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ROLE OF INTERFERON-INDUCED PKR AS PREDICTIVE BIOMARKER AND ANTI-CANCER STEM CELLS PROPERTIES OF INTERFERON

Memoria presentada por **Dña. María Belén García Ortega** para optar a la mención de Doctor Internacional por la Universidad de Granada

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Tati

NOTA

A lo largo del texto se han utilizado las abreviaturas de varios términos en inglés. Esto es debido al uso extendido de la lengua inglesa en publicaciones científicas y al uso de abreviaturas en inglés de forma cotidiana en el lenguaje científico. En el Anexo III, al final de este trabajo, se detalla la descripción en inglés y en castellano de cada una de las abreviaturas. Así mismo, en este glosario se recogen las abreviaturas de los términos usados en castellano.

A mis padres y a mi hermano Adolfo.

En los sentidos pon tu confianza,
nada erróneo verás en la distancia,
si el entendimiento en guardia pones.

Sea alegre tu observancia,
y seguro y flexible tus pasos avanza,
por los valles de un mundo rico en dones.

J. W. Von Goethe

ÍNDICE

ABSTRACT

Colon cancer and malignant melanoma figure among the leading causes of morbidity and mortality whose incidence is increasing worldwide. Currently, available cytotoxic treatment options show low response rates, significant side effects and low/medium impact on survival, thus emphasizing the need for more effective therapies and new predictive biomarkers that could be achieved by a personalized treatment, based on the individual tumours molecular profile.

The correct and early diagnosis of cancer is essential for adequate and effective treatment because every cancer patient requires a specific treatment regimen, which encompasses one or more modalities such as surgery, radiotherapy and/or chemotherapy. Despite the success of new treatments, most patients progress due to the presence of intra- and intertumoral heterogeneity conferring resistance and thus disease relapse. Factors contributing to heterogeneity include interactions with the microenvironment, inflammatory response, genetic mutations and the presence of cancer stem cells subpopulations (CSCs). This small cell subpopulation is characterized, among others, by relative quiescence, longevity, slow cell cycle progression and accordingly high resistance towards radiation and cytotoxic drugs, which finally accounts for metastasis formation and local relapse after long periods of dormancy. Therefore, to prevent cancer relapse, new therapeutic effective strategies against CSCs are required; and the search for specific drugs with low-toxicity and new predictive and pronostic biomarkers are still a very attractive approach.

In this way, with the aim of searching new predictive biomarkers for cancer patients, our group has identified the protein kinase, PKR. The protein kinase R (PKR, also called EIF2AK2) is an interferon-inducible double-stranded RNA protein kinase with multiple effects on cells that plays an active part in the cellular response to numerous types of stress. PKR has been extensively studied and documented for its relevance as an antiviral agent and a cell growth regulator.

PKR has been previously identified by our group as a therapeutic target of the chemotherapeutic drug 5-Fluorouracil (5-FU), inducing tumor cell death by apoptosis in a complementary and independent manner to the tumor suppressor p53. In addition, PKR is regulated by a non-coding pre-mir886 (nc886), which has been identified in serum of patients with metastases having high potential to be a detectable biomarker in liquid

biopsies. Our data show the great variability in PKR expression levels and localization between tumor samples and healthy tissues in patients with metastatic colon cancer (plasma and colon epithelium). The expression levels and localization of PKR together with the expression of nc886 have allowed the identification of groups of patients with different responses to treatment based on the use of 5-FU, supporting the great potential of this protein and the pre-microRNA that regulates it as possible predictive biomarkers in different types of cancer.

In addition, PKR is induced by Interferon type I (IFN), a cytokine widely known for their antiviral and antitumor activity. In fact, non metastatic high-risk melanoma is still treated with high dose of Interferon alpha (IFN-α) with a significant improvement in Disease Free Survival in patients through a mechanism that is not yet fully understood. In this thesis, we have analyzed the effect of low and high dose of IFN-α-treatment over melanospheres enriched in CSCs subpopulations, demonstrating that this cytokine has a potent antitumor action against these subpopulations both *in vitro* and *in vivo,* which contributes to deep in the mechanism of action of these cytokines and to devise new therapies based on effective combinations against these resistant populations.

Since new immunotherapies are being imposed in melanoma and other solid tumors, as well as different combinations are under clinical trial to avoid resistances, the efficacy of interferons over CSCs even at low doses with fewer side effects, should be considered as a potentially important combination treatment against the relapse of the disease in oncology.

Recently, cellular communication by extracellular vesicles has gained great importance for its likely involvement in cancer. Tumor-derived exosomes are abundant in the body fluids of cancer patients, including those with malignant melanoma, and are involved in several processes such as tumor initiation, progression and tumoral metastasis. In this work, we have demostrated the IFN- α modulation on specific traffic vesicles and exosomes production from melanoma stem cell, suggesting their role of potential biomarkers in novel cancer therapies. Moreover, for the first time, we have found significant differences on the metabolomic profile of exosomes derived from melanoma CSCs as compared with the population of differentiated IFN- α - treated cells. The metabolomic characterization of exosomes opens the door for the discovery of prognosis and diagnosis biomarkers of malignant melanoma with a clear translational and clinical application.

RESUMEN

El cáncer de colon y el melanoma maligno figuran entre las principales causas de morbilidad y mortalidad cuya incidencia está aumentando en todo el mundo. En la actualidad, las opciones de tratamientos citotóxicos disponibles muestran bajas tasas de respuesta, importantes efectos secundarios y un bajo/medio impacto en la supervivencia, lo que pone de manifiesto la necesidad de terapias más eficaces y la detección de nuevos biomarcadores predictivos que podrían lograrse mediante un tratamiento personalizado, basado en el perfil molecular individual de los tumores.

El correcto y precoz diagnóstico del cáncer es esencial para un tratamiento adecuado y eficaz, ya que cada paciente requiere un régimen de tratamiento específico, que abarca una o más modalidades tales como la cirugía, la radioterapia y/o la quimioterapia. A pesar del éxito de los nuevos tratamientos, la mayoría de los pacientes progresan debido a la presencia de heterogeneidad intra e intertumoral que confiere resistencia y por tanto, la recaída de la enfermedad. Los factores que contribuyen a la heterogeneidad son principalmente las interacciones con el microambiente, la respuesta inflamatoria, las mutaciones genéticas y la presencia de subpoblaciones de células madre cancerígenas (CSC). Esta pequeña subpoblación celular se caracteriza, entre otras, por su quiescencia, longevidad, lenta progresión del ciclo celular y, en consecuencia, alta resistencia a la radiación y a los fármacos citotóxicos, lo que finalmente explica la formación de metástasis y la recaída local tras largos periodos de inactividad. Por tanto, para prevenir la recaída del cáncer, se requieren nuevas estrategias terapéuticas eficaces frente a CSC; es por ello, que la búsqueda de fármacos específicos de baja toxicidad y de nuevos biomarcadores predictivos y pronósticos sigue siendo un enfoque muy atractivo.

De este modo, con el objetivo de buscar nuevos biomarcadores predictivos para los pacientes con cáncer, nuestro grupo ha identificado la proteína quinasa, PKR. La proteína quinasa R (PKR, también llamada EIF2AK2) es una proteína quinasa de ARN de doble cadena inducida por interferón, con múltiples efectos en las células, que participa activamente en la respuesta celular a numerosos tipos de estrés. PKR ha sido ampliamente estudiada y documentada por su importancia como agente antiviral y regulador del crecimiento celular.

PKR ha sido identificada previamente por nuestro grupo como una diana terapeútica del fármaco quimioterapéutico 5-Fluorouracilo (5-FU), induciendo la muerte de las células tumorales por apoptosis de una forma independiente y complementaria al supresor de tumores p53. Además PKR está regulada por un pre-mir886 no codificante (nc886), que ha sido identificado en suero de pacientes con metástasis teniendo alto potencial para ser un biomarcador detectable en biopsias líquidas. Nuestros datos muestran la gran variabilidad en los niveles de expresión y localización de PKR entre muestras tumorales y los tejidos sanos en pacientes con cáncer de colon metastásico (plasma y epitelio de colon). Los niveles de expresión y localización de PKR junto con la expresión del nc886 han permitido identificar grupos de pacientes con diferentes respuestas al tratamiento basado en el uso del 5-FU apoyando el gran potencial de esta proteína, y el pre-microRNA que la regula como posibles biomarcadores predictivos en diferentes tipos de cáncer.

Además, PKR es inducida por el Interferón tipo I (IFN), una citoquina ampliamente conocida por su actividad antiviral y antitumoral. De hecho, el melanoma no metastásico de alto riesgo sigue siendo tratado con altas dosis de Interferón alpha (IFN-α) con una mejora significativa en la Supervivencia Libre de Enfermedad en los pacientes a través de un mecanismo que aun no es entendido del todo. En la presente tesis, hemos analizado el efecto del tratamiento con dosis bajas y altas de IFN-α sobre melanosferas enriquecidas en subpoblaciones de CSCs demostrando que dicha citoquina tiene una potente acción antitumoral frente a estas subpoblaciones tanto *in vitro* como *in vivo,* lo que contribuye a profundizar en el mecanismo de acción de estas citoquinas y a idear nuevas terapias basadas en combinaciones eficaces contra estas poblaciones resistentes.

Dado que se están imponiendo nuevas inmunoterapias en el melanoma y otros tumores sólidos, así como diferentes combinaciones están en fase de ensayo clínico para evitar resistencias, la eficacia de los interferones sobre las CSCs, incluso a dosis bajas y con menos efectos secundarios, debería considerarse como un tratamiento combinado potencialmente importante contra la recaída de la enfermedad en oncología.

Recientemente, la comunicación celular mediante vesículas extracelulares ha cobrado gran importancia por su implicación en cáncer. Los exosomas derivados del tumor son abundantes en los fluidos corporales de los pacientes oncológicos, incluidos los que padecen melanoma maligno, y están envueltos en numerosos procesos como el inicio, la progresión y la metástasis tumoral. En este trabajo, hemos demostrado la

modulación del IFN-α en el tráfico de vesículas específicas y producción de exosomas de células madre de melanoma, sugiriendo su papel como potenciales biomarcadores en nuevas terapias contra el cáncer. Además, por primera vez, se han encontrado diferencias significativas en el perfil metabolómico de los exosomas derivados de las CSC de melanoma en comparación con la población de células diferenciadas tratadas con IFN- α. La caracterización metabolómica de los exosomas abre una puerta al descubrimiento de biomarcadores de pronóstico y diagnóstico del melanoma maligno para su enfoque clínico y traslacional.

1. INTRODUCTION

1. CÁNCER

1.1. Definición y Origen

Actualmente, el cáncer se define como el conjunto de enfermedades caracterizadas por el crecimiento excesivo y descontrolado de células, las cuáles invaden y dañan tejidos u órganos propagándose a partes adyacentes del cuerpo y extendiéndose más allá de sus límites habituales a través del sistema linfático y torrente sanguíneo en un proceso conocido como metástasis (Boyle & Levin, 2014). La metástasis es la diseminación de células tumorales primarias a lugares distantes, siendo la causa de muerte de la mayoría de casos de cáncer. Numerosos estudios son constantemente publicados acerca de los diversos elementos involucrados en este proceso tan importante, y que sin embargo, sigue siendo uno de los aspectos más enigmáticos de la enfermedad.

El cáncer abarca un grupo heterogéneo de trastornos con propiedades biológicas muy diferentes teniendo como resultado mutaciones en genes que regulan la proliferación y la homeostasis, incluida la muerte celular. Entre ellos se encuentran los oncogenes y los genes supresores de tumores. Alteraciones en éstos, provocan la aparición del cáncer de manera directa, ya que se produce la pérdida de control de procesos básicos celulares como por ejemplo la pérdida de la función de un gen supresor que afecta a la progresión del ciclo celular o proliferación descontrolada debido a la ganancia de la función de un oncogén (Hanahan & Weinberg, 2000). En este caso, podríamos nombrar el gen *c-myc* (un oncogen involucrado en la proliferación celular) que está alterado en la mayoria de los casos de cáncer, o la pérdida de expresión del gen supresor de tumores *PTEN*, localizado en el cromosoma 10q23 que actúa como un regulador negativo de *AKT*, activando la ruta metabólica *PIP3K-AKT*, bloqueando la apoptosis (como vía de escape celular) e incrementando la progresión tumoral (Jinushi, 2014; Quintana et al., 2008).

Dentro del proceso del desarrollo del cáncer o carcinogénesis se conoce como oncogénesis o tumorogénesis a la transición de una célula normal a una célula tumoral capaz de iniciar un tumor, mientras que se denomina progresion tumoral a la evolución del tumor hacia la metástasis y diseminación por el organismo (De Palma et al., 2017). La importancia del microambiente tumoral en la progresión del cáncer y su papel, no sólo en el mantenimiento del tumor con el que está estrechamente vinculado, sino en el

desarrollo de resistencia a las terapias se ha convertido hoy día en el mayor obstáculo para el tratamiento con éxito de esta enfermedad (Hamidi & Ivaska, 2018).

1.2. Carcinogénesis

El desarrollo del cáncer o carcinogénesis es caracterizado por la acumulación de alteraciones fundamentales en la fisiología celular que van a determinar profundos cambios metabólicos y genéticos dando lugar a una proliferación excesiva y a una transformación maligna (Hanahan & Weinberg, 2011).

Se ha propuesto que la carcinogénesis supone la adquisición de diez características por parte de la célula no tumoral para convertirse en célula cancerosa (Figura 1). Son las siguientes principalmente:

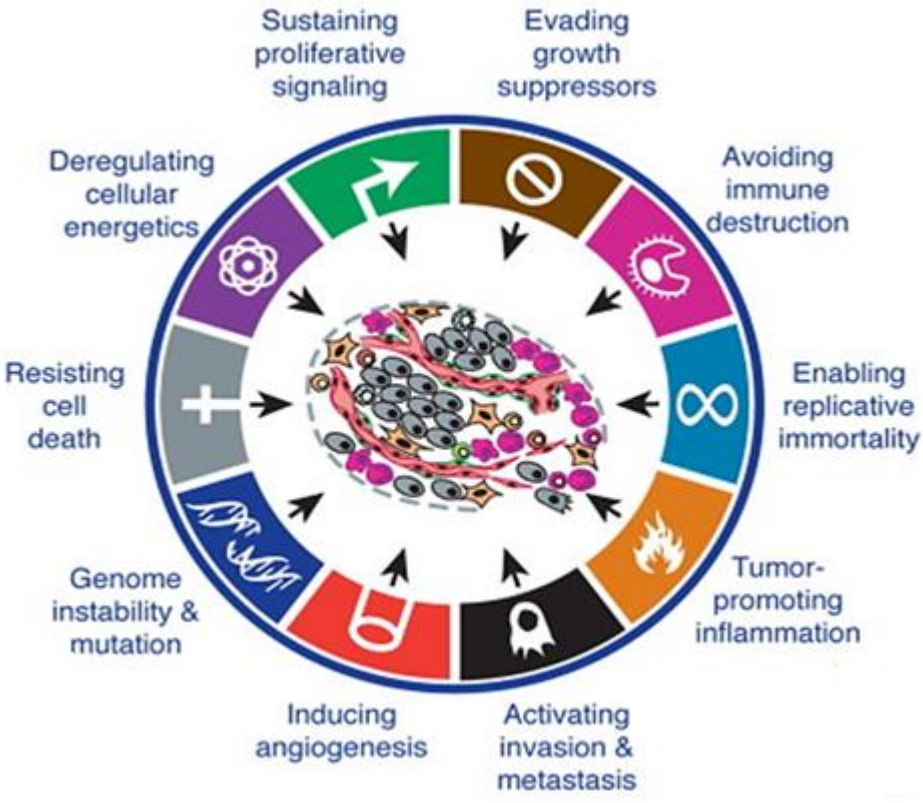


Figura 1. Características generales de las células tumorales
(Hanahan & Weinberg, 2011).

- *Mantenimiento de la señalización proliferativa*

Para el mantenimiento de la arquitectura y función normal de los tejidos hay un control entre la poducción de señales que favorecen el crecimiento, asegurándo una constante homeostasis celular. En células tumorales este proceso de homeostasis está

desregulado, sosteniendo su crecimiento y proliferación de manera incontrolada (Hanahan & Weinberg, 2011). Esto es debido a sobreexpresión de protooncogenes, producción de sus propios factores y aumento de receptores de factores de crecimiento celular (Sonnenschein & Soto, 2013).

Los protooncogenes, o también conocidos como oncogenes, están implicados en el proceso de proliferación celular dentro del genoma humano. Las funciones celulares de estos genes, o sus productos conocidos como oncoproteínas, son muy variadas. Por ejemplo, algunos de estos genes codifican receptores de factores de crecimiento como el receptor *EGF* (*EGFR*) o coactivadores de crecimiento como la β-catenina nuclear, o bien, transducen señales como las proteínas *RAS -p21-* (Collado & Serrano, 2010). El estado alterado de la proteína RAS induce alteraciones en la expresión de integrinas y participa en la activación constitutiva de rutas de señalización promoviendo la migración celular, e incluso senescencia (Figura 2).

La oncoproteína *c-MYC* está también desregulada en el desarrollo tumoral (Y. Li et al., 2015). *MYC* es de gran importancia en la regulación de la expresión de un gran número de genes involucrados en apoptosis, ciclo celular, diferenciación y angiogénesis, metabolismo y renovación de las células madre (Linchong Sun et al., 2015).

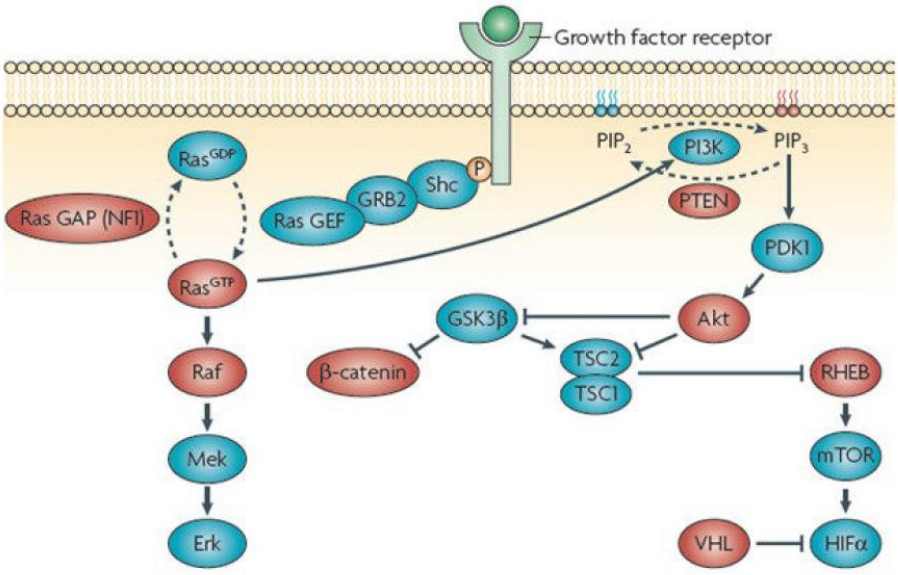


Figura 2. Rutas oncogénicas inductoras de proliferación celular
(Collado & Serrano, 2010)

Cabe destacar la existencia de pequeños fragmentos de ARN denominados microARNs (o mirRNAs) que pueden tener ambas funciones: o bien supresores de tumores o bien oncogenes, según la conformación que adopten y al gen diana al que se unan, interviniendo de manera activa en el mantenimiento de la proliferación celular (Kiselev, 2014; Y. Liu et al., 2015).

- *Evasión de los mecanismos supresores del crecimiento*

Además de inducir crecimiento celular descontrolado, las células cancerosas eluden programas que regulan negativamente la proliferación celular. Muchos de estos programas dependen de funciones en proteínas supresoras de tumores, tales como *RB* y *TP53* (Shigdar et al., 2014). Ambos están mutados en la mayoría de los cánceres humanos y son los responsables del arresto del ciclo celular, apoptosis y senescencia.

El gen RB produce un regulador transcripcional que integra las señales procedentes de fuentes extra e intracelulares, en respuesta a si una célula debe continuar a través de su ciclo de crecimiento y replicación (Burkhart & Sage, 2008); el factor de transcripción *TP53* (*P53*, conocido como *guardián del genoma*) está mutado en más del 50% de casos de cáncer. Esta proteína responde a diferentes señales de estrés celular o daños en el ADN, deteniendo el ciclo celular, activando sistemas de reparación como por ejemplo PARP, o incluso desencadenando apoptosis cuando el daño es irreparable (Tabori et al., 2010).

La Poli (ADP-ribosa) polimerasa 1 es una enzima nuclear perteneciente a la superfamilia PARP. En humanos, esta familia consta de 18 miembros distintos, siendo PARP-1 la más estudiada y representativa. La función principal de esta enzima es la señalización de daños en el ADN para su posterior reparación y mantenimiento de la integridad genómica (Aguilar-Quesada et al., 2007). El ADN dañado que no se repara de manera apropiada, da lugar a inestabilidad genómica predisponiendo al organismo a cáncer. Así PARP-1, como factor de reparación del ADN, está estrechamente ligado al proceso de carcinogénesis (Figura 3) (Rodríguez et al., 2013).

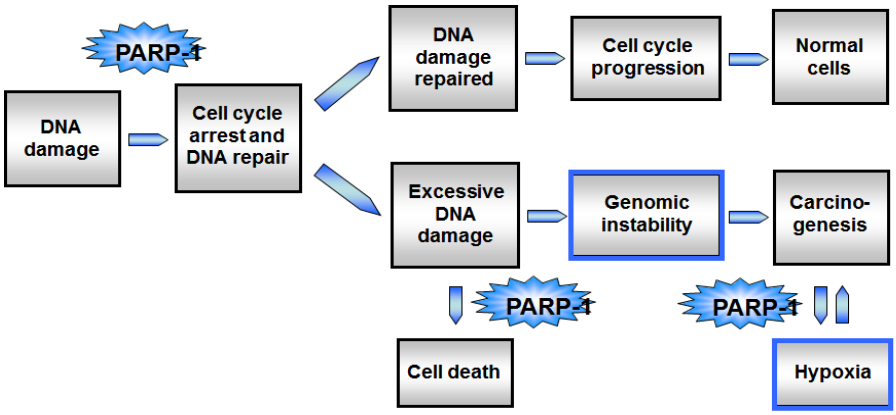


Figura 3. Relación de PARP con carcinogénesis
(Martin-Oliva et al., 2006)

- *Resistencia a muerte celular programada*

La forma más estrictamente regulada de muerte celular es conocida como apoptosis. Este proceso desempeña un papel fundamental en el desarrollo y proliferación normal de los tejidos así como su homeostasis celular mediante la eliminación de células innecesarias, dañadas o perjudiciales (Elmore, 2007; Lowe & Lin, 2000). Existen dos vías principales por las que las células sufren apoptosis: a) la *ruta extrínseca*, que procesa señales extracelulares inductoras de apoptosis principalmente por receptores de la superfamilia TNF (S. Wang & El-Deiry, 2003); b) la *ruta intrínseca*, que integra señales detectadas a nivel intracelular. En esta ruta podemos destacar la familia BCL-2.

Ambas vías de apoptosis tienen como resultado la activación de un grupo de proteínas intracelulares con función hidrolítica denominadas caspasas (CASP) siendo las efectoras en último término de ejecutar la apoptosis (Plati et al., 2011). La familia de proteínas BCL-2 incluye miembros tanto pro-apoptóticos como anti-apoptóticos. Modificaciones genéticas en estas proteínas conllevan a la inactivación de caspasas o a la sobreexpresión de proteínas anti-apoptóticas permitiendo a las células tumorales evadir la apoptosis (Julien & Wells, 2017).

- *Potencial replicativo ilimitado*

La capacidad de proliferación celular ilimitada está mediada por los telómeros, que son secuencias de ADN que se encuentran en los extremos de los cromosomas confiriéndoles protección y garantizando la adecuada replicación. Las células tumorales en cultivo sufren un acortamiento de los telómeros dando lugar a una detección

irreversible del crecimiento conocida como senescencia. El estado de senescencia en una célula es por lo general un estado irreversible, no proliferativo pero viable (Artandi & DePinho, 2009; Shammas, 2011).

Más del 80% de los tumores presentan una activación de los mecanismos para mantener la función de los telómeros mediante la sobreexpresión de una enzima denominada telomerasa (Blasco et al., 2018).

- *Angiogénesis sostenida*

En la formación de nuevos vasos circulatorios que irrigan al tumor están implicados diferentes mecanismos y tipos celulares, como son células mieloides, fibroblastos asociados al tumor o al cáncer (TAF o CAF) o factores pro-angiogénicos. Los tumores requieren alimento en forma de aporte de O2 y nutrientes, al igual que en los tejidos normales. Por ello, la neovasculatura formada y asociada al tumor, proceso conocido como angiogénesis, va a suplir estas necesidades (Chung et al., 2010).

El principal activador de angiogénesis en los tumores es el VEGF (factor de crecimiento endoletlial vascular, de sus siglas en inglés *Vascular Endothelial Growth Factor*) y angiopoyetina-2. Sin embargo, los nuevos vasos formados son a menudo irregulares y menos eficientes (Carmeliet & Collen, 2000). Diferentes terapias anti-cáncer que se aplican hoy día tienen como finalidad bloquear la angiogénesis y el mantenimiento del crecimiento tumoral (Yancopoulos, 2010).

- *Invasión y metástasis*

A medida que las neoplasias avanzan en su grado de malignicidad, las células tumorales adquieren capacidad de migración e invasión desde el tumor primario a órganos distantes, produciendo invasión local, micro-metástasis y metástasis (ver apartado 1.3. Figura 4). La cascada de eventos que suceden está muy regida con la pérdida de E-Cadherina (cadherina epitelial), confiriendo a la célula cancerosa propiedades agresivas y motrices. En procesos de migración, invasión y metástasis, la adhesión célula-célula y célula-matriz extracelular queda desestructurada por cambios genéticos y epigenéticos que ocurren sobre las proteínas cadherinas favoreciendo el proceso de transición epitelio-mesénquima (Folberg & Maniotis, 2004; Thiery et al., 2009).

En la última década, a estas alteraciones se han adicionado nuevos marcadores emergentes de las células tumorales relacionados con la reprogramación del metabolismo

energético, incremento de las tasas de mutación genómica respecto al resto de células del organismo, desarollo de procesos inflamatorios asociados al crecimiento del tumor y la capacidad de evasión del sistema inmune (Figura 1), todo ello lleva a contribuir al mantenimiento y desarrollo del cáncer (Hanahan & Weinberg, 2011).

1.3. Transición Epitelio-Mesénquima y proceso metastásico

El proceso biológico que permite que las células cambien de un fenotipo polarizado y epitelial a uno altamente motriz y fibroblastoide es definido como Transición Epitelio-Mesénquima (EMT, del inglés *Epithelial-Mesenchymal Transition*). Durante la EMT se producen alteraciones en la adhesión célula-célula, en las interacciones célula-sustrato, en la degradación de la matriz extracelular y en la reorganización del citoesqueleto (M. A. Huber et al., 2005). Por lo tanto, la EMT engloba un amplio espectro de cambios moleculares, intercelulares e intracelulares.

Esta EMT se clasifica en tres subtipos diferentes dependiendo del contexto biológico en el cual se producen y las consecuencias que producen en el organismo (Kalluri & Weinberg, 2009). La EMT tipo III está asociada a la progresión tumoral y a la metástasis. La excesiva proliferación de células epiteliales y la formación de nuevos vasos (angiogénesis) son marcadores de la iniciación y crecimiento de un carcinoma primario (Hanahan & Weinberg, 2011). La adquisición de la capacidad invasiva se manifiesta inicialmente por la degradación de la membrana basal, lo que permite la diseminación metastásica con fatales consecuencias para la vida. La activación del programa de EMT se ha propuesto como mecanismo crítico para la conversión del fenotipo maligno por células cancerosas, encontrándose estas en el frente invasivo de tumores primarios (Their, 2002; Thiery et al., 2009). Estas células son capaces de intravasar, de ser transportadas a través de la circulación, de extravasar y de formar micrometástasis o macrometástasis (Figura 4).

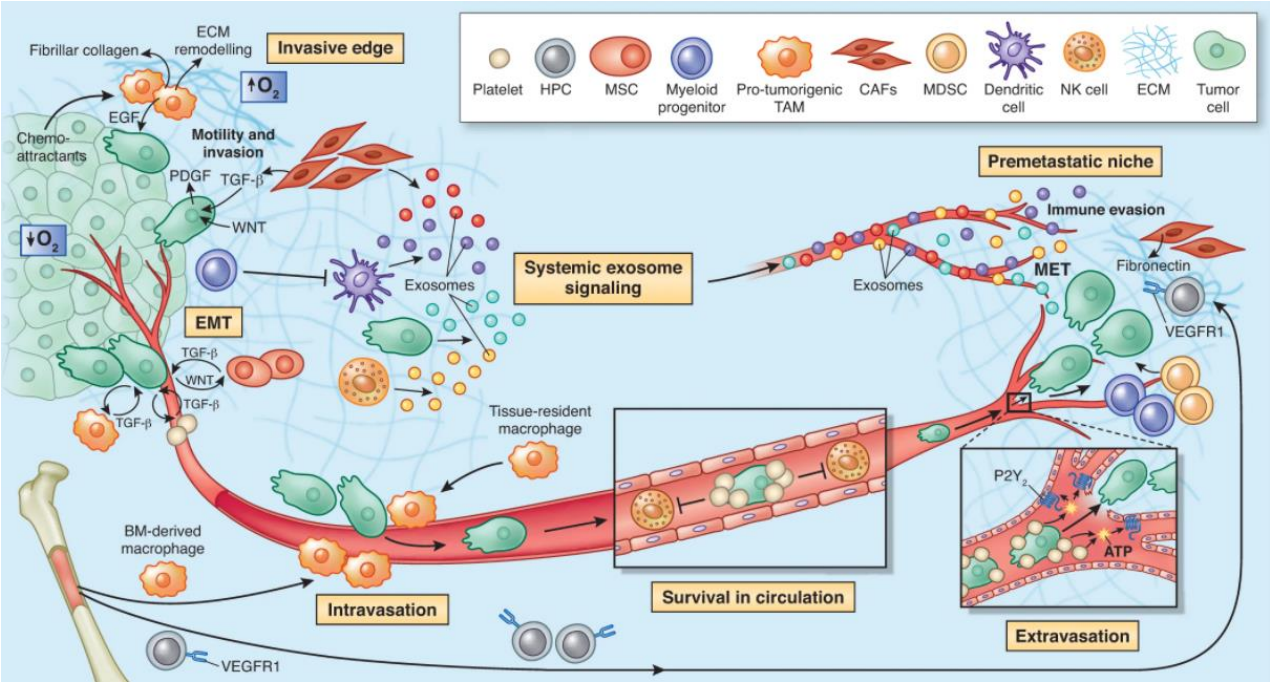


Figura 4. Contribución de la EMT a la progresión de un tumor primario.
(Quail & Joyce, 2013)

La progresión desde un epitelio normal a un carcinoma invasivo pasa por diversos estadios: 1) las células del tumor primario se someten a la EMT adquiriendo propiedades invasivas; 2) ocurre la degradación de la membrana basal y la remodelación de la matriz extracelular por proteinasas que facilitan la migración de células tumorales; 3) las células tumorales invaden los tejidos circundantes como células individuales o colectivamente; 4) ocurre la intravasación de las células tumorales en los vasos recién formados por angiogénesis dentro o cerca del tumor; 5) las células tumorales son transportadas a través de la vasculatura y arrastradas en un lecho capilar donde son extravasados; 6) ocurre la extravasación de las células tumorales a tejidos adyacentes del tumor primario; 7) estas células con capacidad motriz y fenotipo fibroblastoide son capaces de estar latentes durante años; 8) parte de ellas, fuera de su microambiente celular normal experimentan una muerte celular programada cuando se desprenden del anclaje a su matriz extracelular circundante (conocido el proceso como *anoikis);* 9) las células diseminadas crecen fuera de su tumor primario gracias al aporte de nutrientes y factores que le proporcionan los canales creados por células no endoteliales, distinguiéndose dos procesos: *mimetismo*, porque son canales y no vasos sanguíneos, simplemente tienen la misma función; y *vasculogénico*, porque no se forman a partir de un vaso preexistente (Folberg & Maniotis,

2004); 10) estas células proliferan en sitios secundarios gracias al microambiente tumoral generando un nicho pre-metastásico. Esto será clave para el desarrollo de metástasis en órganos distantes y por ello, la diseminación de la enfermedad (Figura 4) (T. R. Geiger & Peeper, 2009; Hanahan & Weinberg, 2000).

1.4. Epidemiología

El cáncer sigue siendo una de las principales causas de morbi-mortalidad en todo el mundo, superando enfermedades cardíacas coronarias y los infartos cerebrales (Mendis et al., 2011). Según los últimos datos recogidos por el programa GLOBOCAN, que tiene como objetivo proporcionar una estimación actual de la incidencia y la mortalidad de los principales tipos de cáncer, en el pasado año 2020 se diagnosticaron aproximadamente 19,2 millones de casos nuevos en el mundo (últimos datos disponibles a nivel mundial). Las estimaciones realizadas por GLOBOCAN y la OMS indican que el número de casos aumentará en las dos próximas décadas hasta 30 millones al año en 2040.

Los tumores más frecuentemente diagnosticados en el mundo en el año 2018 fueron los de pulmón, mama, colon-recto, próstata y estómago, teniendo más incidencia en varones (Figura 5) (Ferlay et al., 2010).

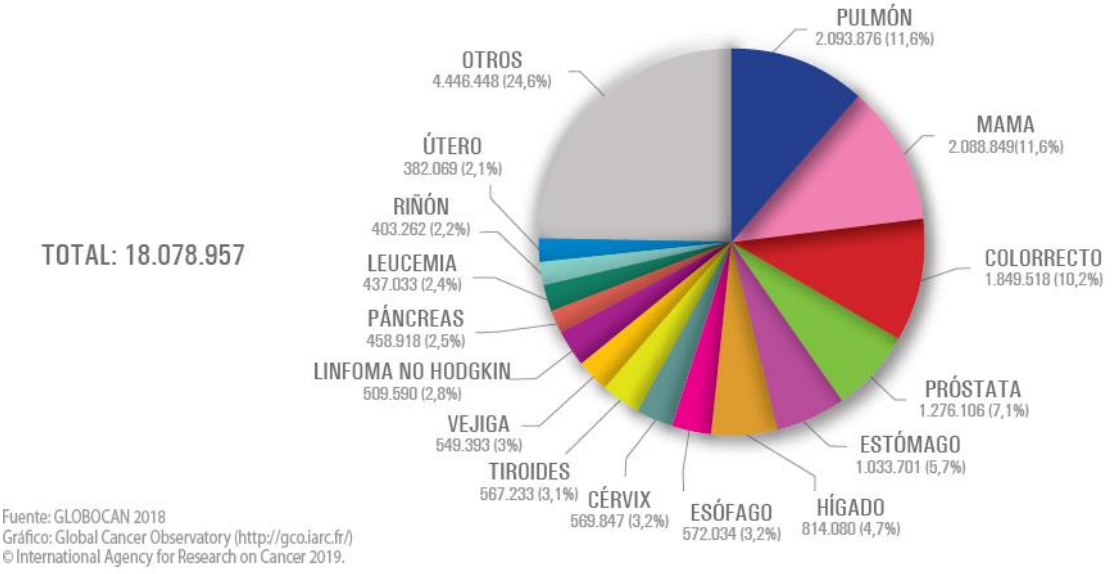


Figura 5. Tumores más frecuentemente diagnosticados a nivel mundial en el año 2019. (International Agency for Research on Cancer, 2019)

En España, el cáncer es una de las primeras causas de muerte. En nuestro país se estimó que el número de nuevos casos alcanzó los 277.394 en el año 2020 según los cálculos de la OMS y la SEOM (Sociedad Española de Oncología Médica). Los cánceres más frecuentemente diagnosticados en España son los de colon-recto (44.231 nuevos casos), próstata (35.126), mama (32.953), pulmón (29.638) y vejiga urinaria (22.350). A más distancia, los siguientes más frecuentes serán los linfomas no *Hodgkinianos* (9.188), y los cánceres de cavidad oral y faringe (8.604), páncreas (8.338), estómago (7.577), riñón (7.300) y cuerpo uterino o cérvix (6.804).

Los cánceres más frecuentes diagnosticados en varones en España en este año serán los de próstata, colon y recto, pulmón y vejiga urinaria. Sin embargo, los cánceres más diagnosticados en mujeres son los de mama, colon y recto, siguiéndoles a mucha distancia los de pulmón y cuello uterino (Figura 6).

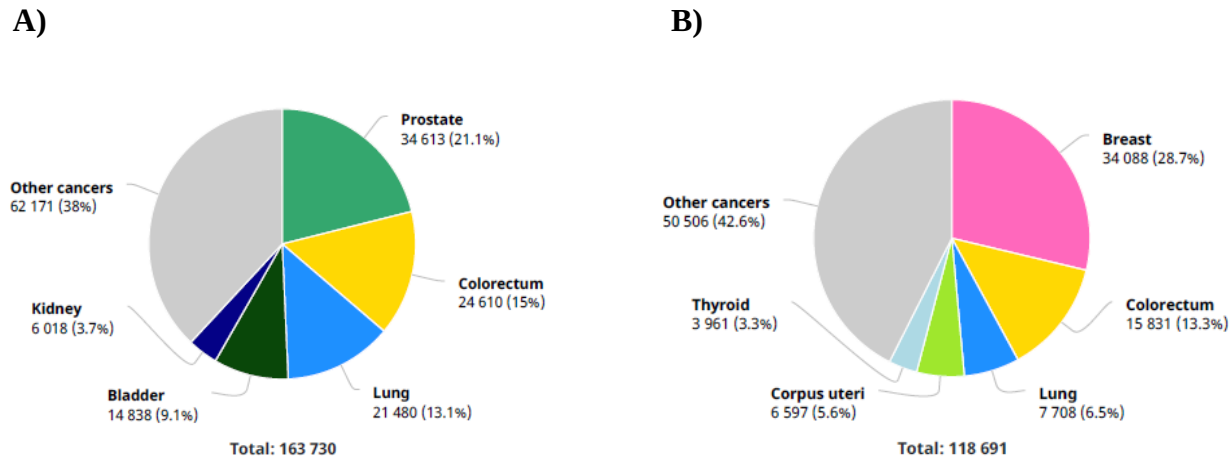


Figura 6. Número de nuevos casos diagnosticados en España en el año 2020 en hombres (A) y mujeres (B) (Imagen adaptada de GLOBOCAN 2020)

De acuerdo con las actualizaciones del Instituto Nacional de Estadística en este último año, los tumores constituyen la segunda causa de muerte en España (26,4% de los fallecimientos) por detrás de las enfermedades del sistema circulatorio e incluso, la pandemia producida por la Covid-19.

Los cánceres que produjeron más defunciones son los de pulmón, colorrectal, mama, páncreas y próstata, todas ellas con más de 5.000 fallecimientos en cada una de ellas, destacándo un aumento de los casos de pulmón en hombres y mama en mujeres (Figura 7).

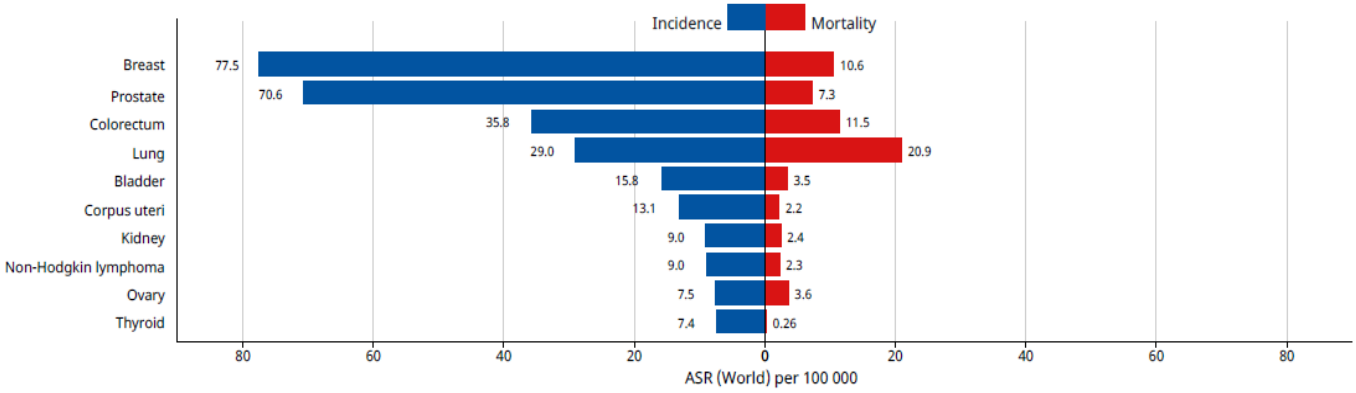


Figura 7. Incidencia y mortalidad en ambos sexos en España en el año 2020 (*The Global Cancer Observatory*)

Esta tendencia se ha ido manteniendo en los años, pero la OMS prevee una disminución de la mortalidad en ambos sexos debido a los planes de detección precoz del cáncer y actividades sanitarias preventivas, hábitos de vida más saludables, avances terapéuticos, así como prevee una reducción de los factores de riesgo, como es la disminución de la prevalencia del tabaquismo.

1.5. Tipos de cáncer

La manera más simplificada de clasificar los tumores, y que es ampliamente utilizada, es la aplicación de una nomenclatura en función de su localización en el cuerpo. Así, se habla coloquialmente de cáncer de pulmón, mama, colon, estómago, próstata... sin embargo, cada uno engloba muchos subtipos diferentes según su expresión molecular, morfología, perfil de marcadores específicos. Esto es conocido como heterogeneidad intertumoral (Hoadley et al., 2018; Visvader, 2011).

Se distinguen tres tipos principales de cáncer según el tejido a partir del cual se origina. Son los siguientes:

i) *Carcinomas.* Representa a los tumores sólidos que se originan a partir de células epiteliales que recubren las superficies internas y externas del cuerpo. Son los más frecuentes, se estima que representan más del 85% de todos los tipos de cáncer. Un tipo especial de cáncer de células epiteliales es el adenocarcinoma, un tumor maligno que deriva de células epiteliales glandulares.

ii) *Sarcomas.* Son tumores sólidos que se desarrollan a partir de células del tejido conectivo. Estos reciben una nomenclatura diferente según el tejido conectivo al que afecte. Por ejemplo, el osteosarcoma se origina a partir del tejido óseo o el condrosarcoma que se origina en el tejido cartilaginoso.

iii) *Leucemias y Linfomas.* Son cánceres de las células de la serie blanca de la sangre. Cuando tienen su origen en las células formadoras de la sangre que residen en la médula ósea se habla de leucemias, y cuando afecta a los nódulos linfáticos de linfomas.

iv) *Blastomas y Mielomas.* Se pueden distinguir otros tipos de cáncer según el tejido de origen: los blastomas derivan de tejidos embrionarios de los órganos, o los mielomas que se desarrollan a partir de tejido medular de los huesos (Pardee & Stein, 2008).

En el ámbito clínico, el grado tumoral y el estadio son factores muy importantes en la clasificación de los tipos de cáncer ayudando a una correcta elección de tratamientos. El grado del tumor hace referencia a la similitud que tienen las células cancerosas con respecto a las células normales en el mismo tejido. Para determinar el grado se utilizan diversos parámetros histológicos tales como la morfología de los núcleos, tamaño, grado de displasia y de división, y se establece numéricamente del 1 al 4 (bajo a alto grado, clasificando la agresividad del tumor).

Existe otro tipo de estatificación de los tumores ampliamente usado por el sistema de extensión anatómica (TNM) desarrollado y mantenido por la Unión Internacional contra el Cáncer (UICC). Esta clasificación se basa en la medida del tumor primario (T), la ausencia o presencia de metástasis en ganglios linfáticos (N) y la ausencia o presencia de metástasis distantes del tumor primario (M) (Tabla 1) (Worrall, 2000).

Muchos carcinomas que presentan el mismo estadio, grado y diferenciación tienen cursos clínicos y respuestas a tratamientos divergentes. En este sentido, se utiliza como factor pronóstico y predictivo de la enfermedad el estudio de los tumores en función de la expresión de determinados marcadores moleculares (Nenclares & Harrington, 2020).

Estadio	Descripción TNM
I	T1/T2 N0 M0
II	T1/T2 N1 M0 T3 N0 M0
III A	T3 N1 M0 T1/T2/T3 N2 M0
III B	T4 N0/N1/N2 M0 T1/T2/T3/T4 N3 M0
IV	T1/T2/T3/T4 N0/N1/N2 M1

Tabla 1. Sistema Internacional de estatificación TNM para los subtipos de cáncer

2. MELANOMA MALIGNO

Cada año se diagnostican en España 3.300 nuevos casos de melanoma, un tipo de cáncer de piel que se origina en los melanocitos encargados de producir melanina. La OMS estima que en el año 2020, este tipo de tumor fue el responsable del 80% de los fallecimientos por cáncer de piel en nuestro país.

La incidencia de melanoma cutáneo ha ido aumentando desde los últimos 20 años, siendo las regiones más afectadas las de origen europeo (población blanca) con 232.000 casos nuevos diagnosticados en el mundo, posicionándose como el tercer cáncer más común entre los hombres en EEUU y Europa (Ferlay et al., 2015; Schadendorf et al., 2018).

2.1. Subtipos de Melanoma

Dada la hetereogeneidad intertumoral y por tanto la gran variabilidad entre tumores del mismo órgano, se han definido gran cantidad de subtipos que son estudiados según su morfología y expresión de marcadores específicos, lo que supone diferencias en la respuesta terapéutica a seguir (Visvader, 2011).

En la actualidad, la Organización Mundial de la Salud utiliza aspectos morfológicos de la fase de crecimiento temprana y lugar de aparición del tumor primario, quedando la siguiente clasificación:

i) *Melanoma de extensión superficial* (SSM, del inglés *superficial spreading melanoma*). Es el subtipo histológico más común con el 70% de los casos diagnosticados. También se conoce como melanoma en fase de crecimiento radial (RGP, del inglés *radial growth phase*) y se caracteriza por una apariencia plana y crecimiento en horizontal lento.

ii) *Melanoma léntigo maligno* (LMM, del inglés *lentigo malignant melanoma*). Es una variante de melanoma *in situ* que afecta a áreas de exposición solar crónica en pacientes de avanzada edad constituyendo entre el 5-50% de los casos diagnosticados (Samaniego & Redondo, 2013).

iii) *Melanoma nodular* (NM, del inglés *nodular melanoma*). Es un subtipo frecuentemente diagnosticado, alrededor del 20% de casos, que tiende a moverse de manera vertical y penetrar en la dermis. Es entonces cuando se conoce como melanoma en fase de crecimiento vertical (VGP, del inglés *vertical growth phase*) (Poliseno et al., 2012).

iv) *Melanoma lentiginoso acral* (ALM, del inglés *acral lentiginous melanoma*). Es la forma menos común de melanoma. Generalmente ocurre en las plantas de las manos y pies o debajo de las uñas, teniendo un comportamiento biológico más agresivo y peor pronóstico (Bastian, 2014).

Los tumores SSM se caracterizan por tener mutado *BRAF*, mientras que los melanomas LMM comúnmente tienen mutado *NRAS*, y a veces, *KIT*. En más del 80% de los melanomas primarios, la activación aberrante de la ruta MAPK y RAS-RAF-MEK está presente (Scolyer et al., 2011). Investigaciones genéticas en pacientes con melanoma han demostrado mutaciones en los genes *c-MYC* y *PTEN* en la mayoría de los casos, estando involucrados en la proliferación celular y progresión tumoral (Jovanovic et al., 2013).

