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“EL RECEPTOR A2B DE ADENOSINA ES UN REGULADOR DE LA MIGRACIÓN EN CÉLULAS DERIVADAS DE CARCINOMA OVÁRICO”

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PRESENTA:
M. EN C. ANAÍ DEL ROCÍO CAMPOS CONTRERAS

DIRECTOR DE TESIS:
DR. FRANCISCO GABRIEL VÁZQUEZ CUEVAS. INB
COMITÉ TUTOR:
DRA. ROCÍO BRENDA ANGUIANO SERRANO. INB
DR. ALEJANDRO GARCÍA CARRANCA. IIB

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A mi familia: Rosa, Federico, Rosy y Abraham.

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“It is not enough to have a good mind. The main thing is to use it well”.

“Divide each difficulty into as many parts as is feasible and necessary to resolve it”.

René Descartes

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RESUMEN

El sistema purinérgico de comunicación intercelular es un componente clave del microambiente tumoral dado que puede actuar de modo autocrino-paracrino para regular diversos procesos celulares como la proliferación. En este trabajo, hemos investigado el papel del receptor de adenosina A2B en la modulación del fenotipo de células derivadas de carcinoma ovárico (LDCOv). Utilizando bases de datos públicas, hemos observado que una alta expresión del receptor A2B está asociada con una mejor prognosis para los pacientes de carcinoma ovárico seroso (COv). Con el fin de elucidar el papel del receptor A2B en la regulación del fenotipo asociado con la migración de células de carcinoma se realizaron experimentos en la línea SKOV-3. Se demostró la expresión del receptor A2B en esta línea celular por RT-PCR, *western blot* e inmunofluorescencia. La estimulación del receptor A2B con BAY-606583 (BAY), un agonista selectivo, indujo la fosforilación en ERK1/2, y este efecto fue atenuado por el antagonista PSB-603. La activación del receptor A2B con BAY disminuyó la migración y la presencia de fibras de estrés, en concordancia, la disminución de la expresión del receptor A2B causó un incremento en la migración celular y promovió la presencia de fibras de estrés. Así mismo, la expresión del marcador epitelial E-Cadherina aumentó en las células tratadas con BAY e indujo su relocalización a las uniones celulares. Finalmente, el análisis transcripcional de cDNA por microarreglos mostró que la activación del receptor A2B regula a la baja la expresión de transcritos asociados con la migración e invasión celular tales como *ADAM12*, *MMP2* y *MMP16*. Asimismo, se encontró la regulación al alta de los transcritos de *PVRL2* y *DSG2*, los cuales están asociados con la adhesión celular. Los resultados muestran que el receptor A2B contribuye al mantenimiento de un fenotipo epitelial en LDCOv, por lo que este receptor es un posible blanco terapéutico para modular la migración de las células de carcinoma ovárico.

Palabras clave: señalización purinérgica, receptor A2B, carcinoma ovárico, migración celular, EMT

SUMMARY

The purinergic system is fundamental in the tumor microenvironment, regulating tumor cell interactions with the immune system, as well as growth and differentiation in autocrine-paracrine responses. Here, we investigated the role of the adenosine A2B receptor (A2BR) in ovarian carcinoma-derived cells' (OCDC) properties. From public databases, we documented that high A2BR expression is associated with a better prognostic outcome in ovarian cancer patients. In vitro experiments were performed on SKOV-3 cell line to understand how A2BR regulates the carcinoma cell phenotype associated with cell migration. RT-PCR and Western blotting revealed that the ADORA2B transcript (coding for A2BR) and A2BR were expressed in SKOV-3 cells. Stimulation with BAY-606583, an A2BR agonist, induced ERK1/2 phosphorylation, which was abolished by the antagonist PSB-603. Pharmacological activation of A2BR reduced cell migration and actin stress fibers; in agreement, A2BR knockdown increased migration and enhanced actin stress fiber expression. Furthermore, the expression of E-cadherin, an epithelial marker, increased in BAY-606583-treated cells. Finally, cDNA microarrays revealed the pathways mediating the effects of A2BR activation on SKOV-3 cells. It was found that transcripts *ADAM12*, *MMP2* and *MMP16* which are associated with cell invasion and migration were down-regulated. In addition, it was found the up-regulation of the transcripts *PVRL2* and *DSG2* which are associated with cell adhesion. Our results showed that A2BR contributed to maintaining an epithelial-like phenotype in OCDC and highlighted this purinergic receptor as a potential target to modulate OCDC migration.

Keywords: purinergic signaling, A2B receptor, ovarian cancer, cancer cell migration, SKOV-3 cells

1. INTRODUCCIÓN

El carcinoma ovárico es la neoplasia ginecológica más letal; en el 2020, se reportó una incidencia a nivel mundial del 4.8% con respecto al total de casos de cáncer en la población femenina y una mortalidad de 6.9% (http://globocan.iarc.fr). En nuestro país, representa el 6.2% de casos de este grupo de cánceres y presenta una tasa de mortalidad de 8.8% (http://globocan.iarc.fr). Este alto índice de mortalidad está asociado con el hecho de que el diagnóstico se realiza en estadios avanzados de la enfermedad, en los cuales se presenta metástasis en la cavidad peritoneal. Por lo que resulta importante estudiar las vías que regulan la migración e invasividad celular.

Un fenómeno que define el desarrollo del cáncer es la metástasis, que es el proceso de diseminación de la enfermedad que conduce a la formación de tumores secundarios en sitios alejados del tumor primario (Senga y Grose 2021). Los mecanismos celulares implícitos en la metástasis involucran plasticidad entre los fenotipo epitelial y mesenquimal. Uno de estos mecanismos es la transición epitelio-mesenquima (EMT, por sus siglas en inglés), un proceso de transdiferenciación celular en el que las células con fenotipo epitelial, adquieren un fenotipo mesénquimal y por lo tanto la capacidad de migrar e invadir órganos distantes. Aunque este proceso fue originalmente descrito en el desarrollo embrionario, un gran cuerpo de información fundamenta su participación en la inducción de metástasis. Dentro de las moléculas que modulan este proceso destacan el factor de crecimiento transformante β (TFG-β), los ligandos de la familia de *wingless* (Wnt) y el factor de crecimiento epidermal (EGF) (revisado en: Dongre & Weinberg, 2019).

Una gran variedad de estudios han demostrado la participación de diversos elementos del sistema de comunicación purinérgico en la regulación de la migración celular y de la EMT (revisado en: Martínez-Ramírez et al., 2017; Campos-Contreras et al., 2020). Estudios en nuestro laboratorio han demostrado que la hidrólisis del ATP producido por las células derivadas de carcinoma ovárico SKOV-3, mediante la adición de apirasa (Apy), disminuye la migración celular y favorece un fenotipo epitelial. La caracterización farmacológica de esta respuesta, permitió concluir que

el efecto es mediado por la producción de adenosina (ADO) extracelular por las ectonucleotidasas de las propias células. En concordancia, la estimulación farmacológica con ADO o NECA, un agonista no selectivo de los receptores a adenosina A2, inhibe la migración de las células SKOV-3 y favorece un fenotipo epitelial (Martínez-Ramírez et al., 2017). Dado que el receptor A2B tiene el mayor nivel de expresión en esta línea celular y que existe diversa evidencia sobre su papel como regulador de la migración celular en distintos tipos de cáncer (Gao & Jacobson, 2019), analizaremos la idea de que estos efectos son mediados probablemente por el receptor A2B.

A partir de estos antecedentes y evidencias generadas por nuestro grupo de trabajo, el objetivo del presente trabajo consistió en la caracterización del efecto de la activación del receptor A2B sobre la migración celular en células de carcinoma ovárico.

2. MARCO TEÓRICO

2.1. Cáncer de ovario

El término cáncer de ovario (COv) se utiliza para hacer referencia a cualquier tumor maligno en el ovario. Sin embargo, en años recientes se ha hecho hincapié en que la patología es un conjunto de neoplasias con características y pronósticos clinipatológicos y moleculares distintos (Prat 2012; Kroeger y Drapkin 2017).

