicin), a clinically active and widely used antitumour anthracycline antibiotic. The purpose of the present study was to investigate the reversal mechanism of doxo resistance by the potent PARP [poly(ADP-ribose) polymerase] inhibitor ANI (4-amino-1,8-naphthalimide) in the p53-deficient breast cancer cell lines EVSA-T and MDA-MB-231. The effects of ANI, in comparison with doxo alone, on doxo-induced apoptosis, were investigated in matched pairs of EVSA-T or MDA-MB-231 with or without ANI co-treatment. Doxo elicited PARP activation as determined by Western blotting and immunofluorescence of poly(ADP-ribose), and ANI enhanced the cytotoxic activity of doxo 2.3 times and in a caspase-dependent manner. The long-term cytotoxic effect was studied by a colony-forming assay. Using this assay, ANI also significantly potentiates the long-term cytotoxic effect with respect to treatment with doxo alone. Decrease in mitochondrial potential together with an increase in cytochrome *c* release, association of Bax with the mitochondria and caspase 3 activation were also observed in the presence of ANI. Therefore PARP inhibition may represent a novel way of selectively targeting p53-deficient breast cancer cells. The underlying mechanism is probably a potentiation of unrepaired DNA damage, shifting from DNA repair to apoptosis due to the effective inhibition of PARP activity.

Key words: apoptosis, Bax, breast cancer, chemotherapy, doxorubicin, mitochondria, p53, poly(ADP-ribose) polymerase-1 (PARP-1).

INTRODUCTION

Dysregulation of normal apoptotic mechanisms provides a growth advantage to cancer cells [1]. In breast cancer, dysregulated apoptotic pathways include down-regulated death receptor pathway function, p53 mutations and abnormal bcl-2 pathway function [2–4]. Furthermore, breast cancer treatments including chemotherapy, radiation therapy and hormone therapy induce apoptotic mechanisms to cause cancer cell death [5]. Therefore activation of specific apoptotic mechanisms in breast cancer cells could be an effective means to treat breast cancer.

PARP-1 [poly(ADP-ribose) polymerase-1] is the principal member of a family of enzymes possessing poly(ADP-ribosylation) catalytic capacity. It is a conserved nuclear protein that binds rapidly and directly to both single- and double-strand breaks. Both processes activate the catalytic capacity of the enzyme, which in turn modulates the activity of a wide range of nuclear proteins by covalent attachment of branching chains of ADP-ribose moieties. Organisms and cellular systems deficient in functional PARP-1 display severely impaired base excision repair and genomic instability, suggesting that the enzyme may play a primary role in the cellular response to DNA damage [6].

Increasing interest in potential clinical applications of PARP inhibition has led to the development of a wide range of new compounds, the more recently developed of which display greatly increased potency and specificity compared with the prototype PARP inhibitor 3-aminobenzamide [7]. In particular, higher potency PARP inhibitors have a greatly decreased effect on mono(ADP-ribosyl) transferase enzymes. ANI (4-amino-1,8-naphthalimide) is a potent PARP inhibitor ($IC_{50} = 180$ nM) and has been reported to increase the sensitivity to radiation in a number of human tumour cell lines, both *in vitro* and when grown as xenografts in mice [8]. Apoptosis is one of the most important pathways through which chemotherapeutic agents inhibit the growth of cancer cells. Thus it is crucial to investigate whether the induction of apoptosis is associated with the molecular mechanism by which inhibition of PARP may exert its biological effects on breast cancer cells.

The objectives of the present study were to investigate whether ANI could potentiate the cytotoxic effect of doxo (doxorubicin) in the p53-deficient human breast cancer cell lines, EVSA-T and MDA-MB-321, and elucidate the molecular mechanism by which ANI and doxo may induce apoptotic cell death in these cell lines. Our results show that doxo induces a rapid PARP activation and moderate cell killing, which is markedly potentiated by co-treatment with the PARP inhibitor ANI by accelerating the mitochondrial steps of apoptosis. In summary, our results suggest that PARP inhibition may represent a novel way of selectively targeting p53-deficient breast cancer cells.

EXPERIMENTAL

Cell culture

EVSA-T and MDA-MB-231 cells (breast cancer cell lines with p53 mutated [9,10]) were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) foetal bovine serum at 37 °C in a humidified 5 % CO_2 atmosphere. Cells were plated for 24 h before doxo treatment.

Abbreviations used: ANI, 4-amino-1,8-naphthalimide; CFA, colony-forming assay; doxo, doxorubicin; NF-κB, nuclear factor κB; PAR, poly(ADP-ribose); PARP, PAR polymerase; PI, propidium iodide; Z-Val-Ala-DL-Asp-CH_2F, benzyloxycarbonyl-valylalanyl-DL-aspartylfluoromethane.

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Drugs

Cells were treated with doxo for 1 h in Dulbecco's modified Eagle's medium supplemented with 10 % foetal bovine serum at 37 °C in a humidified 5 % CO_2 atmosphere. The PARP inhibitor ANI (10 μM) was dissolved in culture medium immediately before use. ANI solutions (10 μM) also contained < 2 % DMSO to improve solubility. ANI is sparingly soluble in water without adding DMSO. ANI was added 1 h before doxo treatment and thereafter present in the culture throughout the experiment. The pan-caspase inhibitor Z-Val-Ala-DL-Asp-CH_2F (benzyloxycarbonyl-valylalanyl-DL-aspartylfluoromethane, also known as Z-VAD-FMK; 50 μM) was added 2 h before doxo treatment and was thereafter present in the culture throughout the experiment.

Analysis of cell death

Cell viability was evaluated as described previously by the sulphorhodamine B method [11]. Measurement of apoptosis was determined by annexin V staining. After drug treatments, cells were harvested using trypsin-EDTA, washed once with ice-cold PBS and resuspended in 1 ml of annexin V binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$). Then, 75 000 cells were stained with 5 μl of annexin V FLUOS (Roche Molecular Biochemicals) in 100 μl of annexin V buffer at 4 °C. After 30 min, 100 μl of binding buffer was added to each tube and samples were analysed using a tri-laser FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software (Becton Dickinson).

Sub-G_1 analysis was examined by flow cytometry using the PI (propidium iodide) DNA-staining method. Cells were harvested with trypsin-EDTA, washed once with ice-cold PBS and resuspended in 100 μl of PBS. Ice-cold ethanol (70 %, 900 μl) was added to the cells for 5 min, washed with 2 ml of PBS and the cells were resuspended in 250 μl of PI/RNase solution (PBS, 100 μg/ml RNase and 40 μg/ml PI). After 30 min, samples were analysed using a tri-laser FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software (Becton Dickinson).

CFA (colony-forming assay)

Semi-confluent culture flasks were trypsinized, and adequate number of cells were seeded in 25 cm^2 tissue culture flasks. One day later, cells were exposed to doxo at the indicated concentrations for 1 h. Cells were treated with the PARP inhibitor ANI (10 μM) for 24 h. Cells were stained with crystal violet, 15 or 20 days later, and colonies of 50 or more cells were scored. Surviving fractions were determined from colony counts and were corrected for the plating efficiency of the non-treated controls.

Immunofluorescence

Immunostaining for PAR [poly(ADP-ribose)] was performed on the cells, which were grown on glass coverslips and fixed in ice-cold methanol/acetone (1:1, v/v) for 10 min. PAR was detected by immunofluorescence using the monoclonal antibody 10H (Alexis, Grünberg, Germany) and FITC-conjugated goat anti-mouse immunoglobulins (Sigma, St. Louis, MO, U.S.A.). Nuclear counterstaining with PI was performed after removal of excess secondary antibody. Immunostaining was visualized with a Leica Spectral confocal laser microscope.