Por otro lado, destacar que no sólo existe el melanoma cutáneo o cancer de piel, sino que además existen diferentes tipos de melanoma según la localización del tumor primario. En este caso, los melanomas no cutáneos son: el melanoma ocular o uveal y el melanoma intestinal. El melanoma uveal es un tumor maligno que afecta al ojo en sus

tres estructuras (iris, cuerpo ciliar o coroides) ocupando el primer lugar de los tumores intraoculares diagnosticados. Su gravedad es tal, que la propia lesión ocasiona pérdida de la función visual y lesiones extraoculares por su rápida expansión. Se han descrito formas familiares, lo que hace suponer la posible herencia con un patrón autosómico dominante (Jovanovic et al., 2013). El melanoma intestinal afecta al estómago y al intestino delgado, siendo poco habitual en clínica y de dificil diagnóstico. Suele presentarse en la mayoría de los casos en pacientes sin tumor primario conocido o melanoma primario, teniendo peor pronóstico y mayor agresividad (Lens et al., 2009).

2.2. Factores de riesgo en Melanoma

Sin duda, el mayor factor de riesgo en melanoma viene dado por una exposición intensa intermitente a radiación UV y quemaduras solares durante la niñez. Aunque este es el principal determinante, múltiples quemaduras solares y una alta exposición a lo largo de la vida dan lugar a un riesgo significativo en la etapa adulta (Wilkins et al., 2006). La radiación UV tiene múltiples efectos en la piel y ojos, debido a cambios genéticos e inducción de especies recativas de oxígeno (ROS, del inglés *Reactive Oxygen Species*), que tienen como consecuencia alteraciones en la función cutánea y producción de factores de crecimiento (International Agency for Research on Cancer (IARC), 2018). La exposición solar ocupacional es asociada con frecuencia a padecer melanoma ocular o uveal.

Otros factores de riesgo de melanoma son la historia familiar, múltiples lunares, piel clara o inmunosupresión. Los melanomas cutáneos que aparecen en cuello, nuca y rostro se relacionan con una exposición crónica al sol, mientras que los que aparecen en el tronco ocurren en pacientes con múltiples nevus melanocíticos y con una proliferación melanocítica innata. Además, se conoce un patrón de herencia familar que sólo representa menos del 1% de los casos de melanoma cutáneo debido a mutaciones heredadas en genes supresores de tumores tales CDKN2A (P. A. Ascierto et al., 2012).

2.3. Tratamiento del Melanoma

El melanoma cutáneo es uno de los tumores con más probabilidad de producir metástasis, y desgraciadamente es el cáncer que ofrece mayor resistencia a la quimioterapia, presentando los pacientes metastásicos una supervivencia de 4 a 12 meses.

El plan terapéutico se inicia con la extirpación del tumor primario y reconstrucción de la zona siguiendo los principios básicos de cirugía dermatológica. En general se recomienda hacer una extirpación-biopsia del tumor y en función de los resultados, se amplían los márgenes (Zuluaga-Sepúlveda et al., 2016). La exploración de las áreas ganglionares es necesaria para estadiar correctamente el tumor y realizar una disección ganglionar si están invadidos (Serrano Ortega & Serrano Falcón, 2017).

Las terapias comunes usadas en clínica para el melanoma metastásico se dividen en tres grupos: quimioterapia citotóxica, terapia molecular dirigida e inmunoterapia. Los dos primeros grupos abarcan drogas que van directamente frente a la célula tumoral interfiriendo en procesos relevantes en la proliferación y división celular; la inmunoterapia por otro lado, ejerce su efecto mediante la modulación de la respuesta inmune frente a las células tumorales (Cipriani et al., 2020). Los agentes citotóxicos usados en clínica incluyen agentes alquilantes (dacarbazina, temozolomida, nitrosoureas), toxinas microtubulares (*Paclitaxel*) y análogos al platino (DeSantis et al., 2014).

En pacientes metastásicos se utiliza especialmente la radiación junto a quimioterapia o inmunoterapia. Pacientes en estadio II/III con alto riesgo de recaída se tratan con Interferón-alpha-2b como tratamiento adyuvante. Se ha demostrado que mejora la supervivencia en estadios avanzados y es el único aprobado por la FDA y CEE en pacientes de alto riesgo. El régimen más usado es el de *Kirkwood* que suministra Interferón a altas dosis (20 millones UI/mL) tres días a la semana vigilando toxicidad en el paciente (Davar et al., 2012; J. M. Kirkwood et al., 1996).

Sin embargo, en los últimos años ha cambiado el tratamiento en pacientes con melanoma en estadio IV, e incluso en estadios III con alto riesgo de recaída sustituyendo el interferón por inmunoterapia o terapia molecular dirigida. Entre los fármacos inmunoterapéuticos se encuentra el *Ipilimumab*, un inhibidor de CTLA-4 que se encuentra en las células T y ayuda al organismo a mantener bajo control las respuestas inmunitarias aumentando la supervivencia en pacientes con melanoma avanzado (Hodi, 2010) o el *Nivolumab* o *Pembrolizumab*, ambos receptores de muerte programada PD1 e inhibidores PDL-1, que se usan no sólo en melanoma avanzado sino también en primera

línea del cáncer de pulmón no microcítico al potenciar la respuesta de linfocitos T incluyendo una respuesta antitumoral (Passiglia et al., 2018).

Entre los fármacos usados como terapia molecular dirigida se encuentran los inhibidores de BRAFv600 (*Vemurafenib*, *Dabrafenib*) e inhibidores de MEK (*Trametinib*) (Bollag et al., 2010; Sullivan & Flaherty, 2013).

3. CÁNCER DE COLON

El cáncer colorrectal (CCR) es el tumor más común en el tubo digestivo, siendo la neoplasia más común entre los hombres (por detrás de pulmón y próstata) y la segunda más común en mujeres (detrás del de mama). En España, se diagnostican aproximadamente 35.000 casos nuevos anuales, de los que 13.000 fallecen, representando el 15% del total de muertes con respecto a todos los tumores (Galceran et al., 2017).

La mayoría de los CCR se localizan en el colon descendente o izquierdo, colon sigmoide y en el recto, teniendo más incidencia en hombres que en mujeres y siendo la edad media de presentación 70 años (la mayoría de los pacientes tienen más de 50 años en el momento del diagnóstico) (SEOM, 2018, 2020).

3.1. Subtipos de Cáncer de colon

Podemos encontrar diferentes tipos de cáncer que afectan tanto al colon como al recto, según la *American Cancer Society* (American Joint Committee On Cancer, 2009) podemos destacar:

i) *Adenocarcinomas.* Constituyen más del 95% de los canceres de tipo colorrectales.

ii) *Adenocarcinomas Mucinosos o Coloides.* Es un subtipo histológico de cáncer de colon, en el que las células tumorales producen mucina extracelular en abundancia (puede representar hasta el 50% del volumen total del tumor). La mucina es producida por el colon para ayudar a lubricar a los tejidos. Este tipo de adenocarcinomas suele presentarse en el colon derecho, y generalmente en fases avanzadas de la enfermedad.

iii) *Tumores gastrointestinales carcinoides.* Este tipo de tumores suelen presentarse tanto en el colon como en el recto. Cuando lo hacen en el

colon, suelen tener peor pronóstico por presentar una mayor probabilidad de metástasis; en cambio, cuando se presentan en el recto, al estar muy localizado, tienen mejor prognosis y no suelen metastatizar (Memon & Nelson, 1997).

iv) *Tumores gastrointestinales estromales.* Su desarrollo está relacionado con mutaciones en los genes *KIT* y *PDGFRA* (del inglés, *platelet-derived growth factor receptor alpha*) pertenecientes a la superfamilia de receptores tirosina-quinasa. Estos tumores representan las neoplasias de tipo mesenquimal más frecuentes en el tracto intestinal (Rutkowski et al., 2011).

v) *Linfomas y Sarcomas.* Los linfomas que suelen afectar al tracto gastrointestinal son los linfomas No-Hodking; por su parte, los sarcomas suponen menos del 1% de los tumores en colon, teniendo su origen en células del músculo liso de la pared intestinal (Aksu et al., 2004).

En lo referente a la clasificación molecular del cáncer colorrectal, existen tres subtipos principales (CCS, del inglés *cancer subtypes*) según características moleculares y genéticas (Figura 8) (Linnekamp et al., 2015). Son los siguientes:

i) *Tumores CCS-1.* Se caracterizan por estar mutados los genes *KRAS* y *TP53* y tener una marcada inestabilidad cromosómica (CIN, del inglés *chromosomal instability*).

ii) *Tumores CCS-2.* Característicos por estar fuertemente enriquecidos en inestabilidad de los microsatélites (MSI, del ingles *microsatellite instability*) e islas CpG metiladas (CIMP, del inglés CpG *island methylator phenotype*) y por tener una gran infiltración de células inmunes. Según su grado de inestabilidad, los MSI se dividen a su vez en tres grupos: alta inestabilidad de microsatélites (MSI-H); baja inestabilidad de microsatélites (MSI-L) y por último, microsatélites estables (MSS). Esta clasificación está siendo hoy día muy usada en clínica para la caracterización de los tumores en colon. Tanto los tumores CCS-1 y CCS-2 son los más frecuentes y se caracterizan por tener mejor evolución clínica (Brenner et al., 2014).

iii) *Tumores CCS-3.* Presentan MSI y CIN, además de mutaciones en BRAF y PIK3CA, con fenotipo mesenquimal (Figura 8) (Linnekamp et al., 2015).

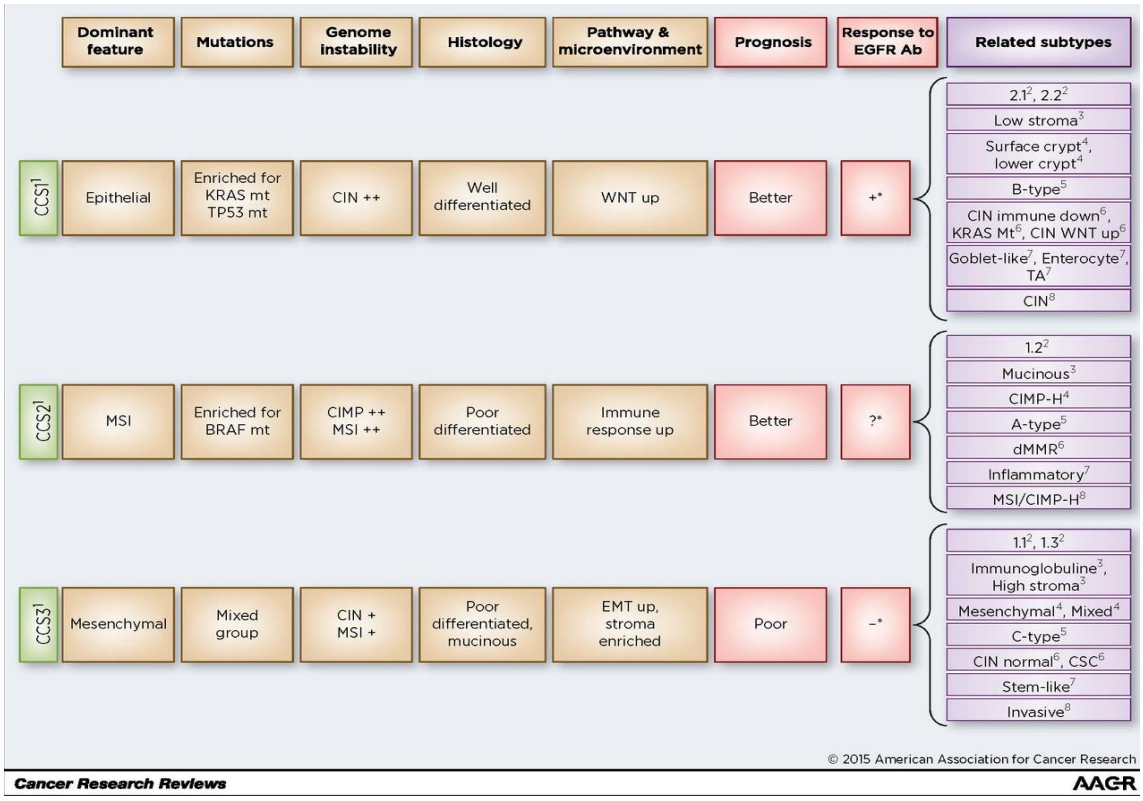


Figura 8. Clasificación molecular del cáncer colorrectal
(Linnekamp et al., 2015)

3.2. Factores de riesgo en Cáncer de colon

Los factores implicados en la aparición de cáncer de colon se pueden dividir en factores genéticos o factores no genéticos. En ellos podemos destacar:

a) Factores genéticos.

La suceptibilidad genética constituye un factor de riesgo importante, ya que, aproximadamente el 20% de los nuevos casos diagnosticados tiene un patrón de herencia familiar. El cáncer de colon hereditario se categoriza como polipósico (siendo los más comunes la poliposis adenomatosa familiar -FAP- y la poliposis asociada a MUTYH -MAP-); y no polipósico (siendo el más común el síndrome de Lynch) (Esplin & Snyder, 2014). FAP es el segundo cáncer colorrectal hereditario con más prevalencia, y está causado por la mutación en APC en la línea germinal (un supresor de tumores implicado en la regulación de la cascada Wnt). Por su parte, el síndrome de Lynch, es el cáncer hereditario más común (aproximadamente el 3% de los nuevos casos

diagnósticados). Tiene un patrón autosómico dominante y está asociado a mutaciones en genes de reparación MMR (MMR, del inglés *mismacth repair*) encargados del mantenimiento del material genético y de roturas de la doble cadena del ADN (Stoffel et al., 2018).

b) Factores no genéticos.

El tabaco y consumo de alcohol son los factores de riesgo ambientales más importantes para sufrir cáncer de colon. En los últimos años, la incidencia de esta patología ha disminuido por la adquisición de hábitos de vida más saludables por parte de la población. Otros de los factores que aumentan considerablemente el riesgo son la dieta y el estilo de vida: alimentación procesada altamente calórica y un estilo de vida sedentario, ausencia de actividad física deportiva y tratamientos prolongados de antiinflamatorios no esteroideos, provocan en órganos como el colon, inflamación crónica (Bray, 2018).

3.3. Tratamiento del cáncer de colon

El tratamiento del cáncer colorrectal varía según el estadio en el momento del diagnóstico y la localización del tumor. De este modo, en estadios más tempranos (I y II) la cirugía es el tratamiento más común para eliminar el tumor. Entre las técnicas quirúrgicas más usadas son las resecciones intestinales y las colectomías. Para los pacientes en estadio III, y algunos en estadios II con alto riesgo de recaída, la cirugía es seguida por tratamiento adyuvante, y en los casos más avanzados o metastásicos la quimioterapia (DeSantis et al., 2014).

La quimioterapia del cáncer de colon comenzó con el desarrollo en 1957 del 5-fluorouracilo (5-FU) (Figura 9) (Heidelberger et al., 1957). Desde entonces la quimioterapia ha avanzado hasta los últimos años con la combinación del 5-FU con otros agentes como leucovorin, oxaliplatino, irinotecan, capecitabina (Figura 10) (Gustavsson et al., 2015).

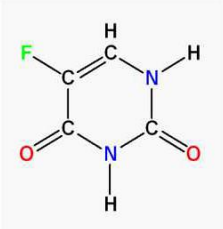


Figura 9. Estructura del 5-Fluorouracilo
(Lvarez et al., 2012)

En pacientes en estadio III y metastásicos suele usarse de manera estándar y en primera línea el FOLFOX (5-FU/leucovorin y oxaliplatino) o capecitabina más oxaliplatino (XELOX), siendo este último de preferencia en pacientes de más de 70 años en menor dosis. En pacientes metastásicos en estadio IV suele utilizarse como tratamiento combinado FOLFOX junto a irinotecan (FOLFIRI), aumentando la tasa de supervivencia. En pacientes jóvenes, en buen estado, se utiliza la combinación del 5-FU, irinotecan y oxaliplatino (FOLFOXIRI), lo cual ha demostrado mayor eficacia, además junto con tratamiento molecular dirigido: anticuerpos para VEGF (bevacizumab) y para EGFR (cetuximab o panitumumab) (Figura 10) (Stintzing, 2014).

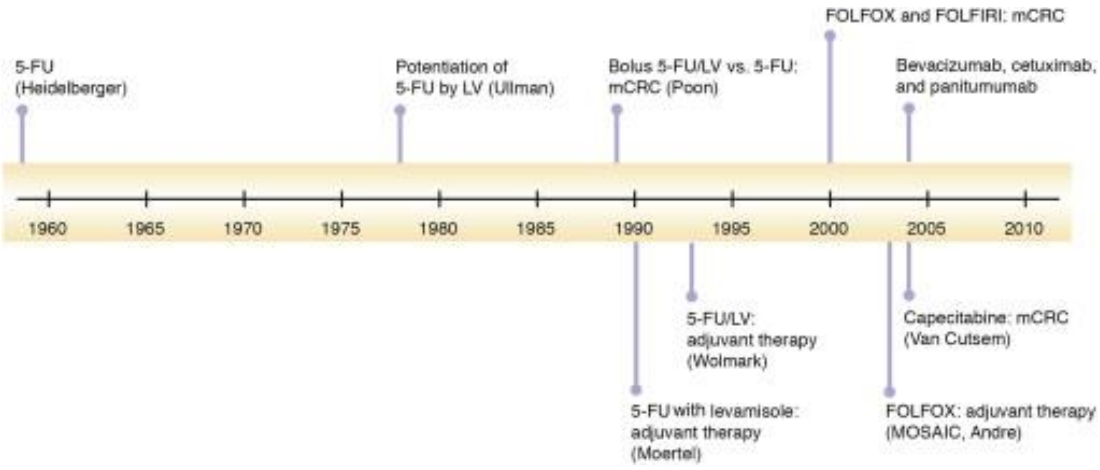


Figura 10. Evolución de los nuevos agentes quimioterapéuticos en pacientes con cáncer colorrectal. 5-FU: 5-Fluorouracilo; FOLFIRI: 5-FU/LV con irinotecan; FOLFOX: 5-FU/LV con oxaliplatino; LV: leucovorina; mCRC: cáncer colorrectal metastásico (Gustavsson et al., 2015).

4. CÉLULAS MADRE CANCERÍGENAS

4.1. Definición y Origen

El punto de vista clásico del cáncer ha ido cambiando y evolucionando gradualmente, puesto que diversos estudios han identificado subpoblaciones de células dentro de los tumores que impulsan el crecimiento, la recurrencia tumoral y la metástasis. Dentro de cada tumor individual existe una alta variabilidad de poblaciones de células que lo conforman, esto es conocido como hetereogeneidad intratumoral.

Existe una pequeña subpoblación de células tumorales con propiedades funcionales de célula madre, como es la capacidad de autorrenovación y de diferenciación multipotente hacia todas las células cancerígenas diferenciadas que componen el tumor. A esta subpoblación de células se las ha denominado células madre cancerígenas (CSCs, del inglés *cancer stem cells*) (Jinushi, 2014; Shigdar et al., 2014; Visvader, 2011).

Estas CSCs, presentes en una pequeña proporción en la masa tumoral, se caracterizan por permanecer quiescentes durante largos periodos de tiempo manteniendo el crecimiento y la hetereogeneidad del tumor, evadiendo los agentes quimioterapéuticos por la presencia de transportadores de membrana ABC (ABC, del inglés *ATP-binding cassette*), sobreexpresando genes antiapoptóticos y de reparación de ADN y aportando capacidad de propagar y sostener la tumorogénesis (Shigdar et al., 2014).

Los modelos que explican la hetereogeneidad intracelular en los tumores han evolucionado gracias a los numerosos avances en la oncología experimental, permitiendo el desarrollo de teorías que explican el proceso de formación del tumor.

El modelo estocástico o modelo de evolución clonal propone que los tumores proceden de una única célula anormal, y que con el tiempo, esta célula ancestral va adquiriendo cambio oncogénicos o mutaciones que hacen que adquieran ciertas ventajas con respecto a sus células vecinas. Esta variabilidad genética es la responsable del desarrollo y mantenimiento del tumor y de la existencia de diferencias entre las células según este modelo (Figura 11) (Gottschling et al., 2012).

El modelo jerárquico o modelo de las células madre cancerígenas postula que una pequeña subpoblación celular con propiedades de CSCs es la encargada de iniciar el tumor. Esta característica esclusiva de las CSCs se ve corroborada por la capacidad que

tienen de iniciar un tumor en animales de experimentación si son inoculadas en bajo número, además de poder dividirse en células hijas que se diferencian otorgando hetereogeneidad en el tumor (Figura 11) (Gottschling et al., 2012; Prince et al., 2007).

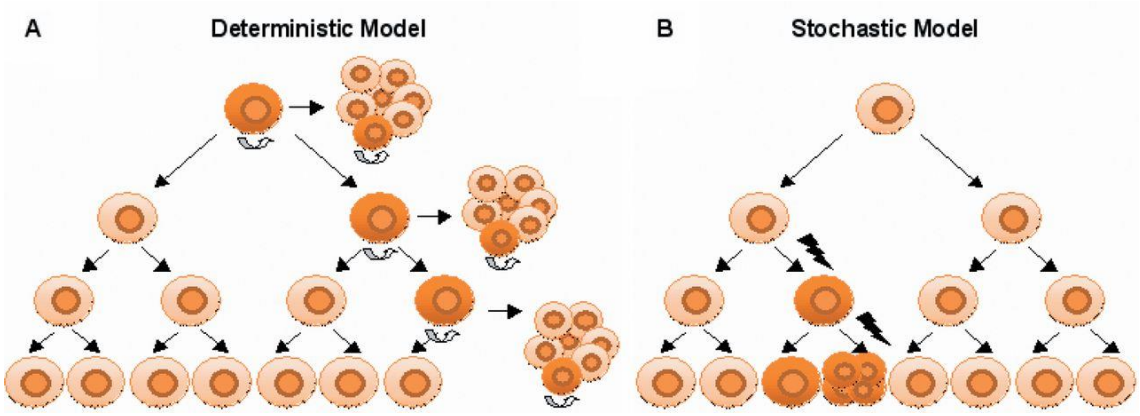


Figura 11. Modelos del formación del cáncer. A) Modelo jerárquico. El tumor se inicia a partir de una CSC, la cuál genera a su vez células tumorales y CSCs mediante división asimétrica; B) Modelo estocástico. El tumor se inicia a partir de células alteradas genéticamente, y mediante evolución clonal, generan clones dominates mediante división simétrica (Gottschling et al., 2012).

4.2. Características de las células madre cancerígenas

En la actualidad, el concepto de CSCs es de gran interés en parte a los avances biológicos conseguidos que explican sus características y su importante papel en el desarrollo y mantenimiento de la tumorogénesis (Batlle & Clevers, 2017; Dalerba et al., 2007). Las características más destacables de las CSCs que las hace fundamentales y las diferencia del resto de poblaciones celulares son:

4.2.1. Autorrenovación y Pluripotencia de las CSCs.

La capacidad de autorrenovación y diferenciación en linajes específicos de célula madre viene regulado por factores y señales ambientales presenten en el nicho en el que se desarrollan. Estos factores vienen dados por la alteración y desregulación de cascadas de señalización alteradas en cáncer, como son: vía Notch, vía Wnt/β-catenina y la vía Sonic Hedgehog (SHh). Estas rutas juegan un papel crucial en el desarrollo de células tumorales con propiedades de célula madre (Figura 12) (Malaguarnera & Belfiore, 2014).

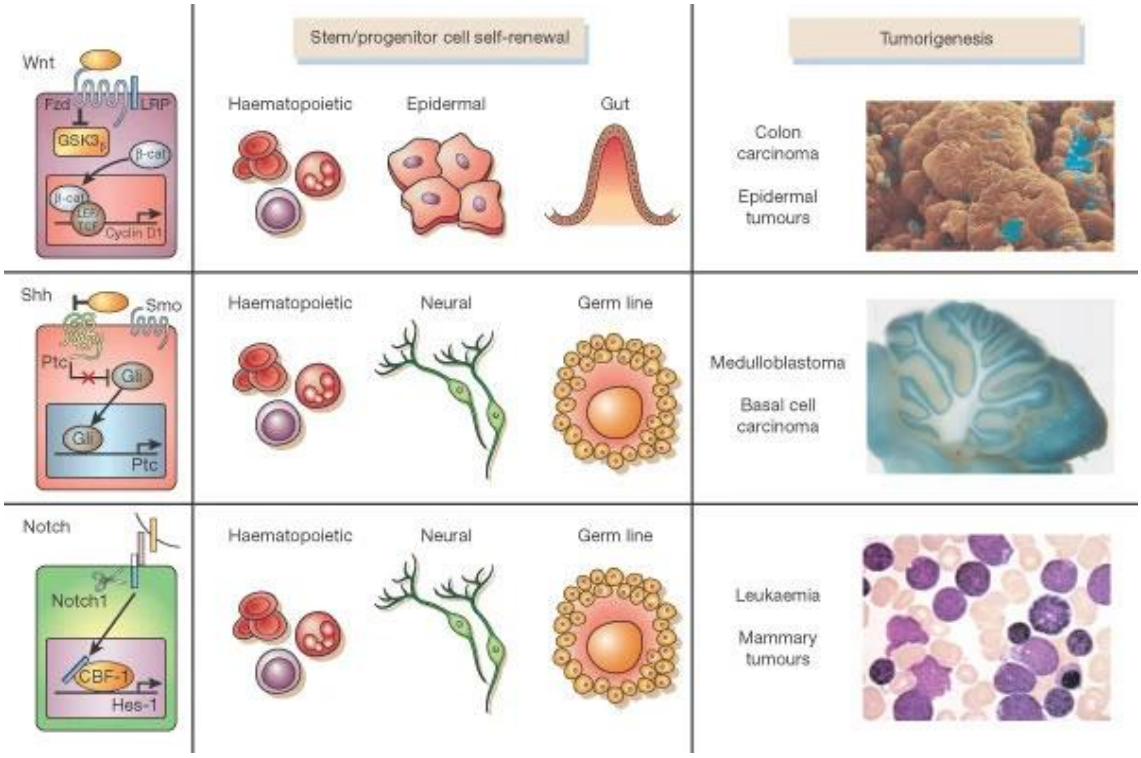


Figura 12. Principales rutas de señalización y de pluripotencia expresadas en la formación de las células madre y en la tumorogénesis (Reya et al., 2001).

La ruta de señalización Notch está implicada en la proliferación, supervivencia, diferenciación celular y angiogénesis. Se ha demostrado que su alteración da lugar al mantenimiento de las CSCs en diferentes tipos de cáncer como mama, colon o glioblastoma (Radtke & Raj, 2003). La ruta Wnt/β-catenina es la más conservada evolutivamente implicada en la diferenciación y embriogénesis. La activación de esta vía está relacionada en los cánceres epiteliales y en el proceso de EMT (Murugan et al., 2012). Estas dos rutas participan de manera sinérgica en la homeostasis de las células madre. La vía de señalización Hedgehog (Hg) juega un papel muy importante en el organismo adulto, ya que está implicada en el mantenimiento de la homeostasis celular y tisular, regeneración y mantenimiento de las células madre. Cuando esta ruta está activada de forma aberrante se da un mantenimiendo de las propiedades de las CSCs, además de activar otros factores como VEGF o TAF (TAF, del ingles *Tumor Associated Fibroblasts*) para la angiogénesis y vasculatura sostenida, formación y mantenimiento del tumor (M. A. Huber et al., 2005; Taipale & Beachy, 2001).

4.2.2. Plasticidad de las CSCs.

Numerosos estudios han demostrado que tanto las células madre como las CSCs tienen características de plasticidad y son capaces de experimentar transiciones fenotípicas y morfológicas para llegar a adquirir cualquier linaje celular. Esta plasticidad se da en respuesta a diversos factores microambientales y depende en gran medida del proceso de EMT, llegando a conferirles alto potencial invasivo y metastásico (Hernández-Camarero et al., 2018).

4.2.3. Quimio y Radiorresistencia de las CSCs.

Otra característica muy importante en las CSCs es la multiresistencia a drogas (MDR, del inglés *Multidrug Resistance*) por la alta presencia de transportadores ABC en sus membranas que utilizan la energía producida de la hidrólisis del ATP para expulsar fármacos al medio extracelular y conferirles protección. Entre la familia de transportadores ABC destacamos la proteína P-gP que codifica el gen ABCB1 (MDR-1) que son capaces de expulsar hasta 20 tipos diferentes de fármacos citotóxicos usados en las terapias convencionales (Leonard et al., 2003; Robey et al., 2018). En cuanto a la radiorresistencia, las CSCs poseen menor capacidad de producir especies ROS (ROS, del inglés *Reactive Oxigen Species*), por lo que desarrollan menor daño en el ADN (Hittelman et al., 2010).

4.2.4. Quiescencia.

El estado de quiescencia es el estado en el que la célula no se divide permaneciendo en la fase G_0 del ciclo celular. Esta es una característica muy importante de las CSCs, ya que les permite sobrevivir a la mayoría de los fármacos antitumorales (Reya et al., 2001). Este fenómeno se encuentra implicado en la reaparición del cáncer décadas después del tratamiento inical, generando recaídas o recidivas (Dick, 2008).

4.2.5. Incremento de la capacidad de reparación del ADN.

Las CSCs poseen una alta capacidad de reparación de daños en el material genético confiriendoles protección y estabilidad del genoma. En los mecanismos de reparación del ADN se han encontrado proteínas implicadas tales como ATM, BRCA-1 o p53 que inducen mecanismos de control regulados por dos vías quinasas distintas: la vía ATM-Chk2 y la ATM-Chk1 (ATM, del inglés *Ataxia Telangiectasia Mutated* gen). Ambas vías de señalización son puntos control que evitan la progresión del ciclo celular,

incrementando la eficiencia en la reparación del ADN provocada por los fármacos antitumorales y con ello, aumentando la supervivencia (Ronco et al., 2017).

4.2.6. Aumento de su capacidad de invasión y metástasis.

A medida que las neoplasias avanzan en su invasión y malignidad, las células tumorales adquieren la capacidad de difundir a tejidos u órganos desde el tumor primario provocando una invasión local, micrometástasis y finalmente, metástasis (Hanahan & Weinberg, 2000). Este proceso de invasión a tejidos adyacentes y diseminación a otros órganos se produce a través del proceso de EMT. Este proceso es clave en la tumorogénesis (vease epígrafe 1.3. Transición Epitelio Mesénquima).

Las células cancerosas pierden la expresión de e-cadherina, una proteína implicada en la polaridad celular y adhesión célula-matriz, y adquieren rasgos invasivos y motrices por la expresión de marcadores mesenquimales tales como vimentina, SNAIL, SLUG y N-cadherina (Malaguarnera & Belfiore, 2014). La pérdida de expresión de e-cadherina y ocludinas da lugar a la translocación de β-catenina al núcleo, modulando la transcripción de varios genes clave en el desarrollo y progresión del cáncer, tales como CD44, MYC y VEGF (Cui et al., 2014).

En este proceso, la respuesta inflamatoria juega un papel importante, ya que produce modificaciones en el microambiente por la secreción de factores (citoquinas) y células inmunes (macrófagos, fibroblastos) proinflamatorios y oncogénicos, que activan rutas necesarias para la migración de las CSCs (Shigdar et al., 2014). Una vez que las células metastatizan en órganos diana se produce el proceso contrario, transición mesénquima-epitelial (MET, del inglés *Mesenchymal Epithelial Transition*) para proliferar en ese nicho y establecer nuevos tumores que sigan el proceso metastásico (Britton et al., 2011).

4.3. Aislamiento y caracterización de las células madre cancerígenas

Las células madre cancerígenas se pueden aislar e identificar en la mayoría de los tumores en base a diferentes características que han dado lugar a cuatro metodologías. Son las siguientes: aislamiento por citometría de flujo de marcadores de superficie celular específicos de CSCs, determinación de la habilidad de formación de esferas en suspensión, capacidad de expresar altos niveles de actividad aldehído-deshidrogenasa

(ALDH) y detección del fenotipo *Side Population* (SP) mediante la exclusion del colorante vital Hoechst. Otra característica fundamental de las CSCs es su capacidad de formar tumores en animales de experimentación tras la inoculación de un menor número de células que el resto de poblaciones del tumor. Hipotéticamente, una única célula debe sercapaz de regenerar el tumor original (Tirino et al., 2013).

4.3.1. Expresión diferencial de marcadores de superficie

Las subpoblaciones de CSCs expresan marcadores celulares de superficie específicos que las diferencia del resto de poblaciones del tumor. Los marcadores habitualmente relacionados con un fenotipo de CSCs en un gran número de tumores son por ejemplo, CD44 (Yan et al., 2015), CD20 (Schlaak et al., 2012), CD133 (Brescia et al., 2013), CD326 (Patriarca et al., 2012) y ABCB5 (Ksander et al., 2014). Estos marcadores son usados conjuntamente para una mejor caracterización. Así, las CSCs de mama expresan altos niveles de CD44 pero no de CD24 y CD133 (Lorico & Rappa, 2011); en melanoma maligno, se caracterizan por expresar CD44, CD20 y CD271 (Magnoni et al., 2014); para las CSCs de colon se usa esta combinación de marcadores CD44, CD133 y CD326 (EpCAM o ESA) (Testa, 2012).

4.3.2. Formación de colonias

Este método se basa en las propiedades de las CSCs de formar esferas en suspensión cuando son cultivadas en bajo número y en ausencia de suero bovino fetal, así como la presencia de factores de crecimiento como EGF (EFG, del inglés *Epidermal Growth Factor*) y FGF (FGF, del inglés *fibroblast Growth Factor*) (Morata-Tarifa et al., 2016a). Las células presentes en estas esferas tiene una alta capacidad de autorrenovación al compararlas con células adherentes (Magee et al., 2012).

4.3.3. Actividad aldehído deshidrogenasa

La familia de enzimas ALDHs está implicada en la síntesis de intermediarios aldehído como el ácido retinoico, el folato y la betaína. Esta metabolización de los rectivos ALDH confiere a las CSCs protección a agentes citotóxicos y con ello, aumento de la proliferación, diferenciación y supervivencia de las células. En humanos existen 11 familias siendo la isoforma ALDH-1 la más presente en las CSCs de múltiples cánceres (Ginestier et al., 2007).

4.3.4. Side Population (SP)

El aislamiento de la subpoblacion de *Side Population* se basa en la capacidad de las células madre cancerígenas a expulsar colorantes vitales tales como el Hoechst 33342. Esta capacidad de expulsión se debe al elevado número de transportadores ABC presentes en su membranas plasmáticas. Esta SP es aislada mediante citometría de flujo y está formada por células con una alta capacidad de autorrenovación (Shimoda et al., 2018; C. Wu & Alman, 2008).

4.4. Estrategias terapéuticas dirigidas a células madre cancerígenas

Las células madre cancerígenas son consideradas como la raíz del cáncer, por lo que la identificación y eliminación de ellas es crucial para el diseño de terapias dirigidas frente a esta población. La mayoría de terapias anticancerígenas que se usan habitualmente en clínica están destinadas a las células tumorales, por lo que las CSCs están protegidas por sus características de quiescencia y lento crecimiento (Tirino et al., 2013).

Dado que las CSCs presentan características moleculares diferentes del resto de poblaciones en el tumor y de las células tumorales, y que son las responsables de la recurrencia en pacientes, un tratamiento dirigido a esta subpoblación puede ser más eficaz para prevenir futuras recaídas (Ghaffari, 2011).

Como se muestra en la Figura 13, las terapias más efectivas consistirán en radiación localizada y quimioterapia contra la masa tumoral, combinado con nuevos fármacos dirigidos a dianas moleculares de CSCs (Cojoc et al., 2015).

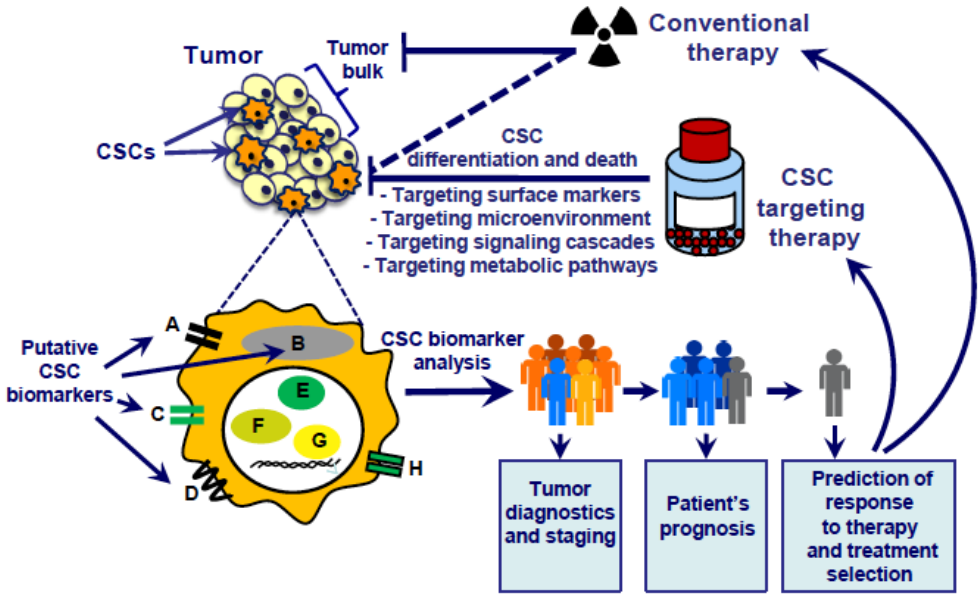


Figura 13. Esquema de posibles dianas terapéuticas y tratamientos contra CSCs (Cojoc et al., 2015)

5. VESÍCULAS EXTRACELULARES: EXOSOMAS

5.1. Definición y Características

La comunicación intercelular es crucial para el desarrollo y funcionamiento apropiado de los tejidos. Las formas clásicas de comunicación entre células están representadas por uniones celulares, contactos de adhesión y factores solubles que actúan sobre las células donde se producen, o sobre células vecinas o incluso pueden actuar de forma endocrina en otros tejidos. Además de estos medios ya conocidos, recientemente se ha descubierto otro mecanismo de comunicación de célula a célula a través de vesículas extracelulares (EV, del inglés *extracellullar vesicles*) (Tetta et al., 2013). El término EV es un término genérico que se refiere a un grupo heterogéneo de organelas unidas a la membrana (microvesículas, exosomas, ectosomas, vesículas desprendidas) con diferentes tamanos que varían entre 30 nm a 1 µm.

Los exosomas son un subconjunto homogéneo de EV de tamaño nanométrico, con diametros de 40 a 120 nm) que se desprenden de manera activa por la mayoría de tipos celulares, incluyendo células tumorales y CSCs (Pegtel & Gould, 2019; Raposo & Stoorvogel, 2013). Los exosomas se identificaron por vez primera en 1983 a partir de

reticulocitos de oveja en un estudio sobre la externalización del receptor de transferrina, es por ello que acuñaron el primer concepto de vesículas externalizadas (B. T. Pan & Johnstone, 1983). El término exosoma fue propuesto años después por Johnstone, siendo el primero en aislarlos y caracterizarlos mediante microscopia electrónica. Estas pequeñas partículas provenían de eritrocitos de rata y eran exocitadas al medio extracelular a partir de cuerpos multivesiculares (MVB, del inglés *Multi-Vesicular Bodies*) (Johnstone et al., 1987).

Numerosos grupos siguieron identificando exosomas y MVB comparandolos con otras vesículas con el fin de hacer una clasificación más exacta (Raposo & Stoorvogel, 2013). De esta manera, se ha tratado de diferenciar las variadas vesículas por su tamaño, densidad, fuente de formación siendo discutida su nomenclatura por la comunidad científica en la actualidad. En la siguiente tabla se expone a modo de resumen las diferentes vesículas clasificadas hasta el momento (Tabla 2) (Jeppesen et al., 2019).

Los exosomas además son marcadamente diferentes a las microvesículas y ectosomas por sus características biológicas: al microscopio electrónico tienen una forma ovalada o de copa observándose su doble membrana lipídica, flotan en un gradiente de densidad de sacarosa 1.13-1.19 g/mL y son ricas en marcadores específicos como las tetraspaninas CD63 y CD81 o proteínas de choque térmico (HSP70) (Jeppesen et al., 2019; Pegtel & Gould, 2019; Tetta et al., 2013).

Característica	Exosomas	Microvesículas	Ectosomas	Partículas de membrana	Párticulas similares a exosomas	Vesículas apoptóticas
Tamaño	30-100nm	100-1.000nm	50-200nm	50-80nm	20-50nm	50-500nm
Densidad en sacarosa	1,13-1,19 g/ml	ND	ND	1,04-1,07 g/ml	1,1 g/ml	1,16-1,28 g/ml
Apariencia en microscopio electrónico	Ligeramente alargada	Forma irregular	Estructura redonda bilaminar	Forma redondeada	Forma irregular	Heterogénea
Sedimentación	100.000 g	10.000 g	160.000 g-200.000 g	100.000 g-200.000 g	175.000 g	1,200 g 10,000 g 100,000 g
Composición lipídica	Enriquecido en colesterol esfingomielina y caramida contiene balsas lipídicas exporen fostatidilcoina Tetraspaninas (CD63, CD9) Alix y TSG101	Exponen fostatidilcoina	Enriquecido en colesterol y diacilglicerol; exponen fostatidilcoina	No determinado	No contiene balsas lipidicas	No determinado
Principales marcadores	Tetraspaninas (CD63, CD9) Alix y TSG101	Integrinas selectinas y CD40 ligando	CR1 y enzimas proteolíticas; no CD63	CD133; no CD63	TNFRI	Histonas
Origen intracelular	Compartimentos internos (endosomas)	Membrana plasmática	Membrana plasmática	Membrana plasmática	Compartimentos internos	No determinado

Tabla 2. Características biológicas de los diferentes tipos de vesículas extracelulares
Adaptado de (Jeppesen et al., 2019)

5.2. Biogénesis y composición de los exosomas

Los exosomas y otras EVs son originados en diferentes compartimentos celulares y por multitud de células como son células sanguíneas, células dendríticas, neuronas, linfocitos T y B, células madre embrionarias, células tumorales, células epiteliales, células madre cancerígenas...; es por ello, que los exosomas se pueden aislar en diferentes fluidos biológicos tales como plasma y suero (Alegre et al., 2016), saliva y orina (Hoshino et al., 2015), leche materna y calostro (Lässer et al., 2011), líquido amniótico, semen, ascitis, lavado broncoalveolar... (Madison et al., 2015; Taylor et al., 2013).

Las EVs son generadas en los MVB a partir de endosomas tempranos existentes, que pueden estar almacenados durante algún tiempo en el citoplasma, y en respuesta a estímulos y factores se fusionan con la membrana plasmática liberando la carga (exosomas) al medio extracelular (Figura 14) (Jeppesen et al., 2019). Los exosomas son,

por tanto, las vesículas liberadas al espacio extracelular que contenían los endosomas ya formados en el citoplasma (Kowal et al., 2014).

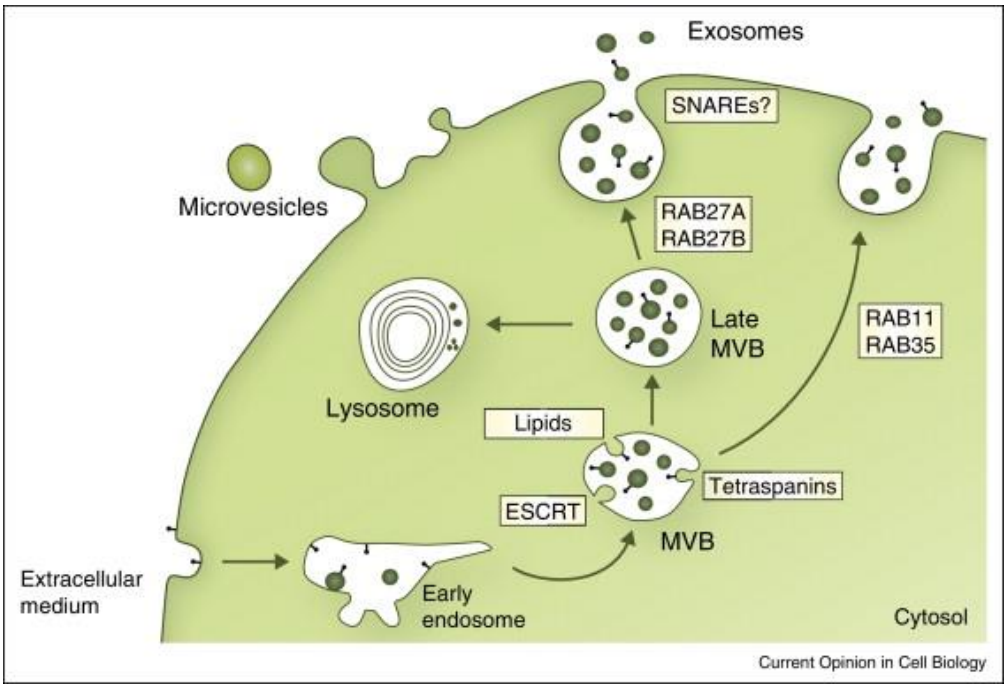


Figura 14. Exosomas liberados al medio extracelular tras la fusión de MVBs con la membrana (Kowal et al., 2014)

Los MVBs que son destinados a degradarse por la ruta exocítica (exocitosis mediante la cuál se fusionan con la membrana plasmática y secretan exosomas) presentan una composición lipídica diferente (alta proporción de colesterol) con respecto a los MVBs destinados a la ruta degradativa (ruta clásica celular de degradación de lisosomas). Este mecanismo de externalización de exosomas se encuentra muy conservado y dirigido por cuatro complejos protéicos denominados complejos clasificadores endosomales responsables del transporte o ESCRT (ESCRT, del inglés *Endosomal Sorting Complex Required for Transport*) (M. Colombo et al., 2013; Larios et al., 2020). Algunos de estos complejos necesitan la interacción mediada por ubiquitina (ESCRT-0, 1, y 2 participando en la vía endosomal). Otros procesos de fusión, en cambio, no dependen de estos complejos de proteínas y lo hacen sólo con el aporte de esfingomielinas o ceramidas en la membrana.

A pesar de que aún se sabe poco sobre el mecanismo exacto por el cuál sucede la fusión de los MVBs con la membrana, se ha descubierto que la presencia de altos niveles

intracelulares de calcio juega un papel muy importante. La familia de proteínas Rab-GTPasas ejercen un papel regulador de la vía secretora de exosomas. Rab 11 y Rab 27 se descubrieron por primera vez en células HeLa y poseen funciones claves en el control de la secrección en cada compartimento celular (Hsu et al., 2010).

Se sabe que la familia de proteínas SNAREs (SNARE, del inglés *soluble N-athylmaleimidesensitive fusion attacment protein*) son las encargadas en el paso final de la fusión de MVBs y membrana (Kowal et al., 2014).

En cuanto a la composición molecular de los exosomas varía según el origen celular de donde deriven, a pesar de ello, y de los avances metodológicos en aislamiento y caracterización de estas poblaciones, encontramos componentes comunes (Raposo & Stoorvogel, 2013; Théry et al., 2018). Cuando se eliminan al entorno extracelular, contienen moléculas biológicamente activas e importantes como son proteínas, ARN mensajeros (ARNm), ADN, lípidos y otros metabolitos (Figura 15) (Farooqi et al., 2018). Las moléculas presentes en los exosomas son activas en las células diana y pueden influir y reprogramar las células receptoras, por ejemplo, silenciando genes a través de miRNAs (micro ARN) (M. Colombo et al., 2013). De hecho, diversos estudios han demostrado que ARN extraído de exosomas procedentes de suero de pacientes oncológicos expresan un mayor número de miRNAs con respecto controles sanos (J. Pan et al., 2017).

Los exosomas también muestran moléculas en su superficie como Alix, Tsg101, Hsc70, integrinas, tetraspaninas superficiales específicos como CD63, CD81 o CD9, además de las clases I y II del Complejo Mayor de Histocompatibilidad (MHC) (Figura 15) (Kalra et al., 2016). Además se han destacado en los últimos años a través de lipidómica la presencia de esfingolípidos, colesterol, ácido lisobifosfatídico (LBPA, del inglés *lysobisphosphatidic acid*), ceramidas y lípidos de tipo raft, que se exponen en la capa externa lipídica del exosoma (De Gassart et al., 2003; Mathivanan et al., 2010).