Debido a su localización anatómica y sintomatología inespecífica y a pesar del uso de los métodos de diagnóstico rutinario, como el ultrasonido pélvico o transvaginal y la inmunodetección de la proteína CA-125, no se ha logrado desarrollar un estudio de tamizaje con alto valor predictivo, lo que conlleva al diagnóstico en estadios avanzados de la enfermedad.

La Federación Internacional de Ginecología y Obstetricia (FIGO) ha desarrollado una clasificación para determinar el grado de diseminación del COv, basada en métodos de imagen y a los hallazgos durante el procedimiento quirúrgico. En el estadio 1, el tumor está limitado al ovario; en el estadio 2, el tumor se ha extendido hacia la pelvis; en el estadio 3 el tumor se ha diseminado hacia el peritoneo y/o nódulos linfáticos y en el estadio 4 hay metástasis distales como lesiones hepáticas y/o efusiones pleurales (Prat y FIGO Committee on Gynecologic Oncology 2015). Otro concepto importante en el COv es el grado del tumor, el cual es una medida de que tan “desdiferenciadas” lucen las células cancerosas en comparación al tejido normal. De manera general, entre más se pierde el fenotipo del tejido normal (desdiferenciación) en las células tumorales, se designa un grado mayor (O’Shea 2022).

Los tumores de ovario se clasifican de acuerdo al lugar de origen en: epiteliales, del estroma y de las células germinales. Aproximadamente el 90% de los tumores ováricos tienen un origen epitelial (carcinomas) y estos a su vez comprenden diversos tipos histológicos: seroso de alto grado (**HGSC**), seroso de bajo grado (**LGSC**), endometroide (EC), de células claras (**CCC**), mucinoso (**MC**), de Brenner y el carcinoma epitelial mixto (Kossaï et al., 2018). En la Tabla 1 se

enlistan algunas características sobre los distintos tipos histológicos de carcinoma epitelial ovárico (CEOv).

Tabla 1. Características de los distintos subtipos de carcinoma ovárico

	LGSC	**HGSC**	**MC**	**EC**	**CCC**
Frecuencia	<5%	70%	2-3%	10%	5-10%
Inmunofenotipo	CK7+, WT1+, ER+	CK7+, CK20-, PAX8+, WT1+	CK7+, CK20+, ER-, PR-, WT1-	CK7+, PAX8+, CK20-, WT1-	Napsina A+, WT1-, p53-, ER-
Lesión precursora	Lesiones malignas de bajo grado	Fimbrias de la trompa de Falopio; STIC	Lesiones *borderline* mucinosas	Endometriosis	Endometriosis
Mutaciones	*KRAS, BRAF*	*TP53, BRCA1/2*	*KRAS, HER2*	*ARID1A, PTEN*	*ARID1A, PIK3CA*
Prognosis	Intermedia	Pobre	Buena	Favorable	Intermedia

Carcinoma seroso de bajo grado (LGSC); carcinoma seroso de alto grado (HGSC); carcinoma mucinoso (MC); carcinoma endometroide (EC); carcinoma de células claras (CCC); CK7, citoqueratina 7; CK20, citoqueratina 20; ER, receptor de estrógenos; PAX8, *paired box* 8; WT1, *Wilms tumor 1*; WT2, *Wilms tumor 2*; STIC: carcinoma tubárico intraepitelial seroso*. ARID1A,* Dominio de interacción rico en AT 1A; *BRAF*, Protooncogén B-Raf, serina/treonina cinasa; *BRACA1/2*, gen del cáncer de mama línea germinal 1/2; *HER2*, receptor 2 del factor de crecimiento epidermal; *KRAS*: Homólogo del oncogén viral del sarcoma de rata de Kirsten; *PI3KCA*: fosfatidilinositol-4,5-bisfosfato 3-cinasa subunidad catalítica α; *PTEN*: homólogo de fosfatasa y tensina; *TP53*: proteína tumoral 53. El signo + y – indican la presencia y ausencia respectivamente, la cual fue detectada por inmunohistoquímica. Modificado de Kossaï et al., 2018.

Una clasificación más actual divide los tumores epiteliales ováricos en dos grupos: tipo I y tipo II, (Kurman y Shih 2016). Los tumores tipo I son carcinomas de bajo grado y están diferenciados, entre estos se encuentran el LGSC, el EC, el MC y el CCC. Se caracterizan por presentar mutaciones somáticas en los loci *KRAS/BRAF/ERK, PI3KCA, PTEN, ARID1A* y *ERBB2*. Son tumores de baja agresividad y con mejor pronóstico. Los tumores tipo II incluyen carcinomas de alto grado, como el HGSC, el carcinoma indiferenciado y el carcinosarcoma. Estos tumores tienen mutaciones características del cáncer como *TP53* y son altamente desdiferenciados. Son tumores más agresivos, de peor pronóstico, pobre respuesta clínica y una alta recidiva. Cabe mencionar, que aproximadamente el 75% de los carcinomas de ovario son de este subtipo.

Tomando en cuenta los distintos fenotipos del CEOv, se han propuesto distintos orígenes tales como las células del epitelio superficial ovárico (OSE), las células epiteliales atrapadas en quistes de inclusión dentro del estroma ovárico, células provenientes de la porción distal de la trompa de Falopio, células originadas en alguna lesión del endometrio e incluso a partir de la transformación de células troncales del hilio ovárico (Klymenko et al., 2017). De igual modo, se ha planteado que el carcinoma tubárico intraepitelial seroso (STIC) es la transformación precursora del HGSC (Vang et al., 2013).

Finalmente, es importante mencionar que el diagnóstico oportuno es el factor más importante para una buena prognosis en el CEOv. No obstante más del 70% de las pacientes son diagnosticadas en estadios avanzados (3 y 4) y menos del 10% se diagnostican en estadios tempranos (1 y 2). A pesar de la existencia de múltiples tratamientos antineoplásicos la supervivencia es baja (O'Shea 2022). Por lo que el estudio de los reguladores de procesos biológicos clave como la migración y diseminación de este cáncer es clave para identificar métodos de diagnóstico oportunos y de este modo plantear una terapéutica más efectiva.

2.2. Mecanismos de diseminación del CEOv

Como se ha comentado anteriormente, el CEOv suele ser detectado cuando el tumor primario ha hecho metástasis intraperitoneal. A diferencia de la gran mayoría de los tumores, el CEOv sigue preferencialmente una ruta de diseminación transcelómica por lo que los sitios más frecuentes de metástasis son el peritoneo, el mesenterio, el omentum y las vísceras pélvicas y abdominales. No obstante, se han planteado dos modelos de diseminación para el CEOv: la vía transcelómica y la vía hematógena, los cuales serán expuestos a continuación (Yousefi et al., 2020) (Figura 1).

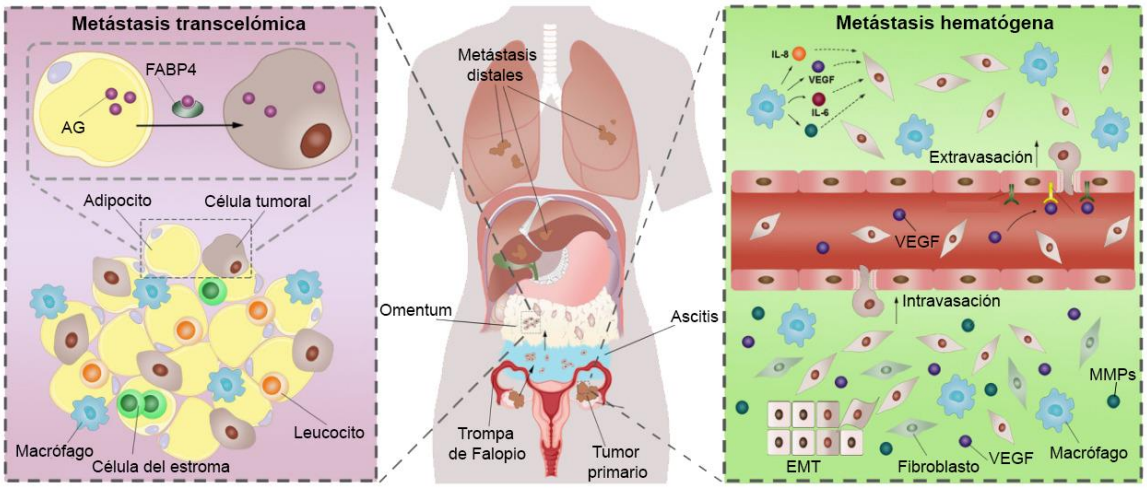

Figura 1. Modelos de la migración de las células tumorales en el cáncer de ovario. La metástasis transcelómica (izquierda) y la metástasis hematógena (derecha). Los adipocitos del omentum proveen ácidos grasos (AG) a las células tumorales para su proliferación y crecimiento. Los AG son transportados mediante el transportador de ácidos grasos FABP4. En la derecha, se ejemplifica el modelo de metástasis hematógena. EMT, transición epitelio mesénquima; IL-6, interleucina 6; IL-8, intereleucina 8; MMPs, metaloproteasas; VEGF, factor de crecimiento vascular endotelial. Modificado de Yousefi et al., 2020.