Western-blot analysis

Cells were detached from the culture flask, washed with PBS and resuspended in 100 μl of lysis buffer (50 mM Tris/HCl, pH 8, 0.1 mM EDTA, 0.5 % Triton X-100 and 12.5 mM 2-mercaptoethanol) for 30 min on ice. The pellet was eliminated and a sample buffer [50 mM Tris/HCl, pH 6.8, 6 M urea, 6 % 2-mercaptoethanol, 3 % (w/v) SDS and 0.003 % Bromophenol Blue] was added to the supernatant. Proteins were resolved by SDS/PAGE (12 % gel) and transferred on to Immun-Blot PVDF membranes (Bio-Rad). The blot was blocked with 5 % (w/v) milk powder in PBS containing 0.1 % Tween 20 for 30 min, washed with PBS/Tween, incubated overnight with the antibodies PAR (Biomol, Hamburg, Germany), anti-PARP-1 (EGF-69), anti-Bax and anti-cytochrome *c* (Pharmingen, San Diego, CA, U.S.A.) and anti-α-tubulin (Sigma) and incubated for 2 h with appropriate secondary antibodies. Bands were visualized by ECL-PLUS (Amersham Biosciences) and pictures were taken with the imaging system ChemiDoc XRS System (Bio-Rad).

For the measurements of cytochrome *c* and Bax, cells were washed with PBS and lysed for 5 min in 30 μl of ice-cold lysis buffer (80 mM KCl, 250 mM sucrose, 500 μg/ml digitonin, 1 μg/ml each of the protease inhibitors leupeptin, aprotinin, pepstatin and 0.1 mM PMSF in PBS). Then, cell lysates were centrifuged for 5 min at 10 000 ***g***. Proteins from the supernatant (cytosolic fraction) and pellet (membrane fraction) were mixed with sample buffer and resolved by SDS/PAGE (12 % gel). Cytochrome *c* and Bax were determined by Western-blot analysis as described above.

Detection of mitochondrial membrane potential

Cells were plated in 6-well plates (2×10^5 cells) and grown for 24 h. After this period, the cells were treated with doxo (1 μg/ml) and doxo (1 μg/ml) + ANI (10 μM). Cells were harvested using trypsin-EDTA, washed once with ice-cold PBS and resuspended in PBS with 40 nM $DIOC_6$ (3,3′-dihexyloxacarboxyanine iodide; Molecular Probes, Eugene, OR, U.S.A.) for 30 min at 37 °C. Samples were then analysed with an FACScan cytometer (Becton Dickinson) and the fluorescence was detected in FL1. During analysis of the flow-cytometric data, a marker indicating a cell population having lower $\Delta\psi_m$ was applied to histograms, and the percentage of cells in the region was determined. The number of cells with lower membrane potential (cells treated with doxo, with or without ANI) were expressed as a percentage of the cells without treatment (control cells).

Electrophoretic mobility-shift assay

The double-stranded oligonucleotides 5′-TGCTAGGGGGATTTTCCCTCTTCTTGT-3′ [12] with the sequences of the binding sites for NF-κB (nuclear factor κB) of the inducible nitric oxide synthase promoter were purchased. These oligonucleotides were end-labelled with [γ-^{32}P]ATP and T4 DNA polynucleotide kinase and used as probe. Nuclear extracts were obtained according to a previous report [13]. Nuclear extracts (3 μg of protein) were incubated with 2 μl of ^{32}P-labelled probe (6×10^4 d.p.m.) in a final volume of 20 μl of reaction mixture for 15 min at 4 °C as described previously [14]. The DNA–protein complexes were separated on native 5 % polyacrylamide gels in 0.5 Tris/borate/EDTA buffer.

RESULTS

Doxo treatment induces PARP activation

Many different cell insults infringing DNA damage have been shown to be capable of activating PARP. Doxo is a powerful DNA-damaging agent but there is no evidence in the literature of a direct effect on PARP activation in tumour cells. We hypothesized that the activation of PARP may counteract the doxo-induced cytotoxicity by promoting DNA repair. First, we demonstrate that

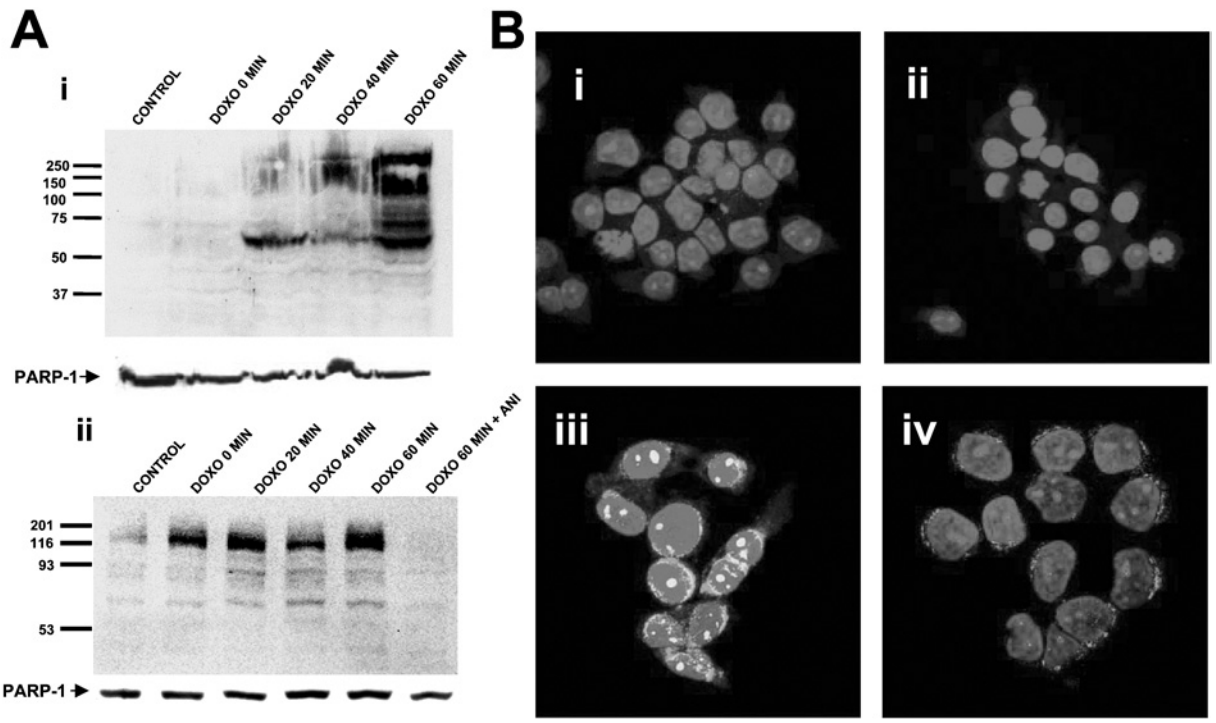

Figure 1 PARP activation after doxo treatment

(**A**) Time course of PAR formation in (i) EVSA-T and (ii) MDA-MB-231 cell lines. After treatment with 1 μg/ml doxo (1 h), cells were harvested and analysed by immunoblotting for PAR formation. PARP-1 was used as the loading control. (**B**) Immunofluorescence staining of EVSA-T cells for PAR formation. Nuclei are counterstained with PI: (i) control, (ii) 10 μM ANI, (iii) treated with doxo (1 μg/ml, 1 h), (iv) EVSA-T treated with doxo (1 μg/ml) + ANI (10 μM). Overlaid images show that doxo induces PAR formation in nuclei (white), and it is reduced with ANI treatment. Cells grown on coverslips were fixed for 40 min after treatment with doxo.

doxo induced PARP activation. In Figure 1, we show that doxo (1 μg/ml) is capable of inducing a rapid PARP activation (20 min after treatment) measured by Western blotting [Figure 1A(i) (EVSA-T) and 1A(ii) (MDA-MB-231)] or immunofluorescence [Figure 1B(iii), EVSA-T cells]. Pretreatment with 10 μM ANI completely prevented doxo-induced PARP activation [Figure 1B(iv)]. The action of ANI as a PARP inhibitor has been extensively described elsewhere [15].