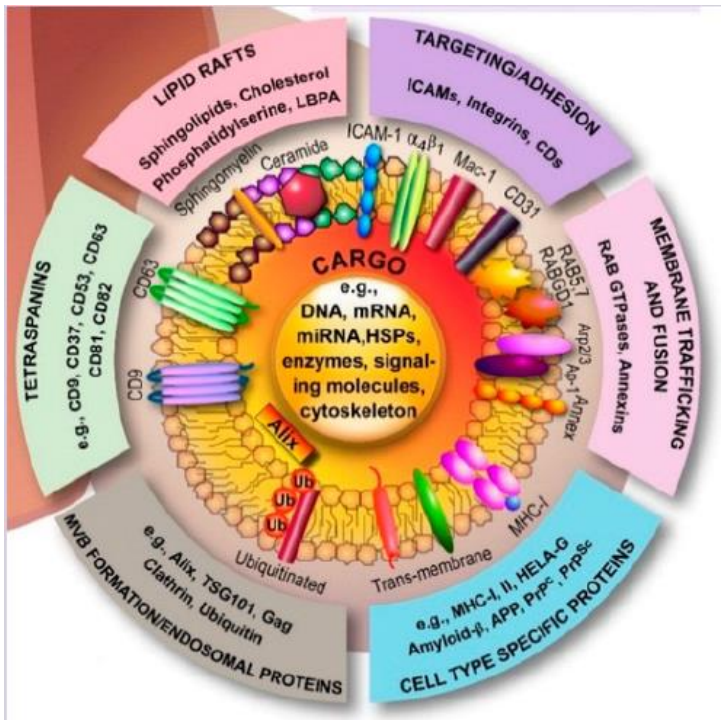


Figura 15. Composición específica de exosomas
(Kalra et al., 2016)

Los datos obtenidos hasta la actualidad de numerosos estudios sobre la composición molecular de los exosomas se recogen en una base de datos de acceso libre llamada *ExoCarta* (www.exocarta.org). A día de hoy estos datos son complementados para sentar las bases acerca de las normas de nomenclatura y métodos de purificación en una base llamada *Vesiclepedia* (www.exocarta.org/vesiclepedia).

5.3. Funciones de los exosomas en procesos biológicos

Los avances y estudios en exosomas relacionan estas vesículas con importantes funciones biológicas involucrados en procesos de coordinación claves para mantener la homeostasis celular y modificar el entorno. Estas funciones biológicas son:

- *Modulación del sistema inmune.*

  El papel de los exosomas como presentadores de antígeno está ampliamente demostrado al jugar un papel clave en la inmunidad celular y regulación de la respuesta inmunológica. Sin duda, esta función es clave en el desarrollo y progresión tumoral, en la que los exosomas actúan como células presentadoras de antígeno y como mediadores de respuesta a través de diversos factores que modulan el microambiente tumoral (Héctor Peinado et al., 2011).

- *Regulación de la respuesta inflamatoria.*

  Se ha demostrado la capacidad de los exosomas de modular la respuesta inflamatoria en diferentes tejidos del sistema nervioso y digestivo. Por ejemplo, en pacientes asmáticos y con inflamación de las vías aéreas, se ha demostrado que los exosomas aislados a partir de líquido bronco-alveolar muestran una alta cantidad de citoquinas que modulan o incrementan la respuesta inflamatoria en en ese tejido (Prado et al., 2008).

- *Reparación y crecimiento de tejidos.*

  Los exosomas liberados al medio procedentes de células madre de tejidos neuronales o musculares muestran la capacidad de contribuir a procesos de reparación y regeneración tisular (Lai et al., 2010).

- *Papel en procesos biológicos como fertilización y desarrollo embrionario.*

  Los exosomas parecen jugar un papel importante en el mantenimiento y protección del esperma en el semen regulando el pH y aumentando su motilidad (Barceló et al., 2019).

- *Transferencia de agentes infecciosos.*

  Estudios recientes han revelado un papel inesperado de los exosomas al tener un papel activo en la propagación celular de priones. Las enfermedades por priones producen en humanos alteraciones neurológicas agresivas y letales. El grupo de Raposo y col. han demostrado que la proteína priónica celular (PrP) utiliza cuerpos multivesiculares y vesículas extracelulares para ser liberados en exosomas (Lo Cicero et al., 2015).

- *Intercambio de proteínas patógenas.*

  Otra de las funciones aceptadas de los exosomas es la vehiculización de proteínas potencialmente patógenas en diferentes patologías, como por ejemplo el péptido β-amiloide relacionado con enfermedades neurodegenerativas como es el Alzheimer (Rajendran et al., 2006).

- *Exosomas en la comunicación intercelular.*

  Sin duda, esta es la función por excelencia de los exosomas, vesículas extracelulares, micropartículas y MVBs. Los exosomas secretados al medio extracelular constituyen una vía de comunicación entre células y tejidos facilitando funciones de homeostasis. Por ejemplo, los queratinocitos de la epidermis secretan exosomas que contactan con los melanocitos para modular la

pigmentación y la actividad de las proteínas que sintetizan la melatonina (Schorey & Bhatnagar, 2008).

La transferencia de material genético a través de exosomas desde una célula donadora a otra célula diana juega un papel clave en la comunicación célula-célula. Los exosomas secretados al espacio extracelular pueden permanecer en las proximidades o recorrer largas distancias a través de los fluidos biológicos. Esto explica la presencia de exosomas en plasma, orina, saliva, leche materna.... Entre las diferentes formas de ARN y ADN que encontramos en el contenido exosomal destacamos en mayor proporción a ARN ribosómico, microARN y ARN de transferencia (Tabla 3) (Lässer et al., 2012). Esta presencia de ARN y fragmentos de ADN juega un papel relevante en la regulación transcripcional de procesos de proliferación o tumorogénesis, inflamación, desarrollo de nicho pre-metastásico o metástasis (Shao et al., 2018).

RNA	functions	coding	typical size
messenger RNA (mRNA)	protein translation	yes	400–12000 nt average ~2100 nt
microRNA (miRNA)	post-transcriptional gene silencing	no	17–24 nt
Y RNA	component of Ro60 ribonucleoprotein particle; initiation factor for DNA replication	no	~100 nt
signal recognition particle RNA (SRP RNA)	component of SRP ribonucleoprotein complex that directs protein trafficking	no	~280 nt
transfer RNA (tRNA)	adapter for matching amino acid to mRNA	no	76–90 nt
ribosomal RNA (rRNA)	RNA component of ribosome	no	18S (1.9 kb) 28S (5.0 kb)
small nuclear RNA (snRNA)	RNA processing such as mRNA splicing	no	~150 nt
small nucleolar RNA (snoRNA)	guiding chemical modifications of other RNAs	no	20–24 nt
long noncoding RNA (lncRNA)	many putative functions, including in-transcription and post-transcription regulations	no	>100 nt

Tabla 3. Diferentes tipos de RNA encontrados en exosomas
(Shao et al., 2018)

5.4. Aislamiento y caracterización de los exosomas

La técnica de aislamiento en exosomas y EVs más frecuentemente usada en el laboratorio está basada en la separación de subpoblaciones por ultracentrifugación diferencial. Este método sencillo y barato, tiene la peculiaridad de que el pellet final siempre va a presentar agregados protéicos que ensucian la muestra. Para evitar esto, se suele recurrir al uso de gradiente se sacarosa para separarlos por su densidad característica. Otro método rápido y selectivo es la captura de exosomas o inmunocaptura

mediante bolitas magnéticas específicas a marcadores clásicos de superficie (Tabla 4) (Lobb et al., 2015).

Las técnicas usadas comúnmente para la visualización y caracterización de exosomas consisten en el estudio de su morfología mediante microscopía electrónica de transmisión y de barrido, microscopía de fuerza atómica y en la inmunodetección de anticuerpos específicos mediante Western Blot o citometría de flujo asociado a bolitas magnéticas (Théry et al., 2006).

Method	Pros	Cons
Ultracentrifugation	Relatively straightforward	Purity/EV integrity
Density centrifugation	High purity	Slow, sucrose toxicity
Ultrafiltration	Quick	Low purity
Immuno-magnetic bead	Specific, high purity	Prior knowledge of EV characteristics required
Affinity purification	Relatively specific, high purity	Prior knowledge of EV characteristics required
Chromatography	High purity	Specialised equipment
Microfluidics	Specific, high purity	Prior knowledge of EV characteristics required
Field flow fractionation	Quick	? purity
Precipitation techniques	High yield	Low purity

Tabla 4. Métodos de aislamiento de exosomas
(Adaptado de *ISEV2014 Guidelines*)

5.5. Exosomas en cáncer

A pesar de que se descubrió recientemente que los exosomas eran liberados por prácticamente todos los tipos de células, muchos estudios se centran en exosomas secretados por células tumorales y CSCs. Es de particular interés el hecho de que las células tumorales exhiben una producción mejorada de exosomas, como lo demuestran los estudios de vesículas purificadas a partir de plasma, ascitis y derrames pleurales de pacientes con cáncer.

Las concentraciones de exosomas se incrementan en los fluidos corporales de los pacientes oncológicos si los comparamos con los controles sanos (Iero et al., 2008; Vaiselbuh, 2015). Además, los exosomas de cáncer son muy interesantes porque representan una fuente rica de biomarcadores no invasivos con poder pronóstico y predictivo de la evolución de la enfermdad, marcando un hito importante en la era de la

biopsia líquida (Alegre et al., 2016). De hecho, los exosomas son réplicas cercanas de las células de origen en términos de contenido de proteína oncogénica expresando gran cantidad de antígenos tumorales cuando se secretan por células neoplásicas (Valenti et al., 2007).

El papel de los exosomas en el cáncer viene determinado por la carga interna que vehiculizan, como son lípidos de membrana característicos, microARNs y ARNm y proteínas del complejo mayor de histocompatibilidad I (MHC-I). Esta carga es absorbida por la célula diana induciendo vías implicadas en la iniciación, soporte, progresión y diseminación del cáncer (Greening et al., 2015; Hoshino et al., 2015; Kalluri, 2016; Nogués et al., 2018). Se ha demostrado que los exosomas no circulan por los fluidos biológicos hacia células diana de forma aleatoria, sino que lo hacen de manera regulada por la presencia de integrinas presentes en su membrana. Peinado y cols. han demostrado en pacientes con melanoma que los exosomas derivados del tumor contienen proteínas específicas (TYRP-2, VLA-4, HSP70 y RAB27A) constituyendo la llamada *firma del melanoma*. Estas proteínas juegan un papel en la formación del nicho pre-metastásico, generando y manteniendo el microambiente tumoral en órganos diana (Hector Peinado, 2013).

En cáncer, los exosomas derivados de un tumor primario alteran su ambiente para formar un nicho pro-tumorogénico y dirigir la diseminación en el organismo (Quail & Joyce, 2013), además de inducir la aparición de fenotipos más agresivos en células cancerosas por la transferencia de proteínas pro-oncogénicas, incluyendo resistencia a fármacos y modulación de la respuesta al tratamiento (Ciravolo et al., 2012; Jaiswal & Sedger, 2019).

Se han conseguido aislar exosomas liberados por células malignas mediante selección inmunomagnética que expresan en su superficie marcadores de CSCs como son CD133, EpCam y CD44 (Tauro et al., 2012). Los exosomas pueden inducir efectos angiogénicos y antiapoptóticos a través de la transferencia de ARNm y microARNs estimulando, por ejemplo, la producción de VEGF, bloqueando la vía celular Wnt/β-catenina (Huber et al., 2005) o aumentando el potencial oncogénico en pacientes con cáncer de estómago por la liberación de miRNA-let-7 (Ohshima et al., 2010).

El descubrimiento de las funciones de los exosomas en el entorno y microambiente celular podría ofrecer una herramienta para el desarrollo de tratamientos oncológicos. El encontrar drogas que interfieran en la secrección de EVs por las células tumorales y CSCs, podría presentar una novedosa arma terapéutica en la regresión tumoral (Adorno-Cruz et al., 2015).

6. INTERFERON

6.1. Historia de los Interferones

En 1957, Isaacs y Lindenmann identificaron una glicoproteína secretada por células infectadas por el virus de *Influenza A del Melbourne* que confería a otras células resistencia a ser infectadas por dicho virus (Isaacs & Lindenmann, 1987). En un segundo trabajo, definieron a estos factores como interferones, sustancias muy pequeñas producidas por las células infectadas con el virus de *Influenza A*, estable durante semanas a 2ºC y termosensible a 56ºC durante una hora (Isaacs & Lindenmann, 1988).

A partir de entonces, se han definido a los interferones (IFN) como un grupo numeroso y heterogéneo de glicoproteínas con aproximadamente 150 aminoácidos, con peso molecular entre 5 a 50 KDa y activas a concentraciones muy bajas. Los IFN forman parte de las familias de las citocinas o interleucinas relacionados íntimamente con el envío de señales indispensables en el proceso de comunicación celular y respuesta inmune (Vilček & Feldmann, 2004).

Desde su origen, se ha mantenido su nomenclatura inicial y en la actualidad se publican en *PubMed* una media de 5 artículos, describiéndose hoy día más de 20 glicoproteínas que componen a esta superfamilia de IFN. Estas moléculas, a parte de proteger a la célula de infecciones virales, modulan el crecimiento y diferenciación celular confiriendo actividad antiinflamatoria, antiangiogénica y apoptótica (Meyer, 2009).

6.2. Clasificación de los Interferones

Originariamente, los IFN se clasificaron desde su descubrimiento en tres grandes grupos IFNα, IFNβ e IFNγ; sin embargo, en la actualidad y tras su profundo estudio, se han clasificado en tres clases diferentes atendiendo a su unión a receptores de membrana (Hervas-Stubbs et al., 2011).

a) Interferón Tipo I

El receptor de IFN tipo I está compartido y utilizado por 13 subtipos de IFN destacando los más importantes y conocidos el IFNα e IFNβ. Este receptor tiene dos subunidades (denominadas IFNAR1 e IFNAR2) que se encuentran asociadas a proteínas tirosina-quinasa Janus (Jak PTKs), que al activarse, fosforilan a su vez a STAT1 y STAT2 (miembros de la superfamilia de transductores de señal, STATs, del inglés *signal transducer and activator of transcription protein*) (Darnell, 1997; Dash et al., 2008).

El complejo activador de la transcripción formado está compuesto por IRF9/p48/ISGF3γ, se transloca al núcleo y da lugar a la transcripción de genes inducidos por IFN (ISGs, del inglés *interferon-stimulated genes*) (Marie, 1998). Las proteínas resultantes están implicacdas en procesos tales como la regulación de la membrana, respuesta a estrés celular, apoptosis o ciclo celular (Figura 16) (Sen, 2001).

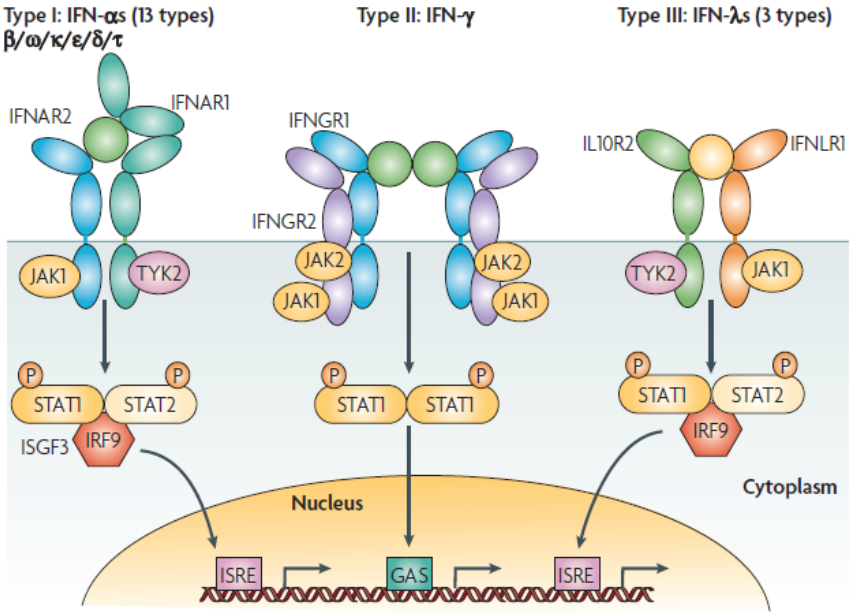


Figura 16. Ruta de señalización regulada por receptores de IFN tipo I, II y III (Borden et al., 2007)

El IFN alfa (IFNα) es el más antiguo de todos, y se ha demostrado que tras la infección de la célula por el virus, su actividad ocurre desde las primeras horas del contacto induciendo antividad antiviral. Así mismo, también tiene capacidad de promover la diferenciación celular, el crecimiento de diferentes células del

sistema inmune y control del ciclo celular provocando una acumulación en fase G_1 y bloqueo de la fase G_2/M y actividad antitumoral (Yanai et al., 2002).

Por su parte, el IFN beta (IFNβ) comparte muchas características funcionales y estructurales iguales a los IFN alfa, es sintetizado fundamentalmente por fibroblastos. Destacan su capacidad de modificar la producción de anticuerpos, estimulación de linfocitos T citotóxicos, células NK (NK, del inglés *natural killer*) y de moléculas del MHC (MHC, del inglés *molecular histocompatibility complex*) (Ramos-Bello & Ramos-Niembro, 2008).

Una de las moléculas con gran relevancia biológica e inducida por IFN es la proteína quinasa dependiente de ARN_{ds} (PKR, del inglés *RNA kinase protein*), que juega un papel fundamental en la defensa y respuesta celular frente a infección por un virus (Garcia-Ortega et al., 2017).

b) Interferón Tipo II

Este grupo está representado por el IFN de tipo γ, es a nivel estructural y funcional diferente a los IFN tipo I. Se une a un receptor diferente compuesto por dos subunidades denominadas IFNGR1 e IFNGR2. Su producción se encuentra regulada por las citoquinas secretadas IL-12 e IL-18 y es producida por los linfocitos Th durante la respuesta inmune adaptativa. Entre sus funciones se destaca la capacidad antibacteriana y antitumoral (Figura 16) (Schroder et al., 2004).

c) Interferón tipo III

El IFN tipo III lo componen el IFNλ1, IFNλ2 y IFNλ3. La expresión celular de este tipo de IFN se realiza en respuesta mediada por receptores tipo *Toll* o TLR (TLR, del inglés *toll like receptor*) provocada por infecciones virales y bacterianas (Figura 16) (Onoguchi et al., 2007).

6.3. Principales aplicaciones clínicas de los interferones

A pesar de que el efecto del IFN comenzó como agente antiviral, se ha convertido con el paso del tiempo como el primer producto biológico para el tratamiento de diferentes patologías como cáncer, infecciones virales, esclerosis múltiple, hepatitis, etc. Como puede observarse en la tabla 5, se encuentran disponibles para su empleo rutinario en clínica numerosos compuestos basados en diferentes tipos de interferones (Tabla 5) (Borden et al., 2007). En años recientes, en un intento de alargar la vida media del IFN en los pacientes, se ha rshecurrido al procedimiento de pegilación (agregando polietilenglicol) a pesar de asociarse efectos adversos (Ramos-Bello & Ramos-Niembro, 2008).

• Interferón α-2a:	hepatitis C crónica, leucemia mielocítica crónica, leucemia de células peludas, leucemia mielocítica crónica cromosoma Filadelfia positiva, sarcoma de Kaposi asociado a infección por virus de inmunodeficiencia humana, linfoma no Hodgkin
• Interferón α-1:	hepatitis C crónica
• Interferón α-2b:	hepatitis C crónica, hepatitis B crónica, condiloma acuminado, linfoma folicular de células B, leucemia de células peludas, sarcoma de Kaposi asociado a infección por virus de inmunodeficiencia humana, melanoma maligno, linfoma no Hodgkin
• Interferón α-n3:	condiloma acuminado
• Interferón β-1a:	esclerosis múltiple, recaídas y esclerosis múltiple remitente
• Interferón β-1b:	esclerosis múltiple, recaídas y esclerosis múltiple remitente
• Interferón γ-1b:	enfermedad granulomatosa crónica, osteopetrosis
• Peginterferón α-2a:	hepatitis C crónica
• Peginterferón α-2b:	hepatitis C crónica

Tabla 5. Interferones disponibles e indicaciones terapéuticas aprobadas
(Ramos-Bello & Ramos-Niembro, 2008)

Los estudios pioneros del IFN en la hepatitis B se iniciaron en los años 70 siendo una de las infecciones virales más comunes alrededor del mundo. La finalidad del tratamiento a corto plazo es disminuir la replicación viral, lo cuál este antiviral ejerce de fármaco preventivo de la progresión de la enfermedad hacia cirrosis o carcinoma hepatocelular (Tang et al., 2018).

Actualmente, tanto para la hepatitis B como para la hepatitis C crónica se usa como primera línea de tratamiento IFNα-1, IFNα-2a e IFNα-2b, aprobados por la FDA desde 1991 junto con otros retrovirales aumentando la supervivencia de los pacientes (Webster et al., 2015).

Los estudios iniciales del IFN en la esclerosis múltiple datan del año 1981 cuando se demostró el efecto del IFNβ producido en fibroblastos humanos reducía la tasa de exacerbaciones de la enfermedad (Fernández et al., 2015). Hoy día se usa el IFNβ-1a e IFNβ-1b por vía subcutánea, sus beneficios derivan del aumento de IL10 en el líquido cefalorraquídeo y de efectos antiinflamatorios regulados por células T (Borden et al., 2007).

Respecto a la toxicidad aguda, subaguda y parte de la toxicidad crónica observada en pacientes en tratamiento con IFN, se acepta que la misma es consecuencia de la inducción de otras citocinas como IL-1, IL-2 e IL-6, sus efectos sobre macrófagos y linfocitos T. Es por ello, que se explican síntomas constitucionales como anorexia, fiebre, mialgia, artralgias, cefaleas, y otros como disfunción cognitiva y depresión (Sleijfer et al., 2005).

Actualmente, el IFN está siendo sustituido por otras terapias más efectivas y con menos efectos secundarios. Ya no se usa, por ejemplo, en hepatitis C, desde que está disponible el *Sovaldi*® o *Virunon*®, nombres comerciales del sofosbuvir, un inhibidor de la enzima polimerasa de ARN del virus de la hepatitis C, esencial para la multiplicación y replicación del virus (Saafan et al., 2020).

6.4. Interferón y cáncer

Una de las necesidades en la lucha contra el cáncer es el desarrollo de nuevos tratamientos que potencien los ya existentes y disminuyan su toxicidad, es por esto, que el IFN se estudia combinado con otras drogas teniendo efectos esperanzadores. Las propiedades antitumorales y antiproliferativas del IFN han sido probadas en diferentes procesos malignos, en alguno de los cuales su empleo ya está aprobado, ya sea en monoterapia o terapia sinérgica combinada, entre ellos leucemia, linfoma no Hodgkin, mieloma múltiple, sarcoma de Kaposi o melanoma maligno (Borden et al., 2007; Gore et al., 2010). Existen numerosos estudios donde la combinación de IFN alfa junto con drogas quimioterapéuticas convencionales como el 5-FU (María Angel García et al., 2011; Juan

Antonio Marchal et al., 2013) o bevacizumab han mejorado tumores como el carcinoma renal o cáncer de páncreas (Conlon et al., 2019; Escudier et al., 2007).

Actualmente, el único agente aprobado por la FDA como agente adyuvante en melanoma de alto riesgo de recaída es el IFN alfa-2b. Se cree que actúa ejerciendo un papel inmunomodulador y antiproliferativo sobre las células tumorales, observándose un aumento de la supervivencia y un aumento en el tiempo de supervivencia libre de recaída (J. M. Kirkwood et al., 1996; Tarhini et al., 2012).

También se está usando en primera línea en el cáncer de pulmón no microcítico, o incluso el IFN alfa-2a-pegilado sigue usándose como tratamiento conservador en leucemia mieloide crónica en mujeres jóvenes en edad fértil, que tras el embarazo se alterna con *Dasatinib* (Abu-Tineh et al., 2020).

A pesar de ello, su uso hoy día como tratamiento adyuvante en el melanoma maligno está muy debatido debido a los efectos secundarios o toxicidad dosis-dependiente de IFNα en los pacientes, haciendo difícil un esquema general de dosis y tiempo como tratamiento adyuvante (Eggermont et al., 2014; Eggermont & Dummer, 2017). Diferentes ensayos han demostrado beneficios del IFNα a altas dosis en adyuvancia y en estadios II y III (con alto riesgo de recaída) aumentando la supervivencia libre de progresión y en menor medida, la supervivencia global (Eggermont & Dummer, 2017). Aunque son diferentes los mecanismos implicados en este efecto, aún no se conoce del todo, y es necesario seguir estudiando sus efectos a diferentes niveles, así como las numerosas y eficaces sinergias que es capaz de establecer con otros fármacos.

2. HYPOTHESIS

The hypothesis of this thesis is based on the following evidences:

- PKR is a first-line defence mechanism against infection and a regulator of cell growth and cell proliferation, been involved in metabolism, inflammatory processes and age-related diseases. Due to its extensive contribution to noumerous signaling pathways, and its marked regulation by several modulators, its dysregulation is implicated in different diseases.

- Colorectal cancer chemotherapy is based on the use of fluoropyrimidines (5-FU) alone or in combination with other drugs such as oxaliplatin (FOLFOX, XELOX) or irinotecan (FOLFIRI). However, there is still a significant group of patients who do not respond to these aggressive therapies, been an urgent need to find predictive biomarkers that improve the selection and therefore, the outcome of treated patients.

- PKR is a molecular target of 5-FU involved in the apoptosis induction of cancer cells in response to the chemotherapeutic drug in a P53- independent manner.

- Recently, several researches identified that PKR is highly regulated by a non coding element called nc886, whose expression in the tumor and in the blood, may determine the predictive value of this kinase.

- Malignant melanoma is the most aggressive and life-threatening skin cancer whose incidence is increasing worldwide. This neoplasia is characterized by an extraordinary propensity for dissemination to distant organs and resistance to chemotherapy in part by the existence of melanoma cancer stem cells (CSCs) subpopulations. Non metastatic high-risk melanoma is still treated with high dose of IFN-α with a significant improvement in Disease Free Survival in patients. This cytokine possesses anti-proliferative, anti-angiogenic and immune-modulator properties; however its specific activity over melanoma cancer is still unknown and its secundary effects are negatives.

- miRNAs and exosomes act as the main regulators of a wide variety of biological processes including cell proliferation, differentiation, apoptosis, metabolism and signal translation. Their dysfunction is related to diverse pathologies, including cancer, controlling important mechanisms such as metastasis and resistance to treatment.

- Metabolomic characterization of exosomes from CSC and patients derived exosomes diagnosed with malignant melanoma may critically contribute to determining their physiological role in the identification of candidate biomarkers that may be applicable for the early detection of malignant melanoma and for their modifications after treatment.

Taking into account these evidences we propose the following hipothesis:

1. Since PKR plays an important role in the apoptotic death of tumor cells in response to various chemotherapeutic agents, including the 5-FU, both PKR and its modulator nc886 could be used as potential predictive biomarkers of response in patients with metastatic colorectal adenocarcinoma treated with fluoropyrimidine-based chemotherapies.

2. Since CSCs have a relevant role in the outcome of malignat melanoma patients and IFN-α has important anti-cancer activities in this pathology, the analisys of its efficacy against CSCs trought the modulation of gene expression changes, including microRNAs profile, and the metobolomics of exosomes, among others, could be useful tools for a more precise and personalized medicine of this disease.

3. OBJECTIVES

Based in the previous hypothesis, the **general objectives** of this thesis are:

1. To analyze **the role of PKR** and its modulator, the pre-microRNA-nc886, as potential predictive biomarkers in metastatic colorectal cancer patients treated with 5-FU-based chemotherapy

2. To explore **the efficacy of IFN-α** against cancer stem cells from malignant melanoma disease.

To this end, the main **specific objectives** of our work are established per chapters:

- *Main objectives of Chapter I:*

I.1. To summarise the role that PKR plays in cancer disease, metabolism, inflammatory processes and neurodegenerative diseases.

I.2. To emphasise the importance of PKR as a molecular target for both conventional and novel chemotherapeutic drugs and its potential as a biomarker and therapeutic target for several pathologies.

- *Main objectives of Chapter II:*

II.1. To analyze the expression levels and location of PKR together its modulator nc886 in frozen tissues and paraffinized tumor samples (endoscopic biopsies and surgical specimen) and in peripheral blood of patients with metastatic colorectal adenocarcinoma treated with fluoropyrimidine-based chemotherapies.

II.2. To correlate PKR and nc886 levels with the Objective Response Rate (based in RECIST criteria) after chemotherapy treatment in order to determine its potential as predictive biomarker of progression-free survival and overall survival in metastatic colon cancer patients.

II.3. To use clustering analyses by the hierarchical agglomerative method (PGMRA) for improving the quality and specificity of the results considering the heterogeneity between samples, genetic and proteomic background of oncologic patients, with the ultimate aim of establishing groups of patients in relation to their response to chemotherapy, assessing the biomarker potential of PKR and nc886.

- *Main objectives of Chapter III:*

III.1. To analyze the effect of IFN-α on stemness properties of melanospheres subpopulations in both a human established cell line and a primary cell line from malignant melanoma.

III.2. To explore the gene expression profile changes, including microRNAs profile, induced by IFN-α treatment in CSCs enriched subpopulations.

III.3. To demostrate the anti-CSCs efficacy of IFN-α *in vivo* using xenograft mice models.

III.4. To analyze and characterize the exosomes from cell culture supernatants after IFN-α treatment of CSC-enriched subpopulations derived from MEL-1 and A375 cell lines to explore potential predictive biomarkers for malignant melanoma.

CHAPTER I:
INVOLVEMENT OF PKR PROTEIN KINASE IN SEVERAL DISEASES

1. Abstract

The protein kinase R (PKR, also called EIF2AK2) is an interferon-inducible double-stranded RNA protein kinase with multiple effects on cells that plays an active part in the cellular response to numerous types of stress. PKR has been extensively studied and documented for its relevance as an antiviral agent and a cell growth regulator. Recently, the role of PKR related to metabolism, inflammatory processes, cancer and neurodegenerative diseases has gained interest. In this review, we summarise and discuss the involvement of PKR in several cancer signalling pathways and the dual role that this kinase plays in cancer disease. We emphasise the importance of PKR as a molecular target for both conventional chemotherapeutics and emerging treatments based on novel drugs, and its potential as a biomarker and therapeutic target for several pathologies. Finally, we discuss the impact that the recent knowledge regarding PKR involvement in metabolism has in our understanding of the complex processes of cancer and metabolism pathologies, highlighting the translational research establishing the clinical and therapeutic potential of this pleiotropic kinase.

2. Introduction

Protein kinase R (PKR), also called eukaryotic translation initiation factor 2-alpha kinase 2 (EIF2AK2), is an interferon (IFN)-inducible double-stranded RNA (dsRNA) protein kinase with multiple effects in cells (Chaves, 2016; M. A. García et al., 2006). PKR actively contributes to the cellular response to numerous types of stress with a critical role in the antiviral defence mechanism of the host induced by IFNs, which are cytokines with a wide range of biological functions, including antiviral, antiproliferative and immunomodulatory properties (Pestka et al., 2004; Samuel, 1991). PKR is expressed constitutively in mammalian cells and has an IFN-stimulated response element (ISRE) in its determined promoter, required for transcriptional induction by type I IFN, but also a kinase conserved sequence motif with an important role in basal transcription in absence of cytokine treatment (Kuhen & Samuel, 1997). PKR is a first-line defence mechanism against infection and a regulator of cell growth and cell proliferation, and recently, it has also been involved in metabolism, inflammatory processes and age-related diseases (B. R. Williams, 2001).

PKR is a serine-threonine kinase composed of a kinase domain, shared by the members of the eukaryotic initiation factor 2α (eIF2α) kinase family and two dsRNA-binding domains that constitute the regulatory domain (Figure 17) (Balachandran & Barber, 2007; Juan A. Marchal et al., 2014). Although the primary PKR activator is dsRNA (produced during infection by several viruses and detected at low doses in mammalian cells), PKR is also activated by a variety of cellular stresses including cytokines, calcium stress, oxidative stress, endoplasmic reticulum stress, lipo stress, amyloid-β (Aβ) peptide accumulation, polyanions such as heparin, and several drugs (Barreto-de-Souza et al., 2015; Bommer et al., 2010; Vaughn et al., 2014), or through the PKR associated activator (PACT) (Marques et al., 2008; Singh & Patel, 2012). PKR, in response to specific stress signals, is activated by autophosphorylation and leads to the phosphorylation of eIF-2α impairing its activity, which results in the inhibition of protein synthesis and the induction of apoptosis (García et al., 2007; Lee & Esteban, 1994).

In addition to its translational regulatory function, PKR has a role in signal transduction and transcriptional control through the regulation of the κB inhibitor (IκB)/nuclear factor κB (NF-κB) pathway (Lee & Esteban, 1994; Zamanian-Daryoush et al., 2000).

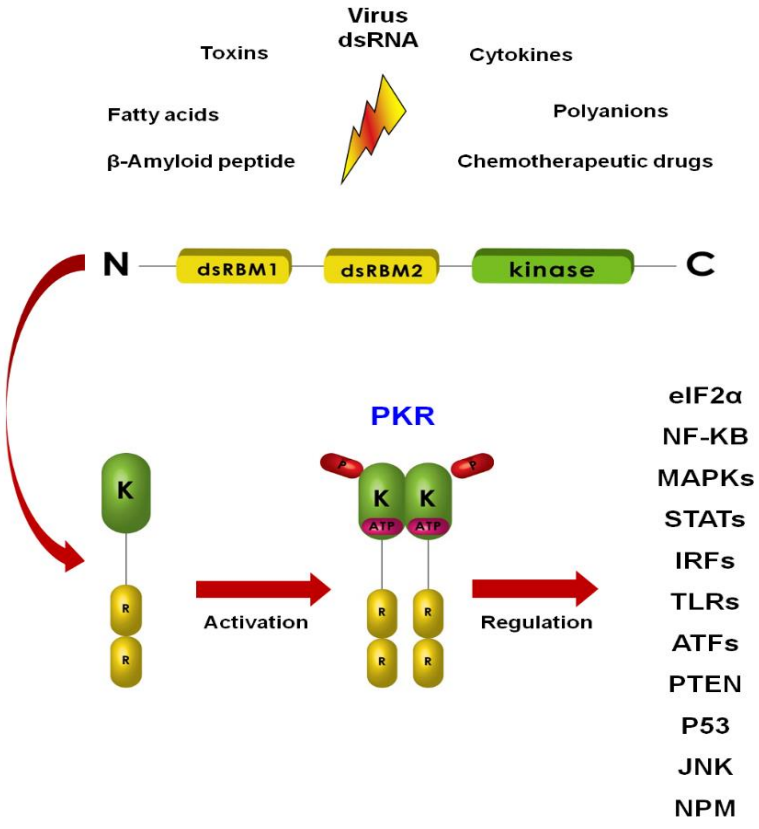


Figure 17. PKR is a sentinel kinase for cellular stress that engages several transcription factors, tumour suppressors and protein kinases involved in cancer signalling pathways. *PKR is a serine-threonine kinase from the eIF2α kinase family composed of a kinase domain and two dsRNA-binding domains that constitute the regulatory domain. Although the main direct PKR activator is the dsRNA produced during infection by several viruses and detected at low doses in mammalian cells, PKR is also activated by a variety of forms of cellular stress including cytokines, calcium stress, oxidative stress, endoplasmic reticulum stress, lipo-stress, Aβ peptide accumulation, polyanions such as heparin, several drugs such as 5-FU and DOX, and fatty acids such as ceramide and palmitate. PKR is activated by autophosphorylation and leads to the phos- phorylation of eIF-2α resulting in the inhibition of protein synthesis. PKR has been also implicated in NF-κB, IRFs, STATs, ATFs, p53, PTEN, MAPKs and TLRs signalling pathways. Aβ, amyloid-β; ATF, activating transcription factors; DOX, doxorubicin; 5-FU, 5-Fluorouracil; eIF2α, eukaryotic initiation factor 2α; IRF, interferon regulatory factor; MAPKs, mitogen-activated protein kinases; NF-κB, nuclear factor κB; PKR, protein kinase R; PTEN, phosphatase and tensin homolog; STATs, signal transducers and activators of transcription factors; TLRs, toll-like receptors.*

PKR also regulates some tumour suppressors and protein kinases involved in cancer pathways such as the signal transducers and activators of transcription factors (STATs), activating transcription factors (ATFs), tumour suppressor p53 (Tp53), the phosphatase and tensin homologue tumour suppressor (PTEN), the mitogen-activated protein kinases (MAPKs) and the toll-like receptors (TLRs) (Figure 17) (Chaves, 2016; García et al., 2007).

As predicted for a translation regulator, PKR is associated with ribosomes, mainly 40S subunits. Ribosomal association of PKR appears to be mediated by its dsRNA-binding domains (dsRBDs), implying a key role of these domains in the regulation of PKR activity (Wu et al., 1998). In addition, a recent study identified a PKR nuclear interactome formed by a complex of proteins involved in ribosome biogenesis, RNA splicing, mRNA stability, gene expression, cell cycle, or chromatin organisation, suggesting PKR roles in ribosome biogenesis, mRNA processing and cell division (Blalock et al., 2014).

These PKR-associated processes support the relevance of PKR's regulation; in fact, numerous studies have found PKR to be dysregulated in most type of cancers, neurodegenerative diseases, metabolic disorders and other pathologies (Sud et al., 2016). Whereas PKR is recognised for its negative effects on neurode- generative diseases, the role of PKR activation in cancer is complex and remains controversial. Alternatively, recent studies have shown that PKR is activated by chronically elevated levels of specific

nutrients and deletion of PKR has a number of effects on whole-body metabolism including protection against high-fat diet-induced obesity, glucose intolerance, insulin resistance and inflammation (Koromilas, 2015).

This review discusses the dual role of PKR in cancer and the involvement of this kinase in metabolic disorders. Moreover, PKR is proposed as a relevant therapeutic target and biomarker.

3. PKR role in cancer

In addition to its well-established role in the IFN response, PKR is involved in many cellular pathways exerting various functions on cell growth and tumourogenesis (Chaves, 2016; Marchal et al., 2014). The role of PKR activation in cancer is complex and remains controversial.

In general, PKR is considered to have a tumour suppressor function, and some clinical data show a correlation between suppressed or inactivated PKR and a poor prognosis for several cancers. The outcome of eIF2α phosphorylation by PKR in cancer is complex where a single phosphorylation event can act as an integrator of diverse stress stimuli and orchestrate different biological outcomes in stressed cells (Koromilas, 2015). In fact, increased eIF2α phosphorylation has been associated with several pathophysiological conditions including cancer, neurodegeneration, virus infection, inflamma- tion, diabetes and obesity (Koromilas, 2015).

Furthermore, the apoptosis induced by PKR activation in cancer cells, in response to several antitumour drugs, indicates a role in potentiating the response to chemotherapy. However, other studies have shown high PKR expression and elevated PKR activation in various cancers, suggesting that PKR might be contributing to neoplastic progression (Marchal et al., 2014). It is well-known that PKR is a potent activator of NF-κB, via interaction with TRAF family modulators; inducing IFN transcription in concerted action with IFN regulatory factors 3 and 7 (IRF-3/7) (Gil et al., 2004). Aberrant activation of NF- κB has been linked to inflammatory and autoimmune diseases, infection and cancer (Gil et al., 2004; Marchal et al., 2014). Therefore, dissecting the regulatory contribution of PKR and identifying components involved in its deregulation have clinical and therapeutic importance.

3.1. PKR involvement in different signalling pathways implicated in cancer

PKR is involved in various pathways that activate and engage a number of transcription factors controlling the expression of multiple genes. PKR has been implicated in the activation of IFN regulatory factor 1 (IRF 1), which acts mainly as a transcriptional activator of IFN-α/β gene expression, and it has been proposed as an inhibitor of oncogenesis in melanoma patients (Kumar et al., 1997; Martinovic et al., 2016). Moreover, PKR controls several pathways by modulation of STAT1 and STAT3 transcription factors. In fact, PKR-knockout cells are defective in STAT1 phosphorylation on Ser727, resulting in a significant decrease in the activation function of STAT1 gene (Ramana et al., 2000). STAT1 is also a target for PKR-mediated activation in response to lipopolysaccharide in glial cells (Williams, 1999).

Recently, PKR has been described to be involved in the differentiation of chondrocytes through the modulation of STAT1 and the transcription factor Sox-9 expression (Wu et al., 1998). Furthermore, PKR also associates with STAT3, and is required for full STAT3 activation in response to platelet-derived growth factor (PDGF) involved in regulation of ERK activation and ultimately involved in STAT3 phosphorylation (Deb et al., 2001). The transcription factor STAT3 plays an important role in cancer cells through the abnormal activation of cell cycle progression, and the deregulation of survival and senescence pathways. A novel tumour suppressor function for PKR was suggested by Koromillas's group via the inhibition of the hypoxia-inducible factor-1α (HIF-1α) expression through STAT3 in an eIF2α phosphorylation independent manner (Papadakis et al., 2010). Interactions between PKR and STAT3 have been shown to regulate autophagic pathways in the cellular metabolism control of fatty acids (Jonchère et al., 2013; Niso-Santano et al., 2013).

Further, PKR is an activator for signalling cascades involved in stress-activated protein kinases, and mediates Jun kinase (JNK) and mitogen-activated protein kinase p38 (MAPK) activation in response to specific stimuli (Shen et al., 2016). Thus, the activation of both p38 and JNK after lipopolysaccharide or cytokine treatments such as IFN-γ, interleukin IL-1, or tumour necrosis factor (TNF)-α, depend, in part, on PKR presence (Goh et al., 2000). Moreover, PKR interacts with and activates mitogen-activated protein kinase kinase 6 (MKK6) in response to dsRNA stimulation (Silva et al., 2004).

We used human cDNA microarrays to identify genes differentially expressed after PKR expression (Guerra et al., 2006). Most regulated genes were classified according to biological function, including apoptosis, stress, defence and immune response. Interestingly, we found upregulation of the ATF3 transcription factor by PKR activation. ATF3 induced apoptosis in cancer cells in response to different stimuli including antitumour drugs (Udayakumar et al., 2016), and was suggested to be a suppressor of invasion and metastasis.

Although the activation of ATF3 induced by PKR has been analysed in the context of viral infection, this link suggests an interesting mechanism to be further explored in cancer. A connection between ATF4 and PKR has been recently discovered to mediate the anti-tumour activity of a small molecule, currently tested in early phase clinical trials, called ONC201 (also known as TIC10) (Kline et al., 2016). PKR is also involved in the tumour suppressor function of p53. A bidirectional and complex regulatory relationship between PKR and p53 has been demonstrated in cancer cells (Marchal et al., 2014). PKR interacts directly with the C-terminal part of p53 and phosphorylates p53 on Ser392 (Cuddihy et al., 1999).

On the other hand, PKR is able to promote the proteosomal degradation of p53 in association with glycogen synthase kinase-3 and mouse double minute 2 homologue, independently of translational control (Harding et al., 1999). Moreover, it has been demonstrated that the ability of p53 to cause cell cycle arrest and regulate transcription of target genes was impaired in PKR-knockout cells (Cuddihy et al., 1999). In fact, p53-mediated tumour suppression can be attributed, at least in part, to the biological functions of PKR induced by p53 in some genotoxic conditions, and PKR could be transcriptionally regulated by p53 activity in response to DNA damage (Yoon et al., 2009).

Recently, it has been proposed that activated PKR inhibits pancreatic β-cell proliferation through sumoylation-dependent stabilisation of p53, implicating both proteins also in the metabolic regulation of diabetes (Song et al., 2015), as we discuss in the next section. Moreover, a sumoylation-dependent mechanism involving p53 phosphorylation through the PACT-PKR stress response induces translational activation leading to G1 cell cycle arrest (Bennett et al., 2012).

The alternative reading frame (ARF) encoded by the INK4a-ARF locus is one of the most frequently mutated genes in cancer (Garcia et al., 2007). The control of cellular proliferation by this tumour suppressor is crucial to restrict tumour development. Several reports describe the activation of ARF after the expression of viral proteins, type I IFN treatment, or after virus infection, suggesting a physiological role for ARF during virus infection (Garcia et al., 2007). We have revealed that ARF can be induced by viral infection and that the expression of ARF reduces viral infectivity.

We also have shown that this antiviral effect depends, in part, on PKR activation mediated by its release from inhibitory complexes with nucleophosmin (NPM) (García et al., 2006), a protein commonly overexpressed, mutated, rearranged and sporadically deleted in cancer (Box et al., 2016). Although the role of the PKR-NPM complex has been only studied in the viral context, at present we are analyzing its involvement in cancer. Tumour suppressor retinoblastoma protein, involved in cell cycle control, differentiation and inhibition of oncogenic properties (Classon & Harlow, 2002), was required for the activation of the NF-κB pathway in response to virus infection, also in a PKR-dependent manner (Garcia et al., 2009).

These results provided a new link between tumour suppression, PKR and the antiviral host defence, indicating an important tumourogenic role of viruses and a crucial potential issue in the forthcoming use of viruses as therapeutic agents. In fact, selective tumour cell replication of oncolytic viruses is thought to depend on infection of neoplastic cells, which harbour low levels of PKR and dysfunctional tumour suppressors and type I-IFN signalling elements (Muñoz-Fontela et al., 2007).

3.2. PKR is a potent pro-apoptotic protein

Cell death by apoptosis is a genetic program in multicellular organisms that conditions the ordered removal of damaged or unwanted cells during development and in adult life. Deregulation of the apoptotic process can lead to pathological conditions such as cancer, autoimmunity and neurodegeneration (Driver et al., 2012). Moreover, apoptosis is an important event during some chemotherapy treatments in cancer.

PKR mediates apoptosis induced by several viruses such us poxviruses, influenza, etc., probably through dsRNA production (Kibler et al., 1997). However, PKR also

regulates apoptosis in the absence of viral infection. Some examples are the apoptosis observed during Alzheimer's disease (Onuki et al., 2004), the apoptosis induced by oncogenes such as IRF1 or E2F-1, or triggered in response to dsRNA, TNFα, lipopolysaccharide, tunica- mycin, serum starvation, or IL-3 withdrawal (García et al., 2006).

Many of the stimuli that trigger PKR-dependent apoptosis in the absence of viral infection rely on PACT/RAX activation. PACT/RAX mediates PKR activation and subsequent apoptosis in response not only to cytokines and serum withdrawal, but also to chemotherapy, ethanol and viral infection (Singh & Patel, 2012). However, PACT-knockout cells and mice did not exhibit significant differences in response to stressful stimuli compared with a wild-type phenotype (Marques et al., 2008). Analysis of the role of PKR effectors in mediating cell death suggests an intricate pathway, in which at least eIF-2α, NF-κB, ATF-3 and p53 have been implicated.

PKR-induced apoptosis mainly involves the FADD/caspase 8 pathway and caspase 9 activation, which correlate with Bax protein translocation to the mitochondria and cytochrome c release to the cytoplasm, resulting in mitochondrion depolarisation (Gil & Esteban, 2000b). Numerous works including ours have highlighted the importance of apoptosis induction in cancer mediated by PKR in response to different stimuli such as IL and antitumour drugs (García et al., 2010; García et al., 2011).

3.3. Does PKR induce cell death or cell survival in tumours?

The balance between cell survival and cell death is essential for cancer initiation and for the control of cancer outcome. PKR is able to induce cell survival through NF-κB activation or cell death via eIF2α/apoptosis induction in a complex temporally regulated manner balancing cell survival and cell death (Donzé et al., 2004). EIF2α phosphorylation has been associated with cell survival, whereas a long-term induction of this factor has been associated with cell death.

Although NF-κB is often classified as a pro-survival factor that prevents apoptosis, there is evidence for NF-κB as either, a proor an antiapoptotic factor, depending on the stimulus that triggers the event (Gil & Esteban, 2000a). Probably, because of this dual ability to produce death or survival, the precise role of PKR in cancer biology remains

controversial. Initially, PKR was thought to be a tumour suppressor. The overexpression of PKR in mammalian, insect and yeast cells indicated that PKR controls cell growth and, consequently, may function as an inhibitor of cell proliferation.

In fact, the expression of several PKR dominant-negative mutants leads to malignant transformation of NIH 3T3 cells, and are able to cause tumourigenesis in nude mice (Koromilas et al., 1992).