2.2.1 Metástasis transcelómica

En la metástasis transcelómica las células tumorales son exfoliadas directamente del tumor primario hacia el fluido intraperitoneal donde sobreviven a la anoiquis y migran hacia la cavidad peritoneal como células únicas o agregados multicelulares

(MCs). Posteriormente, las células tumorales se adhieren e invaden las superficies de los órganos blanco en la cavidad peritoneal, donde proliferan y forman tumores secundarios (Klymenko et al., 2017).

La exfoliación de las células tumorales del epitelio superficial ovárico (OSE) requiere de la pérdida de las uniones célula-célula. Dentro de las moléculas responsables de dichas uniones destaca la E-cadherina (E-cadh), una proteína transmembranal de adhesión dependiente de calcio, que participa en la formación de las uniones adherentes (Bruner y Derksen 2018). Se ha reportado que la disminución de la expresión de E-cadh tiene un papel crucial en la migración celular en distintos tipos de células de cáncer. La disminución de la expresión de la E-cadh está asociada con la inducción de la transición epitelio-mesénquima (EMT), un proceso de transdiferenciación determinado por la pérdida de características epiteliales y de la polaridad basal-apical, la disminución de la expresión de citoqueratinas, de proteínas de la zona occludens 1 (ZO-1) y de las uniones celulares. Por el contrario, en este proceso las células adquieren características mesenquimales como polaridad antero-posterior, incremento de la expresión de las metaloproteinasas (MMPs), de la N-cadherina (N-cadh) y vimentina, así como un reordenamiento del citoesqueleto de actina (Dongre y Weinberg 2019).

En distintas líneas derivadas de carcinoma ovárico (LDCOv) se ha documentado una correlación entre el bajo nivel de expresión de la E-cadh y la capacidad de invasividad (Rosso et al., 2017). De igual modo, la baja expresión de dicha proteína ha sido asociada con las características clinicopatológicas de los tumores, siendo menor en estadios avanzados de la enfermedad (estadios 3 y 4), en el HGSC y cuando hay metástasis peritoneal (Faleiro-Rodrigues et al., 2004).

La regulación de la EMT y la expresión de la E-cadh es mediada por los factores de transcripción ZEB1/2, *Snail* y *Slug*. Estos factores de transcripción pueden ser activados por diferentes vías de señalización, como TGF-β, EGF, WNT e incluso el ATP (Martínez-Ramírez et al., 2017).

Una vez en el líquido peritoneal, las células tumorales necesitan desarrollar resistencia a la anoiquis, como se denomina a la muerte celular debido a la ausencia de interacciones entre células y la matriz extracelular (Paoli et al.,2013). La

expresión de RAB25, una GTPasa pequeña, favorece este proceso en distintas líneas de carcinoma ovárico. De igual modo, se encontró la expresión de altos niveles del transcrito de RAB25, en estadios avanzados (3 y 4) del CEOv tipo seroso (Cheng et al., 2004).

La migración de las células tumorales es facilitada por la acumulación de ascitis en el peritoneo en pacientes con CEOv. Se ha reportado que el aumento de la secreción del factor de crecimiento vascular endotelial (VEGF) (Zebrowski et al., 1999) y la obstrucción de los vasos linfáticos promueven la producción de fluido ascítico. De igual modo, la secreción de VEGF de las células tumorales es favorecida por las MMP2 y MMP9 (Belotti et al., 2003). La ascitis exacerbada característica del CEOv hospeda distintos tipos celulares además de las células tumorales, tales como los fibroblastos asociados al cáncer (CAFs), células mesenquimales *stem* (MSC), células mesoteliales y células del sistema inmune (Ahmed & Stenvers, 2013; Kipps et al., 2013).

Una vez que las células mesenquimalizadas llegan a los sitios secundarios, se da inicio al proceso inverso a la EMT, la transición mesenquima-epitelio (MET), lo que implica el aumento de la expresión de E-cadh e integrinas como la $\alpha v\beta 6$ (Ahmed et al., 2002) y la $\alpha 5\beta 1$ (Sawada et al., 2008) con el objetivo de “colonizar” esos nuevos sitios y por ende formar tumores secundarios. Se ha reportado una regulación a la alta de la MMP-2, MMP-9 y la MMP-14 en la metástasis e invasión de las LDCOv (Kamat et al., 2006).

El sitio predilecto de metástasis de las células de CEOv es el omento, el cual es rico en adipocitos. Se ha demostrado la transferencia directa de lípidos de los adipocitos a las células cancerosas como fuente de energía para su proliferación y crecimiento. Este hecho correlaciona con la regulación a la alta de la proteína de unión a los ácidos grasos (FABP4) (Nieman et al., 2011).

2.2.2 Metástasis hematógena

La metástasis hematógena del CEOv ha ganado importancia en los últimos años. En primer término, se ha reportado la presencia de células tumorales en la

circulación de pacientes de COv, lo que sugiere que este mecanismo se encuentra activo en la patología (Phillips et al., 2012; Kuhlmann et al., 2014). Asimismo, se ha demostrado la migración de LDCov a través de los vasos linfáticos (Feki et al., 2009). Utilizando un modelo de parabiosis, que implica la unión quirúrgica de dos organismos (1 ratón con células de COv y otro libre de células tumorales) de modo que comparten la circulación sanguínea, se demostró la diseminación hacia el omento y órganos del peritoneo del ratón sano por vía sanguínea (Pradeep et al., 2014). Esta aproximación experimental resulta de gran relevancia dado que el modelo utilizado demuestra la diseminación hematógena *in vivo*. De igual forma, se ha demostrado la importancia del ovario en este tipo de migración, ya que la ooforectomía ha resultado en la anulación completa del desarrollo de ascitis y la metástasis peritoneal en los ratones (Coffman et al., 2016). Estas evidencias permiten considerar la diseminación hematógena de las células de COv como un posible mecanismo de migración.

2.3. El sistema purinérgico

La señalización mediada por ATP y adenosina (ADO) han sido de gran relevancia en el estudio del cáncer dado que existen altas concentraciones (en el rango μM) de estos mensajeros celulares en el microambiente tumoral (TME, por sus siglas en inglés) (Ohta et al., 2006; Pellegatti et al., 2008).

Diversas evidencias han demostrado que las purinas pueden regular la proliferación celular, la EMT y la migración en las células tumorales. En el presente estudio nos hemos centrado en el papel de la comunicación purinérgica en la migración de células derivadas de carcinoma ovárico (LDCOv), por lo que describiremos brevemente este sistema de comunicación.

El ATP es liberado al espacio extracelular a través de hemicanales de panexina y conexina, de canales maxianión, de canales aniónicos regulados por volumen, mediante transportadores específicos (transportadores ABC), a través de liberación vesicular, por el receptor purinérgico P2RX7 y por lisis celular (Fredholm et al. 2011), esta última cobra importancia en el cáncer, dado que durante las

terapias anticáncer hay una alta tasa de muerte celular y por ende altas concentraciones de ATP liberado (Figura 2). Una vez en el espacio extracelular, el ATP puede activar receptores específicos denominados P2, los cuales se dividen de acuerdo a su estructura molecular y a sus propiedades farmacológicas en dos subfamilias: los P2RX y los P2RY. Los receptores PR2X son canales iónicos activados por ligando que permiten el influjo de los iones Na^{+} y Ca^{+2} y el eflujo del ion K^{+}. Existen siete genes que codifican para subunidades distintas ($P2X_{1-7}$). Se han descrito 8 subtipos de receptores P2RY en células de mamífero: P2Y1, P2Y2, PY4, P2Y6 y P2Y11-P2Y14. Los receptores P2Y pertenecen a la familia de receptores de siete pases transmembranales acoplados a proteínas G (GPCR) heterotriméricas (Burnstock 2011).