Co-treatment of p53-deficient breast cancer cells with doxo + ANI potentiates apoptotic cell death

EVSA-T breast cancer cells are resistant to treatment with a number of chemotherapeutic agents, including doxo [9]. In a preliminary assay, we performed a dose–response of doxo-induced cell death to determine at which dose these cells start to be sensitive to the drug. In short-term experiments of cell viability (until 72 h), they were completely resistant to doses below 1 μg/ml and partially sensitive to a dose of 1 μg/ml, reaching a 50 % cell death after doxo treatment (Figure 2A). These results show that EVSA-T cells were very poorly sensitive to doxo-induced cytotoxic effect, as has been shown previously [11]. Then, we used annexin V and sub-G1 to evaluate cell death as a measure of short-term cytotoxic effects and CFA as a measure of long-term cytotoxic effects, and we also studied the effect of ANI co-treatment in a second p53-deficient breast cancer cell line, MDA-MD-231, to substantiate further our observation. Pretreatment with 10 μM ANI resulted in a potentiation of doxo-induced cell death measured by all three criteria [Figures 2B (annexin V in EVSA-T), 2C (sub-G_1 in EVSA-T), 2D (sub-G_1 in MDA-MB-231) and 2E (CFA in EVSA-T)]. The extent of potentiation of cell death was 2.3-fold with annexin V (Figure 2B) and 1.7-fold (Figure 2C) or 2-fold (Figure 2D) with sub-G_1. Long-term cytotoxicity using ANI and doxo was also potentiated subsequent to treatment with doxo alone according to the CFA (Figure 2E). The effect of ANI was completely abolished with the pan-caspase inhibitor Z-Val-Ala-DL-Asp-CH_2F (Figure 2B), suggesting that ANI was activating the apoptotic pathway at some point.

In most cases, p53-induced apoptosis proceeds through translocation of the cytoplasmic protein Bax to the mitochondria, where it co-operates with truncated Bid in the release of cytochrome *c*, leading to caspase activation. This pathway is impaired in p53 mutant cells like EVSA-T. To analyse more precisely which steps of apoptosis were altered by ANI, we studied depolarization of mitochondrial membrane potential, mitochondrial Bax translocation, cytochrome *c* release and activation of caspase 3 by PARP-1 cleavage (which have been described as the hallmark of doxo-induced apoptosis) in EVSA-T cells. In Figures 3(A)–3(C), the change in mitochondrial permeability was poorly shifted by doxo alone, and the pretreatment with ANI further increased the decrease in permeability. This change was also accompanied by an increase in Bax migration, cytochrome *c* release (Figure 3D) and caspase 3 activation, as measured by PARP-1 cleavage (Figure 3E), suggesting that PARP inhibition is capable of restoring a p53-like response in these cells.

Another mechanism by which tumour cells may be resistant to chemotherapy is by activation of the transcription factor NF-κB, which is responsible for the activation of anti-apoptotic genes [16] and has been involved in the progression to hormone independence in breast cancer [17]. Several laboratories, including ours, have shown that elimination of PARP-1 impairs the response of NF-κB [13,14]. We assessed the impact of ANI on doxo-induced NF-κB activation using an electrophoretic mobility-shift assay and found that ANI does not affect the ability of

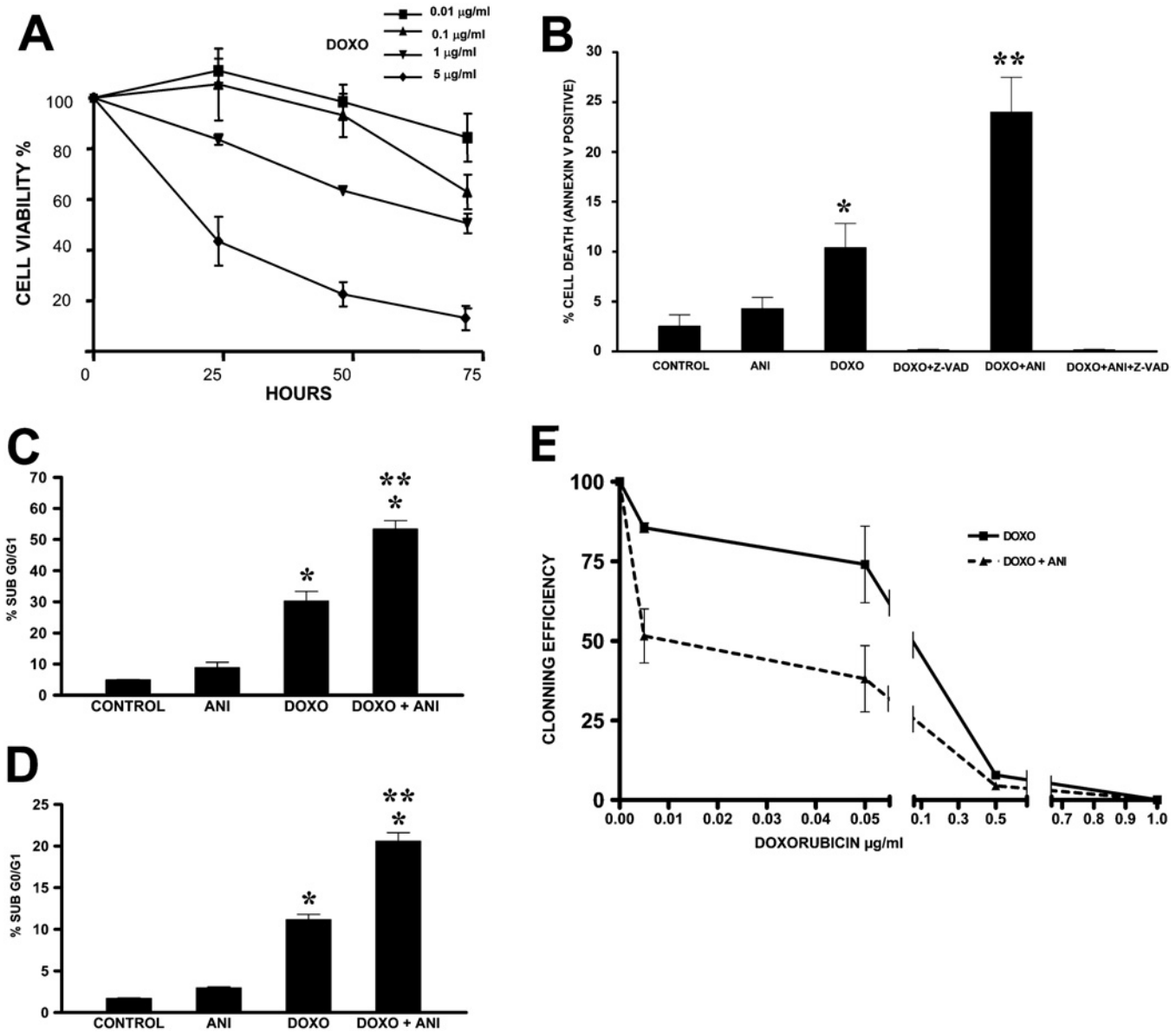

Figure 2 Effects of PARP inhibition on cell death after doxo treatment

(**A**) Cell viability in the EVSA-T cell line was assessed by the sulphorhodamine B assay in the presence of increasing concentrations of doxo until 72 h; results are the average of three separate experiments. (**B**) Induction of apoptosis, 24 h after treatment with 1 μg/ml doxo in the EVSA-T cell line, measured by phosphatidylserine externalization (annexin-V) using flow-cytometry analysis. The PARP inhibitor ANI (10 μM) significantly increased (2.25 times) the cell death induced by doxo. Analysis of doxo-induced cell death with or without the PARP inhibitor (ANI, 10 μm) and the pan-caspase inhibitor Z-Val-Ala-DL-Asp-CH_2F (Z-VAD; 50 μM) showed that the cell death induced by doxo was caspase-dependent. Cytotoxicity of the PARP inhibitor (ANI, 10 μM) was measured and it showed a low toxicity (4 %) 24 h after treatment. Error bars represent the S.E.M. for at least four independent experiments. *$P < 0.05$ compared with control cells, cells treated with ANI and cells treated with doxo + ANI. **$P < 0.001$ compared with control and ANI-treated cells. (**C**, **D**) Cell death 48 h after treatment with 1 μg/ml doxo in EVSA-T (**C**) and MDA-MB-231 (**D**) cell lines, measured by PI staining and flow-cytometry analysis. The cytotoxic effect of doxo was increased by PARP inhibition 1.7 times in EVSA-T and two times in MDA-MB-231 cells. Cytotoxicity of the PARP inhibitor (ANI, 10 μM) was measured and it showed a low toxicity (6 % in EVSA-T and 2.9 % in MDA-MB-231) 48 h after treatment. Error bars represent the S.E.M. for at least four independent experiments. *$P < 0.001$ compared with control and ANI-treated cells. **$P < 0.001$ compared with cells treated with doxo. (**E**) CFA after incubation with different concentrations of doxo and with or without PARP inhibitor (ANI, 10 μM). ANI co-treatment significantly potentiated the cytotoxicity of doxo. CFA with ANI alone gave essentially the same result as the untreated control. Survival was determined from triplicate measurements from three independent experiments and normalized for the plating efficiency or untreated controls. Error bars represent S.E.M.

doxo to induce NF-κB activation, suggesting that ANI-induced potentiation of cell death is independent of NF-κB (Figure 4).