The strong ability of PKR to induce the apoptotic machinery combining intrinsic and extrinsic routes of caspase activation agrees with the notion that this protein could be a tumour suppressor. Moreover, PKR has been suggested to play an essential role in the antitumour activity of tumour suppressors, such as p53 and PTEN (Mounir et al., 2009). Alternatively, data supporting the antitumour role of PKR comes from studies demonstrating the importance of PKR/eIF2α status in cancer response to chemotherapy (discussed above).

The evidence that PKR is suppressed or inactivated in tumours, and the correlation between increased PKR expression and improved prognosis have been shown in head, neck, melanoma, lung and colon cancer (Guo et al., 2013). However, other studies also suggest an antagonistic role of PKR in cancer, challenging the proposed function of PKR as a tumour suppressor. In fact, it has been reported that PKR is overexpressed and linked with malignancy in thyroid carcinoma, broncheoalveolar carcinoma, colon, melanoma, lung, breast and liver cancers (Cheng et al., 2013; Marchal et al., 2014).

Furthermore, it has been suggested that PKR is involved in leukaemia and could play a negative role in some haematological disorders. Increased expression of PKR has been related to the promotion of genomic instability in a mouse model of myelodys- plastic syndrome (Blalock et al., 2011) and high PKR expression in CD34(+) cells of acute myeloid leukaemia patients has been correlated with decreased survival and shortened remission duration (Oshima & Iwama, 2015). More recently, PKR-p38 signalling pathway has been negatively implicated in the suppression of gastric cancer metastasis induced by Gelsolin, an actin-binding protein that regulates cytoskeletal turnover, which is crucial for the migration and invasion of tumour cells (Yuan et al., 2016). Finally, PKR translocation to the nucleus has been involved in lung cancer as a mediator of radiation resistance (Hao et al., 2016).

It is now well established that activation of PKR leads to the induction of pro-survival as well as pro-death pathways depending on the intensity and nature of the activating stimulus, as well as, the activation or level of expression of PKR modulators (Figure 18). Several direct and indirect modulators of PKR protein have been described. For instance, PACT, the transactivation response RNA-binding protein (TRBP), chaperones heat shock protein (HSP90 and HSP70) and the noncoding RNA nc886, among others, have been well characterised as PKR modulators (Chaves, 2016). The specific role that those modulators play in diseases where PKR is deregulated still needs to be elucidated in a clinical context (Figure 18).

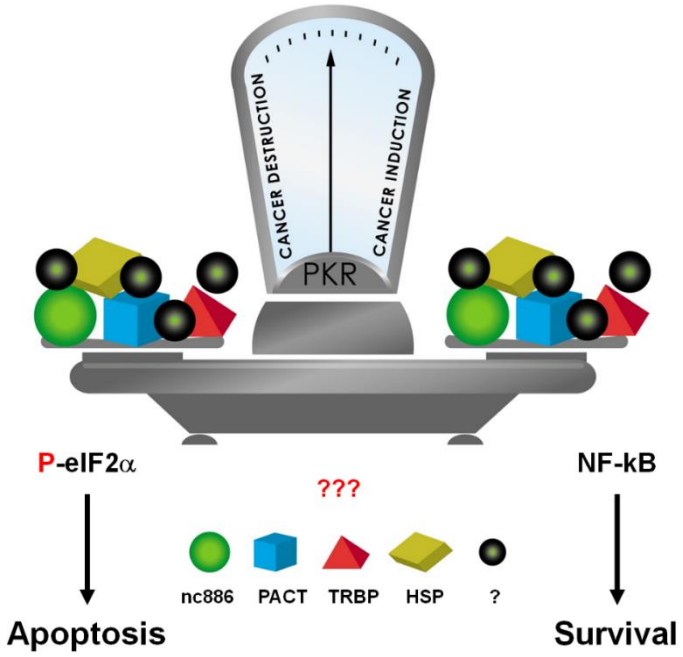


Figure 18. PKR plays an antagonist role in cancer. PKR activation can induce both pro-survival and pro-death pathways. *The nature and the intensity of activation stimuli as well as the level of expression of PKR modulators (PACT, TRBP, HSPs, nc886 among others) could influence the balance between cancer cell survival or destruction by apoptosis of tumour cells. Understanding the cellular factors and signals that regulate PKR in the different diseases would be extremely valuable from a clinical point of view. HSP, heat shock protein; PKR, protein kinase R; PACT, PKR associated activator; TRBP, trans-activation response RNA binding protein.*

For example, the finding of different expression patterns of PKR/eIF2α/NF-κB activity in cancer cell lines derived from different cholangiocarcinoma patients indicates the complexity of the role of PKR and its regulation by the pre-mir-nc886 in cancer. Consistent with these data, an elegant model proposes the escape of virus and cancer cells from the PKR/eIF2α cell death pathway (Lee, 2015).

In summary, clinical studies related to PKR regulation are still insufficient. Furthermore, the few studies that have been conducted did not follow a standardised protocol, differing in the number of tumour samples analysed and in the inclusion criteria of patients, which makes difficult to compare results between studies. Therefore, it is necessary dissect in vivo the involvement of PKR in different cellular pathways, and the importance of its compartment localisation in the presence or absence of certain regulator factors that may affect its activity in several diseases.

3.4. PKR as a molecular target of chemotherapeutics and novel antitumour drugs

The chemotherapeutic drug 5-Fluorouracil (5-FU) is widely used in the treatment of a range of cancers, being the first and second line of combination treatments in colorectal cancer patients, and the third line in the palliative care of numerous cancer types. However, adverse effects and resistance to the drug remain a major clinical problem. Since defects in mediators of apoptosis may account for chemoresistance, the identification of new targets involved in 5-FU- induced apoptosis is of great clinical interest. The p53 tumour suppressor has been reported to be an important protein involved in 5-FU-induced apoptosis. However, several studies have shown that apoptosis can also occur in mutant p53 cell lines by a mechanism still unknown (Longley et al., 2003).

Given the heterogeneous response to 5-FU induced apoptosis and 5-FU sensitivity or resistance, the finding of biomarkers that could predict 5-FU behaviour in patients is of vital relevance to improve personalised cancer therapies. In this respect, we have identified PKR as a key molecular target of 5-FU involved in apoptosis induction in human colon and breast cancer cell lines (García et al., 2011). We analysed PKR distribution and activation, apoptosis induction and cytotoxic effects during 5-FU and 5-FU/IFNα treatment in several colon and breast cancer cell lines with different p53 status. PKR protein was activated by 5-FU treatment in a p53-independent manner, inducing phosphorylation of eIF-2α and cell death by apoptosis.

Furthermore, PKR interference promoted a decreased response to 5-FU treatment and those cells were not affected by the synergistic anti-tumour activity of a 5-FU/IFNα combination. In addition, we showed that PKR is activated in absence of p53 expression and, whereas PKR knockdown decreased 5-FU- mediated apoptosis, cell death was completely abolished in absence of both PKR and p53 proteins. These results suggest the

importance of both proteins in 5-FU-induced apoptosis and the role of PKR in tumour cells where p53 is mutated, considering that more than 50% of colon tumours are deficient in p53 activity (García et al., 2011).

PKR has been also implicated in the anti-tumour activity of anthracycline doxorubicin (DOX), a commonly used treatment for a wide range of cancers. Following DOX application, PKR induces apoptosis in cancer cell lines by mechanisms dependent on eIF2α phosphorylation, p53 phosphorylation and JNK activation (Peidis et al., 2011). Importantly, in a mouse xenograft model, colon cancer cells with reduced PKR expression induced early tumourigenesis and were resistant to DOX or etoposide treatment when compared with control cells. Moreover, PKR negatively regulated leukaemia progression in association with PP2A activation, Bcl-2 inhibition and increased apoptosis in response to DOX treatment in a xenograft model (Cheng et al., 2013). Taken together these results indicate that increased PKR expression in cancer tissues may serve as a biomarker for response to 5-FU or DOX-containing chemotherapy regimens.

Future therapeutic approaches to promote PKR expression/activation and eIF2α phosphorylation may be beneficial for the treatment of some cancers. In this regard, we have analysed PKR/ eIF2α activation in response to new antitumour drugs. For instance, Bozepinib [(RS)-2,6-dichloro-9-[1-(p-nitro-benzenesulfonyl)-1,2,3,5-tetrahydro-4,1 benzoxaze-pin-3-yl]-9H-purine] a potent antitumour compound has been shown to induce apoptosis in breast and colon cancer cells (López-Cara et al., 2011). We identified PKR as a target of Bozepinib, being upregulated and activated by the drug. However, p53 was not affected and was not necessary for Bozepinib induction of apoptosis in either breast or colon cancer cells.

In addition, the efficacy of Bozepinib was improved when it was combined with IFNα cytokine, supporting a role of PKR in Bozepinib-induced apoptosis (Marchal et al., 2013; Ramírez et al., 2014).

4. PKR role in metabolism

The balance between glucose production and utilisation is finely controlled by pancreatic cells secreting insulin, which increases glucose uptake by skeletal muscles and inhibits hepatic glucose production. When the diet provides excess of lipids, the capacity of adipose tissue to store fatty acids is overwhelmed, and fatty acids are accumulated in

collateral tissues inducing the insulin resistance observed during obesity or type 2 diabetes.

Recently, it was observed that PKR is involved in the induction of insulin resistance in peripheral tissues (Nakamura et al., 2014), and antiproliferative activities in pancreatic β cells, indicating a novel role of PKR in metabolism regulation and type 2 diabetes mellitus. Sustained phosphorylation of eIF2α has been observed as a feature of obesity and insulin resistance in experimental systems and humans (Oyadomari et al., 2008). As in the case of pathogen exposure, PKR coordinates eIF2α phosphorylation-mediated suppression of protein translation with the induction of inflammatory responses upon exposure to excess of nutrients and energy. These functions of PKR are attributed to its kinase catalytic activity. Interestingly, an effective therapeutic strategy targeting PKR was confirmed using small-molecule inhibitors of the kinase activity that improved insulin sensitivity and glucose clearance in a mouse model of obesity and insulin resistance.

PKR is also a critical regulator of metabolic homeostasis, placing this enzyme at a critical juncture in immunometabolic pathways. In fact, recently it has been suggested that the association between PKR and TRBP protein integrates metabolism with translational control and inflammatory signalling, playing important roles in metabolic homeostasis and disease. The formation of a complex between PKR and TRBP during metabolic and obesityinduced stress has been critical for the regulation of eIF2α phosphorylation and JNK activation (Nakamura et al., 2015). Therefore, exposure of cells and tissues to lipotoxicity also leads to PKR activation and PKR-dependent activation, raising the possibility that PKR serves as a sensor for metabolic stress signals (Hassan et al., 2016). Indeed, PKR activity and JNK phosphorylation are elevated in multiple tissues in obese humans (Boden et al., 2008), strongly suggesting that this role of PKR is conserved and relevant to human disease.

The translational consequences of the involvement of PKR in metabolism and inflammation are currently present in neurodegenerative diseases such as Alzheimer. For example, it has been suggested that β-amyloid oligomers and Alzheimer-associated toxins activate PKR in a TNF-α-dependent manner, resulting in eIF2α phosphorylation, neuronal insulin receptor substrate (IRS-1) inhibition, synapse loss, and memory impairment (Lourenco et al., 2013). Moreover, cognitive scores in Alzheimer's

participants showed a significant positive relationship with IKKβ and a negative correlation with IRS1, JNK and PKR status.

The complexity of the different activity of PKR found in cancer has also been shown in the context of metabolism and inflammation. The involvement of PKR in metabolism homeostasis is attributed to its kinase catalytic activity; however, PKR can function as an adaptor protein via its protein-binding domain, but not via its regulatory dsRNA-binding domain. In fact, under the conditions where the kinase catalytic activity was defective, the protein-binding function of PKR was shown to promote β-cell proliferation, suggesting a role of the PKR protein-binding domain distinct from its kinase activity, and a pivotal role for PKR protein-binding function on the proliferation of pancreatic β cells through TRAF2/RIP1/NF-κB/c-Myc pathways. Nevertheless, the importance of a functional dsRNA-binding domain in PKR has also been revealed by a study showing that endogenous snoRNAs can activate PKR under metabolic stress (Osama et al., 2015).

Thus, the therapeutic inhibition of PKR at all levels is complicated, and most inhibitor compounds act only on the catalytic domain. Some strategies have implied the use of two structurally distinct small-molecule inhibitors of PKR in the treatment of insulin resistance and type 2 diabetes in both cells and mouse models. These strategies were able to reduce in vitro and in vivo stress-induced JNK activation and IRS-1 serine phosphorylation. In addition, treatment with both PKR inhibitors reduced adipose tissue inflammation, and improved insulin sensitivity, and glucose intolerance in mice after the establishment of obesity and insulin resistance. These findings suggest that pharmacologically targeting PKR may be an effective therapeutic strategy for the treatment of insulin resistance and type 2 diabetes. However, the questioned specificity and toxicity of PKR inhibitors (Naz et al., 2015) together with the ubiquitous and multifunctional properties of PKR, in a catalytic-dependent and independent manner, are still major challenges for future clinical application of these inhibitor compounds.

4.1. Are metabolism and cancer connected by PKR?

The process of transformation in cancer cells is accompanied by profound alterations of cellular metabolism that guarantee the energy required for cancer cell growth and proliferation (Hanahan & Weinberg, 2000). A recent systematic analysis of metabolic gene expression across several cancer types showed that besides glycolysis, other metabolic pathways, including nucleotides, enzymatic activities and protein synthesis

modulation, are activated in cancer. Obesity is a risk factor for many cancers, but the mechanisms by which it contributes to cancer development and patient outcome have yet to be fully elucidated. We have shown the effects of co-culturing human-derived adipocytes with established and primary breast cancer cells on tumourigenic potential.

We found that the interaction between adipocytes and cancer cells increased the secretion of pro-inflammatory cytokines, increasing the proportion of cells expressing stem-like markers and increasing the metastasis potential in vivo (Picon-Ruiz et al., 2017). Since PKR is able to induce cytokines under multiple stress conditions, we are currently exploring its potential role in this process. Previous works have demonstrated that the deregulated inflammation process is a common pathological feature that may negatively contribute to cancer, neurodegeneration and metabolism pathogenesis. Based on studies demonstrating that PKR is induced by IFNs, and the pro-inflammatory NF-κB transcription factor is a target of the kinase and that it contributes to the inflammasome complex formation, the PKR implication in inflammatory processes has been suggested (Lu et al., 2012).

Activation of oncogenes and loss of tumour suppressors promote metabolic reprogramming in cancer, resulting in enhanced nutrient uptake for energetic and biosynthetic pathways. Within the hierarchy of pathways altered in cancer, glucose and glutamine metabolism are consistently reprogrammed by mutations in the tumour suppressor p53, the Myc and Ras-related oncogenes, and the AMP-acivated kinase (AMPK) and PI3 kinase (PI3K) signalling pathways, among others.

Although these metabolic features of cancer are now exploited for diagnostic and therapeutic purposes, their broader clinical implications are still under intense investigation. Epidemiologic studies suggest that people with diabetes, predominantly type 2 diabetes mellitus, had significantly higher risk to develop cancer and greater cancer mortality (Hua et al., 2016). Although, the mechanisms that underlie the associations between insulin resistance, type 2 diabetes mellitus and cancer risk remain far from understood, the insulin-like growth factor axis, inflammation, autophagy, endoplasmic reticulum stress, fatty acid excess stress and other mechanisms have been proposed to be important in this process.

The deregulation of PKR is clearly involved in cancer and metabolism; however, its clinical involvement in the context of the two biological mechanisms is only now

beginning to be explored. An interesting study showing the complexity of PKR in cancer and metabolism in lung cancer cells and tumours, suggests crosstalk among PKR, the AMPK and nutrient depletion (Hardie et al., 2012). AMPK is activated by cellular stresses that deplete ATP and is a key factor in cancer metabolism. The authors showed that PKR causes nutrient depletion, which increases AMP levels and decreases ATP levels, causing AMPK phosphorylation. The inhibition of AMPK expression enhanced PKR-mediated cell death. However, the combination of PKR and p-AMPK expression in non-small cell lung cancer patients predicted a poor outcome for adenocarcinoma patients with high PKR expression and a better prognosis for those with low PKR expression.

In addition, PKR activation has been linked to cachexia, involving loss of muscle, insulin resistance and inflammation in cancer patients, indicating that the kinase may be implicated in metabolic pathways associated with cancer formation (Aoyagi et al., 2015). Finally, in addition to the connection between the transcription factor STAT3 and PKR in cancer pathways, a link it has been established between both proteins in a metabolic context. STAT3 is able to regulate autophagic pathways interacting with PKR kinase in the cellular metabolism control of fatty acids. Several fatty acids, including palmitate, trigger autophagy via a pathway that involves the disruption of the STAT3- PKR complex as well as the phosphorylation of mitogen-activated protein kinase 8/c-Jun N-terminal kinase 1 and eIF2α phosphorylation. This study suggests an interesting crosslink between cellular metabolism (fatty acids), pro-inflammatory signalling (STAT3), innate immunity (PKR) and translational control (eIF2-α) that regulates autophagy and cancer processes suggesting translational and clinical potential.

Although these studies suggest that PKR is involved in signalling pathways common in both processes, the connection between cancer and metabolic disorders needs to be determined in order to support PKR as a relevant therapeutic candidate.

4.2. The PKR role in metabolic diseases

Although numerous studies have concluded that PKR deletion prevents the deleterious consequences of a high-fat diet, with considerable phenotypic variations, recently it has been shown that PKR deletion has no effect on high-fat diet induced obesity or the development of impaired glucose metabolism, having only a modest effect on adipose tissue inflammation (Lancaster et al., 2016). Disappointing results have also emerged from the study of PKR role in cancer.

Despite the demonstration that PKR inhibits cell growth and that mutated forms of PKR results in cellular transformation, other studies using two different mouse models bearing a homozygous disruption of the pkr gene, showed that PKR inactivation did not cause spontaneous tumour development (Y. L. Yang et al., 1995). Curiously, cells derived from knockout mouse models express truncated forms of the protein PKR maintaining either, the catalytic activity (C-PKR KO) or the dsRNA-binding properties (N-PKR KO) depending on the mouse model used.

In fact, metabolic studies from in vivo analysis using the same two knockout models that were used before in cancer studies, suggested differences between groups that may account for the phenotypic differences observed between the two mouse models (Lancaster et al., 2016). Indeed, differences have been reported in the signalling responses of cells isolated from C-PKR KO and N-PKR KO mice exposed to specific stimuli (Abraham et al., 1999). Since PKR may have an important role in both cancer and metabolism in either a catalytic-dependent and independent manner, the total deficiency of the protein PKR must be absolutely confirmed to minimise contradictory results obtained between groups in both pathologies.

5. Conclusions

Numerous studies have confirmed the multifaceted and diverse roles of PKR. The involvement of PKR in IFN-mediated activities, the induction of apoptosis, the activation of inflammatory transcription factors, along with its role in signalling pathways linking numerous biological events, including cell growth and metabolism, are clear indicators of the great potential that this protein may have in the regulation of a variety of pathologies (Figure 19).

The identification of PKR as a target for both conventional chemotherapeutics and novel drugs highlights the need to carry out studies with patients to validate its potential as a biomarker in cancer diseases. Moreover, PKR deregulation in age-related diseases like cancer and Alzheimer's, in inflammatory processes and in metabolism disorders, like obesity and insulin resistance in patients, underlines the need to find applicable inhibitors of this protein. Since PKR is expressed in almost all cells and it is able to act in a catalytic-dependent or independent manner, its therapeutic potential depends on under standing and controlling its regulation factors.

In fact, understanding the cellular factors and signals that regulate PKR in different diseases would be extremely valuable from a clinical point of view. The controversial results obtained from the two knockout mouse models available to study PKR in cancer and in metabolic diseases indicate the need to agree on another mouse model that guarantees the total deficiency of PKR expression.

The use of CRISPR/Cas9 technologies to generate knockout models will allow future studies with human cells from various origins and animal models with complete deletion of PKR in combination with other related host genes to demonstrate beyond doubt its role in biological effects (i.e. cancer and metabolism) in a whole organism.

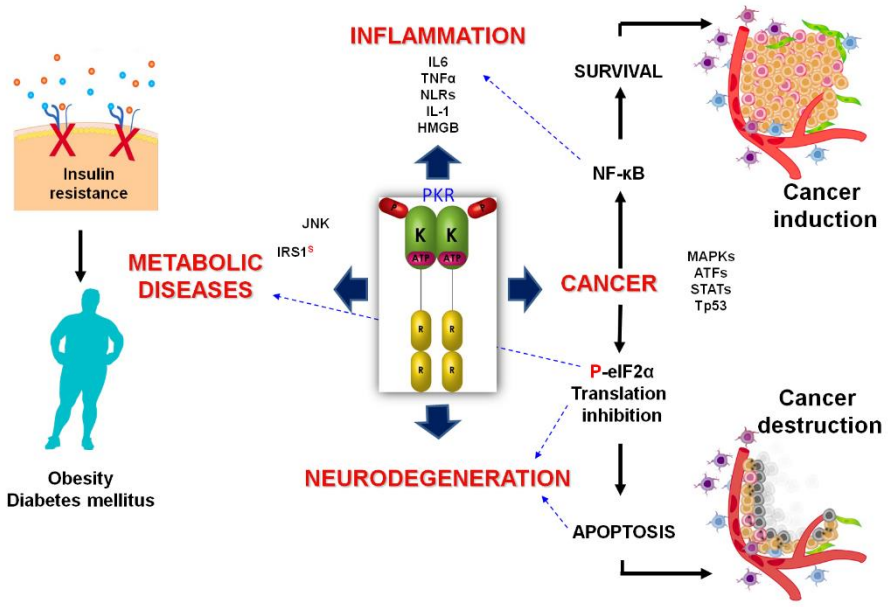


Figure 19. Unbalanced PKR activity is involved in cancer, neurodegeneration, inflammation and metabolism disorders. *PKR coordinates the eIF2α phosphorylation-mediated suppression of protein translation with the induction of inflammatory responses upon exposure to excess of nutrients, energy and different cellular stresses. Sustained phosphorylation of eIF2α has been observed as a feature of obesity and insulin resistance. In addition, PKR is involved in inflammasome activation and cytokine liberation such as IL-6, TNF α and HMGB among others. Moreover, PKR activation can induce both pro-survival via NF-κB activation or cell death by apoptosis via eIF2α phosphorylation playing an antagonist role in cancer. Excess of PKR/eIF2α-P inducing apoptosis has been found in neurodegeneration. Furthermore, an unbalanced effect induced by dysregulated PKR activity could contribute to inflammatory diseases, neurodegeneration, cancer and metabolism disorders. Translational research will help to determine the clinical and therapeutic potential of this pleiotropic kinase. HMGB, high-mobility group box; IL, interleukin; NF-κB, nuclear factor κB; PKR, protein kinase R; TNF, tumour necrosis factor.*

Activation of oncogenes and loss of tumour suppressors promote metabolic reprogramming in cancer, and the crosstalk among cancer and metabolic pathways are now exploited for diagnostic and therapeutic purposes. The present review has shown the importance to conduct continued basic molecular, preclinical and translational research to understand cancer, metabolism, inflammation and even neurodegeneration interplay. With personalised medicine looming in the present/future and the genetic and proteomic background of each patient determining specific approaches, PKR will play in the not too distant future a relevant clinical role.

CHAPTER II:
ANALYSIS OF PKR AND ITS MODULATOR NC886 AS PREDICTIVE BIOMARKERS IN METASTATIC COLON CANCER PATIENTS

1. Abstract

Colorectal cancer treatment has advanced over the past decade. The 5-fluorouracil is still used with a wide percentage of patients who do not respond. Therefore, a challenge is the identification of predictive biomarkers. The protein kinase PKR (EIF2AK2) and its regulator, the non-coding pre-mir-nc886, have multiple effects on cells in response to numerous types of stress, including chemotherapy. In this work, we have performed an ambispective study with 197 metastatic colon cancer patients with unresectable metastases to determine the relative expression levels of both nc886 and PKR by qPCR and the location of PKR by immunohistochemistry in tumour samples and healthy tissues (plasma and colon epithelium). As primary end point, the expression levels were related to the objective response to first-line chemotherapy following RECIST criteria and, as the second end point, with the survival at 18 and 36 months. Hierarchical agglomerative clustering was done to accommodate the heterogeneity and complexity of oncological patient's data. High expression levels of nc886 were related to the response to treatment and allowed to identify clusters of patients. Although the PKR mRNA expression was not associated to chemotherapy response, the absence of PKR location in the nucleolus was correlated with first-line chemotherapy response. Moreover, a relationship between survival and the expression of both PKR and nc886 in healthy tissues was found. Therefore, this work evaluated the best way to analyse the potential biomarkers PKR and nc886 in order to establish clusters of patients depending on the cancer outcomes using algorithms for complex and heterogeneous data.

2. Introduction

Colorectal cancer (CRC) is one of the most common cancer worldwide being the third most commonly diagnosed malignancy and the second leading cause of cancer death in the last years (Keum & Giovannucci, 2019). Although CRC treatment has advanced over the past decades, treatment outcomes depend in part, on tumour and patient specific molecular characteristics (Vacante et al., 2018). Even though many novel drugs have been developed for patients with advanced CRC, 5-fluorouracil (5-FU) is still widely used as the classic and basic drug in adjuvant chemotherapy and palliative cares. 5-FU is used as infusion, oral (capecitabine) or in combination with different drugs (FOLFOX, FOLFIRI) but its efficacy is limited by numerous factors including tumour cells genetics, epigenetics and proteomics, which promote chemoresistance and metastasis (Vacante et al., 2018).

In the last decade, the efficacy of these regimens has been increased by incorporating new biological therapies based on the use of monoclonal antibodies (Ohtsu et al., 2011). Despite the fact of the considerable improvement in the efficacy, there are still a wide percentage of patients who do not benefit from 5-FU-based treatments. Therefore, the identification of biomarkers that associate or predict the benefit of an appropriate selection of candidates for 5-FU-based therapies and combined therapies constitutes a broad area in clinical and translational research.

PKR, also called EIF2AK2, is an interferon-inducible double-stranded RNA protein kinase with multiple effects on cells. This protein kinase plays an active part in the cellular response to numerous types of stress mediating in several biological pathways and with a potent role in the induction of apoptosis in response to numerous compounds (García et al., 2006). PKR is a serine-threonine kinase, composed by the kinase domain shared by the other eIF2α kinases, and two dsRNA binding domains (dsRBD) that regulate its activity. PKR autophosphorylation represents the activation reaction that leads to the phosphorylation of eIF-2α, impairing eIF-2 activity, which results in the inhibition of protein synthesis (Der et al., 1997).

In addition to its translational regulatory function, PKR has a role in signal transduction and transcriptional control through the IκB/NF-κB pathway (Maria A. Garcia et al., 2009). Although the primary PKR activator is dsRNA-produced during infection by several viruses and detected at low doses in mammalian cells, PKR is also activated by a variety of cellular stresses including cytokines, calcium stress, oxidative stress, endoplasmic reticulum stress, lipo-stress, amyloid-β (Aβ) peptide accumulation, polyanions such as heparin, and several drugs, or through the PKR associated activator (PACT). PKR, which is expressed constitutively in mammalian cells, has also been implicated in the control of cell growth and differentiation with debated antitumor role and as an important antiviral agent (Marques et al., 2008). Recently, the role of PKR related to metabolism, inflammatory processes, cancer and neurodegenerative diseases has gained great interest (Garcia-Ortega et al., 2017).

The importance of PKR function in cell growth, differentiation, stress response and immunomodulation are further noted by the existence of numerous modulators. Therefore, it has been identified several PKR regulators involved in cancer outcome, where the non-coding RNA pre-miR-886, also called nc886 or vtRNA 2-1, has been described as a potent regulator of PKR (Jeon et al., 2012; Lee & Esteban, 1994). Nc886 binds to PKR with an affinity comparable to dsRNA and prevents PKR from being

activated, in contrast to the PKR-activating ligand dsRNA. Although nc886 was initially discovered as a PKR inhibitor, recently researchers have demonstrated that nc886 can adopt two structurally distinct conformers that are functionally opposing regulators of PKR (Calderon & Conn, 2017).

We have previously identified PKR as a molecular target of 5-FU in several colon and breast cancer cells lines playing an important role in the cytotoxic effect of 5-FU at least, in part, through the induction of cell death by apoptosis (García et al., 2011). Since PKR has also been implicated in the anti-tumour activity of chemotherapeutic drugs such as doxorubicin (DOX) and etoposide (Lee & Esteban, 1994) and nc886 has been identified as an interesting tumour suppressor (Yoon et al., 2009), we consider of clinical importance the analysis of PKR and the nc886 in patients as potential predictive biomarkers.

For this reason, the aim of this work was to carried out an ambispective study in 197 metastatic colon cancer patients to evaluate the expression levels of PKR and its pre-microRNA-nc886 by qPCR in colon tumour samples and their respective healthy tissues and plasma, analysing its relation with the patient's clinical evolution. The primary end point was the evaluation of these variables with the objective response (OR) to first-line of 5-FU-based chemotherapy determined by RECIST criteria. We have also analysed as the second end point, the relationship of these variables with overall survival (OS) at 18 and 36 months in those patients when the information was available. In addition, we have analysed the PKR location by immunohistochemistry in 76 colon tumours and its respective colon healthy tissues. For all this study, novel bioinformatic analyses have been included in order to distinguish parameters and signals for the identification of different profiles in patients, who have the same diagnostic and disease.

Hence, clustering analyses were done using Hierarchical agglomerative clustering (Statistical Toolbox, Matlab 2007b, Spotfire Decision Site 9.1.2) (Arnedo et al., 2013), with the objective of improving the quality and specificity of the results considering the heterogeneity between samples, genetic and proteomic background of oncologic patients.

3. Materials and Methods

3.1. Patients and samples

The study was approved by the Ethics Committee of the University Hospital Virgen de las Nieves from Granada (Cod Peiba. 0170-N-16) and informed patient consent was obtained. The process of recruitment, traceability of samples and informed consent has been regulated and controlled by the Andalusian Public Health System Biobank (BBSSPA), according to the World Medical Association Declaration of Helsinki analysed (see supplementary document Annex I-*Informed consent for donation of biological samples and associated information to the Biobank*).

The study included a total of 197 colon metastatic cancer patients with unresectable lung or liver metastases from September 2014 through September 2018 and treated with 5-FU-based therapy as first-line using the standard treatment schedule. To avoid discarding as few samples as possible, the missing values were approximated to the median value established for each variable analysed (see supplementary excel document with available data in Annex I-Table S1). Criteria of the RECIST guidelines were used to characterize the response to this treatment (Eisenhauer et al., 2009). According to these criteria, after the first restaging assessment that was generally performed around 3-4 months after the initiation of 5-FU -based treatment, patients with progressive disease were considered as non-responders with primary resistance and those patients with partial response or stable disease under the 5-FU treatment for at least 3-4 months were considered as responders. The survival was considered after 18 months and 36 months in those patients where the data was available (see supplementary Figure S1 in Annex I).

Tumours were classified according to the 2002 TNM classification and the Fuhrman grading system by an experienced pathologist (BR and JLH) (Worrall, 2000). Archived Formalin-Fixed Paraffin-Embedded (FFPE) tissue samples from colon tumour and their corresponding surrounding healthy colon tissues obtained for routine diagnostic purposes were used in this study.

Peripheral blood samples subjects were collected prospectively with one tube for EDTA anticoagulant (3 mL). Samples were centrifuged at 3,000 rpm for 10 minutes and, then, aliquoted and frozen at −80°C until use. The flow diagram shown in Figure 20 outlines the steps performed in this ambispective study.

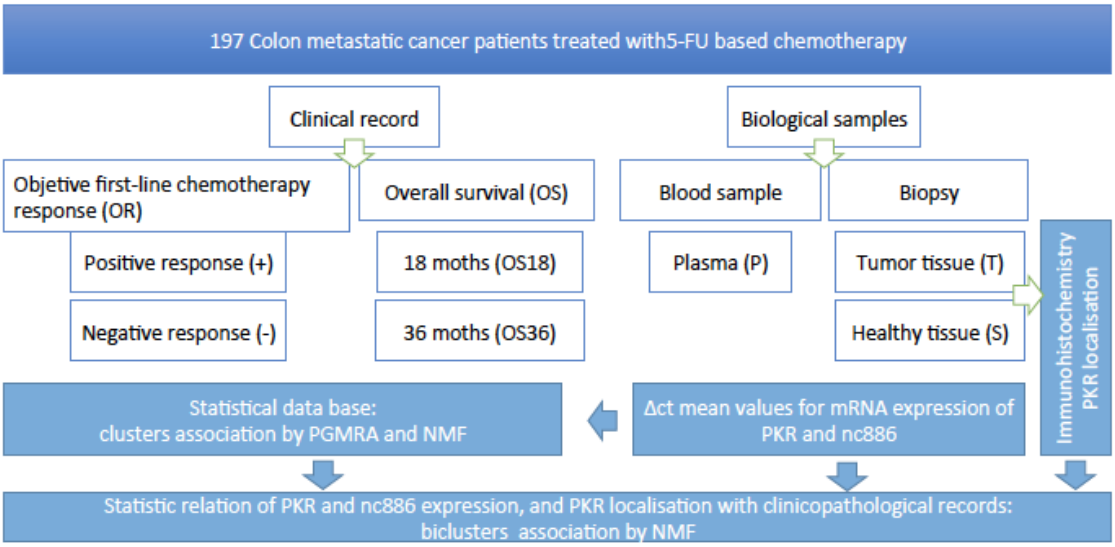


Figure 20. Flowchart of the ambispective study.

3.2. RNA extraction from FFPE tissue and from plasma samples

Haematoxylin-eosin stained histological FFPE sections were prepared to identify areas of normal and tumour tissue. These regions of interest were biopsied by macrodissection after the xylene-alcohol dewaxing performed by specialist pathologists (Atrys Health, Gr, SP). The tissue of interest was scraped with a scalpel and dipped in digestion buffer (ATL) (Qiagen, Hi, GE). Only tumour samples with more than 80% cancer cells were considered for further analysis. Non-malignant tissue with a distance of >100 mm to the cancer tissue was collected from all patients of the study cohort for comparison purposes.

Total RNA was isolated from the dissected FFPE tissue samples using an miRNeasy FFPE Kit (Qiagen, Hi, Ge;) and from plasma using the miRNeasy Serum-Plasma extraction protocol (Qiagen N.V. GE) by previous concentration of 300µl of cold plasma using the Vacufuge™ Concentrator system (Eppendorf ™, GE). The RNA extractions were automated using the robot Qiacube (Qiagen, Hi, GE) according to the manufacturer's instructions. Integrity and quality of RNA (RIN) was tested with Bioanalyzer (Agilent, CA) and diluted to a maximum concentration of 500ng in 14µl. The retrotranscription was performed with the SuperScript ™ VILO ™ cDNA Synthesis Kit (Invitrogen-Thermo Fisher Scientific, USA) on all samples (4µL 5X VILO ™ Reaction

Mix, 2µL 10X SuperScript ™ Enzyme Mix, 14 µL RNA, 10 minutes at 25 ° C, 60 minutes at 42 ° C and 5 minutes at 85 ° C).

3.3. RT qPCR assay

The determination of the expression of PKR gene and nc886 element was carried out in plasma, tumours and healthy colon tissues from patients enrolled in this ambispective study. To determine the expression of the PKR (EIF2AK2) gene and nc886 (VTRNA2) element, specific fluorescent hydrolysis probes TaqMan-MGB (Thermo Fisher Scientific, USA) were used (Hs01091582_m1, Hs04273370_s1 respectively) by Real Time qPCR and by Digital dPCR in different samples. To select the most appropriate endogenous control we analysed the endogenous classic GADPH, HPRT and B2M genes (Huggett et al., 2005) by several tests to assess the stability of them in different types of tissue by qPCR.

Due to the results obtained, B2M (Hs00187842_m1) was considered as endogenous control for plasma and FFPE tissue samples. For the amplification of the samples, the protocol "TaqMan® Gene Expression Master Mix" was adapted to a final volume of 10µl in the QuantStudio 12K equipment (Thermo Fisher Scientific, USA). The mean Ct-values were technically normalized using the endogenous B2M, and the expression level considered as 2-ΔCt (ΔCt = Cttargetgene –CtB2M) (Demes et al., 2012). The missing values were approximated to the median value established for each variable analysed.

3.4. Immunohistochemistry analysis

Formalin-fixed Paraffin-embedded samples (n=76) were cut at 2.5 µm in thickness and placed on a slide. The antigenic retrieval was carried out by incubating the antibody for 30 minutes with hydrogen peroxide (H2O2) at pH 8. The immunohistochemical technique was carried out on the Autostainer 480S immunostains of the Thermo Scientific trademark. For the development of the technique, the Commercial Kit Detection System Master Polymer Plus (Peroxidase) was used. The polyclonal anti-PKR antibody was administered by Santa Cruz Biotechnology, and it was used with a 1:50 dilution in 30 minutes of incubation. The development of the technique was carried out with Diaminobenzidine (DAB) and after, with Hematoxylin-Eosin staining. The immunohistochemical location of PKR protein was determined by two

pathologists that considered the presence of PKR protein in the nucleolus or outside of nucleolus (mostly located in cytoplasm).

3.5. Machine learning and statistical analysis

PGMRA is a deep unsupervised (Geiger et al., 2014) and data-driven machine learning method that combines Model-based, Consensus, Fuzzy, Possibilistic, Relational, Optimization, and Conceptual clustering techniques into a single method. The Model-based approach uses Non-Negative Matrix Factorization to identify candidates for functional clusters (Arnedo et al., 2015) represented as tensors or flattened biclusters (e.g., subjects x symptoms). Biclusters can be learned independently of the number of clusters, and thus, from different granularity partitions (Consensus).

The method separately searches for biclusters in distinct domains of knowledge (e.g., genetics, clinical symptoms) without regard for their calculations in other domain of knowledge. Then, the approach agnostically co-clusters the inter-domain biclusters and identifies natural relationships (associations) among them. Associations result from optimizing the probability of the intersection among biclusters using Hypergeometric statistics or Fisher exact test (Zwir et al., 2005) evaluated by a posterior permutation test instead of using typical inter/intra clustering metrics among dots in the n-dimensional space (Model-based). Biclusters in one domain of knowledge or associations of biclusters from different domains of knowledge can be reorganized into networks at different levels of granularity, connected by sharing observations (subjects) and/or features (Δct mean values, objective first-line chemotherapy response).

This framework constitutes a knowledge base and characterizes architecture of the disease. Methodological basis of PGMRA are available in, and its web server application is online at *http://phop.ugr.es/fenogeno* (Arnedo et al., 2013). Fast parallel software implementations run at the Centre for High Performance Computing (CHPC) facility at WUSM.

3.6. Derivation of the empirical index

First, we calculated a purely empirical (i.e., agnostic and data-driven) indicator of character functioning. We clustered subjects corresponding to the 2 expression variables and assigned each subject the number of the cluster to which they belonged (as described

in the next paragraph). The result was a single empirical index of cluster membership that served as a comprehensive measure of variability in the RNAs expression.

To calculate the cluster rankings, we applied hierarchical agglomerative clustering (Statistical Toolbox, Matlab 2007b) with a complete linkage method and correlation similarity measurement to group value phenotypic, or environmental sets by their shared subjects using hypergeometric statistics. The function that controls the vertical order in which a row is plotted (Spotfire Decision Site 9.1.2) in a hierarchical clustering is defined as follows.

Given two sub-clusters within a cluster (there are always exactly two sub-clusters considered at each step), both sub-clusters are weighted and the sub-cluster with the highest weight is placed above the other sub-cluster. This function is systematically applied until a single cluster containing all rows is obtained. To calculate the weight w3 of a new cluster C3 formed from two sub-clusters C1 and C2 with a weight of w1 and w2, and each containing n1 and n2 rows, the following expression is used:

$$w_3 = \frac{n_1 \times w_1 + n_2 \times w_2}{(n_1 + n_2)}$$

The weight of a sub-cluster with a single row is calculated as the average value of its columns.

3.7. Feature selection process using Non-Negative Matrix Factorization (NMF) in PGMRA

We use Non-Negative Matrix Factorization (NMF) method as a deep autoencoder (Hinton & Salakhutdinov, 2006) in a particular domain of knowledge (qPCR data, clinical data) to identify candidates for functional clusters, represented as tensors or flattened biclusters (e.g., unknown relationships embedded in the data [subjects x ncRNA differential expression]). Our implementation of the NMF, termed Fuzzy NMF method (FNMF), learn, biclusters independently of the number of clusters, and thus, from different granularity partitions (Consensus). The method separately searches for biclusters in distinct domains of knowledge without regard for their calculations in other domain of knowledge (Arnedo et al., 2013). Then, the approach agnostically co-clusters the inter-domain biclusters and identifies natural relationships (associations) among them.

Associations result from optimizing the probability of the intersection among biclusters using Hypergeometric statistics instead of using typical inter/intra clustering metrics among dots in the ndimensional space (Model-based). These associations are learned regardless of any status of the observations (e.g., cases and controls, unsupervised) and are optimized based on multiobjective and multimodal optimization techniques (Arnedo et al., 2014). By incorporating a posteriori, a "supervised" status, the method is able to calculate the risk of the association by the frequency of one status vs. another. Once it occurs, the method becomes semi-supervised, and posterior statistical significance of the association is calculated using Kernel-based and Multivariate statistical analysis (M. C. Wu et al., 2010).

4. Results

4.1. Normalized values of non coding nc886 in plasma and tumor tissues predict the objective first-line chemotherapy response

We tested first the association of the expression level of PKR gene and the nc886 element determined by the Δct mean values identified by qPCR in tumor (T), plasma (P), and healthy (S) tissues (see supplementary Table S2 in Annex I) with the OR to first-line chemotherapy. The response is encoded as a Boolean (positive/responders- negative/non responders) tested after 3-4 months of starting the treatment as indicated in the Material and Methods section.

We identified two biclusters (subjects sharing subsets of features) composed of subjects sharing P-nc886 Δct mean and T-nc886 Δct mean values (Figure 21A). These biclusters exhibit significant different values of their composite features (Figure 21B, p-value <2.44655 e-77 ANOVA Statistics).

Those subjects were significantly associated with the objective first-line chemotherapy response (Figure 21C). The first bicluster displays low P-nc886 and mid/low T-nc886 Δct mean values and was associated with a positive response (Figure 21C, p-value <0.018, Hypergeometric Statistics/ Fisher Tests). The second bicluster exhibited high T-nc886 Δct Mean values, and was associated with a negative response (Figure 21C, p-value <0.016, Hypergeometric Statistics/ Fisher Tests) . The other studied variables that involve the expression of PKR in the different samples were not included in any bicluster significantly associated with the objective first-line chemotherapy

response (see Non-Negative Matrix Factorization (NMF) in PGMRA as a feature selection process in materials and methods section).

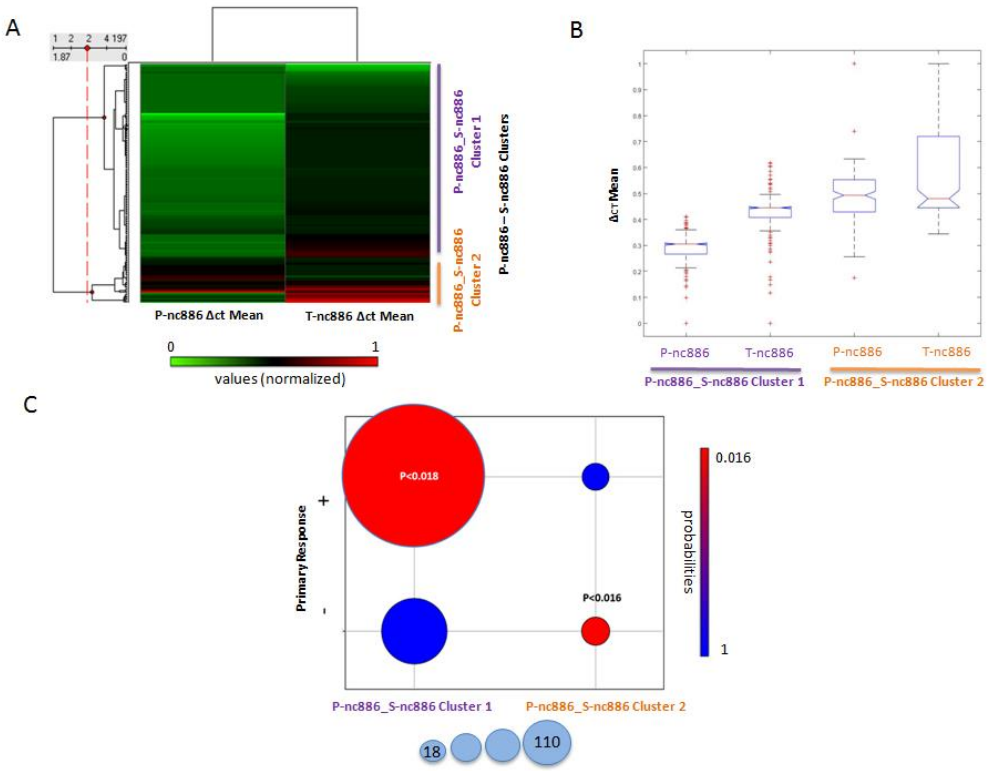


Figure 21. Association of the expression level of PKR and nc886 determined by the Δct mean values identified by RT qPCR in tumor (T), plasma (P), and healthy (S) tissues with the objective response to first-line chemotherapy. *A) P-T_nc886 Clusters: Biclusters of subjects sharing Δct mean values of nc886 in P and T. Δct mean values are normalized between 0 (green) and 1 (red). B) Boxplot of Δct mean values of nc886 in P and T for each cluster. C) Correlation between the objective first-line chemotherapy response and the nc886 Δct mean clusters (Figure 2A). P-values are calculated with the Hypergeometric statistics. Color code for p-values statistical significance indicates high (red) to low (blue). The size of the circles indicates the number of individuals in the relationship.*

One we detected the two highly associated variables, we independently validated the former results by performing a regression analysis between the cluster order (ranking, see Methods) and the individual features with respect to the OR to first-line chemotherapy. Δct values were normalizez between [0,1] due to pGMRA requierements. We determined that the clusters, represented by the order of their observations, were

better associated with the objective first-line chemotherapy response (p-value < 0.00012, F Statistics) than the individual T-nc886 Δct mean values (p-value < 0.0028) and P-nc886 Δct mean values (p-value < 0.013) (data not shown). Moreover, all other variables involved in similar regressions were non-significantly associated with the OR.

Furthemore, since the relative level of gene expression is inversely proportional to the Δct mean value following the 2-ΔCt method (Rao et al., 2013), our data suggest for Cluster 1 (Figure 21A) a significant association between patients who show high level of expression of nc886 in both plasma and tumor samples with a significant positive response to treatments based on the use of 5-FU. In contrast, Cluster 2 (Figure 21A) include patients who mostly and significantly showed lower levels of nc886 expression in the tumor and a negative response to treatment. However, the levels of exppression of PKR gene were not related to the OR to first-line chemotherapy.

4.2. PKR location predicts the objective first-line chemotherapy response

Since the relative levels of expression of the PKR gene mRNA in the colon tumor could not be related to the patient's response to the treatment, we decided to analyze the location of PKR in the tumor and healthy colon tissue cells by immuhistochemistry (n=76). While PKR was located in all healthy tissues analyzed at the level of the cytoplasm of the cells, in tumour samples it could be located restricted to the nucleolus in some cases (Figure 22). Therefore, we considered the two variables: presence or absence (located in the cytotlasm) of PKR in the nucleolus.