En paralelo, el ATP puede ser hidrolizado por ectonucleotidasas de membrana, de tal manera que la acción de este mensajero celular está delimitada por estas enzimas. Los productos de la hidrólisis son ADP, AMP y ADO. Se han descrito cuatro familias de ectonucleotidasas: las ectonucleósido trifosfato difosfohidrolasas (E-NTPD) o CD39, las ectonucleótidopirofosfatasas (E-NPPs), las fosfatasas alcalinas (AP) y las ecto-5´-nucleotidasas (NT5E) o CD73. El ADP a su vez puede activar receptores P2Y y la ADO posee receptores específicos de membrana denominados P1, que son de particular relevancia para este estudio y serán descritos más adelante (al-Rashida y Iqbal 2014). Las ectonucleotidasas clave para regular la concentración de nucleótidos en el espacio extracelular son CD39 y CD73. CD39 hidroliza los nucleótidos de adenosina tri y di-fosfato a AMP y CD73 hidroliza los nucleótidos 5’-monofosfato a adenosina. Finalmente, la ADO extracelular y su papel como molécula de señalización finaliza por la actividad de la enzima adenosina deaminasa (ADA) y también es transportada hacia el interior de la célula por el transportador de nucleósidos (NT).

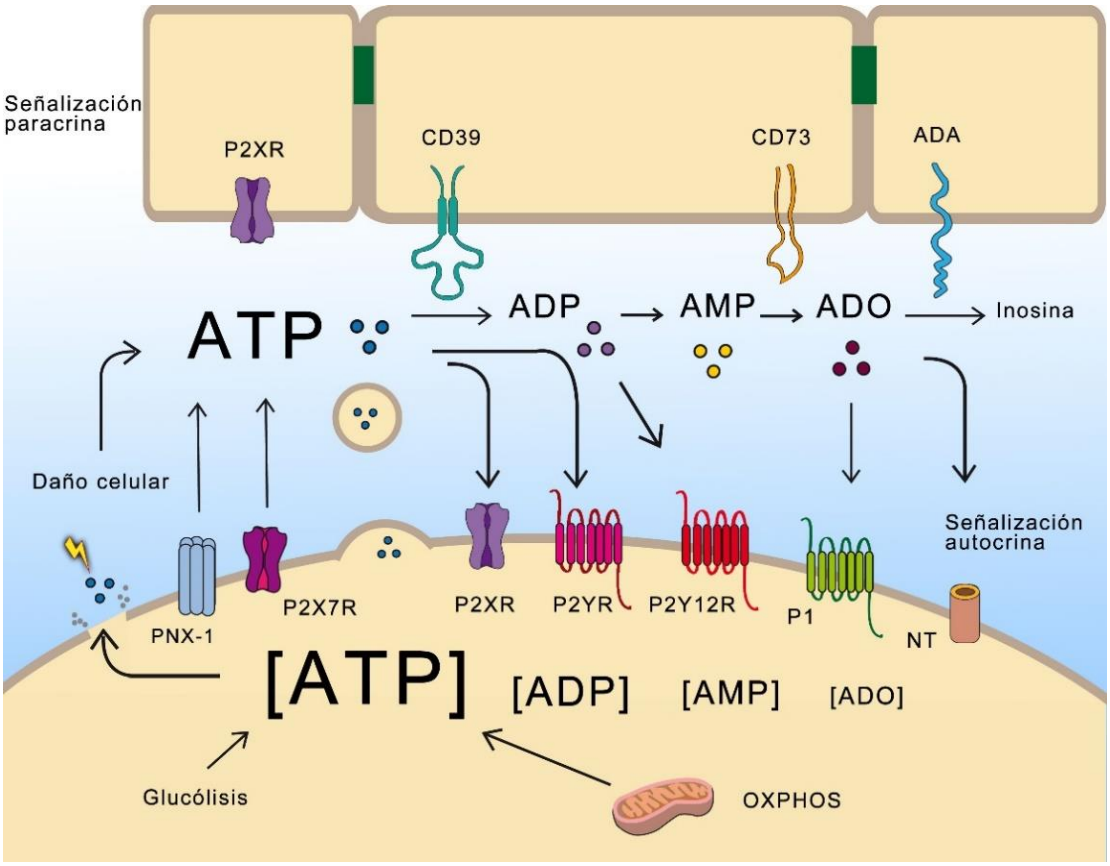

Figura 2. Señalización purinérgica. El ATP se produce en el interior de la célula por glucólisis y por la fosforilación oxidativa. Posteriormente, puede ser liberado al espacio extracelular por diversos mecanismos tales como la lisis celular, hemicanales de panexina (PNX-1), formación de vesículas y el receptor purinérgico P2X7R. Una vez en el espacio extracelular, el ATP a los receptores P2X (canales iónicos activados por ligando) y los receptores P2Y (GPCRs). Asimismo, el ATP es hidrolizado por ectonucleotidasas de membrana tales como CD39 y CD73, lo que lleva a la formación de ADP, AMP y adenosina (ADO). La ADO posee receptores específicos de membrana, los P1. Finalmente, la ADO es hidrolizada a inosina por la adenosina deaminsasa (ADA). Modificado de Campos-Contreras et al., 2020.

2.3.1 El sistema purinérgico en el cáncer

Se ha reportado la liberación de ATP de celulares tumorales y la subsecuente activación de los receptores purinérgicos en distintos modelos de cáncer como en la línea de cáncer de pulmón A549 en respuesta a la estimulación con TFG-β. Se comprobó que esta liberación depende de la expresión del receptor purinérgico P2X7 y el transportador de nucleótidos vesicular SLC17A9 (Takai et al., 2012). De

igual forma, en la línea de carcinoma ovárico SKOV-3 se ha comprobado la liberación de ATP en respuesta a un estímulo mecánico (Vázquez-Cuevas et al., 2014).

En una serie de trabajos realizados por Batra y cols., se demostró en las LDCOv, OVCAR-3 y SKOV-3, la movilización del Ca^{+2} intracelular en respuesta a la estimulación con ATP. Se obtuvo una respuesta bifásica que consiste en un pico seguido de una respuesta en meseta pronunciada, esto sugirió la expresión de receptores purinérgicos P2X y P2Y en estas líneas celulares (Batra, Popper, y Iosif 1993; Popper y Batra 1993).

De la subfamilia de los receptores P2X, el receptor más estudiado en el contexto del cáncer es el P2X7R. En el caso particular del ovario, se ha demostrado la expresión de este receptor en el epitelio ovárico superficial (OSE) así como en biopsias de CEOv, donde se observó una mayor expresión en las zonas de transformación (Vázquez-Cuevas et al., 2014). De igual forma, se ha demostrado la expresión de P2X7R en las líneas celulares de carcinoma ovárico SKOV-3 y Caov-3, donde la activación farmacológica promueve la fosforilación de las cinasas AKT y ERK y la inhibición disminuye la viabilidad celular (Vázquez-Cuevas et al. 2014).

Respecto a los receptores P2Y, existen diversos estudios que describen el papel del receptor P2Y2R en el CEOv. Por ejemplo, en las líneas EFO-21 y EFO-27, se demostró que el ATP regula a la baja la proliferación celular (Schultze-Mosgau et al. 2000). Mientras que en la línea IOSE-29 (preneoplastica) y en la línea IOSE-29EC (neoplástica) el ATP promovió la proliferación celular a través de la activación de las cinasas MAPK/ERK1/2 (Choi et al., 2003). Asimismo, en la línea SKOV-3 se demostró que la activación farmacológica del receptor P2Y2 promueve la expresión de genes asociados a la EMT, como *Snail*, *Twist* y de la proteína vimentina. Estas observaciones correlacionan con el aumento en la migración celular (Martínez-Ramírez et al., 2016).