DISCUSSION

A panel of biological markers including regulators such as p53, Bcl-2 family proteins, caspases and DNA fragmentation factor has been described as having a role in apoptosis. Their assessment in cell lines and in clinical samples, particularly in the neo-adjuvant setting, would help to build a picture of their contribution to the biology of chemoresistance.

EVSA-T and MDA-MB-231 breast-cancer-derived cell lines are deficient in p53 and relatively insensitive to many chemotherapeutic agents [18]. As demonstrated by LD_{50} (median lethal dose) determination and biochemical data, co-treatment with

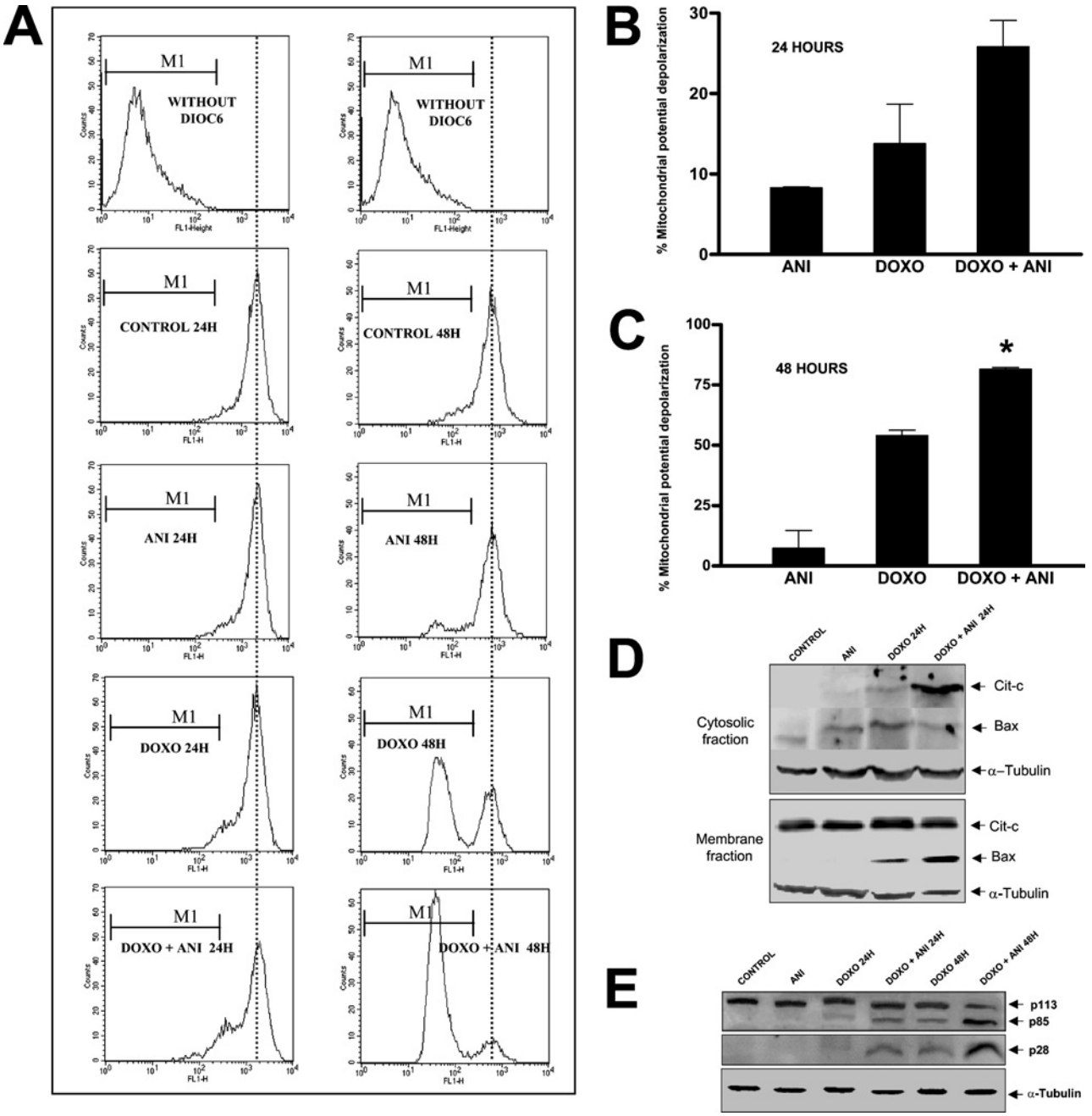

Figure 3 Effects of PARP inhibition on mitochondrial membrane potential and PARP-1 cleavage after doxo treatment

(**A**) Mitochondrial membrane potential in EVSA-T cell line, 24 and 48 h after treatment with 1 μg/ml doxo, detected by $DIOC_6$ staining and flow-cytometry analysis. Marker indicates the region of cell population used for analysis. (**B**, **C**) Analysis of mitochondrial membrane depolarization in EVSA-T for 24 h (**B**) and 48 h (**C**). Mitochondrial membrane depolarization triggered by doxo was increased after PARP inhibition (ANI, 10 μm) for both times. Error bars represent S.E.M. for at least three independent experiments. $^*P < 0.001$ compare with cells treated only with ANI and cells treated only with doxo. (**D**) Cytochrome *c* release from mitochondria to cytosolic fractions and translocation of cytosolic Bax to mitochondrial fractions after treatment with 1 μg/ml doxo or doxo + ANI in EVSA-T cells. (**E**) Caspase-mediated PARP-1 cleavage in EVSA-T was determined by Western blotting. The 85 and 28 kDa fragment of PARP cleavage is shown 24 and 48 h after 1 μg/ml doxo treatment. PARP-1 cleavage triggered by doxo was increased after PARP inhibition for both times.

PARP inhibitors sensitized EVSA-T and MDA-MB-231 cells to doxo-induced apoptosis. Significant increases in the proteolysis of cell death substrates and DNA fragmentation (sub-G_1) further verified a caspase 3-mediated sensitization in doxo-induced apoptosis.

Doxo is an active chemotherapeutic agent used in clinical oncology. Doxo is a key adjuvant drug for breast cancer treatment. It triggers apoptosis through several mechanisms. As with many chemotherapeutic agents, it induces DNA damage by interacting with topoisomerase II, leading to DNA breakage [19]. So far, there are no reports describing PARP activation by topoisomerase II inhibitors. In the present study, we have found a rapid activation of PAR synthesis after doxo treatment, suggesting a direct effect of doxo on PARP activity (Figure 1).

The ability of PARP inhibitors to potentiate drug-induced cell death in tumour cells has been shown in multiple studies due to

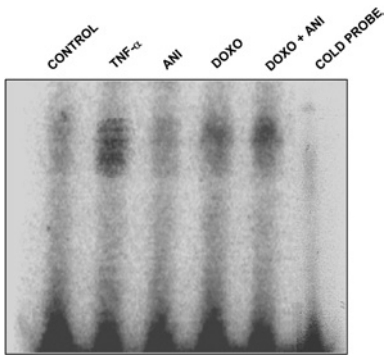

Figure 4 The doxo-induced activation of NF-κB is not counteracted by ANI

Band-shift analysis of NF-κB activation using the κB inducible nitric oxide synthase promoter sequence under different conditions in EVSA-T cell lines: untreated control, internal control for NF-κB activation using TNF-α (20 ng/ml, 2 h), cells incubated with ANI (10 μM), cells treated with doxo (1 μg/ml) and cells treated with doxo (1 μg/ml) + ANI (10 μM), 24 h after treatment. Competition with an unlabelled probe was used to confirm that the shifted complex was NF-κB. NF-κB activation is similar in cells treated with doxo and cells treated with doxo + ANI. The results shown are representative of three independent experiments.

their potential application as chemo- and radiopotentiators [7,20]. Although there are examples showing direct toxic effects of PARP inhibitors in tumour cells [21], most of the studies focus on the potentiating effects of PARP inhibitors on alkylating agents or ionizing radiation-induced tumour cell death.