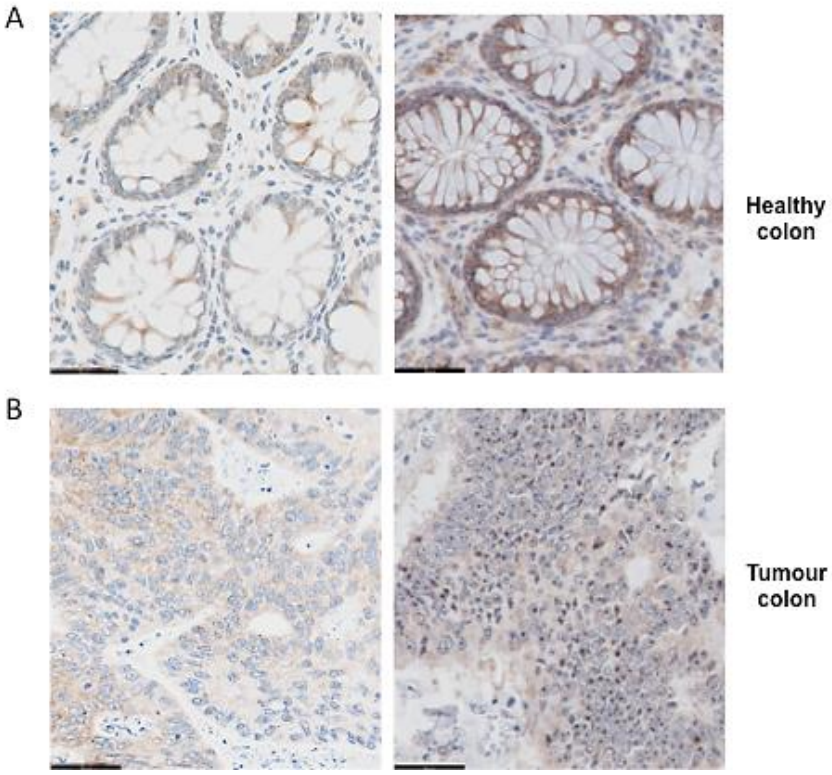


Figure 22. Different location of PKR in healthy colon tissues and tumour tissues. *A) Representative immunohistochemical detection of total PKR in healthy colon tissues. PKR immunostaining is weak (first panel) or strong (second panel), but mostly located in the cytoplasm of cells. Scale bar, 50µm. B) Representative immunohistochemical detection of total PKR in tumour colon tissues. PKR immunostaining is mostly located in the cytoplasm of cells (first panel); However, in several tumours PKR is located strongly in the nucleolus (second panel). Scale bar, 50µm.*

To test the predictive value of the PKR location we analyzed the patients for which this information was available. Two biclusters were obtained by PGMRA when including PKR Δct mean values in P, T and S (P-PKR, T-PKR, S-PKR) and the Δ*ct* mean values of nc886 in P, T and S (T-nc886, P-nc886, S-nc886) (Figure 23A). The first bicluster displays variable Δct mean values (high S-PKR, medium P-PKR, low or medium T-PKR and T-nc886, high or medium S-nc886, and medium P-nc886 values). A second bicluster was composed also of variable Δct mean values (high P-nc886 and T-nc886, medium S-PKR and T-PKR, and low P-PKR and S-nc886 values). These biclusters exhibit significant different values of their composite features (Figure 23B, p-value < 2.05581e-204 ANOVA Statistics). The main differences between clusters include variables in colon tumor and colon healthy tissues (Figure 23B). The first bicluster matches with the absence

of PKR in the nucleolus (Figure 23C, p-value <0.00001, Hypergeometric Statistics/ Fisher Tests). The second bicluster is associated with a presence of PKR in the nucleolus (Figure 23C, p-value <0.00005, Hypergeometric Statistics/ Fisher Tests).

We independently validated the former results by calculating using ANOVA Statistics with the six variables previously selected. We determined that the clusters were significantly better associated with the presence of PKR in the nucleolus (p-value < 0.000002) than the individual values (p-value < 0.035). The bicluster lacking PKR location in the nucleolus showed a relation to the positive first-line chemotherapy response (Figure 23D, p-value <0.006, Hypergeometric Statistics), and the bicluster with PKR location in the nucleolus showed a relation to the negative first-line chemotherapy response (Figure 23D, p-value <0.03, Hypergeometric Statistics).

Therefore, the analysis was able to group patients whose PKR location in the cytoplasm of the tumor cells corresponded with a positive response to the treatment, and in contrast, patients with PKR restricted to the nucleolus could be grouped in clusters that corresponded with the negative response to treatment. Although the expression levels of PKR and nc886 in the different tissues analyzed were necessary to determine these significant clusters, these were highly variable.

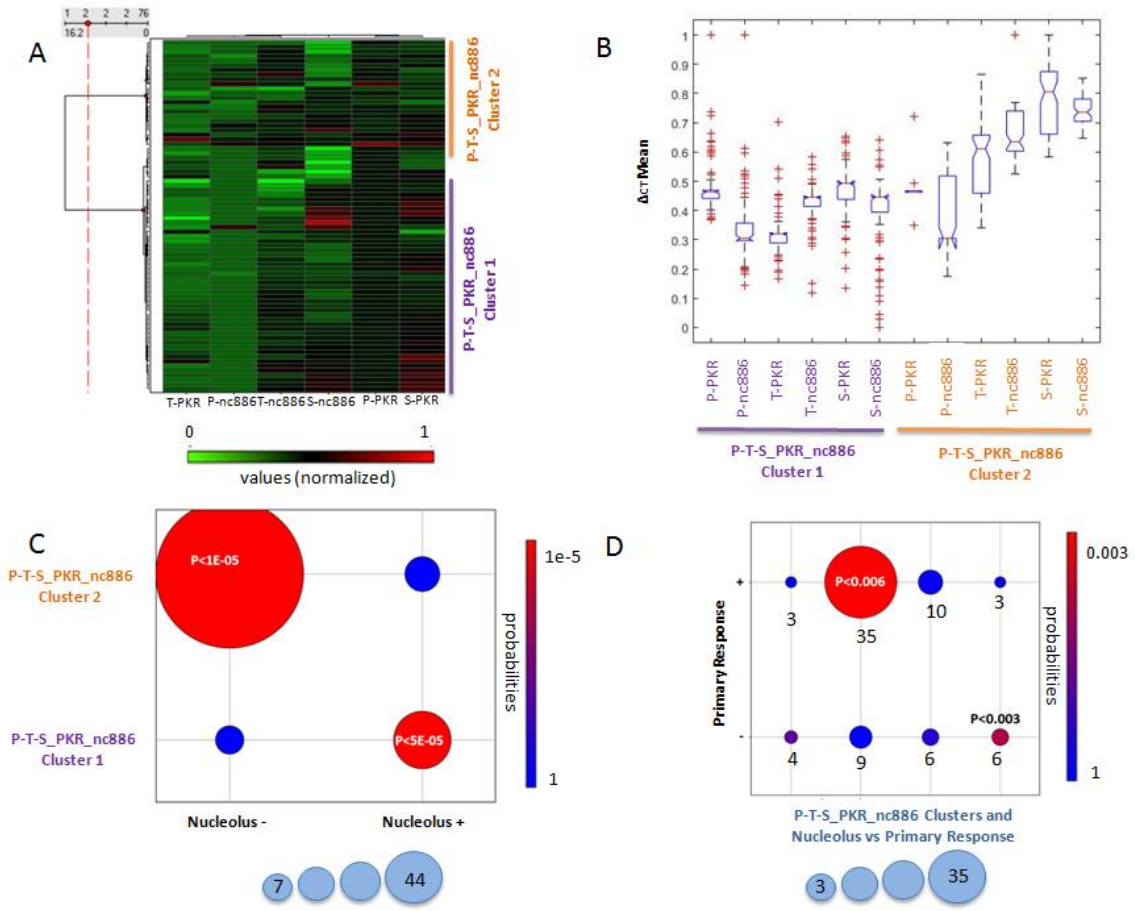


Figure 23. Association of the PKR location with Δct mean values identified by RT qPCR in tumor (T), plasma (P), and healthy (S) tissues and the objective response to first-line chemotherapy. *A) Clusters P-T-S_PKR_nc886: Biclusters of subjects sharing Δct mean values of PKR determined in the analyzed tissues (P-PKR, T-PKR, S-PKR) and Δct mean values of nc886 determined in the analyzed tissues (T-nc886, P-nc886, S-nc886). Δct Mean values are normalized between 0 (green) and 1 (red). B) Boxplot of Δct mean values of PKR determined in the analyzed tissues (P-PKR, T-PKR, S-PKR) and Δct mean values of nc886 determined in the analyzed tissues (T-nc886, P-nc886, S-nc886) for each cluster P-T-S_PKR_nc886. C) Co-clustering between the PKR location in the nucleolus and the Clusters P-T-S_PKR_nc886. P-values are calculated with the Hypergeometric statistics. Color code for p-values statistical significance indicates high (red) to low (blue). The size of the circles indicates the number of individuals in the relationship. D) Co-clustering between the objective first-line chemotherapy response and the co-clusters identified in (C). P-values are calculated with the Hypergeometric statistics. Color code for p-values statistical significance indicates high (red) to low (blue). The size of the circles indicates the number of individuals in the relationship.*

4.3. Final outcome is predicted by the the expression level of PKR and nc886 in healthy tissues

Finally, we raised the question about the correlation between the OR to first-line chemotherapy and the time-range patient survival after 18 and 36 months. To test the predictability effect of the measured variables we first applied the PGMRA method to separately factorize these three measurements using NMF and we uncovered 3 biclusters, now called "Survival clusters" (Figure 24A).

Survival Cluster 1 involved patients who mostly showed a negative response to the first line of treatment and died before 18 months (Figure 24A). Survival Cluster 2 included patients who mostly showed a positive response to the first line of treatment and were alive after 18 months; however, they died before reaching the 36 months (Figure 24A). Cluster 3 involved all patiens who mostly showed a positive response to the first line of treatment and were alive after 18 and 36 months (Figure 24A).

By other hand, independently, PGMRA selected the expression values of PKR and the nc886 in P, T and S for two biclusters (Figure 24B). We found two clusters based in colon healthy tissue values from PKR and nc886, from now on called S-PKR_S-nc886 Clusters. S-PKR_S-nc886 Cluster 1 included a large number of patients mostly with low Δct mean values for nc886 and PKR. S-PKR_S-nc886 Cluster 2 included fewer number of patients mostly with high Δct Mean values for nc886 and PKR (Figure 24B). These S-PKR_S-nc886 biclusters exhibit significant different values of their composite features (Figure 24C, p-value < 2,48689e-136 ANOVA Statistics).

Finally, we co-clustered the S-PKR_S-nc886 Clusters with the Survival Clusters identifying two significant associations. The most representative bicluster displays low nc886 Δct mean values and medium PKR Δct mean values in healthy tissues (Figure 24D). This cluster was associated with a positive objetive first-line chemotherapy response , and a long time-range patient survival (Figure 24D, p-value <0.014, Hypergeometric Statistics/ Fisher Tests). Therefore, higher expression levels of nc886 once again selected patients with better disease outcomes. The other significant bicluster exhibited high Δct mean values in healthy colon tissue of the two variables PKR and nc886 (Figure 24D), and was associated with negative objetive first-line chemotherapy response and short survival (Figure 24D, p-value <0.016, Hypergeometric Statistics/ Fisher Tests). Therefore, lower expression levels of nc886 and medium levels of PKR in

healthy colon tissue selected patients with worst disease outcomes. A non significant survival bicluster exhibiting a mixture survival values, was not significantly associated with nc886 and PKR Δct mean values in healthy tissues (Figure 24D) .

The three survival classes were independently validated using regression analysis with the clusters (rankings, see Methods) of healthy tissue nc886 Δct mean values and the healthy tissue PKR Δct mean values (p-value < 0.0053, F Statistics) (data not shown).

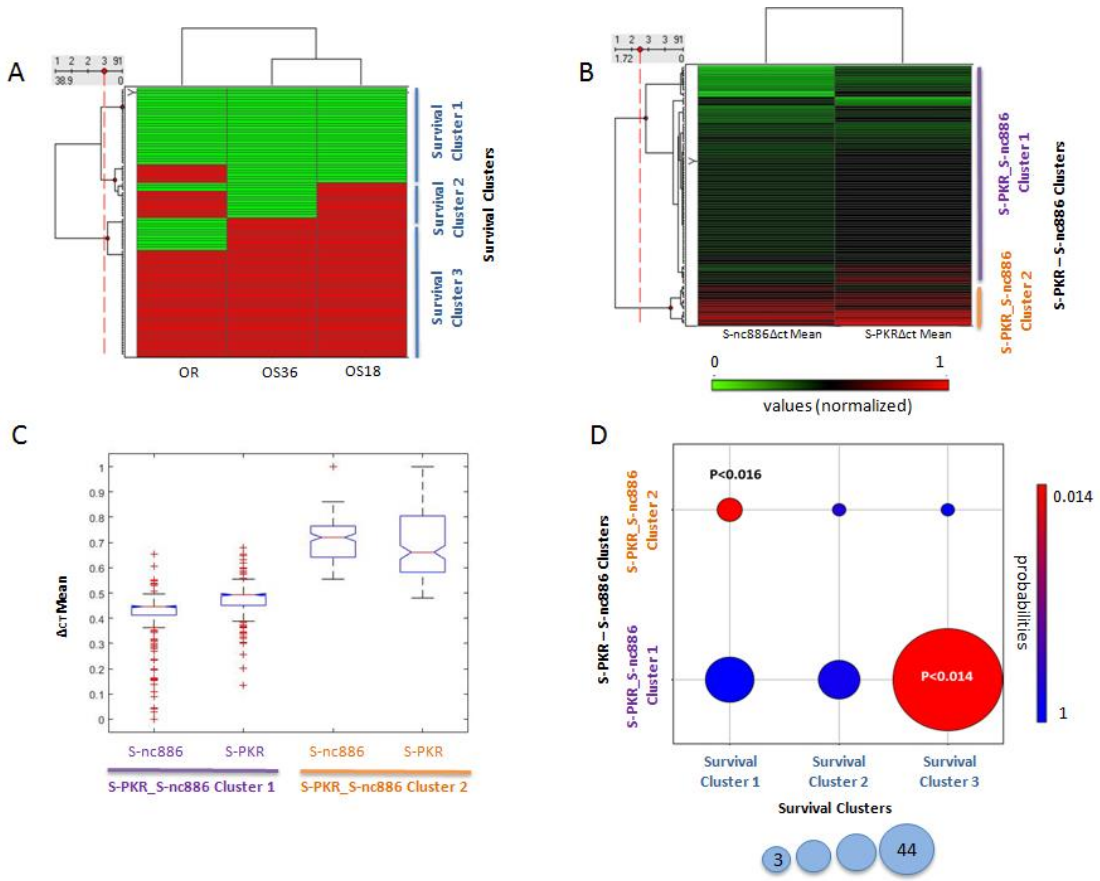


Figure 24. Correlation between the objective first-line chemotherapy response and the time-range patient survival with the Δct mean values identified by RT qPCR in colon healthy (S) tissue. *A) Survival Clusters: Biclusters of subjects according to their objective first-line chemotherapy response, and survival after 18 and 36 months. Values are coded as follow: negative response in green and positive response in red. B) S-PKR-S-nc886 Clusters: Biclusters of patients according to their Δct mean values of nc886 and PKR in healthy tissue (S-nc886 Δct Mean, S-PKR). Δct mean values are normalized between 0 (green) and 1 (red). C) Boxplot of S-PKR and S-nc886 Δct mean values in healthy tissue for each S-PKR-S-nc886 Cluster. D) Co-clustering between Survival Clusters and the S-PKR-S-nc886 Clusters. P-values are calculated with the Hypergeometric statistics. Color code for p-values statistical significance indicates high (red) to low (blue). The size of the circles indicates the number of individuals in the relationship.*

5. Discussion

The identification of biomarkers that associate or predict the benefit of an appropriate selection of patient candidates for both 5-FU-based and combined therapies constitutes a broad area useful in clinical and translational research of CRC disease. However, the low specificity of chemotherapy and the great heterogeneity of the patients and samples analysed make very complex the predictive biomarkers search (Vacante et al., 2018). Although tests for MSI and for the detection of 18qLOH in early stage of the disease are beginning to be evaluated for guiding therapeutic decisions regarding the administration of 5-FU-based treatments, however, these are still under investigation. Then, it is necessary to explore new biomarkers that can increase the portfolio to the oncologists and facilitate to take the better decision in the treatment of CRC patients.

In many cancers, mutation or abnormal expression or activity of protein kinases is correlated with tumorigenesis, metastasis and resistance to chemotherapy. This study identified clusters of metastatic colon cancer patients based on the kinase PKR and its modulator nc886 after the analysis in tumours and healthy samples in relation to the response to chemotherapy based on the use of 5-FU drug (Table 6).

Metastatic colon cancer patients		**Total**	**Sex**				**Age (mean years), SD**	
			Male	**Female**				
		197	127	70			65,1±10,5	
Firs-line Chemotherapy Response clusters (OR)		**Total**	**Responders(+)**		**Non-Responders(-)**		**Δct mean values, SD**	
		197	128		69			
P-nc886_S-nc886							**T-nc886**	**P-nc886**
	Cluster 1	160	110		50		0,427±0,09	0,289±0,05
	Cluster 2	37	18		19		0,567±0,17	0,495±0,14
S-PKR_S-nc886							**S-nc886**	**S-PKR**
	Cluster 1	77	45		32		0,386±0,12	0,465±0,09
	Cluster 2	14	6		8		0,682±0,09	0,703±0,16
Survival clusters (OS)			**First-line Response**		**OS 18 m**		**OS 36 m**	
Survival			**+**	**-**	**Survival**	**Exitus**	**Survival**	**Exitus**
	Cluster 1	26	0	26	0	26	0	26
	Cluster 2	18	15	3	12	6	0	18
	Cluster 3	47	36	11	47	0	47	0

Table 6. Clusters of patients associated to Objective first-line chemotherapy response and to Overall survival at 18 and 36 month.

In order to group patients sharing similar features within the existing heterogeneity, we have approached this ambispective study using NMF techniques encoded into the PGMRA system (Arnedo et al., 2013). This system was successfully used to identify complex genotypic-phenotypic architectures of mental disorders and personality traits (Zwir et al., 2019)**,** and now customized for cancer phenotypes. In contrast to classical clustering techniques, not all features are included in such associations, but those that provide a multifaceted description of groups of patients at risk. These meaningful associations are termed biclusters (Arnedo et al., 2013).

When analysing the Δct mean values of nc886 in P and T we identified two biclusters composed of subjects sharing Δct mean values of T-nc886 and P-nc886 that were associated with the OR to first-line chemotherapy. We found a significant association between patients with high levels of nc886 expression in both, P and T, and a positive primary response to treatments based on 5-FU (Figure 23). Our results replicate previous ones that show that nc886, as a Pol III transcript, is expressed abundantly and ubiquitously in all normal human tissues and that its expression is increased in cancer cells (Park et al., 2017). In fact, most patients in our study expressed high levels of nc886 when considering the Δ*ct* mean values obtained in tumours (Table 6). The tumour suppressor role of nc886 has been already previously related to a better prognosis of the disease in several neoplasia such as lung, ovarian, and breast cancer among others (Cao et al., 2013) but here is the first time that is related to CRC.

Moreover, we identified a smaller second significative cluster associating patients that showed a negative response to first line treatment with lower levels of nc886 expression in the tumour. This result agrees with the previous results where the expression of nc886 was found to be diminished or silenced in a subset of malignant cells by the DNA hypermethylation of its promoter's CpG island (Treppendahl et al., 2012). Although we have not analysed the level of silencing of nc886, our results are consistent with the poor outcome detected during nc886 epigenetic repression in several neoplasms, supporting the role of tumour suppressor of nc886 also in colon cancer disease.

The levels of PKR mRNA expression could not be associated to chemotherapy response in our analysis. The Ser/Thr kinase PKR is a non-canonical kinase involved in many cellular pathways exerting various functions on cell growth and tumorigenesis (Garcia-Ortega et al., 2017). Although the multiple studies over PKR, the exact role in

cancer biology remains controversial. This is due on one side to its ability to induce eIF2α-mediated apoptosis and on the other side to NF-κB-mediated pro-survival effect, involved in both, tumour suppressive or oncogenic roles. Since we previously identified PKR as a molecular target of 5-FU in several colon cancer cells lines playing an important role in the cytotoxic effect of 5-FU, through the induction of apoptosis, in a PKR expression dependent manner, we expect a high expression levels of PKR gene in responder patients. However, the high level of post-translational modifications and regulation of the protein indicate that PKR activity does not necessarily have to correspond to the amount of its messenger RNA. In fact, numerous proteins have been described to regulate their activity (e.g. PACT, TRBP, NPM, etc.) (García et al., 2006) and several post-translational modifications has been showed by SUMOylation, and ISGylation, among others (Kang & Tang, 2012). Nc886 has been described as a PKR inhibitor, being the inhibition of PKR/NFκB in correlation with its tumour suppressor activity. However, recently researchers have demonstrated that nc886 can adopt two structurally distinct conformers that are functionally opposing regulators of PKR existing a second conformation able to activate PKR. Therefore, if the high level of nc886 related to a best response to 5-FU treatment correspond with a high ability of PKR for induce apoptosis is still unknown and need further investigations. In addition, a different location of PKR in the nucleus and nucleolus has been demonstrated with different forms that also suggest differences in its activity. It has been described that PKR localizes in the cytoplasm, strongly in the nucleolus, and diffusely throughout the nucleoplasm (Tian & Mathews, 2001).

Our analysis of total PKR expression by immunohistochemistry in 76 samples of colon tumour and their respective healthy colon tissues have shown expression in the cytoplasm in all healthy tissues and in most of colon tumour analysed. However, a smaller group of tumours have shown diffuse staining in the nucleus and, above all, staining restricted exclusively to the nucleolus. The PGMRA analysis considering presence or absence of PKR in the nucleolus found a bicluster where patients with PKR location in tumour cytoplasm (and absence in the nucleolus) were related with OR to first-line chemotherapy. A cluster included patients whose PKR location in the cytoplasm of the tumour cells corresponded with a positive response to the treatment, and although the levels of expression of PKR and nc886 analysed were variable between samples, were necessary to establish statistically significant clusters.

In contrast, patients with PKR restricted to the nucleolus in the tumour could be grouped significantly in a cluster that corresponded with the negative response to treatment. Although the role that PKR activity may have in the nucleus/nucleolus is not yet known, it has been suggested that PKR exists in leukaemia cell lines and patient's samples in diverse molecular weight forms in the nucleus as result of several post-translational modifications. Whereas cytoplasmic location was detected in leukaemia low-risk patients, nuclear location was restricted to high-risk patients. In addition, intrahepatic PKR nucleolar labelling was observed in PBMCs and liver biopsies, with a suggested ribosome biogenesis role (Piazzi et al., 2019).

Finally, we found different survival clusters in those patients for which information was available (Table 6) allowing to group patients according to the expression of nc886 and PKR in healthy colon tissue. Interestingly we found two clusters significantly related to the outcome of the patients; the most representative cluster included patients with higher expression levels of nc886 and medium expression levels of PKR in the healthy colon tissues whose were alive after 3 years of the first-line treatment. In contrast, lower levels of expression of both, PKR and nc886 in healthy colon tissue were related with patients who died at year and a half after the first-line treatment. Although PKR levels do not appear to be related to their activity, as we have previously commented, for tumour cells, interestingly they remained average in healthy tissue in the cluster of patients with the best outcome, and showed less expression in healthy tissue for the cluster with worse outcome. The role of PKR as a cellular stress response protein is widely known since PKR intervenes against numerous and varied infections, also eliminating damaged cells inducing apoptosis. In addition, PKR is able to allow that the cells live *via* NF-kB activation once the stress has been resolved on time. Moreover PKR also regulates some tumour suppressors and protein kinases involved in cancer pathways such as the signal transducers and activators of transcription factors (STATs), activating transcription factors (ATFs), tumour suppressor p53 (Tp53), the phosphatase and tensin homologue tumour suppressor (PTEN), the mitogen-activated protein kinases (MAPKs) and the toll-like receptors (TLRs), among others. All these data suggest how important is that PKR is expressed at adequate levels in normal tissue where it would be slightly regulated, and the importance that PKR would have in tumours where regardless of its expression, its regulation can be critical.

6. Conclusions

In summary, although it would be convenient to increase the "n", especially for studies where we have had less available data, we can consider that PGMA is a useful system for working with heterogeneous diseases as cancer. PGMA analysis has allowed to identify clusters where the levels of expression of nc886 can be suggested as a potential biomarker for both the first-line response to chemotherapy and the survival of patients for as lest 18 or 36 months. The higher levels of expression of nc886 in tumors, plasma and healthy tissues, have been found in those patients with better outcome. Although it is necessary to analyze a greater number of subjects to know the role of PKR as a biomarker, our data suggest that its location in the tumor cells compartments, but not its mRNA expression level could predict the response to treatment based on the use of 5-FU in metastatic colon cancer patients.

CHAPTER III:
EFFICACY OF IFN-α AGAINST MELANOMA CANCER STEM CELLS

1. Abstract

Malignant Melanoma (MM) is the most aggressive and life-threatening skin cancer whose incidence is increasing worldwide. This neoplasia is characterized by an extraordinary propensity for dissemination to distant organs and resistance to chemotherapy, in part due to the existence of melanoma cancer stem cells (CSCs) subpopulations. Non metastatic high-risk melanoma is still treated with high dose of interferon-α (IFN-α) with a significant improvement in disease free survival in patients, but today there is controversy about continuing its use as treatment. This cytokine possesses anti-proliferative, anti-angiogenic and immune-modulator properties, however its specific activity over melanoma cancer is still unknown, and its adverse effects are negatives. In this study, we have analyzed the effect of low and high dose of IFN-α-treatment over melanospheres enriched in CSCs subpopulations. For this aim, we measured the ALDH activity, the side population, and specific surface markers exppression by flow cytometry in both a established MM cell line and a primary cell line derived from a patient. In adittion, we evaluated the clonogenicity ability of IFN- treated cells by soft-agar assay, the migration capacity of cells by wound-healing assay and the *in vivo* anti-CSCs properties. Moreover, we studied by microRNA sequencing (miRNA-seq) and microarrays the molecular modulation of miRNAs and genes related to stemness properties, respectivley. Our results showed that low and high doses of IFN-α decreased the melanospheres formation and all the stemness properties including a significant reduction in the ability to form tumors in mice xenotransplants. In addition, in this work, we demostrated the effect of IFN-α on exosomes from melanoma MM CSCs by tranmission electron, scanning electron and atomic force microscopies, western blott, NanoSight and LC-HRMS-based metabolomic analysis. Since new immunotherapies are being imposed in melanoma and other solid tumors, as well as different combinations are under clinical trial to avoid resistances, the efficacy of interferons over CSCs even at low doses with fewer side effects, should be considered as a potentially important combination treatment against the relapse of the disease in oncology.

2. Introduction

Malignant Melanoma (MM) is the most aggressive and life-threatening skin cancer whose incidence is increasing worldwide (Schadendorf et al., 2018). The incidence

of cutaneous melanoma has been increasing since the last 20 years, with the most affected regions being those of European origin (white population) with 232,000 new cases diagnosed in the world, positioning itself as the third most common cancer among men in the US and Europe (Ferlay et al., 2015; Schadendorf et al., 2018). The WHO estimates that in 2020, this type of tumor was responsible for 80% of deaths from skin cancer in Spain. Moreover, according to the Medical Oncology Spanish Society, the relapse rate of non-metastasic high risk MM patients is over 80% in Spain. Many treatments have been used to try to reduce this rate (chemotherapy, immunotherapy, radiotherapy, etc.) without any significant improvement.

Several groups have proposed the hypothesis that postoperative adjuvant therapy with IFN-α might improve the recurrence-free survival (RFS) and overall survival (OS) of patients with disease who are at a high risk of recurrence (Berrocal et al., 2018). The first statistically significant improvement in RFS and OS was demonstrated in the Eastern Cooperative Oncology Group (ECOG) study E1684, testing high-dose IFN-α2b *versus* observation in patients with deep primary melanoma or lymph node metastasis. There was 40% of reduction in the hazard ratio of RFS and 28% reduction in the hazard OS (Kirkwood et al., 1996).

Interferons (IFN) are a heterogeneous group of glycoproteins classified into type I (IFN-α, IFN-β), type II (IFN-γ) and the type III (Pestka, 2007). The action of these cytokines is mediated by specific cell surface receptors that activate Jak-STAT pathway. This pathway involves the tyrosine kinase Jak and the STAT transduction factors that result in the stimulation of more than a tree hundred genes transcription (Xin et al., 2020). Modulating these genes, IFN exhibits a wide breadth of biological activities: antiviral, anti-proliferative, anti-tumoral, stimulation of cytotoxic activity of a variety of cells of the immune system (T-cells, natural killer cells, monocytes, macrophages and dendritic cells), increases the expression of tumor-associated surface antigens and other surface molecules such as major histocompatibility complex class I antigens (MHC-I), induction of proapoptotic genes and proteins (e.g. TRAIL, caspases, Bak and Bax), repression of anti-apoptotic genes (e.g. Bcl-2, IAP), antiviral action, etc.

All these activities make IFN a promising agent for treatment of various diseases (Nathan & Eisen, 2002; Pestka, 2007). The Food and Drug Administration approved in 1996 IFN-α for some viral diseases and other kind of tumors such as hairy cell leukemia, MM, follicular lymphoma, *Condylomata acuminate*, AIDS-related, Kaposi sarcoma and chronic hepatitis B and C. In addition, off-label use of IFN-α is prevalent in many cancers,

especially in bladder and renal, often as an adjuvant in conjunction with other therapeutics (Conlon et al., 2019; Meyer, 2009).

High risk MM is characterized by an extraordinary propensity for dissemination to distant organs and resistance to chemotherapy, in part by the existence of MM cancer stem cells (CSCs) subpopulations. CSCs are a type of tumor cells able to initiate tumors, drive their progression and cause metastasis (Davar et al., 2012).

In the context of their clinical importance, CSCs are recognized to be highly resistant to chemo and radiotherapy, and can also escape from the cytotoxic action of the host immune cells. CSCs represent a small fraction of the total cell population in a solid tumor and are defined by their ability of self-renewal and to produce cells that differentiate (Yu et al., 2012). Expression of cell surface markers has been used to isolate and enrich CSCs *in vitro* from different tumors, being also tissue type-specific.

Malignant melanoma CSCs overexpress the following cell surface markers: CD20, CD44, CD133 and the membrane transporter ABCB5, have high activity of the enzyme aldehyde dehydrogenase (ALDH) and possess abilities to form new and complete solid tumors *in vivo* or to metastasize (Jiménez et al., 2018). Besides, CSCs gain the characteristic of epithelial-mesenchymal transition (EMT) that supplies the property of change their phenotype from epithelial to mesenchymal and from mesenchymal to epithelial. This facilitates the metastasis process from the primary tissue to distant organs (Yu et al., 2012; Zhu et al., 2014).

Moreover, the interactions between MM CSCs and their microenvironments are involved in the establishment of permissive niches in distant organs from the primary tissue in order to promote the successful outgrowth of cancerous cells before they arrive at these sites by the secretion of soluble factors and extracellular vesicles (EVs), thus originating the secondary or metastatic tumour (Hernández-Camarero et al., 2018). EVs are a heterogeneous group of small membrane vesicles released by diverse populations of normal and malignant cells. According to the origin and the size, membrane vesicles can be classified in two main groups: i) microvesicles (MVs) that are exosomes budded directly from the plasma membrane and have a size of 100-1500 nm, and ii) exosomes that are released by the fusion of multivesicular bodies with the plasma membrane and have a smaller size comprised between 30 to 150 nm. Thus, these EVs enclose specific lipids, proteins and RNA contents (Raposo & Stoorvogel, 2013), which depend on the physiological state of the producing cell (Pegtel & Gould, 2019). In this study, we analyzed the effect of low and high dose of IFN-α-treatment over melanospheres enriched

in CSCs subpopulations from the A375 MM established cell line and the MEL-1 primary cell line. For this aim we measured the ALDH activity, the side population, the specific stemness surface markers expression by flow cytometry. In addition, we evaluated the clonogenicity ability of treated cells by soft-agar assay, the migration capacity of cells by wound-healing assay and the anti-CSCs properties in mice xenotransplants models. In this work, we demonstrate by miRNAseq and microarrays how IFN-α affects the expression of miRNAs related to stemness properties and how IFN-treatment modulates genes expression involvement in cancer biology. In addition, we studied the effects of IFN-α on exosomes from MM CSCs by NanoSight, TEM, SEM western blot and LC-HRMS-based metabolomic analysis.

3. Materials and Methods

3.1. Cell Culture and CSC enrichment

MM cancer cell lines were obtained from A375 and MEL-1 cells cultures. The line A375 was obtained from American Type Culture Collection (ATCC); whereas the human primary MEL-1 cell line comes from a malignant metastatic melanoma (stage M1a) skin biopsy (BBSPA-Mel#1), and was provided by the Biobank of the Andalusian Public Health System (Spain). This cell line is hipotriploid (complex karyotype with multiple numerical and structural chromosome abnormalities), MelA positive, p53 positive and S100 positive, and has high tumorigenic ability. Melanoma adherents cells lines were maintained at standard culture conditions in a humid incubator at 37°C and 5% CO_2, with DMEM (Dubelcco's Modified Eagle's medium, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich) in 75 cm^2 flask culture (Nunc, Roskilde, Denmark) unless otherwise indicated. FBS was inactivated by heating at 56 °C for 45 minutes. Cells were assayed for mycoplasma contamination.

Enriched MM CSCs subpopulation were obtained after culturing as primary and secondary spheroids in serum free medium and under anchorage-independent conditions as described by Jiménez et al (Jiménez et al., 2018). Briefly, for primary spheroids culture cells were plated in serum-free spheres culture medium (DMEM:F12, 1% P/S, B27, 10 µg/mL ITS, 1 µg/mL Hydrocortisone, 4 ng/mL Heparin, 20 ng/mL EGF, 10 ng/mL FGF,

10 ng/mL IL6, 10 ng/mL HGF) in ultra-low adherence 6-well plates (Corning, Corning, NY, USA) previously collected by centrifugation (1500 rpm for 10min). Then, the pellets were resuspended three twice in PBS (phosphate buffered saline). For the secondary spheres culture, cells from primary spheroids were collected by centrifugation (1500 rpm for 10min), and, then, the pellet was resuspended in DMEM:F12 sphere medium mechanically disrupted with a pipette and by syringing three to five times through a sterile 25-gauge needle. After that, cells were plated, resuspended and incubated for 72 h in spheres culture medium in ultra-low adherence 6-well plates and treated with differents IFN-α concentrations: 2000 IU/mL (low dose) and 20000 IU/mL (high dose). The IFN-α was facilited by Hospital Pharmacy Service at Hospital Univesitario Virgen de las Nieves (Granada, Spain) under the commercial name of INTRON A® (Interferon ALFA-2b for Injection -10million I.U. per 1 mL x 1 vial- of MSD laboratories) (John M. Kirkwood et al., 2000).

3.2. Sphere-Forming Assay

To determine the self-renewal ability of the MM CSCs population, sphere-forming assay was performed (Morata-Tarifa et al., 2016a). For the secondary sphere-forming assay, 2.5×10^5 single cells derived from primary spheroids were plated and resuspended in spheres culture medium in ultra-low adherence 6-well plates (Corning) and treated with the same IFN-α concentration detailed above.

Secondary spheres > 75 µm diameter were counted after 3 days by light microscopy. Diameters were measured using the ImageJ software.

3.3. Colony-Formation Assay

The clonogenic capability of MM CSCs was determined by colony-formation assay in soft agar as previously described (Morata-Tarifa et al., 2016a) with minor modifications and treated with IFN-α. Briefly, 10^4 cells coming from secondary spheroids were seeded in 0.4% cell agar base layer, which was on top of 0.8% base agar layer in 6-well culture plates (Morata-Tarifa et al., 2016a).

Then, cells were incubated for further 27 days at 37 °C and 5% CO_2, adding 100 µL of DMEM (10%FBS, 1% P/S) every 1-2 days. Cell colony formation was then examined under a light microscope after staining with 0.1% Iodonitrotetrazolium Chloride (Sigma-Aldrich). The size of colonies was measure using ImageJ™ software.

3.4. Aldefluor assay and phenotypic characterization by Flow cytometry

The analysis of CD20, CD44 and CD133 surface markers and the ALDH1 activity were done using a Becton Dickinson FACSCanto II flow cytometer from the CIC Scientific Instrumental Centre (University of Granada) as previously described (Morata-Tarifa et al., 2016a). Briefly, ALDEFLUOR assays (Stem Cell Technologies, Vancouver, Canada) to detect ALDH1 activity in viable cells were performed according to manufacturer's instructions. Cells lines were suspended in aldelfuor assay buffer containing ALDH1 substrate (BAAA, 1 μmol/l per 1×10^6 cells) and incubated during 45 minutes at 37°C in darkness. Dethylaminobenzaldehyde (DEAB) was used as an ALDH1 inhibitor to set ALDH1 gates. The brightly fluorescent ALDH1-expressing cells were detected in the green fluorescent channel (520-540 nm).

Cell surface levels of CD20, CD44 and CD133 were determined with anti-human antibodies CD20-allopycocyanin (APC), CD44-phycoerithrin (PE) and CD133-allopycocyanin (APC) (MiltenyiBiotec, BergischGladbach, Germany), respectively. All samples were analysed on a FACS CANTO II (BD Biosciences, San Jose, CA, USA) using the FACS DIVA software (Aktas et al., 2009).

3.5. Side Population Assays

Hoechst 33342 exclusion (Side Population) assays were carried out as previously described (Shimoda et al., 2018) to analyse cells overexpressing ABC transporters. Melanospheres were stained with Hoechst 33342 (Sigma-Aldrich) dye, as negative controls, Verapamil (Sigma-Aldrich) was used for maintaining the efflux channel closed inhibiting the capacity to efflux Hoechst 33342 by cells. The brightly fluorescent cells were measured by flow cytometry in Hoechst blue (440/40) and Hoechst red (695/40) of a FACScan Aria III (BD Biosciences) using FACS DIVA software from the CIC Scientific Instrumental Centre (University of Granada). Cells with the ability to efflux Hoechst 33342 were considered as the side population (SP) (Shimoda et al., 2018).

3.6. Wound-healing assay

A375 and MEL-1 MM cells lines were seeded in 6-well low attachment plates and IFN-α treatment was added at 2000 and 20000 IU/mL. Wounds were created by scraping monolayer cells (after disrupted by syringing and plated in 6-well) with 200 μL pipette tip, and non-adherent cells were washed off with medium. At 0, 24, 48 and 72 hours after

the creation of wounds, IFN-treated and control non-treated cells were observed and photographed with a 10X objective in a by light microscopy.

Wound distances were measured at each time point and expressed as pixels-area^2 migration of wound closure by comparing the zero time. Image-J software was used to quantify the wound area (Perán et al., 2017).

3.7. Cell cycle analysis

Cell cycle analysis was performed using the propidium iodide assay. After 3 days in different culture IFN-α conditions, melanospheres were harvested, washed twice with PBS, and fixed in 70% (vol/vol) cold ethanol for up to 1 week. Next, the cells were centrifuged, and the pellet was washed once with phosphate-buffered saline and resuspended in 250μL of propidium iodide solution (100μL/mL RNAse, 40μL/mL propidium iodide in phosphate-buffered saline) for 30 minutes in the dark at 37°C. All samples were analyzed in a FACS Canto II cytometer (BD Biosciences).

3.8. Apoptosis

After 3 days in different IFN-α dose, MM CSCs were washed and mechanically disrupted with a pipette and by syringing three to five times through a sterile 21-gauge needle. After, spheres were analyzed using an Annexin V-fluorescein isothiocyanate detection kit (eBioscience Inc.). All samples were analyzed in a FACS Canto II cytometer (BD Biosciences).

3.9. Microarray profiling and analysis

A375 and MEL-1 MM CSCs were treated with IFN-α for 24h. Non treated cells were used as control. Total RNA was extracted using Qiagen extraction kit, according to the manufacturer's protocol. Transcriptome microarray profiling was carried out using Clariom™ S Assay, Affymetrix Human arrays according to the Affymetrix standard protocol. Data analysis was performed using TAC 4.0 (Transcriptome Analysis Console) Thermofisher software, R and the CRAN package VennDiagram (Chen & Boutros, 2011). In order to establish relationships between selected genes, String data-base was used (Baglivo et al., 2019).

3.10. miRNA NGS profiling of MM CSCs and differential expression analysis

For each cell line, A375 and MEL-1, three different types of libraries were prepared:untreated MM adhered (non stem-like) cells, untreated MM CSCs and IFN-α stimulated MM CSCs. Treated MM CSCs were exposed to a low dose of IFN-α for 30h prior to RNA extraction. Each of the cell line-condition combinations described was performed and profiled in duplicate (6 conditions, 12 RNA libraries).

For sequencing library preparation 1 μg total RNA was used and libraries were prepared according to the TruSeq Small RNA Sample Prep Kit (Illumina) protocol with automated pooled library size selection using Pippin Prep (Thermo Fisher Scientific). Concentration and size profile of the sequencing libraries were measured using Bioanalyzer (DNA 1000 assay) and KAPA library quantitation kit qPCR determined that the pool concentration was 11.91 nM. The pool of samples was run in one lane on a HiSeq2500 instrument (Illumina) for 50 cycles. Resulting sequencing files were processed with sRNAbench (Aparicio-Puerta et al., 2019) and using miRBase (release 22) as miRNA annotation (Kozomara et al., 2019). Quality control was performed using mirnaQC with all samples passing minimum quality criteria and no outliers detected (Aparicio-Puerta et al., 2020).

Count values were normalized using the Variance Stabilizing Transformation and differential expression analysis was performed using DESeq2 (Love et al., 2014). miRNAs were considered to be differentially expressed for fold changes above 2 or below 0.5 and False Discovery Rate (FDR) corrected p-values below 0.05. Consistently under- or overexpressed genes across cell types were systematically detected for each given comparison and included in downstream analysis.

3.11. Quantitative real time-PCR (qRT-PCR)

To confirm reproducibility of miRNA expression profiles obtained by miRNAseq, qRT-PCR validation experiments were carried out. Technical and biological replicates of RNA were prepared for untreated cells and after IFN-α stimulation for 30 h. cDNA was synthesized by reverse transcription of total RNA using the Reverse Transcription System (Promega) for mRNA, and miRCURY LNA TM Synthesis kit II (Exiqon) for miRNAs.

qRT-PCR assay was done using SYBR Green PCR Master Mix (Promega) and miRCURY LNA TM EXILENT SYBR Green (Exiqon) for miRNAs. Each experiment was performed in duplicate and reactions were performed in triplicate. The comparative threshold cycle (Ct) method was used to calculate the amplification factor as specified by the manufacturer ABI 7500. QRT-PCR consisted of 45 cycles of 95 °C for 10 sec, 60 °C for 40 sec, and 72 °C for 1 sec, after an initial denaturation step (95 °C for 10 min).

Expression levels were normalized to SNORD44 and U6 as the internal controls and quantified by the comparative Ct (ΔΔCt) method. Primer sequences are listed in Annex I (Supplementary data Table S2 in Annex II).

3.12. *In vivo* tumor xenograft assays

For xenograft assays, they were used CSCs from A375 and MEL-1 MM cell lines. After 72 hours of treatment with low doses of IFN-α, 500 cells (after disrupted with a syringing of each condition) were injected in 0.05 ml matrigel and 0.05 ml of culture medium by subcutaneous injections to 8 week old NOD scid mice gamma (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ, NSG).

Tumor growth was assessed twice weekly using a digital calliper and the tumor volume was calculated by the formula $V= length^2 \times width \times \pi/6$. Animal experimentation was performed according to the protocols reviewed and approved by the Institutional Animal Care and Use Committee of the University of Granada (PI730/13). After 40 days, the tumors were sectioned and embedded in paraformaldehyde (PFA).

3.13. Immunohistochemistry

Tumors of different conditions were immersed in 4% PFA in 0.1 M PBS for 4 h at 4°C, washed in 0.1M PBS and embedded in paraffin in an automatic tissue processor (TP1020, Leica, Germany). The paraffin blocks were cut into 4 mm sections and subjected to immunohistochemistry. Sections were deparaffinized with xylene and hydrated with decreasing alcohol concentrations (absolute to 70%) and antigen retrieval was performed at 121°C for 15 min in a sodium citrate buffer solution (pH 6.0). Endogenous peroxidase activity was blocked by incubation for 30 min with 0.3% hydrogen peroxide in methanol.

The tissue sections were then incubated with rabbit anti-p75 antibody (Abcam), rabbit anti-SAMD9 antibody (Abcam), rabbit anti-CD133 antibody (Abcam) and mouse

anti-CD44 (Santa Cruz Biotechnology) in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) overnight at 4°C. Bound antibodies were detected with Envision FLEX+, Mouse, High pH (LINK) and stained with Autostainer Link 48, using diaminobenzidinetetrahydrochloride (Dako) as the substrate.

The sections were then counterstained with Mayer's hematoxylin. Negative control tissue sections were prepared by omitting the primary antibody. Observation under light microscopy and digital image acquisition was carried out with an inverted microscope (Nikon H550s). Its immunofluorescence intensity was qualified using ImageJ software.

3.14. Exosome Isolation and Purification

Exosomes were collected from culture supernatants secondary spheres of A375 and MEL-1 MM CSCs and from serum of MM patients by ultracentrifugation as previously described by Costa-Silva *et al.* with minor modifications (Costa-Silva et al., 2015). We set out from ~200 mL of supernatant fractions collected from cell cultures at 72 h with incubation of IFN-α dose in each purification procedure.

To ultracentrifugation process, first, supernatants were centrifuged at 500 x g for 10 minutes. The pellet was discarded and the supernatant was ultracentrifuged at 12000 x g for 20 minutes in a JS-24-38 rotor (Beckman Coulter Inc., Fullerton, California). The pellet contained microvesicles (MVs), which was resuspended in 100 µL of Dulbecco's Phosphate Buffered Saline modified without calcium chloride and magnesium chloride and sterile-filtered (modified PBS; Sigma-Aldrich, St. Louis, MO, USA); whereas the supernatant was ultracentrifuged at 100000 x g for 70 minutes.

The resulting pellet was washed in 35mL PBS and pelleted again by ultracentrifugation at 100000 x g for 70 minutes. Finally, exosomes were obtained in the pellet, whichwas resuspended into 100 µL of modified PBS and stored frozen at -80˚C for further analyses (Figure 24). Repeated freezing and thawing of the exosomes suspensions was avoided.

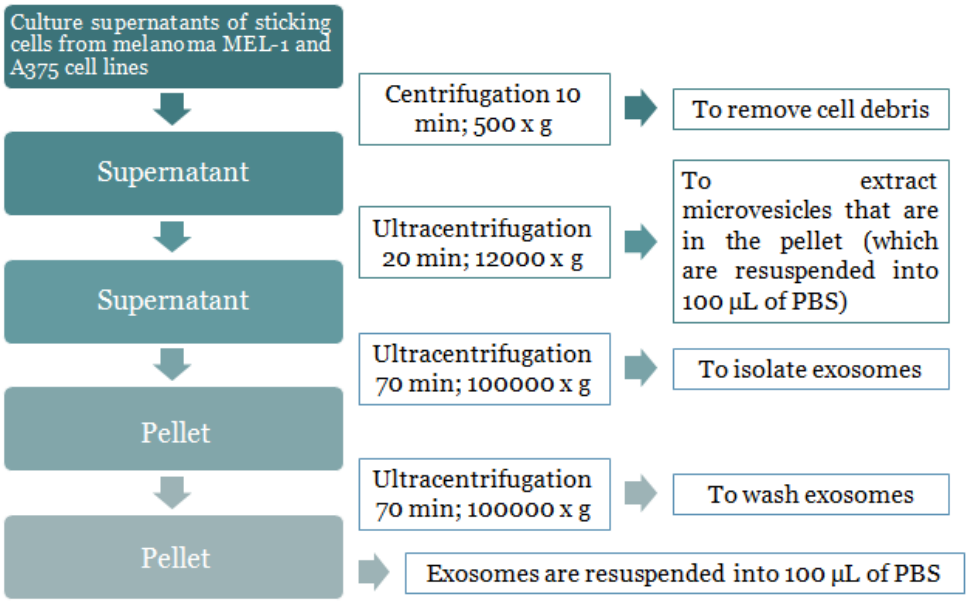


Figure 24. Isolation and purification protocol

3.15. Transmission and Scanning Electron Microscopy

Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) analyses were performed at the Scientific Instrumental Centre (CIC-University of Granada). For TEM and SEM samples were negatively stained with uranyl acetate as follows: a 30 µL drop of the exosome sample was placed on a carbon-coated 300 mesh grid and allowed to adsorb at room temperature for 5 min. The grids were then washed in drops of ultrapure water for 1 min. Adsorbed exosomes were negatively stained by placing the grids on a drop of 1% uranyl acetate in aqueous suspension for 1 min. The excess of fluid was drained with filter paper slightly, and then sample grids were dried at room temperature for 6 min.