La señalización mediada por los nucleótidos extracelulares también tiene un papel relevante en las interacciones entre las células tumorales y las células del sistema inmune. El ATP es reconocido como un patrón molecular asociado al daño (DAMP) dado que recluta neutrófilos, macrófagos y células dendríticas (DCs) (Elliott

et al. 2009). Por otra parte, la ADO ha mostrado tener un papel como inmuno-modulador mediante la activación de los receptores específicos. Se ha demostrado que la generación de ADO por células de carcinoma ovárico atrae células mieloides y favorece la diferenciación hacia el fenotipo M2, macrófagos asociados al tumor (M2-TAM), los cuales poseen un fenotipo anti-inflamatorio y una actividad pro-tumoral al reducir la proliferación de las células T $CD4^+$. Así mismo, se ha demostrado que los TAMs tienen una mayor expresión de CD39 y los fibroblastos del estroma expresan CD73, de este modo ambos tipos celulares colaboran en la amplificación de la síntesis de ADO y por lo tanto en la generación de un efecto de inmunosupresión (Montalbán Del Barrio et al., 2016).

2.4. Receptores a adenosina en el cáncer de ovario

La ADO se sintetiza intracelularmente a partir de la hidrólisis del AMP o de la S-adenosil homocísteina (SAH) por la actividad de la CD73, o la SAH hidrolasa, respectivamente (Bertil B. Fredholm 2014). Sin embargo, la ADO extracelular proviene principalmente de la hidrólisis del ATP extracelular por la acción combinada de las ectonucleotidasas CD39 y CD73. La síntesis de ADO es estrictamente dependiente del estado metabólico de la célula; normalmente, la concentración extracelular de ADO está en el rango nanomolar, sin embargo, estos niveles incrementan de manera significativa en condiciones que involucran una alta demanda metabólica o en condiciones anaeróbicas, como en la inflamación y el cáncer. Se ha demostrado que la ADO extracelular es más abundante en microdializados obtenidos del centro tumoral que en la periferia (Ohta et al. 2006) y que la expresión de las ectonucleotidasas CD39 y CD73 es promovida por el factor inducible por hipoxia 1 (HIF-1), de modo que se promueve la síntesis de ADO (Giatromanolaki et al. 2020).

La ADO ejerce sus acciones como mensajero celular actuando sobre los receptores: A1, A2A, A2B y A3, todos ellos pertenecientes a la familia de GPCRs. Los receptores A1 y A3 se acoplan de manera preferencial a proteínas G heterotriméricas cuya subunidad alfa es Gi/0, lo que se transduce en la inhibición

de la enzima adenilato ciclasa (AC). Por su parte, los receptores A2A y A2B se acoplan principalmente a proteínas Gs y su actividad induce un aumento en la concentración de AMPc al regular a la AC (Fredholm et al., 2011). El receptor A1 además de inhibir a la AC, puede inducir la activación de la fosfolipasa C (PLC); a nivel neuronal y del miocardio estimula canales de K^+ sensibles a la toxina pertussis y los K_{ATP}, mientras que reduce la actividad de los canales de Ca^{2+} tipo Q, P y N (revisado en Borea et al., 2018). La PKA es el efector más común del receptor A2A y esta a su vez activa diversas proteínas como fosfodiesterasas (PDE) y la proteína de unión al elemento de respuesta al AMPc (CREB). El receptor A2B ha mostrado modular la actividad de la PLC y la regulación de canales iónicos mediante las subunidades $\beta\gamma$ (Feoktistov y Biaggioni 1997). El receptor A3 también se acopla con menor eficacia a Gαq y a las subunidades G$\beta\gamma$; también puede regular las vías de PI3K/Akt y NF-κB (revisado en Borea et al., 2018). Los cuatro receptores activan la vía de las MAPK, específicamente la activación de ERK 1/2 (Schulte & Fredholm, 2000; Fredholm et al., 2001). En la Tabla 2 se indican agonistas y antagonistas selectivos para cada receptor P1 (Modificado de Borea et al., 2018).

El papel de la ectonucleotidasa CD73, la principal encargada da la síntesis de ADO a partir de AMP, se ha estudiado en distintos tipos de cáncer y su papel resulta controversial. Se ha reportado que promueve el crecimiento tumoral y la metástasis de células de cáncer de mama (Stagg et al., 2010), en líneas celulares de meduloblastoma, la expresión de CD73 se correlaciona con el fenotipo celular, pues el fenotipo mesenquimal presenta baja expresión de CD73 y por ende baja producción de adenosina (Cappellari et al., 2012) . Específicamente en el cáncer de ovario, algunos reportes sugieren que la expresión de CD73 en el tumor indica un mal pronóstico (Turcotte et al., 2015), mientras que otros grupos han encontrado una correlación entre la mayor expresión de CD73 y un mejor pronóstico en pacientes con carcinomas serosos ováricos (Oh et al., 2012). Posiblemente las diferencias se deban al tipo y grado de los tumores analizados, por lo que se requiere un análisis a profundidad.

Tabla 2. Farmacología y efectores de los receptores ADORA

	Receptor			
	A1	A2A	A2B	A3
Proteína G	Gi/0	Gs	Gs, Gq/11	Gi, Gq/11
Efectores	↓ AC	↑ AC	↑ AC	↓ AC
	↑ PLC	↑ MAPK	↑ PLC	↑ PLC
	Canales iónicos		↑ MAPK	↑ PI3K
	↑ PI3K			↑ MAPK
	↑ MAPK			
Afinidad por la adenosina	1-10 nM	30 nM	1000 nM	100 Nm
Agonistas	CCPA, R-PIA, CPA, IB-MECA, NECA	CGS21680M, UK-432097, HE-NECA, NECA, R-PIA	NECA, BAY606583, IB-MECA, R-PIA	CI-IB-MECA, IB-MECA, NECA, MRS5698, R-PIA, CGS21680
Antagonistas	PSB36, KW-3902, DPCPX, cafeína, teofilina	SCH442416, ZM241385, SCH58261, DPCPX, cafeína, teofilina	PSB-603, ZM241985, MRS1754, DPCPX, cafeína, teofilina	MRS1523, MRS3008F20. DPCPX, ZM241385, cafeína, teofilina

Se ha demostrado la expresión de los receptores P1 en las líneas CAOV-4, SKOV-3 y OVCAR-3, siendo mayor la expresión de los receptores A2B y A3 (Hajiahmadi et al., 2015). Adicionalmente, se ha descrito que la estimulación con NECA, un agonista no selectivo de los receptores A2, disminuyó la viabilidad celular y provocó la muerte celular por apoptosis (Hajiahmad et al., 2015). De igual forma

se demostró la expresión de los receptores A1, A2A y A2B en las líneas de carcinoma ovárico A2780 y HEY. En estas líneas celulares, la ADO indujo una disminución en la viabilidad celular y apoptosis a altas concentraciones y además incrementó la sensibilidad al tratamiento con cisplatino (Sureechatchaiyan et al., 2018).

2.5. El receptor A2B

El receptor A2B fue identificado y clonado en el hipotálamo de rata e hipocampo de humano (Pierce et al., 1992; Rivkees & Reppert, 1992), es una proteína de 328 aminoácidos con un peso aproximado de 36-37 kDa. El análisis de la secuencia de aminoácidos indicó que el receptor pertenece a la familia de GPCR (Feoktistov y Biaggioni 1997). El receptor A2B presenta dos residuos de cisteína (Cys) que se encuentran conservados en los GPCRs clase A ($Cys78^{3.25}$ en el TM3 y $Cys171^{45.50}$ en el ECL2), los cuales tienen un papel esencial en la unión del ligando y la activación del receptor. Mediante estudios de mutagénesis dirigida, se ha demostrado que los residuos de cisteína presentes en el ECL2 del receptor A2B: Cys154, Cys166 y Cys 167 disminuyen ligeramente la activación del receptor, lo que sugiere que los puentes disulfuro formados por estos residuos de cisteína no juegan un papel decisivo para la función del receptor (Schiedel et al., 2011).