Exposure of cells to ionizing radiation leads to hydroxyl radical-mediated DNA injury, whereas alkylating agents directly damage DNA. Other types of cytotoxic drugs such as topoisomerase I and II inhibitors may also lead to DNA breakage. A previous study has shown that the DNA strand breaks induced by the topoisomerase I inhibitor, camptothecin, were increased by the PARP inhibitor NU1025 and on exposure to camptothecin-activated PARP. In contrast, NU1025 did not increase the DNA strand breakage or cytotoxicity caused by the topoisomerase II inhibitor etoposide [22]. However, in our model, we have found that PARP is involved in the cellular response to doxo-mediated DNA damage. This is probably the first report showing that PARP inhibition increases the cytotoxic effects by topoisomerase II inhibitor.

Owing to this, deficient repair of DNA breaks after the inhibition of PARP leads to accumulation of DNA damage and shift from a repair response to an apoptotic one. This apoptotic response is, moreover, p53-independent, since EVSA-T and MDA-MB-231 contain a mutant-inactive p53. The mechanism by which ANI facilitates doxo-induced apoptosis is related not to a decreased NF-κB response (Figure 4) but rather to an acceleration of apoptosis due to an increased loss of the mitochondrial potential, leading to activation of the final caspase 3, as revealed by the increase in PARP-1 cleavage and oligonucleosomal DNA fragmentation (sub-G_1, Figures 2C and 2D). Moreover, the CFA assay shows a striking potentiation of doxo-induced cell death after co-treatment with ANI, suggesting that the long-term effect of doxo is amplified with the use of PARP inhibitors, minimizing clonal expansion of resistant tumour cells. A recent work has also reported that the use of PARP inhibitors together with doxo reduces doxo-induced cardiac dysfunction by avoiding necrotic cell death [23].

On the basis of these results, PARP inhibitors may be potentially useful, in combination with topoisomerase II inhibitors, in anticancer chemotherapy in p53-deficient tumours, which is the direct cause of resistance against chemotherapy.

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Therapeutic Effect of a Poly(ADP-Ribose) Polymerase-1 Inhibitor on Experimental Arthritis by Downregulating Inflammation and Th1 Response

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Poly(ADP-ribose) polymerase-1 (PARP-1) synthesizes and transfers ADP ribose polymers to target proteins, and regulates DNA repair and genomic integrity maintenance. PARP-1 also plays a crucial role in the progression of the inflammatory response, and its inhibition confers protection in several models of inflammatory disorders. Here, we investigate the impact of a selective PARP-1 inhibitor in experimental arthritis. PARP-1 inhibition with 5-aminoisoquinolinone (AIQ) significantly reduces incidence and severity of established collagen-induced arthritis, completely abrogating joint swelling and destruction of cartilage and bone. The therapeutic effect of AIQ is associated with a striking reduction of the two deleterious components of the disease, i.e. the Th1-driven autoimmune and inflammatory responses. AIQ downregulates the production of various inflammatory cytokines and chemokines, decreases the antigen-specific Th1-cell expansion, and induces the production of the anti-inflammatory cytokine IL-10. Our results provide evidence of the contribution of PARP-1 to the progression of arthritis and identify this protein as a potential therapeutic target for the treatment of rheumatoid arthritis.

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INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease that leads to chronic inflammation in the joints and subsequent destruction of the cartilage and erosion of the bone. Although its etiology is unknown, evidences indicate 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. Although the contribution of Th1 responses in RA is not completely understood, several studies in animal models point to a pathogenic role for Th1-derived cytokines [1–3]. Th1 cells reactive to components of the joint, infiltrate the synovium, release proinflammatory cytokines and chemokines, and promote macrophage and neutrophil infiltration and activation. Inflammatory mediators, such as cytokines and free radicals, produced by infiltrating inflammatory cells, play a critical role in joint damage [3,4]. The fact that the inflammatory process in RA is chronic suggests that immune regulation in the joint is disturbed. This disturbance is probably caused by an excessive inflammatory response together with a deficiency in the mechanisms that control the immune response. 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 (5,6). However, they neither reduce the relapse rate nor delay disease onset, and because a continued treatment is required to maintain a beneficial effect, they have multiple side effects [5,6]. This illustrates the need for novel therapeutic approaches to prevent the inflammatory and autoimmune components of the disease and to promote immune tolerance restoration.

Poly(ADP-ribose) polymerase-1 (PARP-1) is a nuclear DNA-binding protein activated by DNA damage, belonging to a family of more than seventeen members, that catalyzes the attachment of ADP-ribose to target proteins, acts as a component of enhancer/promoter regulatory complexes and participates in the regulation of DNA repair and genomic integrity maintenance [7]. Numerous studies have also involved PARP-1 in the regulation of the inflammatory response [8]. Thus, animals treated with PARP-1 inhibitors or PARP-1 deficient mice showed decreased tissue damage and inflammatory mediators in several models of ischemia-reperfusion and heart transplantation (reviewed in [9]). Moreover, mice lacking PARP-1 are extremely resistant to endotoxin-induced septic shock [10]. The involvement of PARP-1 in the pathogenesis of autoimmune disorders has been previously suggested. Thus, PARP-1 inhibition prevented progression of type 1 diabetes, inflammatory bowel disease and experimental autoimmune encephalomyelitis [11–14]. Interestingly, a promoter haplotype for PARP-1 has been shown to confer higher susceptibility for systemic lupus erythematous and RA [15,16]. Therefore, the aim of this study is to investigate the potential impact of selective PARP-1 inhibition in an experimental model of RA. Here we show that delayed treatment with a novel PARP-1 inhibitor has great benefit at the clinical and pathological levels, as its therapeutic action was exerted at multiple levels, being associated with the downregulation of inflammatory and Th1-mediated autoimmune components of the disease. These results provide support for the contribution of PARP-1 in the pathogenesis of arthritis and open the possibility that specific PARP-1 inhibitors might become attractive therapeutic tools in RA.

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RESULTS

PARP-1 inhibition decreases severity of experimental arthritis

Collagen-induced arthritis (CIA) is a murine experimental disease model that shares a number of clinical, histologic and immunologic features with RA, and it is used as a model system to test potential therapeutic agents. 5-aminoisoquinolinone (AIQ) is a new and selective inhibitor of PARP-1 previously used for the treatment of various ischemic/reperfusion injuries [17,18]. A single administration of AIQ (1.5 mg/kg) at the onset of the disease or to mice with established clinical signs of arthritis progressively attenuated the severity of CIA and decreased the percentage of mice with arthritis, as compared to untreated mice (Fig. 1A). We did not observe significant differences in the therapeutic effect of AIQ at 1.5 mg/kg and 3 mg/kg doses (not shown). Histopathological analyses of joints showed that AIQ treatment significantly abrogated CIA-characteristic chronic inflammation of synovial tissue (infiltration of inflammatory cells into the joint cavity and periarticular soft tissue, consisting in lymphocytes, plasma cells, macrophages and neutrophils), pannus formation, cartilage destruction and bone erosion (Fig. 1B). The AIQ-mediated inhibition of neutrophil infiltration was confirmed with decreased joint myeloperoxidase (MPO) activity (Fig. 1B).

AIQ inhibits inflammatory response in CIA

We next investigated the mechanisms underlying the decrease in severity of CIA following PARP-1 inhibition. Several evidences have involved a wide array of cytokines and chemokines in joint inflammation and the arthritis progression [1,3,19]. We evaluated the effect of AIQ treatment on the production of inflammatory mediators that are mechanistically linked to CIA severity. AIQ administration significantly reduced protein and gene expression of inflammatory cytokines (TNFα, IFNγ, IL-6, IL-1β and IL-12), chemokines (Rantes and MIP-2) in the joint of arthritic mice (Fig. 2A). In addition, joints of AIQ-treated mice showed increased levels of the anti-inflammatory cytokine IL-10 (Fig. 2A). The broad anti-inflammatory activity of AIQ in the inflamed joint was accompanied by downregulation of the systemic inflammatory response. In vivo inhibition of PARP-1 decreased CIA-induced serum levels of the proinflammatory cytokines TNFα and IL-1β (Fig. 2B).