The preparations were examined with a LIBRA 120 PLUS transmission electron microscope (Carl Zeiss SMT, Oberkochen, Germany) at an acceleration voltage of 120 kV and the HITACHI, S-510 scanning electron microscope. Then, samples were determinated with the Edwin-Róntec microanalysis system (Palacios-Ferrer et al., 2021).

In addition, pellets obtained from CSCs cultures were immersed in 4% paraformaldehyde, 0.1 M PBS for 4 hours at 4ºC and washed in sucrose in 0.1 M PBS overnight. The fractions were incubated by increase alcohol concentrations and were cut in semithin sections at 0.5 µm with tissue processor (TP1020, Leica, Germany).

3.16. Atomic Force Microscopy

Atomic Force Microscopy (AFM) analyses were performed at the Scientific Instrumental Centre (CIC-University of Granada). For AFM purified exosomes were diluted 1:10 in deionized water. A 10µL drop of exosome suspension was adsorbed to freshly cleaved mica sheets at room temperature for 10 min and rinsed with deionized water to remove salt precipitates.

The sheets were then completely dried under a gently stream of argon gas (Ar). The preparations were examined with a NX20 Atomic Force Microscope (Park Systems, Suwon, South Korea) and images were visualized and processed using the Park Systems XEI software. Measurements were carried out with ACTA cantilevers (40 N·m-1) and in Non Contact Mode (Palacios-Ferrer et al., 2021).

3.17. Exosome Size Analysis

Analyses were performed on NanoSightNS500 instruments (Malvern Instruments, UK). The instrument was equipped with a 488 nm laser, a high sensitivity CMOS camera and a syringe pump. Exosomes were diluted 1:1000 in PBS buffer to obtain a concentration range (1-10 x 10^8 particles/mL). The measurements were analysed using the NTA2.3 software (Malvern) after capture 3 videos of 60 sec.

3.18. Inmunogold Labeling by Transmission Electron Microscopy

Inmunogold labeling of exosomes was performed at the Andalusian Centre for Nanomedicine (Bionand, Spain). Exosomes suspensions were put on copper grid with Formvar-Carbon and incubate 15 min at RT. Dried slightly and was passed to a drop of 15 µl of 2% paraformaldehyde in 0.1 M PBS and incubate 10 min. The samples were transfered to a drop of 15 µL of 2% BSA in 0.1 M PBS, plus the primary antibody Anti-Human CD63 Clone H5C6 (RUO) (Becton Dickinson) diluted 1/500 and incubated 1.5 hour at room temperature and in a humid chamber (Palacios-Ferrer et al., 2021).

After several PBS washed, the grid was incubated with secondary antibody Anti-Mouse IgG (Whole molecule)-gold antibody 10 nm (Sigma Aldrich) and incubated 1 hour at room temperature and in a humid chamber. The samples were maked with a negative stain by passing a drop of 15 µL of 1% uranyl acetate in Milli-Q water for 15 seconds and the preparations were examined with a LIBRA 120 PLUS transmission electron microscope (Carl Zeiss SMT, Oberkochen, Germany).

3.19. Western Blot analysis

The final pellets of cell culture supernatants of CSCs were resuspended in 100 µL of PBS and stored at 4 °C for further protein quantification. The protein concentrations were measured using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) according to manufacturer's instructions.

Proteins extracts (30 µg) were denatured at 95 °C for 5 min in loading buffer (containing Tris – pH 6.8, SDS, glycerol, β-mercaptoethanol and bromophenol blue). Proteins were subjected to 4-20% Mini-PROTEAN TGX (Bio-Rad, USA) gel together with Precision Plus ProteinTM Kaleidoscope Prestained Protein Standards (Bio-Rad, USA). The samples were transferred to a nitrocellulose membrane (Trans-Blot, Mini Format, Bio-Rad) using a transfer apparatus according to the manufacturer's protocols (standard program: 25 V for 30 minutes) (Bio-Rad).

After incubation with 5% skimmed milk in PBS-Tween 0.1% for 1 hour at room temperature, the membranes were incubated overnight with antibodies against CD9 (dilution 1/1500, eBioscience), CD63 (dilution 1/500, Santa Cruz Biotechnology), p75 (dilution 1/500, Abcam), Hsp-70 and Alix (dilution 1/1000, Cell Signaling). Membranes were then incubated with conjugated goat anti-mouse secondary antibody and goat anti-rabbit secondary antibody for 2 h and signals were detected using the ECL-PLUS (Amersham Biosciences).

The bands were visualized with medicals photographic films (AGFA) or detected using the Infrared Odyssey Imager (LI-COR Biotechnology, Lincoln, NE, USA).

3.20. LC-HRMS analysis of exosomes

The metabolomic analyses of exosomes isolated from cell culture supernatant were performed in Fundación MEDINA (Centro de Excelencia en Investigación de Medicamentos Innovadores en Andalucía) as described by García-Fontana, et al. with minor modifications (García-Fontana et al., 2016). Sample preparation for LC-HRMS analysis was performed as follows.

Exosome samples were thawed on ice, vortexed and kept at 4˚C during the analytical process. Proteins were withdrawn using methanol (1:3), shaken, sonicated (1 min) and shaken again. Samples were then centrifuged at 13,300 rpm for 15 min at 4°C. Supernatants were collected and dried under an N_2 air stream. Dried samples were

reconstituted in 90 μL of mobile phase (50% H_2O and 50% acetonitrile at 0.1% of formic acid) and transferred to the analytical vials.

Then, samples were analyzed in triplicate using AB SCIEX TripleTOF 5600 quadrupole-time-of-flight mass spectrometer (Q-TOF-MS) (AB SCIEX, Concord, Canada) coupled to a high performance liquid chromatography (HPLC) system, in positive electrospray ionization (ESI) mode.

Previous to HRMS analysis, chromatographic separation was carried out by an Agilent Series 1290 LC system (Agilent Technologies), equipped with a reverse phase Atlantis T3 HPLC C18 column (C18: 2.1mm x 150mm, 3mm) (Waters). Samples were injected randomly (5 μL per sample) into the HPLC system. Blank solvent (BS) and quality control (QC) samples were also injected throughout the sequence run. The QC samples were prepared by pooling an equal volume of all exosome samples and injected every five samples in order to assess the stability and performance of the system.

The BS samples were also run interspersed in the sequence to detect possible impurities of the solvents or extraction procedure and to check carryover contamination from intense analytes. Generic parameter settings for chromatographic separation and MS detection were used to obtain specific metabolomic fingerprints of the exosome preparations. HRMS analysis was performed using an information-dependent acquisition (IDA) method to collect full scan MS and MS/MS information simultaneously. The method consisted of high-resolution survey spectra from m/z 50 to m/z 1600 and the 8 most intense ions were selected for acquiring MS/MS fragmentation spectra after each scan. An Automated Calibration Delivery System performed an exact mass calibration prior to each analysis.

Data set creation: PeakView software (AB SCIEX) was used in order to evaluate the analytical drift in terms of mass and retention time shift. MarkerView software (version 1.2.1.1, AB SCIEX) was used for processing the LC-HRMS raw data. This software performs peak detection, alignment and data filtering, generating a feature table which defines measured m/z, retention time (RT) and integrated ion intensity. An automated algorithm in the RT range 0.8–19 min and m/z range 50–1600 was used for data mining. The intensity threshold of extraction was established at 50 counts per second. RT and m/z tolerances of 0.1 min and 15 ppm respectively were used for peak alignment.

Background noise was removed by using a specific tool of MarkerView software. The analytical replicates of each sample were averaged.

Analytical validation: QC distribution on PCA plot was used for analytical validation prior to the following analysis. Variables with unacceptable reproducibility (RSD > 30%) or detected in less than 50% of QC samples were also rejected from the data matrix.

Data treatment: Statistical analyses were carried out using MetaboAnalyst 4.0 Web Server (Xia & Wishart, 2011) as previously described (García-Fontana et al., 2016). Briefly, after dataset creation, raw data were normalized (median normalization), transformed (cube root transformation) and scaled (Pareto scaling) in order to achieve a more Gaussian type distribution (Godzien et al., 2013). Then, filtering according to significant differences was done based on statistical analysis including both univariate (UVA) and multivariate (MVA) in order to identify variables (metabolites) that are significantly different between the groups compared. For UVA, a first double filtering procedure with t-test (p-value < 0.05) and fold-change (FC > 1.5) was applied to identify differentially expressed mass signals between BS and exosome samples and therefore discard them as background noise, preserving the peaks from true biological samples.

Then, a t-test based filtering (p-value < 0.05) was used to detect differential metabolites between the sample groups, providing a quality criterion to evaluate variable relevance for further data analysis. For MVA, principal component analysis (PCA) and partial least squares regression (PLS-DA) were carried out after t-test filtering. PCA was applied to assess quality of the analytical system performance. PLS-DA allowed discriminating variables that are responsible for variation between the comparison groups.

For statistical validation, quality description by goodness of fit (R^2) and goodness of prediction (Q^2) was used. A powerful model for diagnostics should show high values of R^2 and Q^2 but also not vary more than 0.2–0.3. For metabolomics data, $R^2 > 0.7$ and $Q^2 > 0.4$ are considered acceptable values (Godzien et al., 2013). The models were also validated using 10-fold cross validation.

3.21. Statistical analysis

All data are presented as the mean ± standard deviation. Differences between groups were analyzed for statistical significance using the two-tailed Student's t-test. P-

value of 0.05 was accepted as the statistical significance level. The different statistical studies have already been reflected in each section of materials and methods.

4. Results

4.1. IFN-α reduces melanospheres proliferation and colony formation capacity of MM CSCs.

After CSCs enrichement of the established A375 MM cell line and a primary patient-derived tumour cell line (Mel1) we analyzed the effect of IFN-α over CSCs characteristics. We studied first, the sphere forming ability, the proliferation rate of secondary melanospheres and the clonogenic capacity by colony-formation assay in soft agar in presence or absence of low and high dose of IFN-α (Figure 25). Cells growing as melanospheres treated with diferent doses of IFN-α had a significantly lower proliferation rate when compared to the control culture.

Secondary spheres from A375 and MEL-1 cell lines treated with low and high doses of IFN-α during 72 h showed an important and significant decrease of number cells with respect to mock spheres as showed in Figure 25 A-B.

In addition, we obseved a significant decrease of diameter in a concentration-dependent manner in both melanospheres from A375 and MEL-1 after IFN-α treatment (Figure 25 C-H). In A375 cells, the size was smaller in secondary spheres treated with IFN-α with an average diameters of 5.7 (low dose) and 2.3 mm (high dose) respectively in comparison to non-treated spheres (6.3 mm) (Figure 25D). Similar trend was found in MEL-1 spheres with a reduction up to 6.7 and 4.8 mm in low and high IFN-α respectively compared to control cells (12.3 mm) (Figure 25G).

Representative optical images for all conditions culture are showed in (Figure 25H and 25E). In concordance with these results, melanospheres showed a high capacity to form colonies in soft agar assay that was significantly reduced after IFN-α treatments as shown for both cell lines (Figure 25I, J).

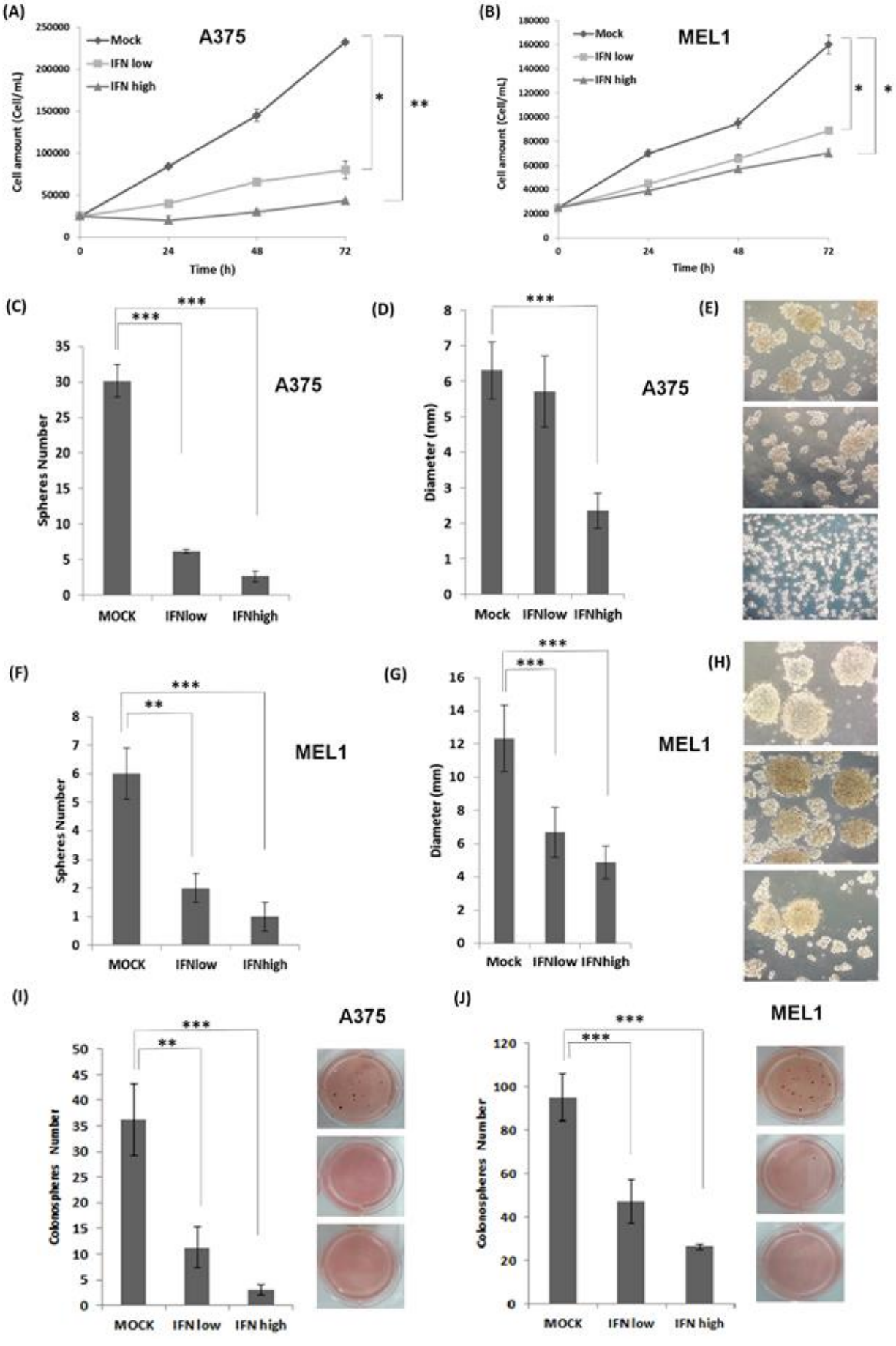

(A)
A375
Mock
IFN low
IFN high
Cell amount (Cell/mL)
Time (h)
(B)
MEL1
(C)
Spheres Number
MOCK
IFNlow
IFNhigh
(D)
Diameter (mm)
(E)
(F)
(G)
(H)
(I)
Colonospheres Number
(J)

Figure 25. Proliferation assay, tumorsphere and colony-forming ability in MM CSCs enriched subpopulations. *(A-B) Proliferation curves of A375 (A) and MEL-1 (B) in suspension cultures after treatment with two differents IFN-α concentrations, 2000 IU/mL (low dose) and 20000 IU/mL (high dose) compared with controls non-treated cells (Mock). The initial cell number plated was 25000 cells per well in all conditions; (C-H) Number of secondary spheres formed by A375 (C) and MEL-1 (F) cell lines growing under anchorage-independent and serum-free conditions and after treatment with different doses of Interferon. Melanospheres were counted after 3 days under light microscopy; Diameter of spheres from A375 cell line (D) and MEL-1 cell line (G) were measured by ImageJ software; Representative light microscopy (4x) images of spheres formed from A375 cell line (E) and MEL-1 cell line (H) with IFN-α: Mock (up), low dose (in the middle) and high dose (down); (I-J) Representative optical image of the colonies formed by A375 (I) and MEL-1 (J) cells from secondary spheroids after 37 days of soft agar culture in P6 well plates, stained with 0.1% Iodonitrotetrazolium Chloride. Data are graphed as mean ± SD from experiments carried-out by triplicates (***P < 0.001; **P < 0.01; *P < 0.05).*

Moreover, the analysis by TEM showed that IFN-α treatment induced modifications and lost of the compaction in cellular and nuclear ultrastructure of MM CSCs (Figure 26).

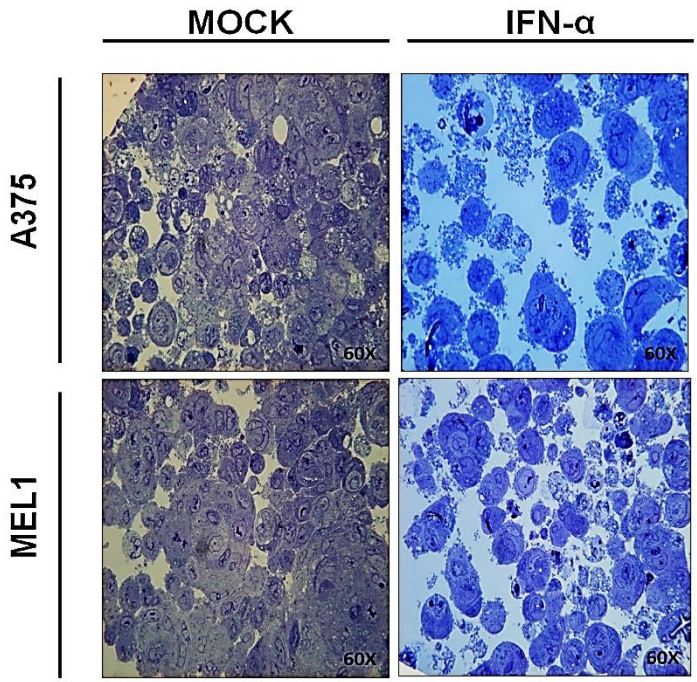


Figure 26. Effects of IFN-α on CSCs ultrastructure. *Confocal microscope images show representative organization of cellular ultrastructure in MM CSCs after low dose of IFN-α treatment. Spheres were observed with toluidine blue (original magnification: 60X) from A375 (up) and MEL-1 (down) cell lines.*

Therefore, IFN-α treatments has a potent antiproliferative effect over both, established and primary patients derived MM CSCs subpopulations.

4.2. IFN-α reduced stemness properties.

To evaluate the effect of IFN treatment over the MM CSCs properties, A375 and Mel 1 secondary spheres were characterized using specific markers such as CD20, CD44 and CD133 expression and ALDH1 activity, in presence and absence of IFN-α treatments (Figure 27). For A375 spheres, ALDH1 activity significantly decreased after IFN-α treatments showing 61.5% (low dose) and 24.4% (high dose) compared with mock non-treated cells (80.2%). Also, stemness surface markers expression was modified after IFN-α treatments, being the reduction more significant after high dose, 14% for CD20, 23% for CD44 and 30.2% for CD133 respect to mock non-treated spheres, which showed an expression of 39.2% (CD20), 52.5% (CD44) and 79.5% (CD133) respectively (Figure 27A).

Similarly, MEL-1 CSCs enriched subpopulations showed an important decrease in ALDH1 activity after IFN-α treatments showing 19.3% (low dose) and 12.1% (high dose) compared to ALDH1 activity in control cells (26.8%). Moreover, stemness surface markers expression decreased after IFN-α treatments, being more significant for the highest dose with 3.95% (CD20), 2% (CD44) and 12.14% (CD133) respectively and in the low dose for CD44 (7.9%) versus mock non-treated spheres (24.15% for CD20, 14.5% for CD44 and 42% for CD133) (Figure 27B).

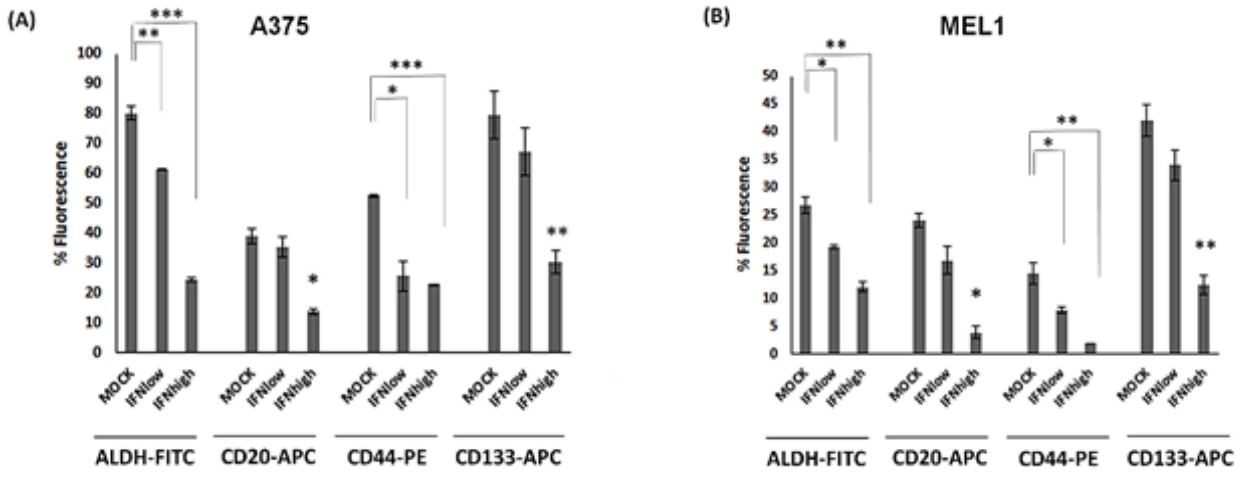


Figure 27. Phenotypic properties of melanoma CSCs. *(A-B) Comparative analysis for ALDH1 activity, CD20+, CD44+ and CD133+ expression in A375 (A) and MEL-1 (B) in spheres cells cultured by flow cytometry after treatment with low and high dose of* IFN-α. *The data were analyzed by T-test, ***P < 0.001; **P < 0.01; *P < 0.05.*

In addition, we decide to analyze the side population (SP) fraction using the Hoechst 33342 staining protocol in melanospheres in absence and presence of the IFN-α treatments. Whereas A375 non-treated secondary spheres showed a 9.9% of SP, however, after treatment SP decreased percentage up to 5.8% and 3.3% for low and high IFN-α dose respectively (Figure 28A). Accordingly, the rate of SP in melanospheres from MEL-1 spheres was significantly higher (20.2%) in control cells respect to treated cells (9.4% for low dose and 6.3% for high dose, respectively (Figure 28B).

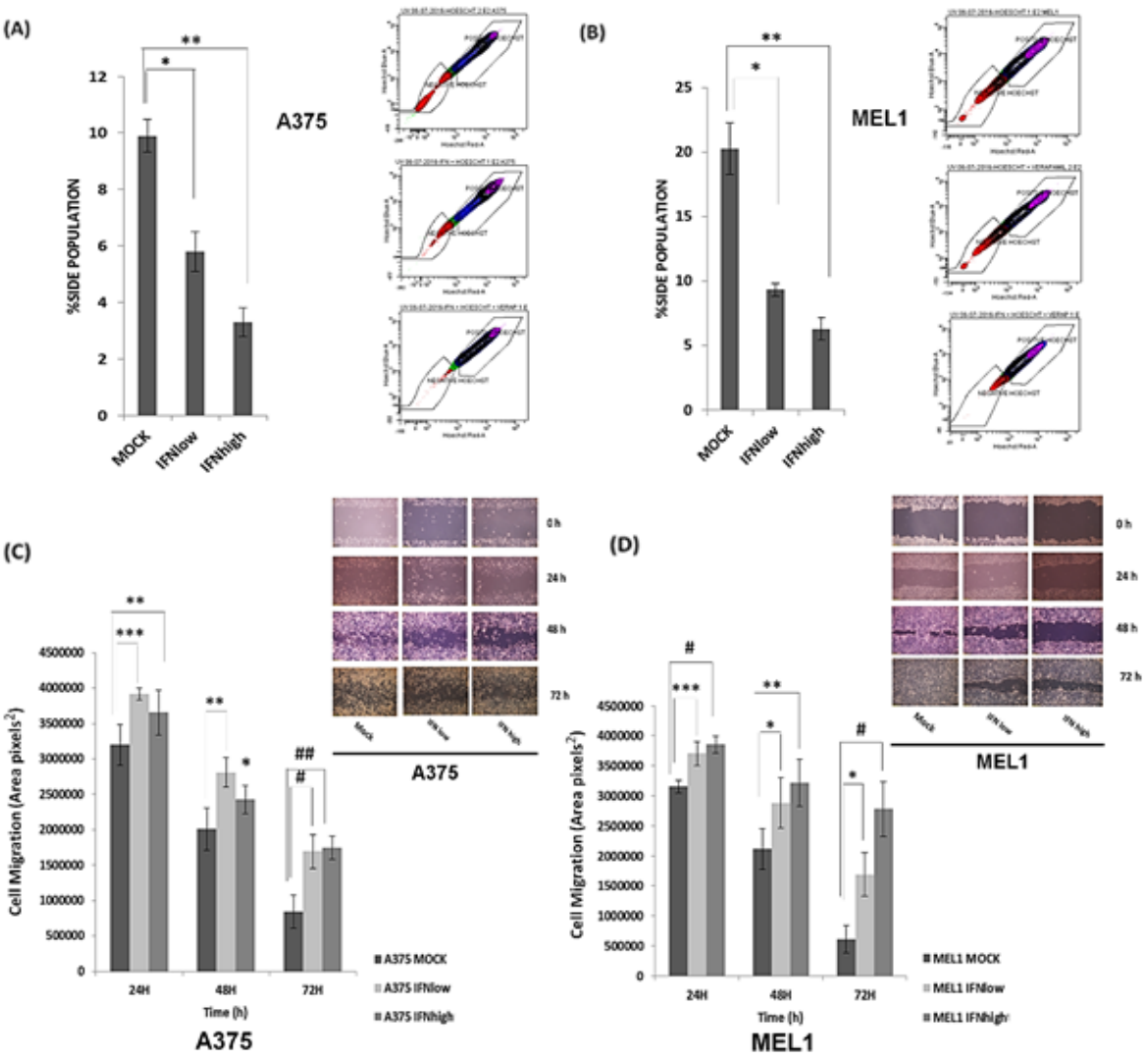


Figure 28. Effects of IFN-α on ability of drug efflux (side population) and cell migration. *(A-B) Effect of IFN-α over SP percentage in (A) A375 and (B) MEL-1 melanospheres; (C-D) Wound healing assay on A375 (C) and MEL-1 (D) MM cancer cell lines. Cell migration was quantified by measuring the wound closure area in pixels^2 at 24 h, 48 h and 72 h with ImageJ software. Representative optical images (10X) show the migration by cells coming from secondary spheroids. The data were analyzed by T-test, ##P < 0.0001; #P < 0.0005; ***P < 0.001; **P < 0.01; *P < 0.05.*

Since CSCs have higher ability to invade and migrate than other cancer cells (Hernández-Camarero et al., 2018), we evaluated the effect of IFN-α treatments on cell migration performing a wound healing assay on A375 (Figure 28C) and primary MEL-1 (Figure 28D) MM cancer stem cell enriched spheres at 24, 48 and 72 hours. Data showed a dose-dependent inhibition of cell migration after both treatments with IFN-α comparing to non-treated cells, which migrated faster to close the gap of a scratch than IFN-α-treated cells for both cell lines.

Altogether, these results indicate that IFN-α inhibits phenotypic and functional stemness properties in a dose-dependent manner for both established and primary MM cell lines.

4.3. Cell cycle regulation and anti-apoptotic effect of IFN-α over MM CSCs.

Cell cycle analyses of melanospheres cultured with both doses of IFN-α showed an accumulation of cells predominantly in S phase, with a concomitant restriction of cells on G1 and G2 phases for A375 and in G1 for MEL1-1 CSCs subpopulations (Figure 29A-B). Moreover, a higher apoptosis rate was found after treatments with high and low doses of IFN-α in comparison with mock non-treated spheres in both CSCs subpopulations (Figure 29C-E).

A375 secondary spheres in control condition showed a percentage of apoptotic (9.3%) that was significantly increased after IFN-α treatments with an average percentage of 17.3% and 24.1% for low and high IFN-α dose respectively (Figure 29C and E). Accordingly, the rate of apoptosis in melanospheres from MEL-1 was significantly lower in control cells respect to treated cells (Figure 29D and E).

Thus, mock cells displayed an average percentage of 4.7%, whereas IFN-α-treated melanospheres showed apoptotic rates of 11.3% (low dose) and 18.8% (high dose).

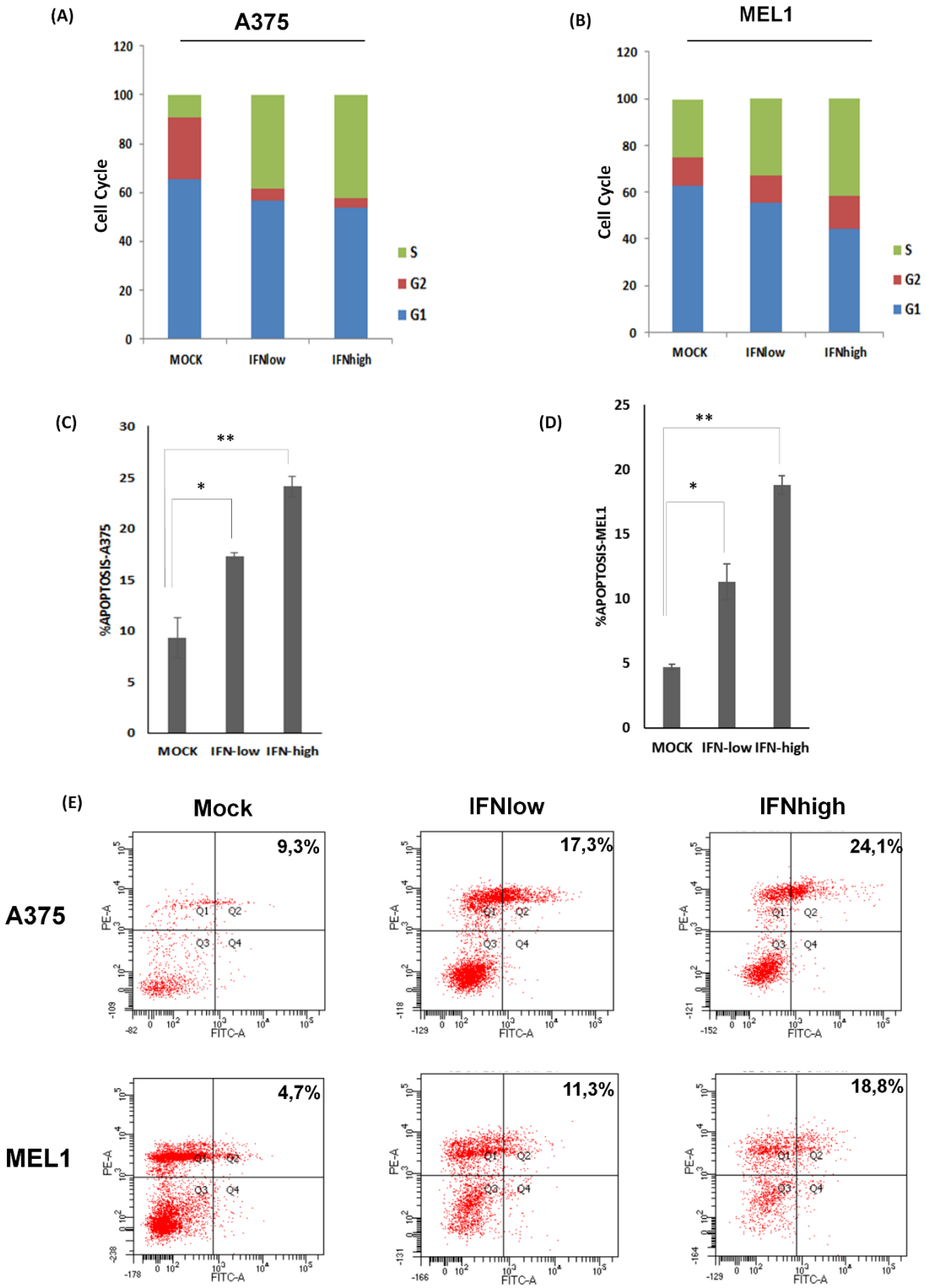


Figure 29. Effects of IFN-α on CSCs cell cycle and apoptosis. *(A-B) Cell cycle analysis in CSCs enriched subpopulations. Flow cytometry analysis of cell cycle distribution of A375 (A) and MEL1 (B) cells upon cultured in spheres medium; (C-D) Apoptosis assay in CSCs enriched subpopulations. Cell viability and apoptosis analysis of cells A375 (C) and MEL1 (D) after treatment with IFN-α; (E) Scatter representation of PI/Annexin V analysis. Statistical significance indicated *($p < 0.05$), **($p < 0.01$), *** ($p < 0.001$) and $^{\#}$ ($p < 0.0001$).*

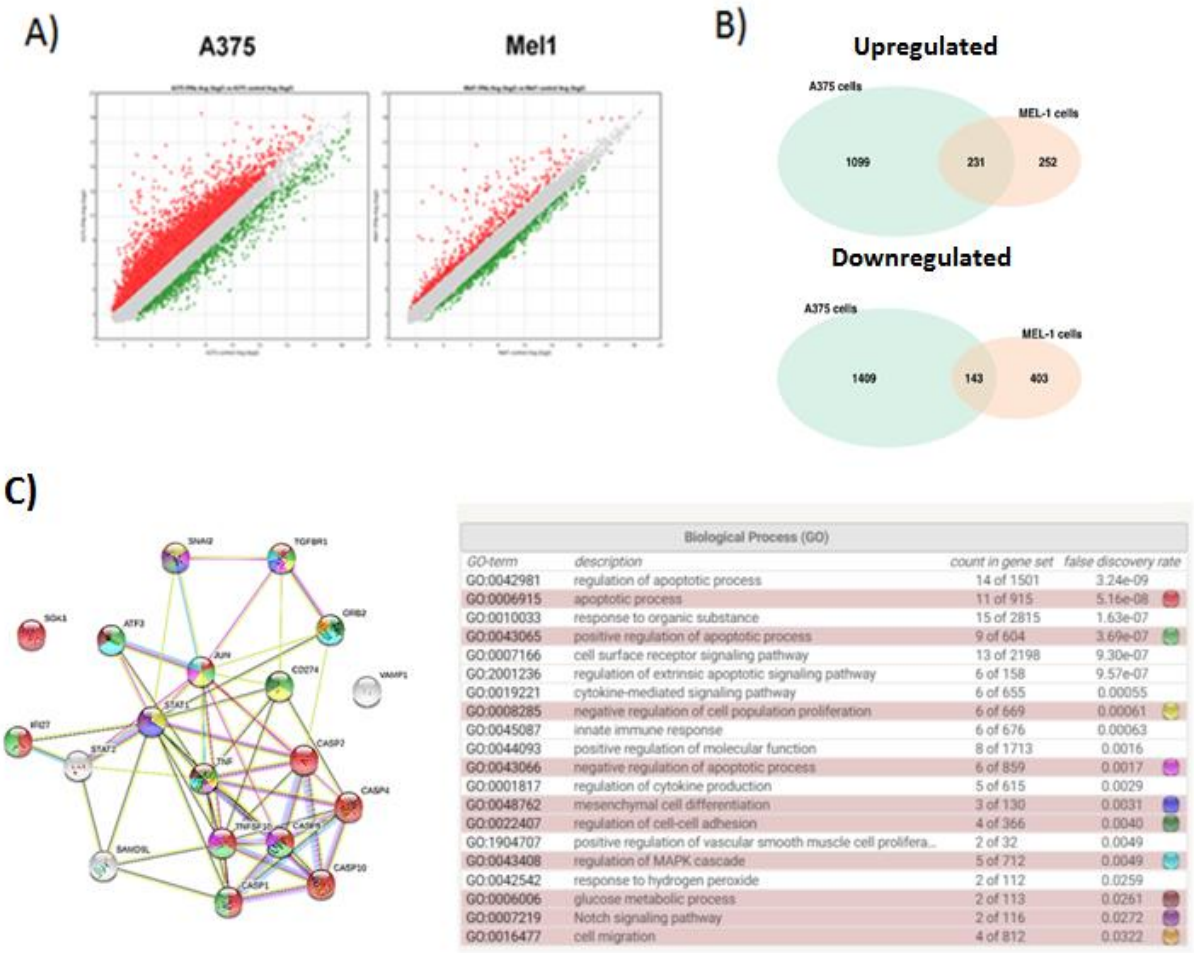
4.4. Gene expression profile changes and effects on selected miRNAs after IFN-α treatment.

Gene expression profiling of melanoma cells was used to understand the molecular changes underlying IFN-α modulation. To this end, gene expression of cells exposed to a low dose of IFN-α for 30h, and untreated A375 and MEL-1 spheres was measured using a cDNA microarray platform with probes for 21448 different genes. Differentially expressed genes were identified (cut-off values >2 or <-2 fold change and p <0.05).

After analyzing the secondary spheres treated with low doses of IFN-α versus mock-treated spheres for A375 cell line, 8692 genes passed filter criteria; among these genes, there were 1099 up-regulated genes and 1409 down-regulated genes. For MEL-1 spheres, 1198 genes passed filter criteria; among these genes, there were 252 up-regulated genes and 403 down-regulated genes. As can be observed in the expression profile heatmap (Supplementary Figure 2, see Annex II), IFN-α treatment induced more changes in A375 CSCs that in MEL-1 secondary spheres. Figure 30A shows scatter plots with up-regulated genes in red and down-regulated genes in green for A375 and MEL-1 spheres-treated cells. After comparing differentially expressed genes between A375 and MEL-1 (Figure 30B), 231 and 143 genes were found to be commonly up- and downregulated, respectively. To make this list shorter, we selected genes related to cancer, apoptosis (CASP1, CASP4, CASP7, CASP8, CASP10, SGK1 and TNFSF10), proliferation (STAT1, CD274, TNF, JUN), migration (GRB2), MAPK (ATF3, JUN, GRB2) or Notch signaling pathways (STAT1). Functional relationships among selected genes was assesed using the String resource (Figure 30C) (Szklarczyk et al., 2021).

The final list was made up of 17 genes (Supplementary Table S4 and Table S5 in Annex II), being the gene SAMD9 the one that showed the most expression change with a 523,4- and 305,22-fold change in A375 and MEL-1 respectively. Moreover, several membrane vesicle-associated members of VAMP family were differentially modulated

in A375 and MEL-1. The most prominent genes in this study involved in CSCs pathways (IFI27, TNFSF10, SAMD9) have been validated by qPCR showing the same trend expression in both cell lines after IFN-α treatment (data not shown).



Biological Process (GO)			
GO-term	*description*	*count in gene set*	*false discovery rate*
GO:0042981	regulation of apoptotic process	14 of 1501	3.24e-09
GO:0006915	apoptotic process	11 of 915	5.16e-08
GO:0010033	response to organic substance	15 of 2815	1.63e-07
GO:0043065	positive regulation of apoptotic process	9 of 604	3.69e-07
GO:0007166	cell surface receptor signaling pathway	13 of 2198	9.30e-07
GO:2001236	regulation of extrinsic apoptotic signaling pathway	6 of 158	9.57e-07
GO:0019221	cytokine-mediated signaling pathway	6 of 655	0.00055
GO:0008285	negative regulation of cell population proliferation	6 of 669	0.00061
GO:0045087	innate immune response	6 of 676	0.00063
GO:0044093	positive regulation of molecular function	8 of 1713	0.0016
GO:0043066	negative regulation of apoptotic process	6 of 859	0.0017
GO:0001817	regulation of cytokine production	5 of 615	0.0029
GO:0048762	mesenchymal cell differentiation	3 of 130	0.0031
GO:0022407	regulation of cell-cell adhesion	4 of 366	0.0040
GO:1904707	positive regulation of vascular smooth muscle cell prolifera...	2 of 32	0.0049
GO:0043408	regulation of MAPK cascade	5 of 712	0.0049
GO:0042542	response to hydrogen peroxide	2 of 112	0.0259
GO:0006006	glucose metabolic process	2 of 113	0.0261
GO:0007219	Notch signaling pathway	2 of 116	0.0272
GO:0016477	cell migration	4 of 812	0.0322

Figure 30. Array Analysis of genes in both cell lines. *(A) Gene expression scatter plots of control versus 30h IFN-α treated CSCs in A375 and MEL-1 cell lines. Up- and downregulated genes are shown in red and green respectively.; (B) Number of common up- and downregulated genes (Fold Change > 2 and FDR < 0.05) between A375 and MEL-1 cells after 24h of IFN-α treatment; (C) Functional relationships among selected genes as displayed by String database and the biological processes implicated.*

After miRNAseq we selected miRNAs considered to be differentially expressed for fold changes above 2 and those involved in different tumor processes and stemness properties (Figure 31). In A375 and MEL-1 melanospheres treated with low-dose of IFN-

α, miR-7-5p, miR-141-3p, miR-425, miR-550a, miR-3614-5p, miR-4521 and miR-4645 were overexpressed in comparison to Mock condition.

In contrast, we observed a lower expression in melanospheres of miR-98-5p, miR-191, miR-744-3p and let-7e-3p in both lines after IFN-treatment (Figure 31). These miRNAs have been validated by qPCR showing the same trend expression in both lines after IFN-α treatment (data not shown). Therefore, we demonstrated that low dose of IFN-α modulates numerous genes and miRNAs involved in several tumor processes and stemness properties in MM CSCs subpopulations.

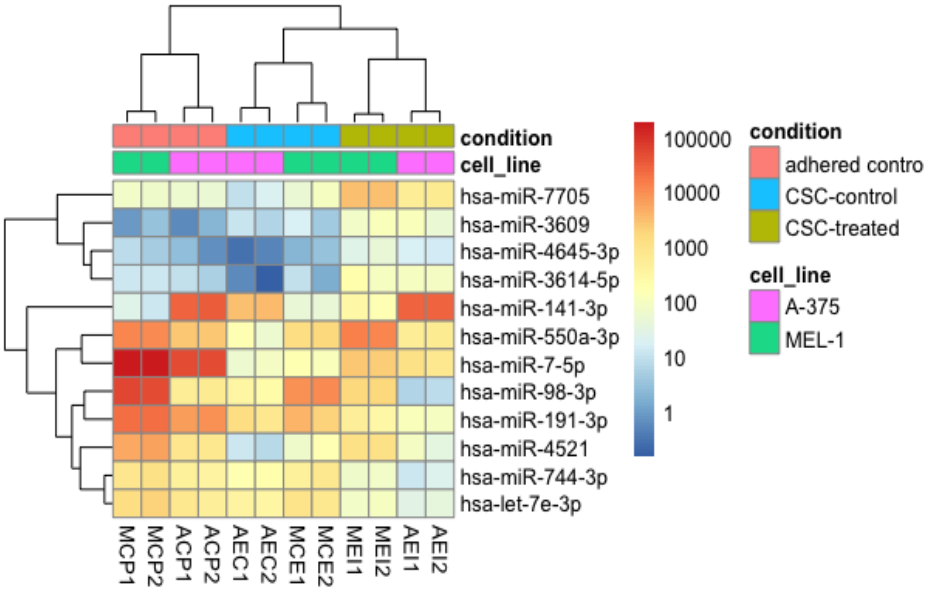


Figure 31. Heatmap of expression values (Reads Per Million) of selected miRNAs in A-375 and MEL-1 cell lines

4.5. IFN-α reduces the tumorigenicity of melanospheres in xenograft mice.

To test *in vivo* the ability of IFN-α to inhibit the initiating tumor capacity of MM CSCs, secondary melanospheres of A375 and MEL-1 were treated with the low dose of IFN-α during 72 h and after that 500 viable cells/mL, were injected into both subcutaneous flanks of 20 NSG mice for both cell lines. The effect of the treatment on tumor volume and weight is graphed in Figure 32.

Tumors generated by A375 CSCs cells emerged 27 days after the injection in control and IFN-α pre-treated cells; however, control CSCs displayed significantly higher tumors volume than treated cells, which displayed a significant ($p<0.01$) tumor mass reduction in more than 65% respect to controls (Figure 32A). Control group were

euthanized on day 35 due to adverse clinicals signs unrelated to treatment in comparison with treated group.

In contrast, mice injected with MEL-1 non-treated CSCs tumors were developed at day 53 (Figure 32B), whereas mice injected with IFN-α treated cells tumor volumes were practically undetectable along the treatment ($p<0.001$). In fact, after 91 days after the injection, tumors in mice injected with MM CSCs treated with a low dose of IFN-α showed a reduction of volume of 90% in comparison to mice injected with control non-treated MM CSCs. These results indicate that CSCs subpopulations treated with IFN-α at low doses significantly decreased the ability to form tumors in comparison with mock-treated cells.

Accordingly, for A375 there was a significant reduction in tumor weight in mice injected with MM CSCs treated with IFN-α (0.8 ± 0.18g) compared to controls (1.08 ± 0.41g). Furthermore, for MEL-1 the reduction in tumor weight was higher than A375 cells with a significant reduction in mean tumour weight in IFN-α-pre-treated injected group (0.035 ± 0.01g) in contrast to mock group (0.196 ± 0.11g) (Figure 32C-D).

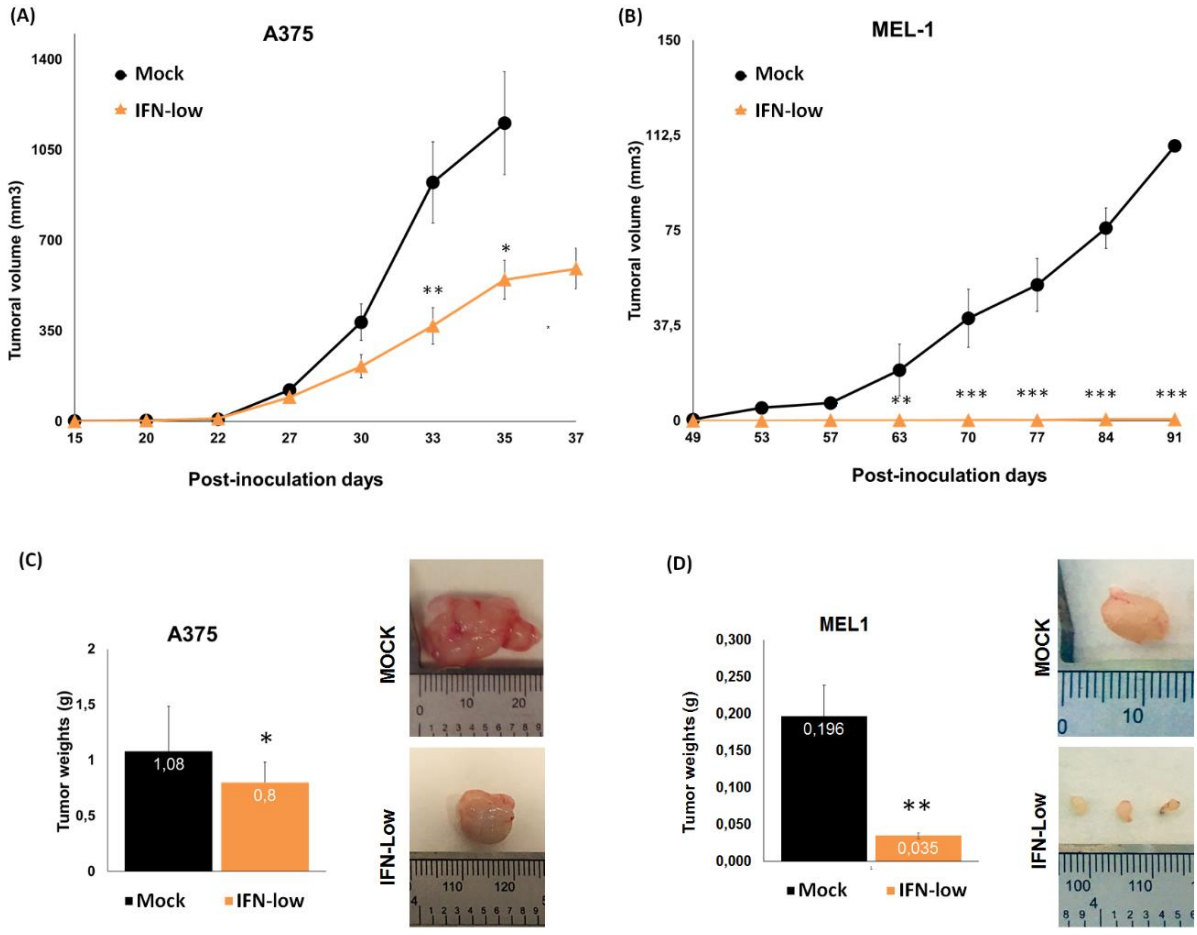


Figure 32. *In vivo* tumour formation by IFN-α pre-treated spheres. *Tumor volume of MM tumors formed in NSG mice after inoculation of (A) A375 CSCs subpopulations and (B) MEL-1 CSCs subpopulations. Data is shown as mean ± SEM statistical analysis Student's test comparison IFN vs Mock; (C-D) Images of excised tumors and mean tumor at the end of the experiments.*

Finally, hematoxilin & eosin staining and immunostaining was done in excised tumors to detect the expression of the classical stemness markers (CD44, CD133, p75) and the previously identified SAMD9 protein (Figure 33A). Results showed a lower level of fluorescence in the stemness markers for IFN-α pre-treated cells (A375 and MEL-1 CSCs subpopulations) which was significantly for CD44 and p75 in A375 CSCs-injected mice and for CD133 and CD44 for pre-treated MEL-1 CSCs-injected mice. In contrast, a significant increase of expression was found for SAMD9 in tumors induced by IFN-α-treated cells (Figure 33B-C). Thus, the anti CSCs activity of IFN-α showed *in vitro*, was also detected *in vivo* analysis.