A2B es el receptor P1 con menor afinidad por la adenosina (EC_{50}= 24 μM) (Fredholm et al., 2001). La vía de señalización propuesta para este receptor involucra la activación de la AC y la consecuente síntesis de AMPc que conlleva a la activación de efectores como PKA y EPAC (Fang y Olah 2007). Sin embargo, también se han demostrado la vía de señalización que involucra Gq-PLC (Feoktistov y Biaggioni 1997) y además el acoplamiento de las vías de MAPK (Schulte y Fredholm 2000), del ácido araquidónico (Donoso et al., 2005) y la regulación de canales iónicos mediante la modulación de las subunidades $\beta\gamma$ (Jiménez et al., 1999).

El transcrito de A2BR se encuentra en una amplia diversidad de órganos y tejidos, como en vasos sanguíneos, músculo liso, el ciego, el intestino grueso, el

cerebro y la vejiga. Asimismo, se ha detectado la expresión del receptor A2B en diversos tipos celulares, como células del sistema inmune, células alveolares tipo II, células endoteliales, células cromafines, astrocitos, neuronas y corpúsculos gustativos (revisado en Borea et al., 2018). Por otra parte, se ha reportado el incremento de la expresión del receptor A2B por diversos factores tales como la inflamación, el estrés celular, el daño (Eckle et al., 2014) y la hipoxia (Kong et al., 2006), procesos importantes durante el cáncer.

En comparación con el tejido sano, se han demostrado mayores niveles de expresión del receptor A2B en biopsias y líneas celulares derivadas de carcinomas de hígado (Xiang et al., 2006), colorectal (Ma et al., 2010), oral escamoso (Kasama et al., 2015) y urotelial (Zhou et al. 2017). Diversos estudios han mostrado que la inhibición farmacológica o genética del receptor A2B disminuye la proliferación celular, por ejemplo en líneas derivadas de carcinomas de próstata (Wei et al. 2013), de mama (Fernandez-Gallardo et al., 2016) y de células escamosas de cabeza y cuello (Wilkat et al., 2020). La inhibición de la expresión del receptor A2B en las líneas EJ y T24, derivadas de carcinoma urotelial, inhibió la proliferación celular y arrestó las células en la fase G1 del ciclo celular (Zhou et al., 2017). En las líneas celulares OVCAR-3 y Caov-4, de carcinoma ovárico, la activación del receptor A2B con NECA, redujo la viabilidad celular y promueve la apoptosis (Hajiahmadi et al, 2015).

Por otro lado, el papel de la activación del receptor A2B sobre la EMT ha sido estudiado en células epiteliales de cáncer de pulmón y se han descrito dos efectos de regulación. En el primero, se observó que la actividad del receptor A2B indujo una EMT parcial donde solo unas células en respuesta al estímulo presentaron un fenotipo mesenquimal. Este proceso involucró las vías de transducción AMPc/PKA y MAPK/ERK. El segundo consistió en la habilidad de atenuar la inducción de EMT inducida por TFG-β (Giacomelli et al., 2018). Estas evidencias sugieren que el mantenimiento o la inhibición de la EMT se basan en el balance de las señales extracelulares. Adicionalmente, en la línea MDA-MB-231, la ADO incrementa la migración celular mediante el eje A2B/AC/PKA/AMPc (Fernandez-Gallardo et al. 2016) y la inhibición farmacológica del receptor disminuye la migración celular en

células de carcinoma de pulmón (Giacomelli et al. 2018) y de riñón (Zhou et al., 2017). Estas evidencias señalan el papel del receptor A2B en distintos tipos de cáncer.

3. ANTECEDENTES

En nuestro laboratorio se ha demostrado que la hidrólisis del ATP extracelular, liberado por el propio cultivo, mediante la adición de la ectonucleotidasa apirasa (Apy), inhibe la migración y favorece un fenotipo epitelial en las células de carcinoma ovárico SKOV-3 (Martínez-Ramírez et al., 2016). La caracterización farmacológica de dicho efecto demostró que la inhibición de la migración es una consecuencia de la acumulación de ADO en el espacio extracelular (Martínez-Ramírez et al., 2017). No obstante, el subtipo de receptor para este nucleósido y el mecanismo de señalización implicado no habían sido estudiados con detalle. Nuestra propuesta es que los efectos están mediados por el receptor A2B y se basan en las siguientes evidencias: 1) recientemente, hemos documentado la expresión de los receptores A2B, A1 y A3 en las células SKOV-3, siendo el más abundante A2B (Martínez-Ramírez et al., 2017); 2) La incubación de las células con NECA, un agonista de los receptores A2, induce un fenotipo epitelial en las células SKOV-3, al ser el A2B el receptor más abundante se puede sugerir que el efecto es mediado por este receptor. 3) Se ha demostrado que la proteína cinasa A (PKA), una cinasa que forma parte de la cascada de transducción del receptor ADORA2B pero no de ADORA 1 ó 3, participa en la inhibición de la EMT (Pattabiraman et al., 2016) y 4) El único receptor ADORA encontrado en las células SKOV-3 que tiene la capacidad de regular a la PKA es el A2B. Estas evidencias nos llevaron a considerar al receptor A2B como el responsable de la inhibición de la migración y probablemente de la EMT en las células SKOV-3.

4. JUSTIFICACIÓN

El cáncer de ovario es la enfermedad ginecológica más letal. La supervivencia promedio de las pacientes de carcinoma ovárico es muy baja e incluso desciende cuando las pacientes son diagnosticadas en estadios avanzados, cuando el tumor ha hecho metástasis. Por lo que el estudio de los mecanismos de la migración celular es de gran relevancia y puede tener un impacto en la práctica clínica. El desarrollo de nuevas estrategias terapéuticas depende de la identificación de mecanismos moleculares y celulares novedosos implicados en la progresión de la metástasis de células de carcinoma ovárico. El estudio de la comunicación mediada por ATP y ADO y sus respectivos receptores es de suma importancia dado que se han reportado altas concentraciones de estos mensajeros en el TME que contribuyen a la progresión tumoral, por lo que sus receptores representan potenciales blancos farmacológicos.

5. HIPÓTESIS

La activación del receptor A2B para adenosina, inhibe la migración en células derivadas de carcinoma ovárico.

6. OBJETIVOS

6.1. General

Entender el papel del receptor A2B de adenosina en la regulación de la migración en células derivadas de carcinoma ovárico.

6.2. Particulares

- Evaluar el efecto de la estimulación farmacológica del receptor A2B en la migración de las células SKOV-3.
- Evaluar el efecto de la manipulación de la expresión del receptor *ADORA2B* sobre la migración de las células SKOV-3.
- Evaluar el papel de la estimulación farmacológica del receptor A2B en el fenotipo (epitelial o mesenquimal) de las células SKOV-3.
- Analizar la expresión de genes en respuesta a la estimulación con BAY-606583, un agonista selectivo de los receptores A2B.

7. MATERIALES Y MÉTODOS

7.1. Cultivo celular

Las células SKOV-3 (HTB-77) y Caov-3 (HTB-75) se obtuvieron de *"American Type Culture Collection"* (ATCC, Manassas, VI, USA). Las células SKOV-3 fueron cultivadas en medio RPMI suplementado con 10% de suero bovino fetal (SBF) y 1X de solución de antibiótico-antimicótico (el cual contiene por mL: 100 U de penicilina, 100 µg estreptomicina y 0.25 µg de fungizona) (Thermo Scientific, MA, USA). Las células Caov-3 fueron cultivadas en medio DMEM-alta glucosa suplementado con 10% de SBF y 1X de antibiótico-antimicótico. Los cultivos celulares fueron mantenidos a 37°C en una atmósfera húmeda al 5 % de CO_2.

7.2. Transcripción reversa y reacción en cadena de la polimerasa

Con la intención de corroborar la expresión del transcrito de *ADORA2B* en las LDCOv utilizadas en el presente trabajo, se obtuvo el RNA total de lisados celulares siguiendo el método de isotiocianato de guanidina (Chomezynski & Sacchi, 1987). La integridad del RNA fue evaluada por electroforesis y la concentración fue determinada por espectrofotometría (NanoDrop 1000, Wimington, DE). Se sintetizó el cDNA utilizando 1 µg de RNA tratado con DNAsa de alta pureza, la cual se inactivó por calentamiento a 65 °C durante 10 min. Posteriormente, se adicionó oligodT, mezcla de hexanucleótidos y retrotranscriptasa reversa RT (Promega, WI). La reacción se incubó durante 1 h a 42°C.