In vivo inhibition of PARP-1 downregulates Th1-mediated CII-specific response in CIA

Although macrophages and neutrophils are the major sources of inflammatory mediators, CD4 T cells play a key role in the initiation and perpetuation of CIA by producing IFNγ, a potent inducer of the inflammatory response. In fact, CIA is considered an archetypal of Th1-type cell-mediated autoimmune disease [1]. Therefore, PARP-1 inhibition could ameliorate CIA by reducing autoreactive T-cell responses and/or migration to the joints. We determined proliferation and cytokine profile of draining lymph node (DLN) cells isolated from AIQ-treated arthritic mice in response to antigen (CII) in vitro. DLN cells obtained from CIA mice showed marked CII-specific proliferation and effector T cells producing high levels of Th1-type cytokines (IFN-γ, IL-2 and TNF-α) and low levels of Th2-type cytokines (IL-4 and IL-10) (Fig. 3A). In contrast, DLN cells from AIQ-treated mice proliferated much less, produced low levels of Th1 cytokines (Fig. 3A). The Th2-type cytokines IL-4 and IL-10 were not significantly affected (Fig. 3A). This effect was antigen-specific, because AIQ treatment did not affect proliferation and cytokine production by anti-CD3-stimulated spleen cells (Fig. 3A). This suggests that inhibition of PARP-1 during CIA progression partially inhibits CII-specific Th1-cell clonal expansion. In order to distinguish whether the decrease in Th1 cytokine production induced by AIQ treatment is consequence of either down-regulation of cytokine release or inhibition of Th1 cell expansion, we determined the intracellular expression of these cytokines by flow cytometry in sorted CD4 T cells. AIQ significantly decreased the number of IFNγ-producing Th1 cells, although did not affect the number of IL-4/IL-10-producing CD4 T cells in DLN (Fig. 3B). We observed similar effects on synovial cells (Fig. 3C). Thus, in vivo inhibition of PARP-1 on CIA mice regulates the expansion of autoreactive/inflammatory Th1 cells.

High levels of circulating antibodies directed against collagen rich joint tissue invariably accompany the development of RA and CIA, and their production is a major factor in determining susceptibility to the disease [20]. AIQ administration resulted in reduced serum levels of CII-specific IgG, particularly autoreactive IgG2a antibodies (Fig. 3D), generally reflective of Th1 activity. These data provide further evidence that PARP-1 inhibition during CIA reduces the Th1 autoreactive responses both in the joint and periphery.

DISCUSSION

The initial stages of RA and CIA involve multiple steps, which can be divided into two main phases: initiation and establishment of autoimmunity, and later events associated with the evolving immune and inflammatory responses. The crucial process underlying disease initiation is the induction of autoimmunity to collagen rich joint components; later events involve a destructive inflammatory process [1,3]. Progression of the autoimmune response involves the development of autoreactive Th1 cells, their entry into the joint tissues, and future recruitment of inflammatory cells through multiple mediators. Certain therapeutic approaches address the autoimmune component of CIA and RA, complementing existing anti-inflammatory therapies. In this study we show that a specific and potent inhibitor for PARP-1 provides a highly effective therapy for CIA. The therapeutic effect of AIQ is associated with a striking reduction of the two deleterious components of the disease, i.e. the autoimmune and inflammatory response. In vivo inhibition of PARP-1 decreased the presence of autoreactive Th1 cells in the periphery and the joint. In addition, AIQ strongly reduced the inflammatory response during CIA progression by downregulating the production of several inflammatory mediators, such as various cytokines and chemokines in the joints. As a consequence, treatment with AIQ reduced the frequency of arthritis, ameliorated symptoms and avoided joint damage. From a therapeutic point of view, it is important to take in account the ability of delayed administration of AIQ to ameliorate ongoing disease, which fulfills an essential prerequisite for an anti-arthritic agent, as treatment is started after the onset of patient arthritis. The fact that we did not observe a loss in its beneficial effect with time suggests that an initial treatment with AIQ could induce remission of the disease. Therefore, a long-term treatment may not be required with AIQ, avoiding the appearance of potential side effects. AIQ offers therapeutic advantages over other PARP inhibitors, such as benzamide and the phenanthridinone derivate PJ34, used in several autoimmune models [11–14]. Thus, PJ34 showed a protective effect on experimental autoimmune encephalomyelitis, CIA and experimental type 1 diabetes only when administered on a prophylactic regime before the disease onset, loosing its therapeutic effect in animals with established clinical signs [13]. In addition, contrary to AIQ, the

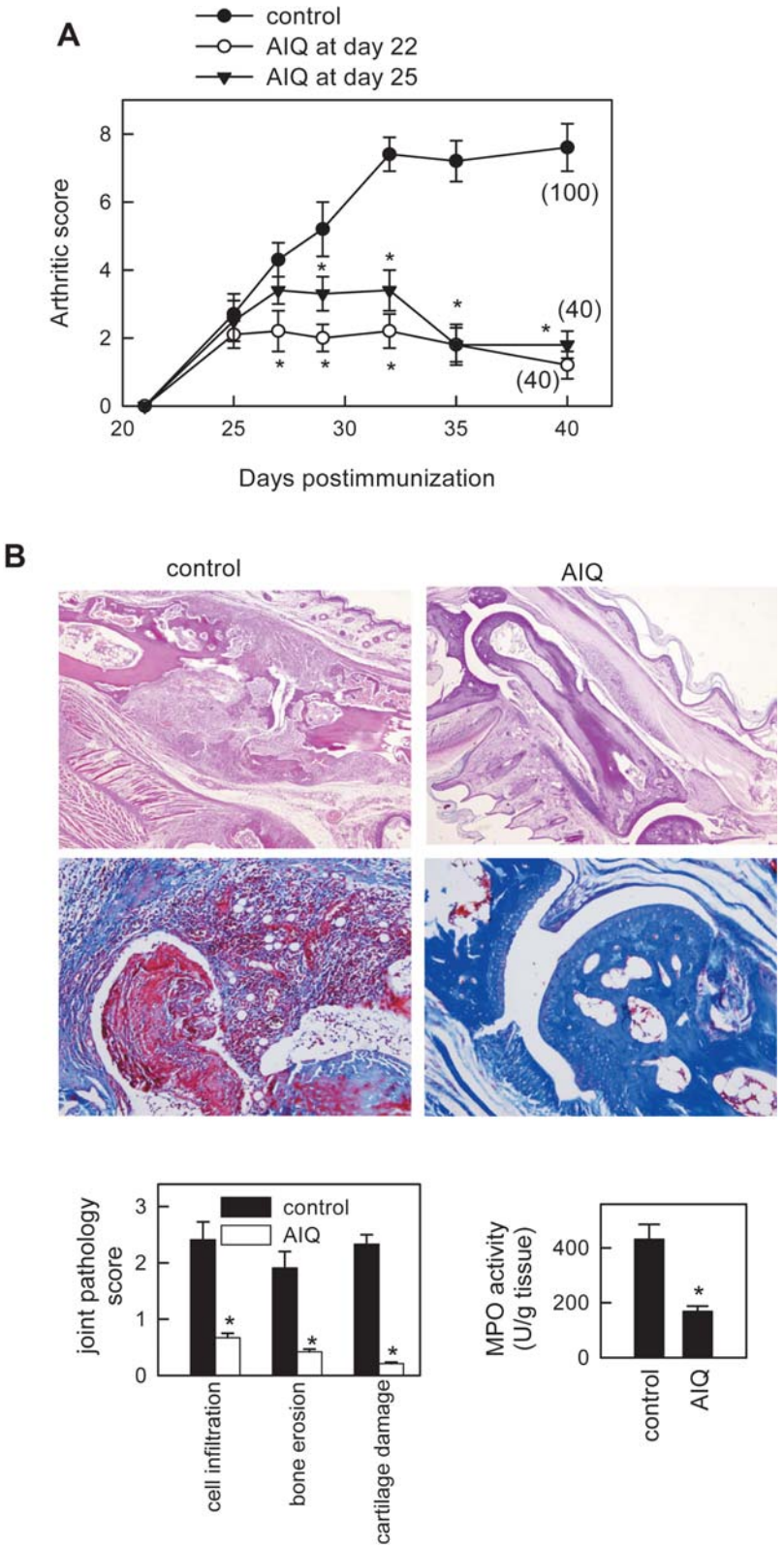

Figure 1. AIQ decreases CIA severity. DBA1 mice with established CIA were injected i.p. either with PBS (control) or with AIQ (1.5 mg/kg) on day 22 or on day 25. **A.** Severity of arthritis was assessed by clinical scoring. Numbers in parenthesis represent frequency of arthritis (% mice with arthritis score>2 at day 35). **B.** Histological analysis of trichromic-stained (lower) or H&E-stained (upper) sections of joints obtained at day 45 was performed. Scoring of inflammation, cartilage damage and bone erosion of paws from untreated (control) and AIQ-treated CIA mice is shown. Neutrophil infiltration in the joints was determined by measuring MPO activity in protein extracts isolated at day 35. n = 6–8 mice per group. *p<0.001 versus control.