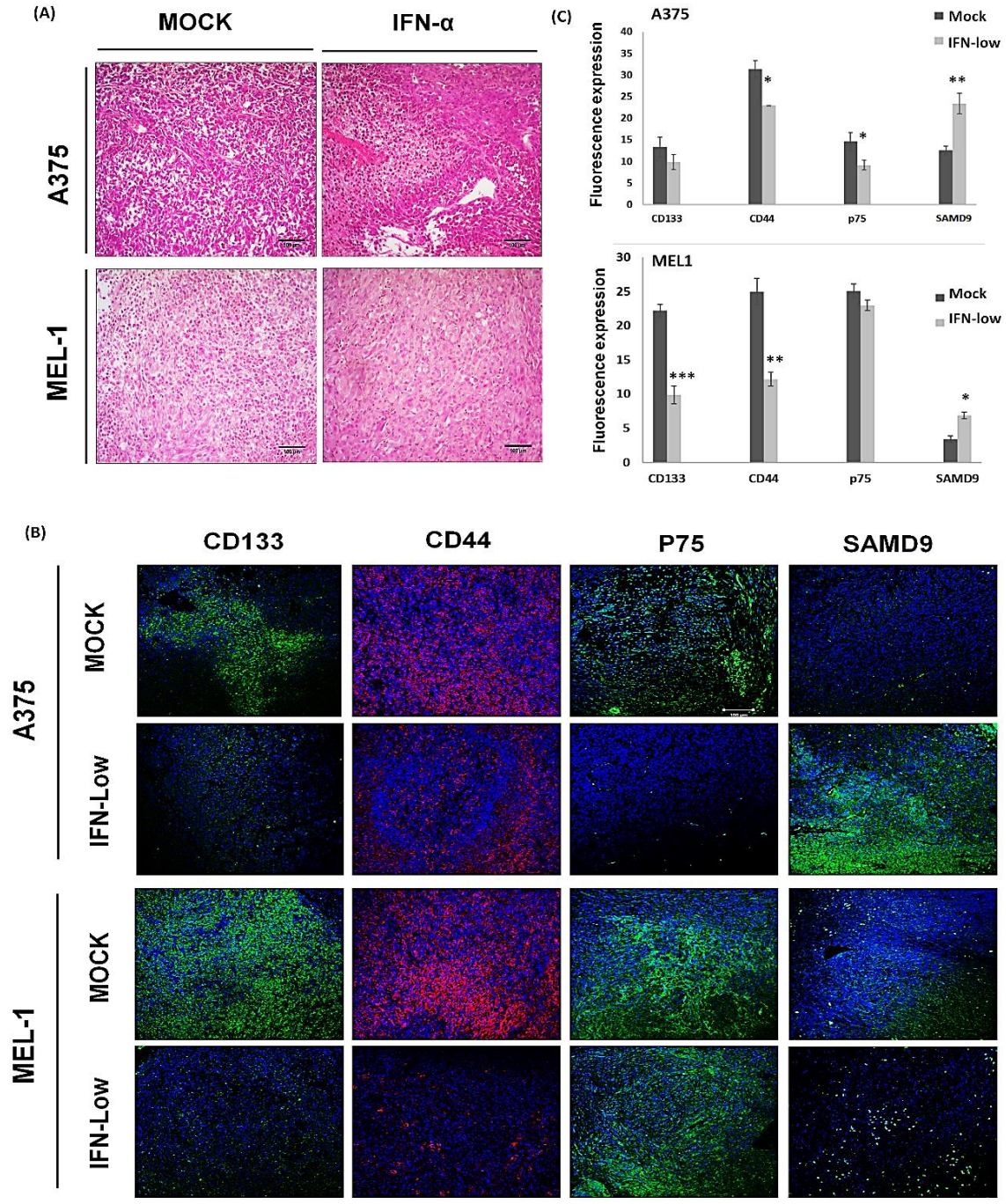


Figure 33. *Histopathology of tumors formed by IFN-α pre-treated spheres. (A) Representative images of hematoxylin/eosin staining of tumors proceeding from A375 (up) and MEL-1 spheres (down) Mock and IFN-α pre-treated. Original magnification: 20X. Scale bar = 100 μm; (B) Representative immunofluoresence images for CD133, CD44, p75 and SAMD9 of tumors. Original magnification: 20x. Scale bar = 100 μm; (C) Quantification of the fluorescence intensities. The average fluorescence intensities were calculated from three parallel immunofluorescence images. Statistical significance indicated *($p < 0.05$), **($p < 0.01$), *** ($p < 0.001$).*

4.6. IFN-α interferes over EVs secretion *via* exosomes in MM CSCs subpopulations.

Based on their unique size and density, we isolated EVs from the culture supernatant of A375 and MEL-1 mock CSCs subpopulations and IFN-α-treated spheres following the ultracentrifugation protocol described in the *Material and Method* section. Exosomes purification was confirmed by TEM, western blot, NanoSight, AFM and SEM (Figure 34 and Figure 35). To collect supernatants, we used the same volumen in each purification procedure (15 mL) and we adjusted the initial number of cells taking into account the different degree of growth in the presence or absence of IFN-α, in order to have the same total number of cells for both conditions (8×10^6 cells) at the time of collection of the supernatants.

As shown in TEM images (Figure 34A) EVs obtained from A375 and MEL-1 secondary melanospheres have a characteristic saucer-like ultrastructure with diameters ranging from 40 to 130 nm and crescent shaped membrane invaginations limited by a lipid bilayer, while vesicles obtained from IFN-α-treated cell cultures had a minor number and diameter ranging from 30 to 90 nm.

Western blot analysis showed that these EVs from A375 and MEL-1 CSCs subpopulations were positive to known exosome classic markers including Alix, Hsp70, CD9 and CD63 (Figure 34B). Finally, to gain additional insight into the potential role and relevance of IFN-α against CSC-melanoma-markers, CD133, PKR, p75, SAMD9 and CD44 markers were analyzed (data not shown) but we were able to detect only CD44 expression, which was significantly reduced in exosomes obtained from IFN-α-treated spheres. Exosomes size distribution determined by NTA Software confirmed a decrease of particles with nanometric size in supernatants from both A375 and MEL-1 spheres treated with IFN-α. We detected an average concentration of 8.26×10^7 particles/mL in A375 spheres-derived exosomes mock-treated cells in comparison with A375 IFN-α-treated spheres which presented an average of 1.22×10^7 particles/mL (Figure 34C). For MEL-1, we detected 3.24×10^7 particles/mL in mock conditions in comparison to IFN-α treated spheres which presented an average of 2.12×10^7 particles/mL (Figure 34D).

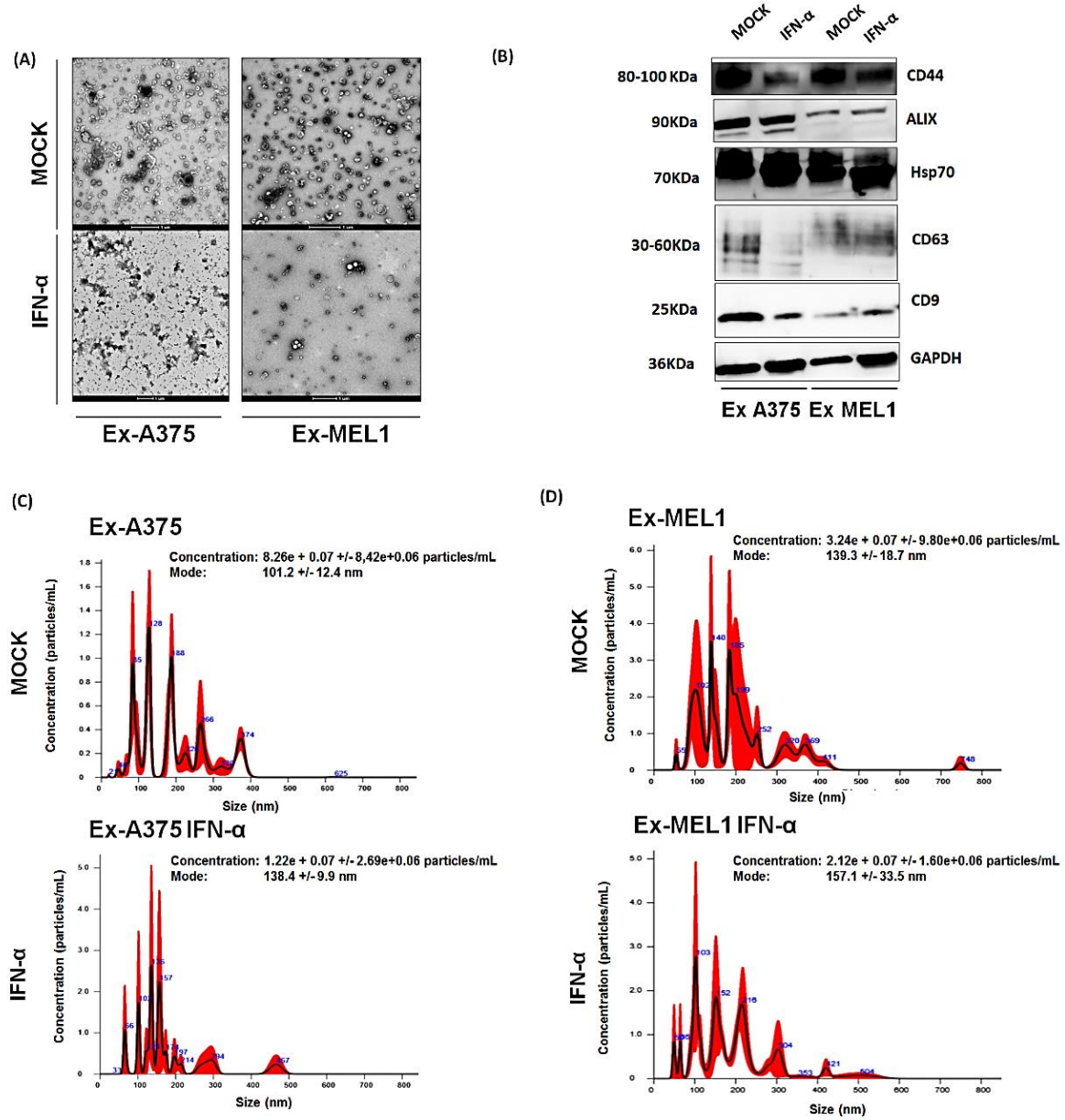


Figure 34. Characterization of exosomes derived from melanospheres cultures under IFN-α conditions. *(A) Transmission electron microscopic images of isolated exosomes with a saucer-like shape limited by a lipid bilayer. Vesicles isolated from culture supernatant (diameter ranging from ~40–130 nm). Black arrows point to exosomes; (B) Western blot analysis of representative CD9, CD63, Alix and Hsp70 exosomes markers and the CD44 MM stem cell marker in melanospheres-derived exosomes treated under IFN-α conditions. GAPDH was used as a positive control; (C-D) The size distribution of exosomes obtained from (C) A375 spheres and (D) MEL-1 spheres was analyzed by NTA.*

Furthermore, the morphology and size of exosomes were also verified by immunogold using beads coated with an anti-CD63 antibody. Black punctate regions indicate a positive staining for CD63 around the exosome membranes from A375 and MEL-1 CSC-derived exosomes (Figure 35A). In addition, we were able to detect multivesicular bodies with spheroid structures surrounded by a phospholipid bilayer inside the CSCs secondary melanospheres. In contrast, melanospheres treated with IFN-α displayed an unstructured morphology appearing an increased number of non-viable CSCs (Figure 35B).

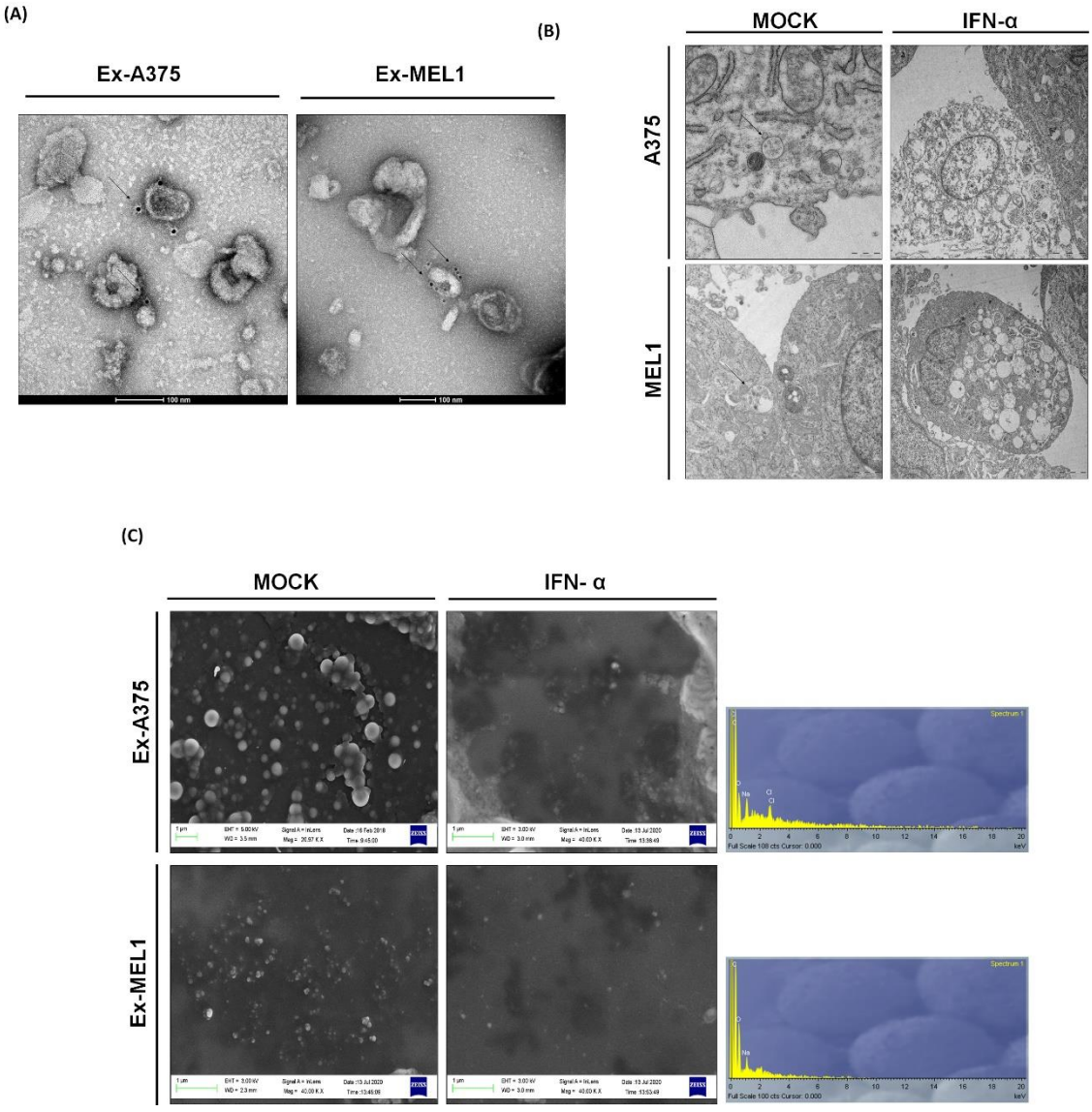


Figure 35. Characterization of exosomes derived from melanospheres cultures under IFN-α conditions. *(A) Inmunogold with Anti-CD63 marker of exosomes derived from spheres secondary cultures in both cell lines. Black arrows point to exosomes; (B) Multivesicular bodies observed by transmission electron microscopic in A375 and MEL-1 CSCs. Images obtained from paraffin sections. Black arrows point to EVs; (C) Scanning electron microscopy images of CSCs derived-exosomes aggregated from both A375 (up) and MEL-1 spheres supernatants (down). The micro-analysis determined the proportion of carbon and discriminated the sales.*

The morphology and arrangement of the exosomes were also verified by scanning electron microscopy and the organic composition was checked by microanalysis on carbon surface in both cell lines (Figure 35C).

AFM images showed a heterogeneous organization of exosomes, in terms of the wide variation in shape and size as demonstrated in both 2-dimensional (2D) images and topographic profiles, regardless of their origin. In IFN-α-treated samples, we observed low density of exosomes population in comparison with exosomes released from non-treated melanospheres (Figure 36). In addition, we analyzed the number of exosomes counts (Figure 36C) and the exosomes quantification of area (nm^2) and volume (nm^3) from exosomes in AFM Grain Mode (Figure 36D). We observed a significant increase in the counting of CSCs-mock exosomes in comparison to treated CSCs derived exosomes, whereas the area in exosomes derived from IFN-α-treated CSCs was significantly higher than untreated CSCs for both cell lines (Figure 36C-D). Moreover, exosomes derived from treated cells displayed a deformed membrane appearance in comparison to the mock sample. Therefore, our results indicate that IFN-α interferes the production, size and morphology of exosomes.

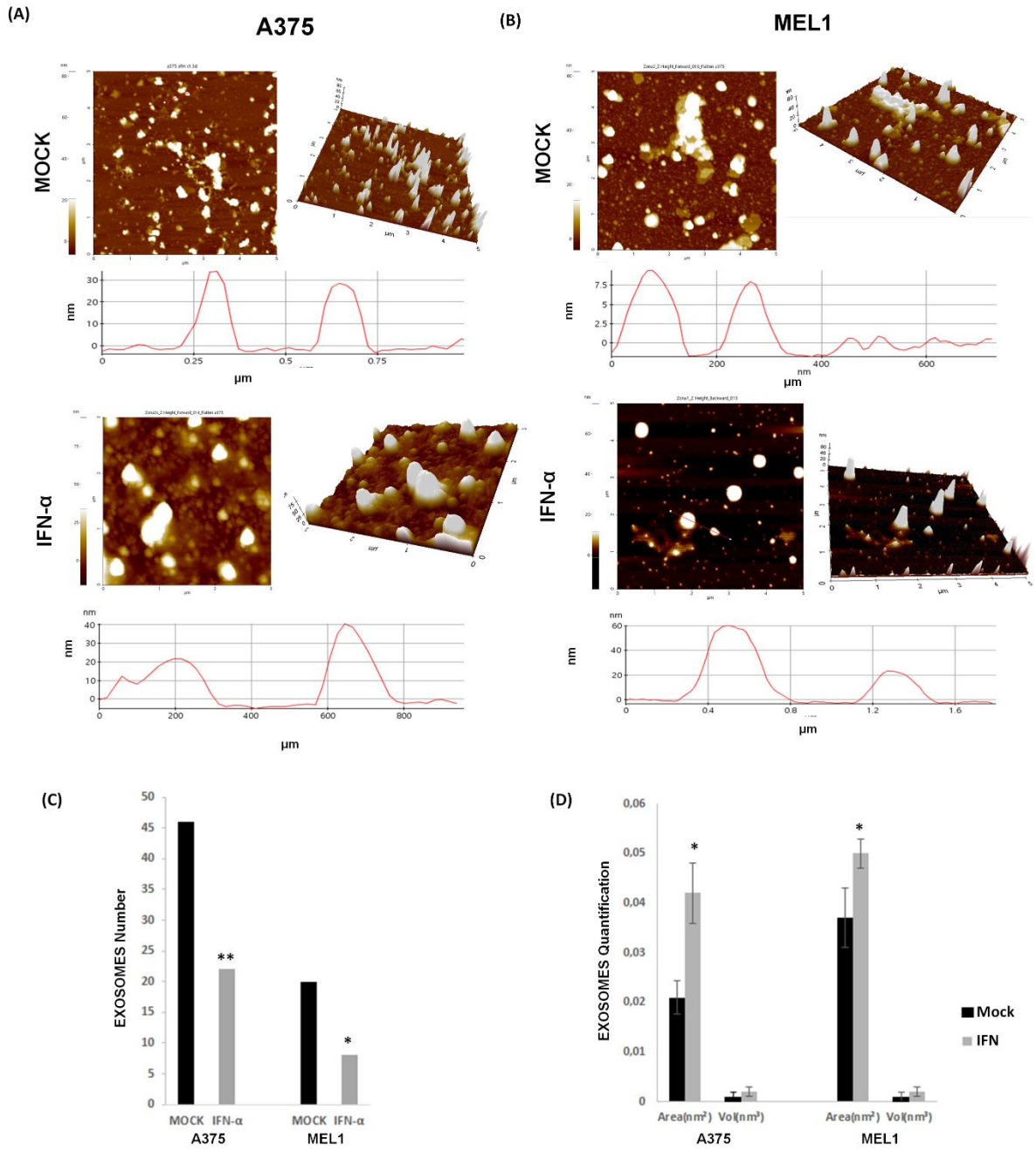


Figure 36. Topography of exosomes derived from *(A) A375 secondary spheres and (B) MEL-1 secondary spheres observed under atomic force microscopy (AFM). Exosomes on a mica surface revealed heterogeneity in size and shape as well as forming aggregates in both 2-dimensional 2D (above) images and 3D profiles (below). Acquisition areas were 5x5 µm2 and 5 µm long profile lines are shown in red; (E) Number of exosomes counts in AFM Grain Mode; (F) Quantification of area (nm2) and volume (nm3) from exosomes in AFM Grain Mode.*

4.7. LC-HRMS metabolomic analysis of exosomes derived from MM CSCs treated with IFN-α.

In order to explore potential biomarkers for the diagnosis of this disease we peviously reported significant metabolomic differences in exosomes derived from melanoma CSCs from MEL-1 cell line, and also in serum-derived exosomes from melanoma patients compared to those from healthy controls (Palacios Ferrer et al., 2021).

Based on these findings, we were interested in checking if some of those metabolites reported in that study were also differentially found between IFN-α-treated and control CSC-derived exosome samples from A375 and MEL-1 cell lines.

The HPLC-QTOF-MS total ion chromatograms (TIC) showed excellent reproducibility with regards to retention time and signal intensity, indicating a low analytical drift across the whole set of samples (data not shown). A positive ionization data matrix of 4300 mass signals was obtained as an outcome of the peak picking and alignment procedures, but only 676 peaks representing monoisotopic ions were considered and subjected to the chemometric analysis. A first filtering process was performed in order to discard those signals coming from mobile phase solvents.

Next, multivariate analyses such as PCA and PLS-DA were carried out in order to assess the quality of the analytical system performance and discriminate those variables that are responsible for variation between the comparison groups. The PCA score plots for all the analyzed sample groups in both A375 and MEL-1 conditions are shown in Figure 37A and 37C, respectively. In both cases, the close clustering of QC samples reflects the quality of the analytical system performance. BS samples were also clearly separated from biological samples. Samples of CSC-derived exosomes treated with IFN-α were clearly separated from control samples in the PCA score plots in both cell lines (Figures 37A y 37C). In PLS-DA models, the different groups of samples were discriminated with an R^2 of 0.99 and Q^2 of 0.99 in A375 cell line and an R^2 of 0.99 and Q^2 of 0.96 in MEL-1 cell line.

After that, a *T-test* (*p*-value < 0.05) filtering was performed for comparison on the 2 groups of samples (IFN-α-treated and mock treated CSC-derived exosomes) for both MM cell lines. As an outcome, 73 and 76 differential metabolites were found in A375 and MEL-1, respectively.

The corresponding heatmaps showing the differential abundance of those metabolites found as statistically different are shown in Figure 37B and 37D for A375 and MEL-1 conditions, respectively.

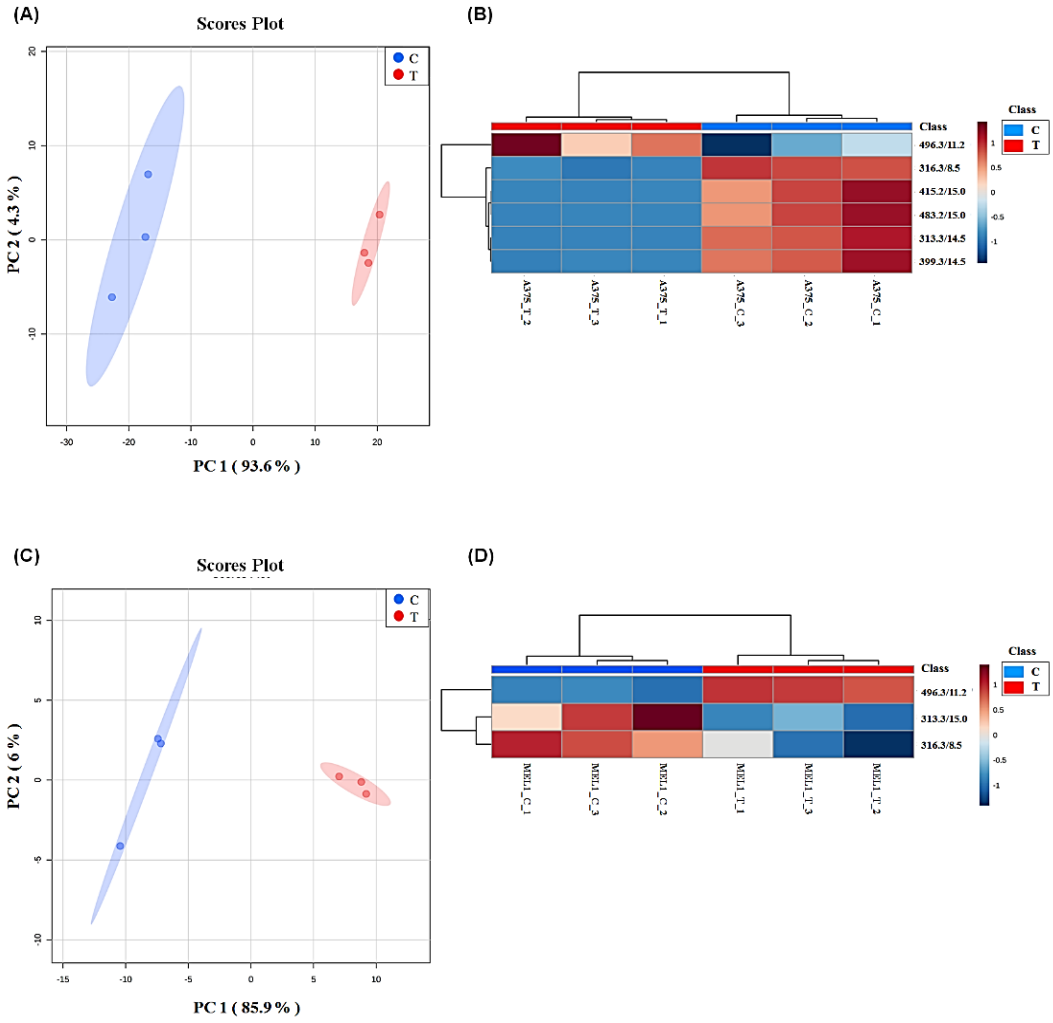


Figure 37. The PCA score plots for all the analyzed sample groups from both A375 and MEL-1 cell lines

As previously described, we checked if some of the differential metabolites found by Palacios-Ferrer *et al*. (2021) in exosomes derived from MM CSCs compared to those from differentiated tumor cells in MEL-1 cell line were also differential when comparing IFN-α-treated and mock CSC-derived exosomes from A375 and MEL-1 secondary spheres. For that, we followed a targeted approach in the same experimental conditions and specifically searched for those metabolites in our samples. Curiously, 6 and 3 of them

in A375 and MEL-1 spheres, respectively, were also differentially found when comparing IFN-α-treated and mock exosome samples.

These signals were analysed by PCA, obtaining a clear separation of exosome samples derived from IFN-treated and control CSCs from A375 and MEL-1 spheres in the PCA score plots along PC1 and PC2, which describe most of the total data variability. In PLS-DA models, exosome samples were discriminated with an $R^2 > 0.99$ and $Q^2 > 0.96$, exceeding the threshold values accepted in metabolomic experiments (R2 > 0.7 and Q2 > 0.4) (Godzien et al., 2013).

The heatmaps representing the differential abundance of those metabolites are shown in Supplementary Figure 3 in Annex II for A375 and MEL-1 cell lines, respectively. As can be observed, in both cell conditions, most of those metabolites were more abundant in exosome samples derived from control CSCs, compared to those from IFN-α-treated CSCs, but 1 metabolite was higher IFN-α-treated samples in comparison to controls (Supplementary Figure 3 in Annex II). Interestingly, that metabolite with m/z 496.3381, which corresponds to 1-hexadecanoyl-sn-glycero-3-phosphocholine (PC 16:0/0:0), was previously found by our research group (Palacios-Ferrer et al., 2021) to be overexpressed in exosomes derived from both healthy controls serum and adherent MEL-1 cells, compared to MM patients and CSC-enriched subpopulations from MEL-1 cells, respectively.

5. Discussion

CSCs are resistant to chemo- and radiotherapy and are involved in the recurrence of the disease. Currently, there is a great amount of clinical trials with new selective drugs attacking this tumor subpopulation. IFN-α might improve the recurrence-free survival (RFS) and overall survival (OS) of patients with high risk melanoma (P. a Ascierto et al., 2014). Despites of these benefices, the questions over the optimal treatment scheme (dosage and duration) and the great amount of side effects of IFN-α are still subjects of controversy and debate. Some oncologists feed this controversy emphasizing in the questionable effect of IFN-α over OS. Despite the intense debate and the great amount of studies, the exact mechanism of action of IFN-α has not been established (Berrocal et al., 2018).

In view of these results and the clinical trials performed to evaluate the effects of IFN-α over melanoma, we hypothesize that this adjuvant probably has an unknown effect over CSCs. In this work, we have analyzed CSCs features establishing phenotypic effects of IFN-α over this aggressive subpopulation in both an established and a primary MM cell lines.

We first performed the sphere-forming assay in the two MM cell lines (A375 and MEL-1) to analyze the effect of IFN-α over secondary melanospheres, which has been proved to be enriched in CSCs (Morata-Tarifa et al., 2016b; Smart et al., 2013). Our results showed an inhibition over melanospheres formation in IFN-α-treated cultures, observing a decrease in the number of spheres in a concentration–dependent manner. Besides, the proliferation of the forming-sphere abilty cells was reduced too, showing smaller sizes. Thus, we suggest that IFN-α could affect at self-renewal ability of CSCs, avoiding their normal cell proliferation.

Analysis of characteristics CSC markers in MM has been based on increased ALDH1 activity, CD20+, CD44+ and CD133+ expression among others (Vargas et al., 2012). Our results show that IFN-α treatment reduced ALDH-positive population and the exppression of CD20, CD44 and CD133 markers.

The effect of IFN-α over CSCs in others solid tumors models is under investigation with opposite effects depending on model analyzed (Martin-Hijano & Sainz, 2020). One of the first evidences of IFN-α effect over CSCs was showed in a model of ovarian cancer in rat primary tumors, where the authors suggested the effect of IFN-α over the SP. The SP comprises cells with stem cell features and show higher proliferation rates and less apoptotic levels compared with non-SP cells, generating also tumors more rapidly than non-SP cells (Moserle et al., 2008). IFN-α demonstrated to exert anti-proliferative and proapoptotic effects on primary cultures containing high numbers of SP cells. Furthermore, IFN-α treatment caused a dramatic reduction in SP rate in tumor cell lines (Moserle et al., 2008). In our study, we observed that IFN-α caused a significant reduction in SP CSCs populations (Figure 28). These results on proliferation and SP together the signficant decrease in MM CSCs markers indicate the selective anti-CSCs effect of IFN-α treatments. In agreement with our results, the anti-proliferative effects of IFN-α on triple negative breast CSCs has been recently described. Triple-negative breast cancer, the most agressive form of this disease, currently lacks a targeted therapy and is characterized by

increased risk of metastasis and presence of therapeutically resistant cancer stem cells. Recent evidence has demonstrated that the presence of an interferon genes signature correlates with improved therapeutic response and overall survival in these patients (Doherty & Jackson, 2018a, 2018b).

Other work in which the effect of IFN-α over CSCs has been evaluated was performed by Motawi et *al.,* who evaluated the combined effect of IFN-α and 5-fluorouracil (5-FU) on CSC markers expression (OV6 and CD90) and on specific pathways that contribute to propagation of CSCs in hepatocellular carcinoma (TGF-β, IL-6 and STAT3) and on angiogenic factor (VEGF) in rats. It was demonstrated that IFN-α treatment suppressed the hepatic expression of CSC markers, proliferation CSCs related molecules and VEGF. In contrast, 5-FU failed to reduce the three CSCs characteristics (Motawi et *al*., 2016). These results proved the anti-proliferative and anti-angiogenic effect of IFN-α over CSCs in hepatocellular carcinoma and the benefit of combining IFN with other conventional therapies.

CSCs have been suggested as the only tumor cell subpopulation able to lead a new complete tumor. This ability has been studied *in vitro* by the clonogenicity assay in soft-agar (Morata-Tarifa et al., 2016), which is based on the ability of CSCs to form colonies in hostile conditions. Our results showed a reduction in the colonies number in melanosphers treated with IFN-α (Figure 26). Migration ability is other important feature related with stemness properties and this ability is studied *in vitro* by the wound-healing assay (Yu et al., 2012). In our study we observed a rapid wound closure (48 hours) in control non-treated cells, whereas IFN-α treatments inhibited the migration capacity in a dose dependant manner in both MM cell lines (Figure 28).

In this sense, previously it has been reported that IFN-α inhibits both the colony-forming and migratory ability of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) cells, by inducing p53-dependent apoptosis, down-regulating the expression of survivin and inactiving NF-κB (J. Q. Yang et al., 2008). Moreover, IFN-α in combination with epigenetic drugs inhibits the proliferation and the metastatic behavior of KRAS-mutated highly metastatic SW620 cells and patient-derived CSCs from colon cancer, by shutting down metastatic cellular pathways, including CXCR4, ERK1/2 and AKT signals and inducing apoptosis (Buoncervello et al., 2016). Since our results indicate the inhibitory effect of IFN-α over important CSCs characteristics in both established and

patient-derived MM cells we were interested in decphering the main molecular mehcanisms related with its potent anti-CSCs properties.

The demonstration of the activity of IFN-α against CSCs could have consequences in patients and can provide biomarkers analysis with potential diagnostic and predictive value (Su et al., 2015; Zhu et al., 2014). For this reason, we performed microarrays and miRNA sequencing to determine gene expression and miRNAs regulation in pre-treated CSCs subpopulations (Figure 31). We observed that IFN-α treatment induced interesting changes in the expression of numerous genes and miRNAs related to CSCs, proliferation and metastasis, apoptosis and immune pathways, as shown in Supplementary Table S6 (see Annex II) . We selected the following miRNAs implicated in different tumor processes and stemness properties in both MM cell lines. In our analysis, miR7-5p, miR141-3p, miR425, miR550a, miR3614-5p, miR4521 and miR4645 were shown up-regulated after IFN-α-treatment. Recent works demonstrated that these miRNAs are involved in several cancer processes acting on many pathways as tumor suppressors. In prostate cancer, the expression of several miRNAs such as miR-34a, let-7b, miR-7-5p, and miR-141-3p has been higher detected in CSCs (Khan et al., 2019). MiR-425 inhibits melanoma metastasis through repression of PI3K-Akt pathway by targeting IGF-1 and inhibits cell proliferation and induced cell apoptosis (P. Liu et al., 2015). The novel miR-550a-3-5p acts as a tumor suppressor and reverses BRAF inhibitor resistance through the direct targeting of YAP. miR-550a-3-5p suppressed cell proliferation, metastasis, and tumor sphere formation through the direct inhibition of YAP and its oncogenic pathway in various cancer cell types (Choe et al., 2018). In the same way, the transfection of the miR-4521 reduced proliferation and invasion of several medulloblastoma cell lines and induced programmed cell death through activation of caspases 3/7 and cyclin B1 (Senfter et al., 2019).

In contrast, we found that miR98-5p, miR-191, miR-744-3p and let-7e-3p were down-regulated after IFN-α treatment. MicroARN-744 promotes carcinogenesis in osteosarcoma and accelerate progression Wnt/β-catenin pathway and EMT (Liangzhi Sun et al., 2019). In fact, the low expression of miR-98-5p has been associated with melanoma patients survival (F. Li et al., 2014); MicroRNA-191, an estrogen-responsive microRNA, functions as an oncogenic regulator in human breast cancer and its mediated enhanced cell proliferation and migration are partly dependent on targeted downregulation of SATB1 (Nagpal et al., 2013).

According to data obtained through the microarrays analysis, we observed that IFN-α treatment induced changes in the expression of numerous genes involved in several cancer process as migration, apoptosis process, vesicles regulation, angiogenesis and CSCs signaling pathways.

All these results suggest the great importance of validating both gene and miRNA signatures after IFN-α treatment as a way for explore novel predictive and prognostic biomarkers in MM patients, although studies carried out with microARNs have also shown contradictory data regarding their role as tumor suppressors or oncogenes in cancer and their value as biomarkers is therefore being widely questioned (H. Wang et al., 2018).

Moreover, we studied the inhibition of the *in vivo* tumorigenic capacity of MM CSCs after IFN-α. Our results showed that CSCs subpopulations treated with IFN-α at low doses significantly decrease the ability to form tumours in comparison with mock-treated cells, in accordance to the *in vitro* results (Figure 32).

The immunoanalysis of tumors showed a significant increase in SAMD9 expression according to data obtained through the microarrays analysis. SAMD9 could be a key molecule to control cancer cell death by IFN-α receptor blocking antibody or JAK inhibitor treatment. In fact, human SAMD9 expression can be downregulated by tumor necrosis factor (TNF) and upregulated by type I and type II interferons, being it classified as an interferon stimulated gene. In agreement with our results, it has been demonstrated that when SAMD9 expression was higher in apoptotic cells apoptosis in malignant glioma cells (Tanaka et al., 2010). Moreover, SAMD9 is involved in numerous types of cancer and its deregulation is related to virus infection. Recent studies demonstrated that expression of mutant SAMD9 caused a significant decrease in proliferation and increase in cell death of the cancer transfected cells (Formankova et al., 2019). In addition, the low expression of SAMD9 has been demonstrated to be associated with aggressive fibromatosis, breast, and colon cancers (Tanaka et al., 2010). Also, it was shown that SAMD9 exhibits higher expression levels in normal tissues that in breast tumors (Ma et al., 2014).

EVs favour cancer progression and, consequently, the metastatic process changing the microenvironment at distant sites (Hood et al., 2011; Hoshino et al., 2015; Iero et al., 2008) by promoting angiogenesis and tumor cell migration (Rak, 2010). Since EVs are present in body fluids (e.g. blood), from a clinical perspective, they can be isolated by non-invasive liquid biopsy and have utility as prognostic and predictive sources of biomarkers. For this reason, we studied the effect of IFN-α-treatment on EVs. Our results

showed that IFN-α decreases EVs secretion *via* exosomes in MM CSCs subpopulations with a lesser concentration and a deformed membrane appearance than non-treated melanospheres in both cell lines. Although we have not deeply into the mechanism involved in the dysregulation found in exosomes, we have identified for the first time that exosomes from melanoma CSCs express the CD44 CSCs-related marker, which was reduced in exosomes derived from CSCs after IFN-α treatment (Figure 34). The gene expression analysis carry out in melanospheres founded a modulation in mRNA of various members of the VAMP family after treatment with IFN-α, suggesting that it could be one of the mechanisms involved in this dysregulation. In fact, some members of VAMP family such as VAMP1 and VAMP8 are essential components of the exocytic machinery and regulate various secretory processes in the intestinal, immune, and exocrine systems (F. Colombo et al., 2021). Numerous studies suggest the importance of VAMP family in the modulating vesicles traffic. VAMP8 is necessary for this latter event, allowing the completion of the autophagic pathway. Furthermore, VAMP1 and VAMP8, participate in the fusion between multivesicular bodies with the plasma membrane to release the internal vesicles (i.e., exosomes) into the extracellular medium (Fader et al., 2009). Few recent studies have demonstrated the effect of IFN-gamma on vesicular trafficking from neural CSCs by inducing the generation of altered exosomes (Zhang et al., 2020). Furthermore, our results suggest that modulation of exosomes from CSC could be involved in the efficacy of IFN-α treatment, being important to deepen and identify the underlying mechanisms in future works.

Our previous study, focused on searching potential biomarkers for the diagnosis of this disease, reported significant metabolomic differences in exosomes derived from MM CSCs compared to those from differentiated tumor cells in A375 and MEL-1 cell line (Palacios Ferrer et al., 2021). We show in this work the differential abundance of those metabolites in exosomes from IFN-treated CSC-A375 and CSC-MEL-1 (Figure 37). Interestingly, the metabolite with m/z 496.3381, which corresponds to 1-hexadecanoyl-sn-glycero-3-phosphocholine (PC 16:0/0:0), was previously found by Palacios-Ferrer et al. to be expressed in exosomes derived from both healthy controls serum and adherent MEL-1 cells, compared to MM patients serum and CSC-enriched subpopulations from MEL-1 cells, respectively. Curiously, PC 16:0/0:0 was higher in IFN-α -treated samples in comparison to controls. The over-expression of this metabolite can been in accordance with previous studies demomonstrating its relationship with lower

risks of breast, prostate and colorectal cancer (Kühn et al., 2016). In fact, it has been suggested that the rapid extracellular hydrolysis of phospholipids like PC 16:0/0:0 by metastatic tumor cells and the subsequent cellular uptake of the resulting free fatty acids (FFA) seems to be a necessary prerequisite for metastatic potential of epithelial tumor cells, probably for generating pro-metastatic lipid second messengers (Palacios-Ferrer et al., 2021; Raynor et al., 2015).

In conclusion, the assays performed in this work show that IFN-α treatment has potent effects against CSCs by reducing melanospheres proliferation, decreasing CSC markers such as CD20 and CD44, the ALDH activity, reducing the SP percentage, the ability to form agar colonies, and the migration capacities of this resistant subpopulations, as well as modulating interesting microRNAs and genes involved in pathways involved in CSC properties. Moreover, IFN-α-treated CSCs decreased their ability to form tumors in nude mice. Thus, we can suggest that this therapy has a clear effect over the MM CSCs enriched subpopulations, also reflected in an interesting modulation of exosomes that can contribute to inhibit the ability to form pre-metastatic niches or the tumor cell communication with metastatic sites, being necessary its study more deeply. Since the most of the anti-CSCs effects were shown with the low dose of IFN-α, our work identifies mechanisms involved in the benefits of continuing IFN-α-based therapy in patients with MM in combination with novel therapeutic strategies such as immunotherapies, which can contribute to a considerable advance in the improvement of survival and better response rates.

7. CONCLUSIONS

1. The known abilities of the interferon-induced PKR as a stress response protein able to inhibit the cellular translation, to induce apoptosis, and contribute to several molecular pathways involved in proliferation, tumor suppression and inflammation, higlight the importance of analyzing PKR in different pathologies such as neurological, metabolic, inflammatory diseases and cancer as a potential biomarker and an interesting therapeutic target.

2. The identification of PKR as a target for both conventional chemotherapeutics and novel drugs, highlights the need to carry out studies with patients to validate its potential as a biomarker in cancer diseases.

3. PGMA analysis has allowed to identify clusters of patients where the levels of expression of the PKR modulator nc886 in tumors and blood can be suggested as a potential biomarker for both the first-line response to chemotherapy and the survival of metastatic colon cancer patients treated with 5-FU based regimens.

4. PGMA analysis has allowed to identify clusters of metastatic colon patients whose PKR location in the cytoplasm of the tumor cells corresponded with a positive response to the 5-FU based treatments, and in contrast, patients with PKR restricted to the nucleolus could be grouped in clusters realted to the negative response to chemotherapeutic treatments.

5. PKR and nc886 have potential as biomarkers of response to chemotherapy based on the use of 5-FU that should be evaluated in a greater number of patients and different pathologies where 5-FU continues to be used as standard therapy.

6. IFN-α showed a potent effect against MM CSCs by reducing melanospheres proliferation, decreasing CSC markers such as CD20, CD44 and CD133, the ALDH activity, reducing the SP percentage, decreasing the forming-colony ability and the migration and invasion capacities in A375 and MEL-1 CSCs enriched subpopulations.

7. IFN-α modulates CSCs-related genes such as SAMD9 and VAMP family members among others, and microRNAs with described ability to modulate several cancer processes such as miR-7-5p, miR-141-3p, miR-425, miR-550a, miR-3614-5p, miR-4521 and miR-4645, miR-98-5p, miR-191, miR-744-3p and let-7e-3p, in CSCs melanoma subpopulations.

8. MM CSCs treated with IFN-α reduced significantly the tumor formation ability showing decreased tumor size and weight in xenotransplants in mice model.

9. Tumors induced by CSCs treated with IFN-α showed low expression levels of several CSCs markers such as CD44, CD133 and p75 and increased the SAMD9 protein expression analyzed by immunohistochemistry.

10. IFN-α modulated EVs secretion *via* exosomes in MM CSCs subpopulations from A375 and MEL-1 lines as demostrated by TEM, SEM, AFM, NanoSight and western blott analysis.

11. We identified for the first time that exosomes derived from MM CSCs subpopulations express the CD44 marker, which was significantily reduced in CSCs treated with IFN-α.

12. Exosomes derived from both A375 and MEL-1 CSCs subpopulations treated with IFN-α presented significant metabolomic differences respect to exosomes from mock treated melanospheres, higligthing the previously described 1-hexadecanoyl-sn-glycero-3-phosphocholine (PC 16:0/0:0) metabolite as potential biomarker.

CONCLUSIONES

1. El papel de PKR inducido por Interferón como proteína de respuesta a estrés es capaz de inhibir la traducción celular, de inducir la apoptosis y de contribuir en varias vías moleculares implicadas en la proliferación, la supresión de tumores y la inflamación, poniendo de manifiesto la importancia de analizar PKR en diferentes patologías como las neurológicas, metabólicas, inflamatorias y oncológicas teniendo un potencial como biomarcador y diana terapéutica.

2. La identificación de PKR como diana tanto para los quimioterápicos convencionales como para los nuevos fármacos pone de manifiesto la necesidad de realizar estudios con pacientes para validar su potencial como biomarcador en procesos oncológicos.