La amplificación de los transcritos de *ADORA2B*, *GAPDH* y *CYC1* fue realizada mediante la reacción en cadena de la polimerasa (PCR) de punto final. Las secuencias de los nucleótidos son las siguientes: A2BR-*forward* 5'-TCC ATC TTC AGC CTT CTG GC-3', A2BR-*reverse* 5'-AAA GGC AAG GAC CCA GAG GA-3'; GAPDH-*forward* 5'-CAA GGT CAT CCA TGA CAA CTT TG-3', GAPDH-*reverse* 5'-GTC CAC CAC CCT GTT GCT GTA G-3'; CYC-1-*forward* 5'-CTC CTG CCA CAG

CAT GGA C-3', CYC1-*reverse* 5'-CAT GCC TAG CTC GCA CGA T-3' y SOD2-*forward* 5'-TGG ACC CTC ACA TCA ACG C-3'; SOD2-*reverse* 5'-TTC CAG CAA CTC CCC TTT G-3'. Las reacciones fueron en un volumen final de 20 μl. Todos los cebadores fueron diseñados en OligodT y sintetizados por Sigma-Aldrich, USA. Las reacciones de PCR fueron realizadas en un termociclador de BioRad. Para corroborar la identidad de los amplicones, éstos fueron secuenciados en la Unidad de Secuenciación del CINVESTAV sede Irapuato y las secuencias se analizaron en la plataforma BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

7.3. Biotiniliación de proteínas de la membrana plasmática

Para la biotinilación de proteínas localizadas en las membranas de células SKOV-3, se utilizaron cultivos celulares con una confluencia del 80-90%. Los cultivos fueron incubados durante 20 min con el reactivo Ez-link Sulfo NHS-LC-C-Biotina a una concentración 300 μM (Thermo Scientific, Walthman, MA, USA). El reactivo se disolvió en una solución de fosfatos (PBS, por sus siglas en inglés); la cual contiene las siguientes sales: de 136 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 y 1.8 mM KH2PO4, pH 7.4). Posteriormente, la solución se retiró y se realizaron 2 lavados con PBS. A continuación, las células fueron lisados por la adición de TNTE, dicha solución contiene: 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 EDTA mM y 0.1% Tritón X-100. Se colectó el homogenado celular y se centrifugó durante 10 min a 10000 rpm a 4°C. Se recuperó el sobrenadante. La concentración de proteínas en la fracción recuperada fue determinada por el método de Lowry, 1 mg de proteína se incubó durante 90 min con 50 μl de esferas de sefarosa-streptavidina (Cell Signaling Technology, Danvers, MA, USA) a temperatura ambiente. Posteriormente, las esferas fueron lavadas 3 veces con PBS y se resuspendieron en 100 μl de Laemlli. Las muestras se hirvieron por 5 min y se analizaron mediante la técnica de *Western blot*, que será descrita posteriormente. Para la detección del receptor A2B se utilizó un anticuerpo en una dilución 1:100 contra la epítope extracelular de éste (KDSATNNSTEPWDGTTNESC; Allomone Labs, Jerusalem, Israel, #AAR-003).

7.4. Western blot

Se sembraron células SKOV-3 en cajas de cultivo de 12 pozos, en cada uno de los cuales se colocaron aproximadamente $5x10^4$ células. Cuando las células alcanzaron una confluencia de 70-80% fueron incubadas en medio RPMI sin suero durante 8 horas previas al tratamiento de cada condición experimental, con el fin de sincronizar el ciclo celular. Transcurrido este tiempo, las células fueron incubadas con BAY-606583 (Cayman), PSB-6083 (Sigma-Aldrich), PKAi (Sigma-Aldrich) o U73122 (Tocris) durante cada uno de los tiempos establecidos para cada experimento. Al finalizar el tratamiento, se agregó solución de Laemmli con el propósito de lisar las células.

Las proteínas fueron fraccionadas por electroforesis en geles de acrilamida al 10% o al 8%. Las proteínas fueron transferidas a membranas de polivinil-difluoruro (PVDF, Bio-Rad Laboratories, CA, USA). Posteriormente, las membranas se incubaron durante toda la noche a 4°C con el anticuerpo primario preparado a una dilución de 1:1000: anti-fosfo p44/42 MAPK, anti-total p44/42 MAPK, anti-E-cadh (Cell signaling Techonologies, Danvers, MA, EUA) o anti-A2B (Alomone Labs, Jerusalem, Israel) en TBS-Tween (Tris 20mM, pH 7.4, Tween-20 al 0.05%). Los anticuerpos fueron elaborados en conejo o en ratón. Seguido de la incubación con el anticuerpo primario, las membranas se incubaron durante 1 h a temperatura ambiente con el anticuerpo secundario (anti-IgG de conejo o anti-IgG de ratón) acoplado a la peroxidasa de rábano (HRP) en dilución 1:5000. La señal fue obtenida mediante quimioluminiscencia y autoradiografía. Se realizó el análisis densitómetrico utilzando el software Image J.

7.5. Viabilidad celular

Con el objetivo de evaluar el efecto de la estimulación farmacológica del receptor A2B sobre la viabilidad se realizó el ensayo de reducción de sales de tetrazolio (MTS). Con este propósito, se cultivaron células SKOV-3 en placas de 48 pozos en medio RPMI suplementado con 10% de SBF. Cuando las células alcanzaron una confluencia del 50-60%, fueron incubadas en medio sin suero durante 12 h. Posteriormente, se incubaron con BAY 60-6583 a una concentración específica en el rango de 10 nM a 10 µM durante 24 h. Para medir la viabilidad, se adicionó el reactivo MTS (3-(4,5-dimetIlthiazol-2-yl)-5-(3-carboxImethoxifenil)-2-(4-sulfofenil)-2H-tetrazolio) (Cell Titter, Promega USA) diluido en medio libre de suero a cada pozo de acuerdo a las instrucciones del fabricante y se incubaron de 2 a 4 h hasta observar la formación de un precipitado de color café, cuya intensidad se determinó midiendo la absorbancia a 495 nm. Los datos fueron normalizados en base al control (ausencia del estímulo farmacológico). Se realizaron cuatro experimentos en septuplicados de cada tratamiento.

7.6. Inhibición de la expresión del receptor A2B por infección lentiviral

Con el propósito de evaluar el papel del receptor A2B sobre la migración celular de las células SKOV-3, se realizó la inhibición de su expresión utilizando lentivirus (vector pLKO.1 puro, Figura 3). Se utilizaron plásmidos prediseñados obtenidos con secuencias que codifican para *short-hairpin RNA´s (shRNA´s)* diseñadas por Sigma-Aldrich, las secuencias fueron:

sh1 5’-CCGGGCAGATGTCAAGAGTGGGAATCTCGAGATTCCCACTCTTGAC ATCTGCTTTTTG-3’, (Sigma#TRCN0000065335); *sh2* 5’-CCGGGC AATGAATATGGCCATTCTTCTCG AGAAGAATGGCCATATTCATTGCTTTTTG-3’, (Sigma#TRCN0000065337) o *sh3* 5’-CCGGGCTGGTGATCTACA TTAAGATCTCGAGATCTTAATGTAGATCACCAGCTTTTTG-3’, (Sigma #TRCN0000065334) (SHCLNG_NM000676; Sigma-Aldrich).

Cada uno de los plásmidos, de manera independiente, fueron co-transfectados en conjunto con los vectores de empaquetamiento: pRSV-Rev, pMD2.G y pMDLg/pRRE en células HEK-293T por el método de precipitación con fosfato de calcio. 72h horas después, se colectaron las partículas lentivirales acumuladas en el medio de cultivo, las cuales fueron utilizadas para infectar células SKOV-3. Las células transducidas fueron seleccionadas por la resistencia a la puromocina (1.5 µg/mL) durante 5-7 días. La disminución de la expresión del transcrito de *ADORA2B* se corroboró por medio de RT-PCR de punto final como se describió previamente.