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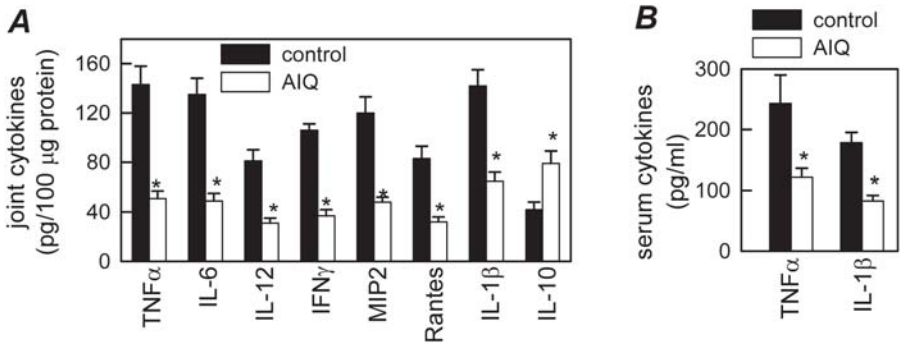

Figure 2. AIQ administration inhibits inflammatory response in CIA. DBA1 mice with established CIA were injected i.p. either with PBS (control) or with AIQ (1.5 mg/kg) on day 25 post-immunization. Systemic and local expression of inflammatory mediators was assayed in protein extracts from joints (**A**) and sera (**B**) isolated at day 35 post-immunization. A paw from an unimmunized mouse was analyzed simultaneously for assessment of the basal response. n = 3–4 mice/group. *p<0.001 versus controls.
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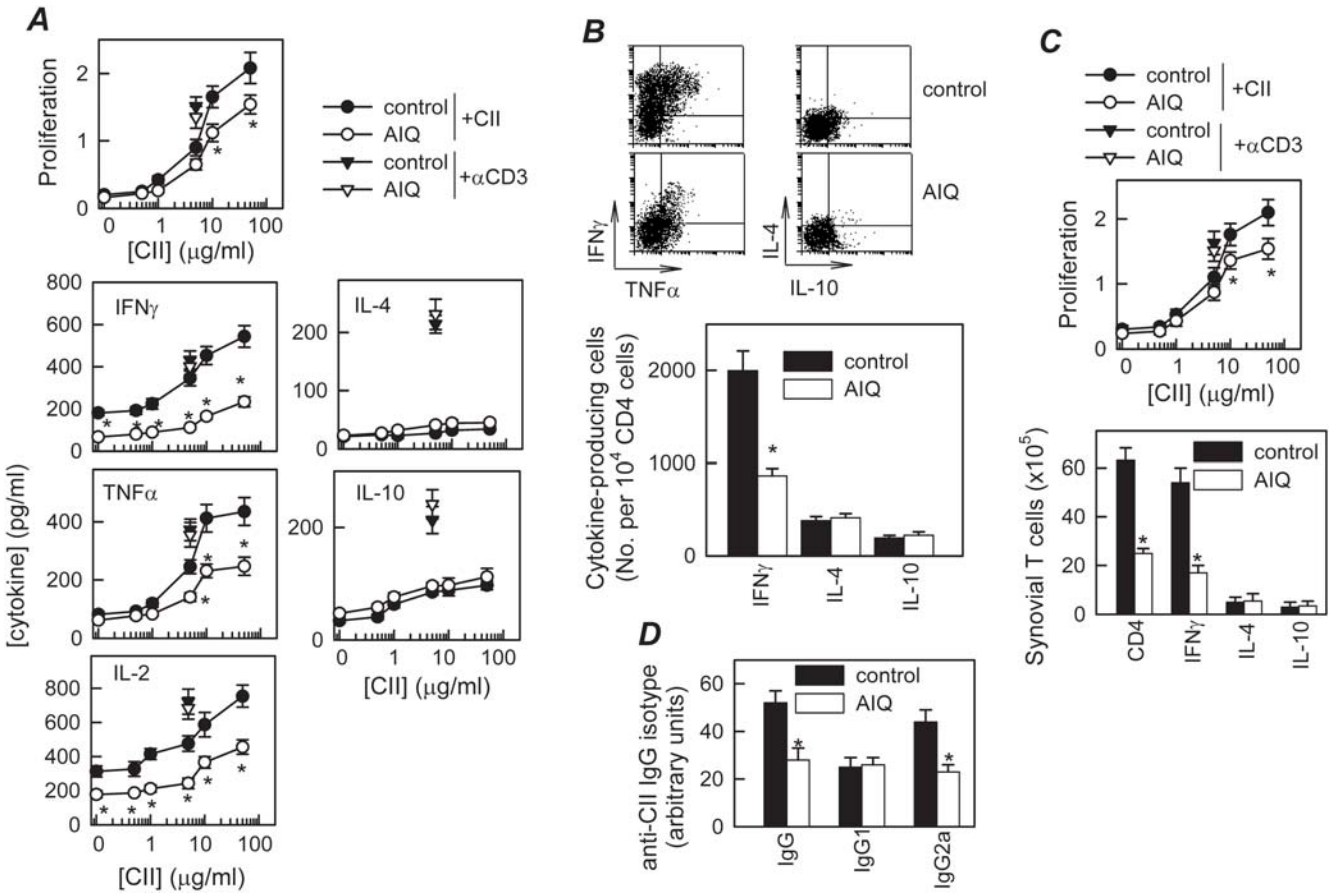

Figure 3. Inhibition of PARP-1 downregulates Th1-mediated response in CIA. DBA1 mice with established CIA were injected i.p. either with PBS (control) or with **AIQ** (1.5 mg/kg) on day 25 post-immunization. **A.** Proliferative response and cytokine production of DLN cells isolated at day 30 from untreated (control) or AIQ-treated CIA mice were determined after in vitro stimulation with different concentrations of CII. Stimulation of DLN cells with anti-CD3 antibodies (▼, for untreated CIA mice; ▽, for AIQ-treated CIA mice) was used for assessment of nonspecific stimulation. A pool of 3 nonimmunized DBA/1 DLN cells was used for assessment of the basal response. No proliferation or cytokine production by T cells was detectable in the presence of an unrelated antigen (OVA). n = 5 mice/group. **B,** Number of CII-specific cytokine-producing T cells. DLN cells from untreated (control) or AIQ-treated CIA mice were restimulated in vitro with CII (10 μg/ml) and analyzed for CD4 and intracellular cytokine expression in gated CD4 T cells by flow cytometry. Dot plots show representative double staining for IFNγ/TNFα or IL-4/IL-10 expression in gated CD4 T cells. The number of IFNγ-, IL-4- and IL-10-expressing T cells relative to 10^4 CD4 T cells is shown in the lower panel. Data shown represent pooled values from two independent experiments. **C,** CII-specific proliferative response and the number of cytokine-producing CD4 T cells were determined in synovial membrane cells isolated from untreated (control) or AIQ-treated CIA mice and stimulated in vitro with CII (10 μg/ml) for 48 h. Data show the results of pooled synovial cells from 3 animals per group. **D.** AIQ decreases titter of autoantigens in CIA mice. The levels of CII-specific IgG, IgG1 and IgG2a antibodies in sera collected at day 35 were determined by ELISA. n = 3-5 mice/group. *p<0.001 versus controls.
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protective effects of these PARP-1 inhibitors disappeared once treatment is terminated [11–14].