3. El análisis PGMA ha permitido identificar grupos de pacientes en los que los niveles de expresión del regulador de PKR, nc886, en los tumores y en la sangre puede presentarse como un posible biomarcador tanto de respuesta a la primera línea de quimioterapia como de la supervivencia de los pacientes con cáncer de colon metastásico tratados con regímenes basados en el 5-FU.

4. El análisis PGMA ha permitido identificar grupos de pacientes de colon metastásico cuya localización de PKR en el citoplasma de células tumorales se correspondía con una respuesta positiva a los tratamientos basados en el 5-FU, y por el contrario, los pacientes con expresión de PKR en el nucleolo podían agruparse en grupos que se correspondían con la respuesta negativa a los tratamientos quimioterapéuticos.

5. PKR y nc886 tienen potencial como biomarcadores de respuesta a la quimioterapia basada en el uso de 5-FU debiendo ser evaluados en un mayor número de pacientes y en diferentes patologías en las que el 5-FU sigue siendo utilizado como terapia estándar.

6. IFN-α actúa frente a CSC de melanoma reduciendo la proliferación de las melanosferas, disminuyendo los marcadores tales como la actividad del enzima ALDH, reduciendo la expresión de los marcadores de superficie celular CD20, CD44 y CD133, reduciendo el porcentaje de SP, así como disminuyendo la formación de colonias y la capacidad de migración e invasión en las subpoblaciones enriquecidas de CSC procedentes de A375 y MEL-1.

7. IFN-α modula genes relacionados con las CSCs, como los miembros de la familia SAMD9 y VAMP entre otros, y microRNAs con capacidad descrita para modular varios procesos cancerígenos tales como miR-7-5p, miR-141-3p, miR-425, miR-550a, miR-3614-5p, miR-4521 y miR-4645, miR-98-5p, miR-191, miR-744-3p y let-7e-3p en las subpoblaciones de CSCs melanoma.

8. Las CSC de melanoma tratadas con IFN-α redujeron significativamente el tamaño y el peso del tumor formado por vía subcutánea en ratones.

9. Los tumores inducidos por CSCs tratados con IFN-α mostraron bajos niveles de expresión de marcadores de CSCs tales como CD44, CD133 y p75 y un aumento de la expresión de la proteína SAMD9 analizados por immohistoquímica.

10. IFN-α disminuye la secreción de EVs *vía* exosomas en las subpoblaciones de CSCs de melanoma de las líneas A375 y MEL-1, como han demostrado los análisis por TEM, SEM, AFM, NanoSight y western blot.

11. Hemos identificado por primera vez que los exosomas derivados de subpoblaciones de CSCs de melanoma maligno expresan el marcador CD44 reduciéndose significativamente en las CSCs tratadas con IFN-α.

12. Los exosomas derivados de las subpoblaciones de CSCs de A375 y MEL-1 tratadas con IFN-α presentaron diferencias metabólicas significativas respecto a los exosomas de las melanosferas no tratadas, destacando el metabolito previamente descrito 1-hexadecanoil-sn-glicero-3-fosfocolina (PC 16:0/0:0) como un potencial biomarcador.

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9. ANNEXS

- **ANNEX I: Supplementary Documents Chapter II**.

INFORMED CONSENT FOR DONATION OF BIOLOGICAL SAMPLES AND ASSOCIATED INFORMATION TO THE BIOBANK (SSPA)

Ejemplar para el Paciente
Consentimiento informado para donación de muestras biológicas e información asociada al biobanco
Biobanco en Red del Sistema Sanitario Público de Andalucía. Versión 6.0 de 25 de febrero de 2020

CONSENTIMIENTO INFORMADO PARA DONACIÓN DE MUESTRAS BIOLÓGICAS E INFORMACIÓN ASOCIADA AL BIOBANCO

Biobanco en Red del Sistema Sanitario Público de Andalucía.

DATOS DEL/DE LA DONANTE Y DE SU REPRESENTANTE (éste último sólo en caso de incapacidad del/de la donante):
Apellidos y nombre del/de la Donante:
……
DNI / NIE: …… **NUHSA:** ……
Apellidos y nombre del/de la representante legal:
……..……
DNI / NIE: ……...

PROFESIONALES QUE INTERVIENEN EN EL PROCESO DE INFORMACIÓN Y/O CONSENTIMIENTO:
Los siguientes profesionales declaran que se ha explicado la información relativa a la donación de muestras biológicas al Biobanco:
Apellidos y nombre
……
DNI / NIE: ……...

CONSENTIMIENTO:
Yo, D./Dña. …….. declaro bajo mi responsabilidad que **he leído y comprendido el Formulario de Información**, del que se me ha entregado un ejemplar.

He **recibido suficiente información** sobre la donación de muestras biológicas de (*detallar tipo de muestras a recoger y posibilidad de recogida en diferentes puntos de muestreo*)……e información asociada, al Biobanco, y sobre la posible realización de análisis genéticos sobre las mismas. He podido hacer preguntas sobre la información recibida y hablar con el profesional indicado, quien me ha resuelto todas las dudas que le he planteado.

Dichas muestras son:
□ Excedentes del procedimiento asistencial al que va a someterse o se ha sometido:
……
□ Tomadas mediante el procedimiento expreso (*indicar procedimiento*):
……

Las muestras biológicas e información asociada serán recogidas para la línea o área de investigación según los capítulos recogidos en la Clasificación Internacional de Enfermedades (CIE-10), indicado a continuación (*indicar capítulo correspondiente*):

- Ciertas enfermedades infecciosas y parasitarias (A00-B99)
- Neoplasias (C00-D49)
- Enfermedades de la sangre y órganos hematopoyéticos y ciertos trastornos que afectan al mecanismo
- inmunológico (D50-D89)
- Enfermedades endocrinas, nutricionales y metabólicas (E00-E89)
- Trastornos mentales, del comportamiento y del desarrollo neurológico (F01-F99)
- Enfermedades del sistema nervioso (G00-G99)
- Enfermedades del ojo y sus anexos (H00-H59)
- Enfermedades del oído y de la apófisis mastoides (H60-H95)
- Enfermedades del aparato circulatorio (I00-I99)
- Enfermedades del aparato respiratorio (J00-J99)
- Enfermedades del aparato digestivo (K00-K95)
- Enfermedades de la piel y del tejido subcutáneo (L00-L99)
- Enfermedades del aparato musculoesquelético y del tejido conectivo (M00-M99)
- Enfermedades del aparato genitourinario (N00-N99)
- Embarazo, parto y puerperio (O00-O9A)
- Ciertas afecciones originadas en el periodo perinatal (P00-P96)
- Malformaciones congénitas, deformidades y anomalías cromosómicas (Q00-Q99)
- Lesiones traumáticas, envenenamientos y otras consecuencias de causas externas (S00-T88)

Así mismo, consiente que las muestras puedan ser utilizadas para otros fines:
– Docencia
– Control de calidad

Deseo que dichas muestras y los datos clínicos asociados sean tratados de forma:
□ **Codificada** (serán identificadas con un código que protege mi identidad, siendo posible volver a ligarlas
conmigo) o
□ **Anonimizada** (no se podrán asociar las muestras conmigo, por haberse eliminado de forma irreversible la vinculación entre las mismas y mi identidad).

Deseo **establecer restricciones** respecto al uso de la muestra, para que no sea utilizada en
……

Autorizo que se pueda **contactar conmigo posteriormente**:
□ SI
□ NO

En caso afirmativo, por favor, indique el medio de hacerlo:
Teléfono: *(indicar número)*……
Correo electrónico: *(indicar dirección)*……
Otros: (identificar)……

Autorizo **recibir información** sobre datos genéticos y datos relevantes para mi salud (Si solicita que las muestras sean anonimizadas, no podrá recibir esta información)
Marque lo que proceda:
□ SI
□ NO

Sé que puedo **revocar**, en cualquier momento, el consentimiento otorgado en este documento.

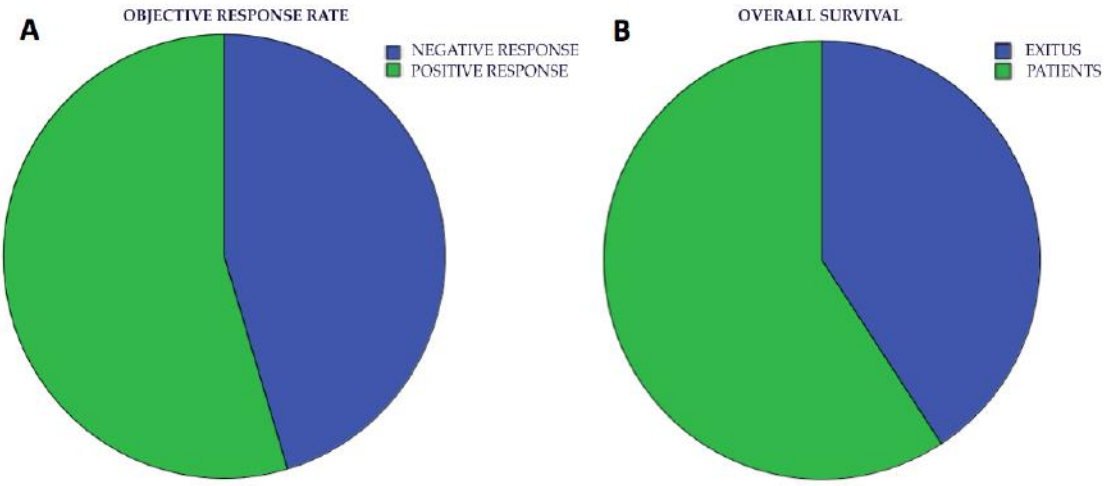
En , a de 2021

EL/LA DONANTE EL/LA REPRESENTANTE LEGAL PROFESIONAL QUE INFORMA
Fdo.: Fdo.:

Supplementary Table S1. Normalized data (from 0 to 1) from qPCR measurement. Nº ID (Identity number for each patient); Final Cluster 1; Cluster 2; Cluster 3); Adult Age (normalized age); Early Age (normalized age); ORR (Objective Response Rate – First Response to 5-FU based chemotherapy treatment (Negative Response 0; Positive Response 1); Plasma samples (P-), measured PKR and nc886; Tumor tissues (T-) measured PKR and nc886; Health Tissues (S-) measured PKR and nc886.

Nº ID	Final-Cluster	Adult Age	Early Age	ORR	P-PKR	P-nc886	T-PKR	T-nc886	S-PKR	S-nc886
1	2	0,72941	0,27059	1	0,41450	0,42929	0,33008	0,34933	0,52365	0,64166
2	2	0,65882	0,34118	0	0,45773	0,38268	0,33379	0,33961	0,34503	0,41328
3	2	0,92941	0,07059	1	0,50000	0,44528	0,42210	0,37173	0,37434	0,38573
4	2	0,74118	0,25882	1	0,42597	0,35712	0,34173	0,39778	0,48678	0,49473
5	2	0,68235	0,31765	1	0,43546	0,47802	0,37243	0,35447	0,46311	0,50748
6	2	0,75294	0,24706	1	0,36944	0,18104	0,50000	0,50000	0,42093	0,45738
7	2	0,84706	0,15294	1	0,45259	0,50000	0,25770	0,20787	0,36124	0,41410
8	2	1,00000	0,50000	1	0,50000	0,26032	0,34078	0,47259	0,41666	0,42135
9	2	0,78824	0,21176	0	0,40776	0,34825	0,34845	0,18495	0,42537	0,46278
10	1	0,88235	0,11765	0	0,41353	0,31369	0,31474	0,39402	0,39367	0,11042
11	1	0,69412	0,30588	0	0,47150	0,36963	0,39984	0,52880	0,38548	0,23527
12	1	0,68235	0,31765	0	0,50000	0,45938	0,36427	0,41123	0,30122	0,19457
13	2	0,70588	0,29412	1	0,44079	0,30123	0,35658	0,28731	0,49465	0,47350
14	1	0,97647	0,02353	0	0,49431	0,29724	0,36738	0,19293	0,38848	0,08915
15	1	0,72941	0,27059	1	0,50000	0,30772	0,33416	0,35235	0,38845	0,13893
16	1	0,81176	0,18824	0	0,63803	0,59546	0,36524	0,36700	0,41114	0,20012
17	3	0,83529	0,16471	1	0,50000	0,50000	0,71231	0,56844	0,56082	0,72342
18	3	0,61176	0,38824	0	0,50000	0,25629	0,41308	0,73820	0,80611	0,85215
19	3	0,98824	0,01176	0	0,50000	0,50000	0,66855	0,47489	0,86813	0,71882
20	3	0,92941	0,07059	1	0,50000	0,46670	0,64563	0,61957	0,80396	0,76500
21	3	1,00000	0,50000	0	0,50000	0,63202	0,88013	0,70587	0,87521	0,78989
22	3	0,65882	0,34118	0	0,50000	0,50000	0,56869	0,49570	0,74207	0,78439
23	3	0,69412	0,30588	1	0,50000	0,50000	0,81127	0,88278	0,91415	1,00000
24	3	0,57647	0,42353	1	0,50000	0,45429	0,50000	0,86174	0,50000	0,50000
25	3	0,67059	0,32941	0	0,34898	0,17577	0,69592	1,00000	0,91735	0,64745
26	3	0,71765	0,28235	1	0,50000	0,40584	0,77312	0,66097	1,00000	0,73626
27	3	0,77647	0,22353	1	0,50000	0,55298	1,00000	0,77097	0,82585	0,73562
28	2	0,81176	0,18824	1	0,37696	0,22301	0,51245	0,33005	0,25673	0,50000
29	2	0,68235	0,31765	1	0,50000	0,30033	0,44620	0,40258	0,41291	0,42248
30	2	0,95294	0,04706	1	0,52839	0,33006	0,56314	0,37520	0,59574	0,38263
31	1	0,70588	0,29412	0,5	0,50000	0,50000	0,58479	0,29995	0,42039	0,12856
32	2	0,85882	0,14118	1	0,60170	0,38924	0,57083	0,40736	0,74794	0,56108
33	2	0,89412	0,10588	0	0,50000	0,44063	0,73516	0,42674	0,50000	0,50000
34	1	0,32941	0,67059	1	0,50000	0,26778	0,48464	0,37907	0,52065	0,41727
35	1	0,60000	0,40000	0	0,41538	0,35206	0,50000	0,50000	0,52712	0,23776
36	2	0,89412	0,10588	0	0,50000	0,49423	0,42790	0,50113	0,64739	0,49613
37	2	0,78824	0,21176	1	0,51513	0,28404	0,39236	0,40276	0,39767	0,28514
38	2	0,94118	0,05882	0,5	0,75798	0,55489	0,43030	0,45481	0,62035	0,45042
39	2	0,95294	0,04706	0	0,42061	0,50000	0,40796	0,39579	0,48460	0,30799
40	1	0,81176	0,18824	1	0,40602	0,28975	0,43077	0,32155	0,50000	0,04563
41	3	0,58824	0,41176	1	0,50000	0,73998	0,86525	0,64276	0,75016	0,73949
42	2	0,71765	0,28235	0	0,50000	0,50000	0,50000	0,50000	0,44547	0,60730
43	2	0,71765	0,28235	0	0,72192	0,51853	0,49322	0,46247	0,68461	0,64724
44	3	0,72941	0,27059	0	0,50165	0,25615	0,54686	0,55281	0,72688	0,68091
45	3	0,96471	0,03529	0	0,46703	0,50000	0,65365	0,56464	0,62854	0,78111
46	3	0,92941	0,07059	0	0,49508	0,55616	0,51803	0,58707	0,58303	0,75101
47	2	0,83529	0,16471	0,5	0,61961	0,45679	0,54029	0,47960	0,57998	0,60774
48	3	0,87059	0,12941	0,5	0,50000	0,50000	0,82750	0,70593	0,58203	0,59249
49	3	0,97647	0,02353	0,5	0,50000	0,50000	0,46006	0,89763	0,57530	0,80907
50	2	0,77647	0,22353	0,5	0,50000	0,50000	0,41634	0,40978	0,53427	0,63085
51	2	0,77647	0,22353	0	0,50000	0,50000	0,55292	0,56796	0,56239	0,61842
52	3	0,74118	0,25882	0	0,50000	0,37539	0,93059	0,75156	0,56429	0,77208
53	3	0,63529	0,36471	1	0,50000	0,37328	0,50462	0,68323	0,63199	0,72500

Nº ID	Final-Cluster	Adult Age	Early Age	ORR	P-PKR	P-nc886	T-PKR	T-nc886	S-PKR	S-nc886
55	2	0,87059	0,12941	0	0,50000	0,50000	0,36198	0,33642	0,46632	0,40195
56	1	0,60000	0,40000	1	0,50000	0,50000	0,41382	0,41955	0,63140	0,55475
57	2	0,87059	0,12941	1	0,50000	0,50000	0,41158	0,45192	0,46920	0,41157
58	2	0,69412	0,30588	0	0,50000	0,50000	0,44590	0,40564	0,30383	0,50000
59	2	0,68235	0,31765	0	0,50000	0,50000	0,47851	0,36322	0,47526	0,41465
60	2	0,89412	0,10588	0	0,50000	0,50000	0,51394	0,34410	0,13263	0,50000
61	2	0,72941	0,27059	1	0,50000	0,50000	0,27757	0,21566	0,38620	0,40652
62	2	0,84706	0,15294	1	0,50000	0,50000	0,44085	0,35183	0,54230	0,34330
63	1	0,45882	0,54118	1	0,50000	0,50000	0,37598	0,44202	0,43795	0,31607
64	2	0,68235	0,31765	1	0,50000	0,50000	0,35470	0,35305	0,42815	0,41504
65	2	0,83529	0,16471	1	0,50000	0,50000	0,36396	0,32570	0,36707	0,36146
66	2	0,70588	0,29412	1	0,50000	0,50000	0,32002	0,26388	0,55537	0,53402
67	2	0,88235	0,11765	1	0,50000	0,50000	0,37959	0,38312	0,63501	0,57795
68	1	0,55294	0,44706	0	0,50000	0,50000	0,31258	0,32392	0,51509	0,39420
69	1	0,54118	0,45882	0	0,50000	0,50000	0,27688	0,29423	0,36377	0,16414
70	2	0,75294	0,24706	1	0,40223	0,24834	0,38766	0,30872	0,43767	0,35198
71	1	0,52941	0,47059	0	0,50000	0,50000	0,39583	0,50000	0,50000	0,50000
72	1	0,49412	0,50588	0	0,50000	0,50000	0,50000	0,50000	0,58057	0,41583
73	2	0,78824	0,21176	0	0,50000	0,50000	0,37831	0,50000	0,53078	0,27872
74	2	0,78824	0,21176	0	0,50000	0,50000	0,36558	0,50000	0,57462	0,36336
75	1	0,54118	0,45882	1	0,50000	0,50000	0,39193	0,50000	0,49483	0,29929
76	2	0,84706	0,15294	1	0,50000	0,50000	0,48392	0,50000	0,68021	0,48332
77	2	0,76471	0,23529	0	0,50000	0,50000	0,49971	0,50000	0,50000	0,50000
78	2	0,81176	0,18824	1	0,50000	0,50000	0,32461	0,50000	0,65457	0,44181
79	2	0,91765	0,08235	0	0,50000	0,50000	0,38001	0,50000	0,57481	0,47922
80	2	0,90588	0,09412	1	0,50000	0,50000	0,40253	0,50000	0,63655	0,57264
81	1	0,77647	0,22353	1	0,50000	0,50000	0,45676	0,50000	0,52656	0,02948
82	1	0,91765	0,08235	1	0,50000	0,50000	0,39387	0,50000	0,34405	0,15563
83	2	0,72941	0,27059	0	0,50000	0,50000	0,59267	0,50000	0,63062	0,45470
84	1	0,62353	0,37647	1	0,50000	0,50000	0,35191	0,50000	0,44881	0,50000
85	2	0,75294	0,24706	1	0,50000	0,50000	0,43227	0,50000	0,55864	0,50000
86	1	0,58824	0,41176	1	0,50000	0,50000	0,36266	0,50000	0,63149	0,50000
114	2	0,67059	0,32941	1	0,50000	0,31323	0,50000	0,50000	0,50000	0,50000
115	2	0,71765	0,28235	0	0,50000	0,50000	0,50000	0,50000	0,50000	0,50000
116	2	0,72941	0,27059	1	0,44564	0,29950	0,50000	0,50000	0,50000	0,50000
117	2	0,71765	0,28235	1	0,50000	0,50000	0,50000	0,50000	0,50000	0,50000
118	2	0,85882	0,14118	1	0,60960	0,50000	0,50000	0,50000	0,50000	0,50000
119	2	0,62353	0,37647	0	0,50000	0,25672	0,50000	0,50000	0,50000	0,50000
120	2	0,64706	0,35294	1	0,66276	0,50000	0,50000	0,50000	0,50000	0,50000
121	2	0,85882	0,14118	1	0,40583	0,25111	0,50000	0,50000	0,50000	0,50000
122	2	0,89412	0,10588	1	0,39230	0,25897	0,50000	0,50000	0,50000	0,50000
123	2	0,84706	0,15294	1	0,46891	0,24310	0,50000	0,50000	0,50000	0,50000
124	2	0,94118	0,05882	0,5	0,46086	0,22159	0,50000	0,50000	0,50000	0,50000
125	2	0,63529	0,36471	0	1,00000	1,00000	0,50000	0,50000	0,50000	0,50000
126	2	0,67059	0,32941	1	0,40659	0,27222	0,50000	0,50000	0,50000	0,50000
127	2	0,70588	0,29412	0	0,50000	0,26660	0,50000	0,50000	0,50000	0,50000
128	2	0,70588	0,29412	0	0,48047	0,22015	0,50000	0,50000	0,50000	0,50000
129	2	0,69412	0,30588	1	0,47108	0,23122	0,50000	0,50000	0,50000	0,50000
130	2	0,62353	0,37647	1	0,50000	0,32380	0,50000	0,50000	0,50000	0,50000
131	2	0,71765	0,28235	1	0,38858	0,20317	0,50000	0,50000	0,50000	0,50000
132	2	0,80000	0,20000	1	0,52269	0,34118	0,50000	0,50000	0,50000	0,50000
133	1	0,50000	1,00000	1	0,50000	0,50000	0,36202	0,37381	0,50000	0,50000
134	1	0,50000	1,00000	0	0,50000	0,50000	0,36707	0,29090	0,40621	0,39910
135	1	0,50000	1,00000	1	0,50000	0,50000	0,31666	0,22798	0,33720	0,39617
136	1	0,50000	1,00000	0,5	0,50000	0,50000	0,34977	0,37588	0,50000	0,50000
137	1	0,50000	1,00000	0	0,50000	0,50000	0,34555	0,33082	0,50000	0,50000
138	1	0,50000	1,00000	0	0,50000	0,50000	0,40732	0,23795	0,50000	0,50000
139	1	0,50000	1,00000	0	0,50000	0,50000	0,33489	0,37588	0,40114	0,54962
140	1	0,50000	1,00000	0	0,50000	0,50000	0,26532	0,47860	0,32829	0,47393



Supplementary Figure 1: Graphics show the frequencies of subject respect to the clinical details. *(A)* Frecuency in the Objetive Response Rate of subjects who had a Positive response, represented in green colour (54,6%) and Negative Response in blue (45,4%); *(B)* Frecuency of the overall survival in a year divided in Patients in green (59,2%). Exitus in blue (40,8%).

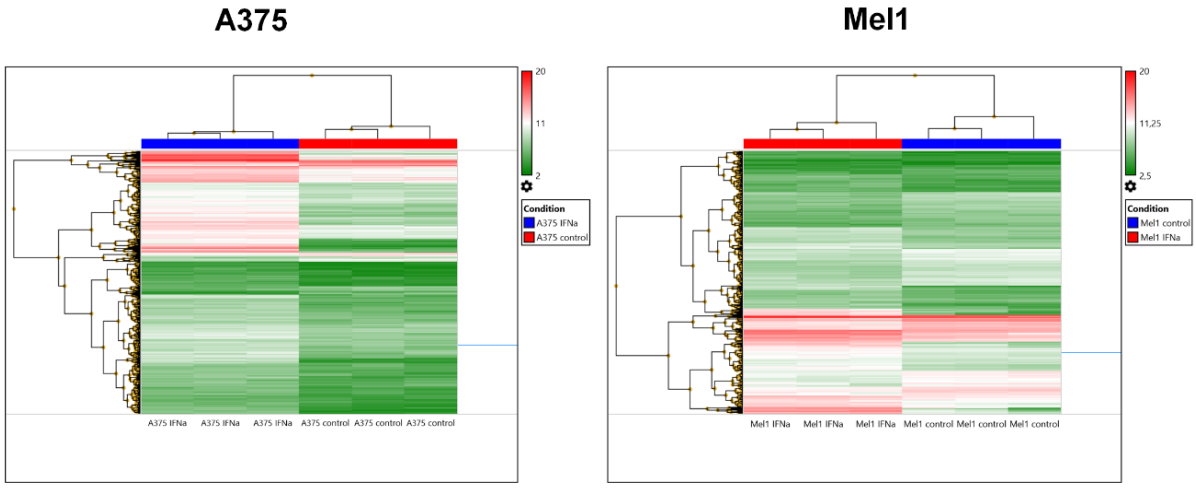
	Relative messenger-RNA measured by Real-Time PCR	
Samples	**Protein Kinase Receptor** (called PKR)	**Micro-RNA-nc886** (called RNA)
Blood (Plasma)	PPKR	P-nc886
Tumor Tissue	TPKR	T-nc886
Health Tissue	SPKR	S-nc886

Supplementary Table S2: Variables used in the statistical study based in clustering analyses. Relative messenger-RNA measured by Real-Time PCR.

- ANNEX II: Supplementary Figures Chapter III.

miRNA	Mature sequence
hsa-miR-7-5p	UGGAAGACUAGUGAUUUUGUUGUU
hsa-miR-98-5p	UGAGGUAGUAAGUUGUAUUGUU
hsa-miR-141-3p	UAACACUGUCUGGUAAAGAUGG
hsa-miR-191	GCUGCGCUUGGAUUUCGUCCCC
hsa-miR-425	AUCGGGAAUGUCGUGUCCGCCC
hsa-miR-550a	UGUCUUACUCCCUCAGGCACAU
hsa-miR-744-3p	CUGUUGCCACUAACCUCAACCU
hsa-miR-3614-5p	CCACUUGGAUCUGAAGGCUGCCC
hsa-miR-4521	GCUAAGGAAGUCCUGUGCUCAG
hsa-miR-4645	UGAUAGGGAAACCAGGCAAGAAAU
Let-7e-3p	UGAGGUAGGAGGUUGUAUAGUU

Supplementary Table S3. Primer sequences used to qRT-PCR for miRNA.



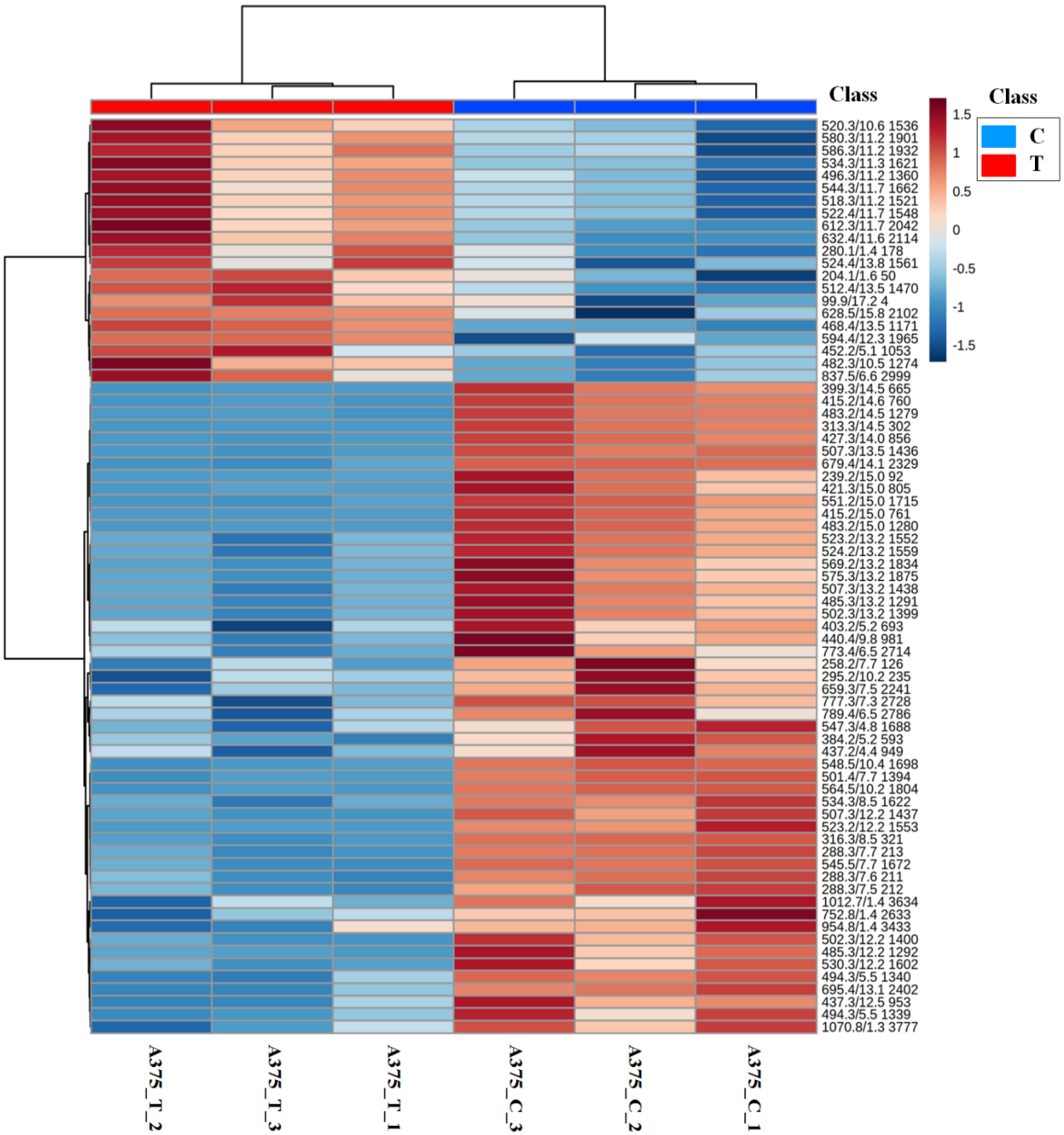
Supplementary Figure 2: Expression profile heatmap of the fold change genes up and downregulated after 24h of IFN treatment in A374 and MEL-1 CSCs.

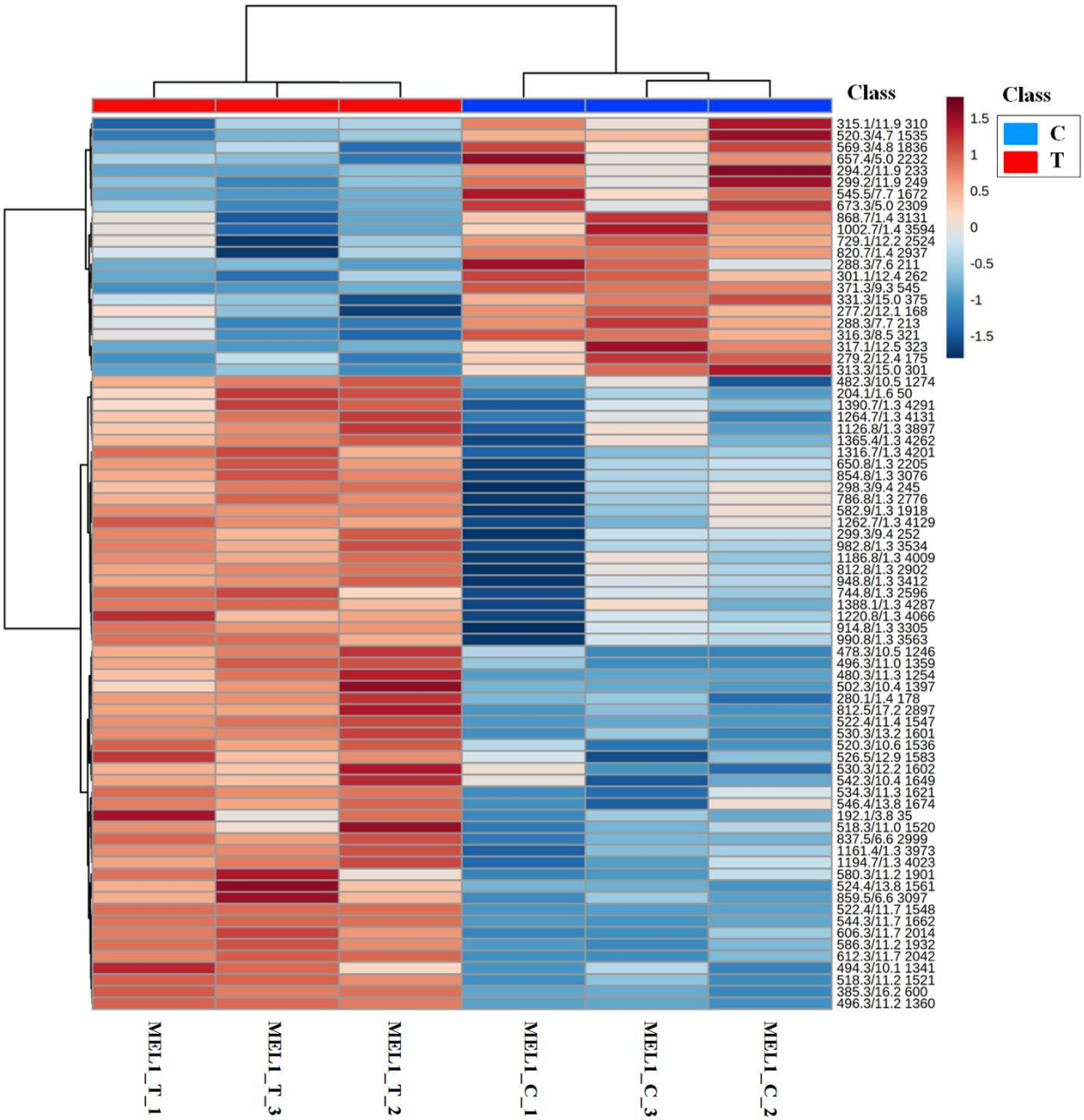
ID count: 16	A375 IFNa Avg (log2)	A375 control Avg (log2)	Fold Change	P-val	FDR P-val	Gene Symbol	Description
TC0700011797....	15,03	5,99	529,84	7,83E-14	1,62E-11	SAMD9L	sterile alpha motif domain containing 9-like
TC1400008056....	12,71	4,09	392,9	3,66E-14	9,80E-12	IFI27	interferon, alpha-inducible protein 27
TC0200015242....	18,26	12,31	61,63	2,59E-13	3,71E-11	STAT1	signal transducer and activator of transcription 1
TC1200010908....	12,43	7,33	34,33	1,90E-13	2,91E-11	STAT2	signal transducer and activator of transcription 2
TC0900006559....	8,73	3,8	30,47	1,28E-13	2,39E-11	CD274	CD274 molecule
TC0300013146....	6,86	3,24	12,29	1,54E-09	3,33E-08	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10
TC0200010447....	11,53	7,93	12,15	1,47E-13	2,62E-11	CASP8	caspase 8, apoptosis-related cysteine peptidase
TC0600013231....	12,33	9,79	5,85	7,48E-09	1,19E-07	SGK1	serum/glucocorticoid regulated kinase 1
TC1200009734....	8,46	6,09	5,14	1,34E-06	9,29E-06	VAMP1	vesicle associated membrane protein 1
TC0200010445....	6,74	4,66	4,23	6,82E-07	5,29E-06	CASP10	caspase 10
TC0900008202....	9,63	7,73	3,74	4,89E-06	2,81E-05	TGFBR1	transforming growth factor, beta receptor 1
TC1000008954....	9,1	7,26	3,56	1,16E-05	5,94E-05	CASP7	caspase 7
TC1100013221....	5,48	3,93	2,92	0,0143	0,0284	CASP1	caspase 1
TC1100012165....	11,6	10,55	2,07	0,0003	0,0010	CASP4	caspase 4
TC0100011533....	5	7,74	-6,68	5,61E-09	9,43E-08	ATF3	activating transcription factor 3
TC0100014349....	6,97	11,16	-18,25	1,74E-13	2,85E-11	JUN	jun proto-oncogene

Supplementary Table S4: List of the selected genes whose expression has changed after 24h of IFN treatment in A375 CSCs.

ID count: 16	Mel1 IFNa Avg (log2)	Mel1 control Avg (log2)	Fold Change	P-val	FDR P-val	Gene Symbol	Description
TC0700011797....	14,8	6,55	305,22	7,31E-13	1,12E-09	SAMD9L	sterile alpha motif domain containing 9-like
TC1400008056....	13,08	6,6	89,31	2,46E-11	1,89E-08	IFI27	interferon, alpha-inducible protein 27
TC0300013146....	13,62	8,18	43,66	2,11E-13	4,83E-10	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10
TC0900006559....	8,86	5,08	13,69	1,14E-11	1,06E-08	CD274	CD274 molecule
TC0200015242....	18,71	15,78	7,62	5,38E-08	1,16E-05	STAT1	signal transducer and activator of transcription 1
TC1200010908....	11,59	8,7	7,4	2,25E-09	8,19E-07	STAT2	signal transducer and activator of transcription 2
TC1100013221....	5,77	3,35	5,34	4,24E-05	0,0027	CASP1	caspase 1
TC1100012165....	11,65	9,63	4,07	2,09E-08	5,00E-06	CASP4	caspase 4
TC1000008954....	10,21	8,41	3,48	5,15E-05	0,0031	CASP7	caspase 7
TC0100011533....	6,68	5,21	2,77	1,15E-05	0,0010	ATF3	activating transcription factor 3
TC0200010445....	5,64	4,21	2,68	0,0002	0,0087	CASP10	caspase 10
TC0200010447....	12,27	11,12	2,22	0,0001	0,0057	CASP8	caspase 8, apoptosis-related cysteine peptidase
TC0600013231....	14,24	13,11	2,2	0,0002	0,0085	SGK1	serum/glucocorticoid regulated kinase 1
TC0100014349....	6,02	4,89	2,18	0,0010	0,0259	JUN	jun proto-oncogene
TC1200009734....	6,85	8,25	-2,63	0,0013	0,0320	VAMP1	vesicle associated membrane protein 1
TC0900008202....	8,43	10,19	-3,38	5,08E-05	0,0031	TGFBR1	transforming growth factor, beta receptor 1

Supplementary Table S5: List of the selected genes whose expression has changed after 24h of IFN treatment in MEL-1 CSCs.


Class
Class
C
T
1.5
1
0.5
0
-0.5
-1
-1.5
520.3/10.6 1536
580.3/11.2 1901
586.3/11.2 1932
534.3/11.3 1621
496.3/11.2 1360
544.3/11.7 1662
518.3/11.2 1521
522.4/11.7 1548
612.3/11.7 2042
632.4/11.6 2114
280.1/1.4 178
524.4/13.8 1561
204.1/1.6 50
512.4/13.5 1470
99.9/17.2 4
628.5/15.8 2102
468.4/13.5 1171
594.4/12.3 1965
452.2/5.1 1053
482.3/10.5 1274
837.5/6.6 2999
399.3/14.5 665
415.2/14.6 760
483.2/14.5 1279
313.3/14.5 302
427.3/14.0 856
507.3/13.5 1436
679.4/14.1 2329
239.2/15.0 92
421.3/15.0 805
551.2/15.0 1715
415.2/15.0 761
483.2/15.0 1280
523.2/13.2 1552
524.2/13.2 1559
569.2/13.2 1834
575.3/13.2 1875
507.3/13.2 1438
485.3/13.2 1291
502.3/13.2 1399
403.2/5.2 693
440.4/9.8 981
773.4/6.5 2714
258.2/7.7 126
295.2/10.2 235
659.3/7.5 2241
777.3/7.3 2728
789.4/6.5 2786
547.3/4.8 1688
384.2/5.2 593
437.2/4.4 949
548.5/10.4 1698
501.4/7.7 1394
564.5/10.2 1804
534.3/8.5 1622
507.3/12.2 1437
523.2/12.2 1553
316.3/8.5 321
288.3/7.7 213
545.5/7.7 1672
288.3/7.6 211
288.3/7.5 212
1012.7/1.4 3634
752.8/1.4 2633
954.8/1.4 3433
502.3/12.2 1400
485.3/12.2 1292
530.3/12.2 1602
494.3/5.5 1340
695.4/13.1 2402
437.3/12.5 953
494.3/5.5 1339
1070.8/1.3 3777
A375_T_2
A375_T_3
A375_T_1
A375_C_3
A375_C_2
A375_C_1



Supplementary Figure 3. The heatmaps representing the differential abundance of those metabolites are shown for A375 (up) and MEL-1 (down) cell lines, respectively. As can be observed, in both cell lines, most of those metabolites were more abundant in exosome samples derived from control CSCs, compared to those from IFN-treated CSCs.

miRNA	Target	mirBASE ID	Role in Melanoma Cancer	Biomarker Type	Regulation in Melanoma	References
hsa-miR-7-5p	KLF4/PI3K/Akt/p21	MIMAT0000252	Tumour Suppressor	Predictive	Up-regulation	A. Khan et al,. 2019
hsa-miR-98-5p	IL6/Stat3/NF-kB	MIMAT0000096	Tumour Suppressor	Diagnostic	Down-regulation	Fei Li et al,. 2014
hsa-miR-141-3p	MITF/CDK6/Akt	MIMAT0000432	Tumour Suppressor	Prognostic	Up-regulation	Horacio-González et al,. 2015
hsa-miR-191	BRAF/MAPK/ERK	MIMAT0001618	OncomiR	Predictive	Down-regulation	Pinto R et al., 2015
hsa-miR-425	IGF1/PI3K/Akt	MIMAT0001343	OncomiR	Prognostic and therapeutic	Up-regulation	Chen X, et al, 2017
hsa-miR-550a	BRAF/YAP	MIMAT0003257	Tumour Suppressor	Prognostic	Up-regulation	Min Ho Choe et al,. 2018
hsa-miR-744-3p	SOX12/c-Myc	MIMAT0004946	Tumour Suppressor	Prognostic and therapeutic	Down-regulation	Wang H, et al,. 2018
hsa-miR-3614-5p	FOX3/Akt/pTEN	MIMAT0017992	Tumour Suppressor	Diagnostic	Up-regulation	Wang Z, et al,. 2018
hsa-miR-4521	FOX1/PLK1/cyclinB1	MIMAT0019058	Tumour Suppressor	Prognostic	Up-regulation	Senfter D. et al,. 2019
hsa-miR-4645	HER2/ERB2/Akt/mTOR	MIMAT0019705	OncomiR	Prognostic	Up-regulation	Persson, et al,. 2011
let 7e-3p	NRAS/BRAF/MEK	MIMAT0000066	Tumour Suppressor	Therapeutic	Down-regulation	Varrone F. et al,. 2020

Supplementary Table S6. Summary outline of the selected miRNAs and its role in biological processes.

- **ANNEX III: Glossary.**

LIST OF ABBREVIATIONS AND ACRONYMS

A

5-FU: 5-Fluorouracilo

ABC: ATP-Binding Cassette

AFM: Atomic Force Microscopy

AJCC: American Joint Committee on Cancer

ALDH1: Aldehyde Dehydrogenase 1

ALM: Acral Lentiginous Melanoma

ATCC: American Type Culture Collection

ATM: Ataxia Telangiectasia Mutated

AUC: Area under the curve

B

BS: Blank solvent

C

CASP: Caspasas

CCR: Cáncer Colorrectal

CCS: Cancer Subtypes

cDNA: Complementary DNA

CIMP: CpG island methylator phenotype

CIN: Chromosomal instability

CSCs: Cancer Stem Cells

CT: Cycle Threshold

CTLA-4: Cytotoxic T-Lymphocyte Antigen 4

D

DMEM: Dubelcco's Modified Eagle's medium

DMEM-F12 Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham

DMSO: Dimetilsulfoxido

DNA: Deoxyribonucleic Acid

E

E-Cadherina: Epithelial Cadherin

EDTA: Ethylenediaminetetraacetic Acid

EFG: Epidermal Growth Factor

EGFR: Epidermal Growth Factor Receptor

EIF2AK2: Eukaryotic Translation Initiation Factor 2 Alpha Kinase 2

EMT: Epithelial-Mesenchymal Transition

ESCRT: Endosomal Sorting Complex Required for Transport

EV: Extracellullar vesicles

F

FACS: Fluorescence-activated Cell Sorting

FAP: Poliposis adenomatosa familiar

FBS: Fetal Bovine Serum

FC: Fold change

FDA: Food and Drug Administration

FGF: Fibroblast Growth Factor

FITC: Fluorescein Isothiocyanate

G

GAPDH: Glyceraldehyde 3- phosphate dehydrogenase

H

H&E: Haematoxylin and Eosin

HCC: Hepatocellular carcinoma

HCs: Healthy controls

Hh: Hedgehog

HIF-1: Hypoxia-Inducible Factor 1

HPLC: High performance liquid chromatography

HRMS: High resolution mass spectrometry

I

IARC: International Agency for Research on Cancer

IFN: Interferon

IFN-α: Interferon- α

IR: Ionizing radiation

ISEV: International society for extracellular vesicles

ISGs: Interferon-stimulated genes

ITS: Insulin transferrin selenium

IU: International Unit

J

Jak- PTKs: Proteínas tirosina-quinasa Janus

L

LBPA: Lysobisphosphatidic acid

LC: Liver cirrhosis

LDH: Lactate dehydrogenase

LMM: Lentigo malignant melanoma

M

MDR: Multidrug Resistance

MET: Mesenchymal Epithelial Transition

MHC: Complejo Mayor de Histocompatibilidad

miR: MicroRNA

MISEV: Minimal information for studies of extracellular vesicles

MM: Malignant melanoma

MMP: Matrix Metalloproteinase

MMPs: Malignant melanoma patients

MS: Mass spectrometry

MMPs: Matrix Metalloproteinases

MMR: Mismacth repair

mRNA: Messenger RNA

MSI: Microsatellite instability

MVB: Multi-Vesicular Bodies

N

NK: Natural killer

NM: Nodular melanoma

P

P/S: Penicillin/streptomycin

P53: Gene tp53

PARP: Poli (ADP-ribosa) polimerasa 1

PBS: Phosphate-Buffered Saline

PC: Phosphatidylcholine

PCA: Principal component analysis

PCR: Polymerase Chain Reaction

PD1: Proteína de muerte celular 1

PDGFRA: Platelet-derived growth factor receptor alpha

PE: Phosphatidyletanolamine

PFA: Paraformaldehyde

PG: Glycerophosphoglycerol

PI: Propidium Iodide

PKR: Protein kinase R

PL: Phospholipid

PLS-DA: Partial least squares discriminant analysis

PS: Glycerophsphoserine

PTEN: Phosphatase and tensin homolog gen

Q

QC: Quality control

qPCR: Quantitative Polymerase Chain Reaction

Q-TOF-MS: Quadrupole time-of-flight mass spectrometer

R

RNA: Ribonucleic Acid

ROC: Receiver operating characteristic curve

ROS: Reactive Oxygen Species

RPM: Revolutions per minute

RT: Radiotherapy

RT: Room Temperature

RT-qPCR: Real-time Reverse-Transcription PCR

S

SEM: Scanning electron microscopy

sEVs: small extracellular vesicles

SEOM: Sociedad Española de Oncología Médica

SNARE: Soluble N-athylmaleimidesensitive fusion attacment protein

SP: Side Population

SSM: Superficial spreading melanoma

STATs: Signal transducer and activator of transcription protein

T

TEM: Transmission electron microscopy

TG: Triacylglicerol

TIC: Total ion chromatograms

TLR: Toll like receptor

TME: Tumour microenvironment

TNM: Classification of Malignant Tumours

U

UICC: Unión Internacional contra el Cáncer

UVA: Ultraviolet Radiation A

UVA: Ultraviolet Radiation B

V

VEGF: Vascular Endothelial Growth Factor

VIP: Variable importance in projection

W

WHO: World Health Organization