The capacity of AIQ to regulate a wide spectrum of inflammatory mediators might offer a therapeutic advantage over other treatments directed against a single mediator, such as the new biologic agents. Chemokines are responsible for the infiltration into the joint and activation of various leukocyte populations, which contribute to CIA pathology (1-3,[21]. The fact that in vivo inhibition of PARP-1 reduced the expression of a plethora of chemokines could partially explain the absence of inflammatory infiltrates in the joint tissues of AIQ-treated mice, being especially relevant for chemokines as MIP-2 (chemotactic for neutrophils) and Rantes (for macrophages and T cells), all involved in CIA pathogenesis [21,22]. Moreover, PARP-1 inhibition reduced the expression of several proinflammatory chemokines receptors involved in arthritis pathology (not shown), reflecting also a decreased joint inflammatory infiltration.

In addition to the regulation of cell recruitment to the joint, AIQ also regulates the activation of inflammatory cells in the joints. Thus, AIQ downregulated the production of the proinflammatory/cytotoxic cytokines TNFα, IFNγ, IL-6, IL-1β and IL-12 in the inflamed joint, and increased the levels of the anti-inflammatory cytokine IL-10, which ameliorates the disease [23]. The decrease in inflammatory mediators could be the consequence of a diminished infiltration of inflammatory cells in the synovium. However, the fact that AIQ inhibited the production of pro-inflammatory mediators by synovial cells isolated from CIA mice, argues against this hypothesis (not shown). This suggests that, in addition to the reduction in inflammatory infiltration, the inhibition of PARP-1 deactivates the inflammatory response.

Two mechanisms have been proposed to explain the role of PARP-1 in inflammatory diseases. One potential mechanism is related to massive PARP-1 activation induced by genotoxic injury developed during the inflammatory process [24]. In this case, hyperactivated PARP-1 would lead to rapid ATP depletion and to irreversible cellular energy failure and necrotic-type cell death subsequent to disruption of oxidative metabolism. However, several lines of evidence suggest that under inflammatory conditions the beneficial effects of PARP-1 inhibition are independent on the prevention of energy failure (12). The "suicide hypothesis", therefore, might be valid only in conditions of massive DNA rupture and intense PARP-1 activation. The other proposed and more plausible mechanism is related to a functional link between PARP-1 and inflammation-related transcription factors. Several *in vivo* and *in vitro* studies have demonstrated the involvement of PARP-1 in the activation of nuclear factor NF-κB [10,25], a transcription factor that plays a central role in the regulation of genes involved in the immune and inflammatory response of RA. 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, YY-1 and Stat-1 [26,27].

CIA is also a Th1-mediated disease, and the bias towards Th1 cytokines (mainly IFNγ and TNFα) is crucial in the establishment of chronic inflammation in the joint (1-3). Our results demonstrate that the administration of AIQ to arthritic mice results in a decreased CII-specific Th1-mediated response. It appears that the inhibition of the Th1 response is caused by a direct action on synovial and DLN cells, since synovial and DLN cells obtained from AIQ-treated animals are refractory to Th1 cell stimulation. Whereas the effects of PARP-1 inhibition on the inflammatory response have been previously suggested by others, the present study is the first evidence describing the involvement of PARP-1 in the Th1-mediated autoreactive response. Although the mechanisms involved in the inhibitory action of AIQ in Th1 activation are unknown, our results suggest that AIQ treatment impairs the proliferation and/or differentiation of antigen-specific Th1 cells. In this sense, NF-kB and AP-1 are involved in the regulation of the proliferative response of T cells, and AIQ inhibition of PARP-1-mediated activation of these transcription factors could play a major role in this effect. Indeed, other PARP-1 inhibitors have been found to decrease NF-kB and AP-1 transduction activity in activated T cells [11].

In summary, this work identifies PARP-1 as a novel therapeutic target for the treatment of RA and other chronic autoimmune disorders and provides a powerful rationale for the assessment of the efficacy of the PARP-1 inhibitor AIQ as a new immunomodulatory factor with the capacity to deactivate the inflammatory response in vivo at multiple levels.

METHODS

Arthritis induction and treatment

Animal experimental protocols were reviewed and approved by the Ethical Committee of the Spanish Council of Scientific Research (CSIC). To induce collagen-induced arthritis (CIA), DBA/1J mice (7–10-wk-old, Jackson Labs) were injected s.c. with 200 μg of type II collagen (CII, Sigma) emulsified in complete Freunds adjuvant (CFA) containing 200 μg of *M. tuberculosis* H37 RA (Difco, Detroit, Michigan). At day 20 after primary immunization, mice were boosted s.c. with 100 μg of CII in CFA. Treatment with PARP-1 inhibitor (AIQ, Alexis) consisted in a single administration i.p. of 1.5 mg/kg of AIQ (in saline) starting at the disease onset (at day 22 post-immunization) or at 25 days post-immunization when all mice showed established arthritis (clinical score>2). The dose of AIQ was selected from previous in vivo studies demonstrating the efficacy and potency of the drug to inhibit PARP-1 activity without any resultant toxicity [17,18]. In each experiment, a control group of mice was injected i.p. with PBS (untreated). Mice were analyzed by two independent, blinded examiners every other day and scored for signs of arthritis by using the following system: grade 0, no swelling; grade 1, slight swelling and erythema; grade 2, moderate swelling and edema; grade 3, extreme swelling and pronounced edema; grade 4, joint rigidity. Each limb was graded, giving a maximum possible score of 16 per animal.

Histopathology analysis

For histological analysis, the paws were randomly collected by two independent experimenters at day 45 after primary immunization, fixed in 4% buffered-formaldehyde, decalcified, paraffin-embedded, sectioned and stained with H&E or Masson-Goldner trichromic stain. Histopathological changes were scored in a blinded manner based in cell infiltration, cartilage destruction and bone erosion parameters as described [28]. Neutrophil infiltration in the joints was monitored by measuring myeloperoxidase (MPO) activity in joint extracts isolated at day 35 post-immunization as described [28].

Cytokine and autoantibody determination

For cytokine determination in joints, protein extracts were isolated by homogenization of joints (50 mg tissue/ml) in 50 mM Tris-HCl, pH 7.4, with 0.5 mM DTT, and proteinase inhibitor cocktail (10 μg/ml, Sigma). Serum samples were collected at peak of disease (day 35) and the levels of anti-CII IgG, IgG1 and IgG2a Abs were measured by ELISA as described [29]. Cytokine and chemokine levels in the serum and joint protein extracts prepared at the disease peak (day 35) were determined by specific sandwich ELISAs using capture/biotinylated detection Abs from BD Pharmingen (San Diego, CA) according to the manufacture's recommendations.

Assessment of T cell autoreactive response

Because T cell autoreactive response precedes to maximal clinical manifestations of the disease, single-cell suspensions (10^6 cells/ml) from draining lymph nodes (DLN) and synovial membrane of knee joints were obtained at 30 days post-immunization. Cells were stimulated in complete medium (RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) with different concentrations of heat-inactivated CII for 48 h (for cytokine determination) or for 72 h (for proliferative response) [29]. Cell proliferation was evaluated by using a cell proliferation assay (BrdU) from Roche Diagnostics GmbH (Mannheim, Germany). Cytokine content in culture supernatants was determined by specific sandwich ELISAs as above. For intracellular analysis of cytokines, DLN and synovial cells were stimulated with inactivated CII (10 μg/ml) for 8 h, in the presence of monensin, and then stained with PerCP-anti-CD4 mAbs at 4°C, washed, fixed/saponin permeabilized, stained with FITC- and PE-conjugated anti-cytokine specific mAbs (BD Pharmingen), and analyzed on a FACScalibur flow cytometer (Becton Dickinson). To distinguish between monocyte/macrophage and T cell sources, intracellular cytokine analysis was done exclusively in the PerCP-labeled CD4 T cell population.

Data analysis

All values are expressed as mean±SD. The differences between groups were analyzed by Mann-Whitney U test and, if appropriate, by Kruskal-Wallis ANOVA test.

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Author Contributions

Conceived and designed the experiments: FOliver FO'Valle EG MD RM. Performed the experiments: FOliver FO'Valle EG RA MD RM. Analyzed the data: FOliver FO'Valle EG CC MD. Contributed reagents/materials/analysis tools: FOliver CC MD. Wrote the paper: FOliver MD.

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