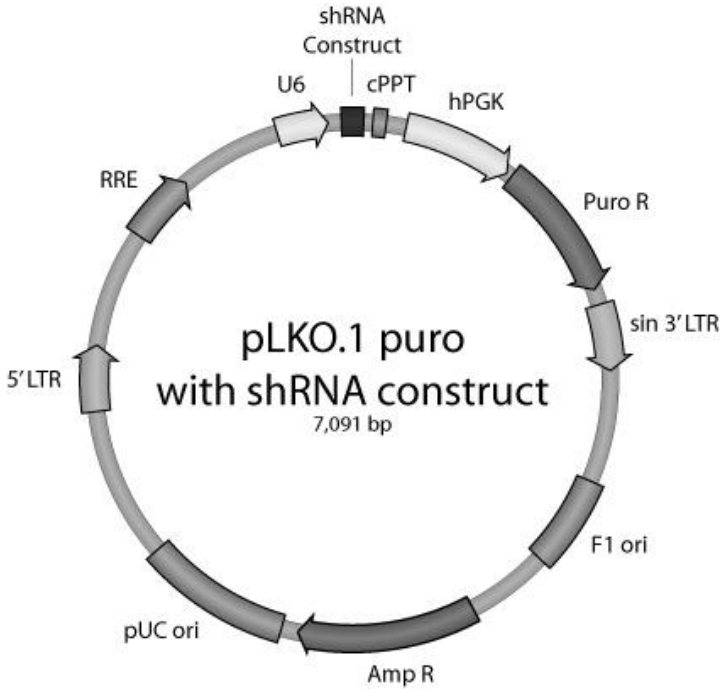

Figura 3. Mapa del vector pLKO.1 puro.

7.7. Tinción del citoesqueleto de actina

Con el fin de observar cambios fenotípicos en el citoesqueleto de actina dependientes del receptor A2B, se cultivaron sobre cubreobjetos células SKOV-3 de tipo silvestre y *sh-ADORA*2B y se realizaron los tratamientos farmacológicos descritos en cada uno de los experimentos. Posteriormente las células se fijaron

con el uso de una solución de paraformaldehído al 4% disuelto en PBS durante 20 minutos y se permeabilizaron con Tritón-X100 al 0.01% durante 5 minutos. Con el objetivo de visualizar los núcleos, los cultivos fueron incubados en una solución de DAPI disuelto en PBS (1:1000). Finalmente, los cubreobjetos fueron montados en medio Vecta Shield con faloidina acoplada a rodamina (Vector Labs, California, USA). Las imágenes fueron obtenidas en un microscopio de fluorescencia (Apotome, Carl Zeiss).

7.8. Ensayo de migración

Para evaluar la migración de las LDCOv, las células SKOV-3 o CAOV-3 fueron cultivadas en placas de 12 pozos en medio suplementado con 10% de SBF. Cuando los cultivos alcanzaron una confluencia del 90%, las células fueron ayunadas durante 8 horas. Posteriormente se realizó una hendidura (herida) en el cultivo utilizando una punta de micropipeta de 200 μl. Se prosiguió a adicionar los tratamientos farmacológicos en la concentración indicada para cada uno de los experimentos. Se tomaron fotografías inmediatamente después de la adición de los fármacos (t=0) y a las 16 h de la incubación (t=16). Se realizó el análisis de la migración de las células mediante las microfotografías utilizando el programa ImageJ. Se midió el área de la herida al t=0 y t=16. Todos los experimentos fueron repetidos al menos tres veces por triplicado.

7.9. Análisis de la expresión del transcrito de *ADORA2B* en la base de datos *Kaplan Meier plotter*

La base de Kaplan-Meier (https://kmplot.com/analysis/) se utilizó para la generación de las gráficas de supervivencia de pacientes de cáncer ovárico. La herramienta permitió obtener los cocientes de riesgo, los valores de P y la media de la

supervivencia comparando grupos de pacientes con alta vs. baja expresión del transcrito de *ADORA2B*.

7.10. Análisis de los microarreglos de cDNA

El experimento del microarreglo de cDNA se llevó a cabo en la Unidad de Microarreglos del Instituto de Fisiología Celular (Universidad Nacional Autónoma de México, CDMX, México). Para esto, se cultivaron células SKOV-3 en cajas Petri de 10 cm con medio suplementado con SBF al 10%. Cuando las células alcanzaron una confluencia del 70-80% se ayunaron para posteriormente ser incubadas con el agonista del receptor A2B, BAY-606583 10 µM durante 24 h. El control consistió en células en ausencia del estímulo farmacológico. Posteriormente, se obtuvo el RNA total utilizando Trizol (Thermo Fischer Scientific, Waltham, MA, USA) y siguiendo el protocolo previamente descrito. Se prosiguió con la síntesis del cDNA a partir de 1 µg de RNA total. Siguiendo las instrucciones de un kit comercial (First-Strand cDNA labeling kit, Thermo Fischer Scientific, Waltham, MA, USA), se incorporaron dUTP-Alexa 555 o dUTP-Alexa 647 en esta etapa. Se analizó la emisión de la fluorescencia a 555 nm para Alexa 555 y a 650 nm para Alexa 647.

El cDNA marcado se hibridó contra una librería de 35 K de transcritos del genoma humano total. La adquisición y cuantificación del arreglo de imágenes se realizó con el sofware GenePix 4100 (OMICtools, RRID:SCR_002250; Molecular Devices, San José, CA, USA). Se calcularon los valores de la densidad promedio de ambas fluorescencias y el fondo promedio. El análisis de los datos del microarrelo se realizó con el software de código abierto genArise (RRID:SCR_001346; http://www.ifc.unam.mx/genarise/), desarrollado por la Unidad de Cómputo del Instituo de Fisiología Celular (UNAM, México). El software calcula la intensidad dependiente del Z-score de las imágenes para identificar los patrones diferenciales de expresión de genes. El valor de Z representa el número de desviación estándar que un punto tiene sobre la media. Los genes que tienen un Z-score > 1.5 desviaciones estándares fueron definidos como transcritos expresados

de manera diferencial. Los datos fueron depositados en in ArrayExpress-EMBL-EBI (accession number: E-MTAB-11130).

7.11. Análisis estadístico

Los resultados se expresaron como la media ± el error estándar de la media (E.E.M). Las diferencias significativas entre los grupos fueron evaluadas mediante una *t de student*. Se utilizó el programa *GraphPad Prism* para las pruebas estadísticas. Los * indican la diferencia significativa entre el control y los grupos, *$p \leq 0.05$, **$p \leq 0.01$ y ***$p \leq 0.001$.

8. RESULTADOS

8.1. Expresión del receptor *ADORA2B* y su relación con la probabilidad de supervivencia de pacientes con carcinoma ovárico

Como primera aproximación se decidió evaluar la correlación entre los niveles de expresión (bajo o alto) del receptor *ADORA2B* con el tiempo de supervivencia de pacientes de carcinoma ovárico. El análisis se realizó con datos obtenidos de 1435 pacientes de cáncer de ovario mediante el uso de la base de datos “Kaplan-Meier Plotter” (K-MPdb) (kmplot.com) (Gyorffy, et al., 2012).

El primer análisis fue realizado tomando todos los datos disponibles para CEOv, sin distinción de subtipo histopatológico, grado o etapa; se observó una correlación entre la expresión alta del transcrito de *ADORA2B* y una probabilidad de supervivencia significativamente mayor (n=996 con baja expresión y n=439 con alta expresión; $p \leq 0.001$; Figura 4A). Además, se encontraron diferencias significativas en el tiempo de supervivencia de los pacientes, donde el grupo de pacientes con baja expresión de *ADORA2B* muestra una menor probabilidad de supervivencia (18.23 meses) y el grupo con alta expresión del transcrito (n=439) mostró una mayor supervivencia (25.1 meses).

Posteriormente, se hizo el análisis en la K-MPdb de acuerdo al tipo histopatológico de CEOv. Tomando los datos de los pacientes de CEOv endometroide no se encontraron diferencias significativas entre los grupos (n=15 con baja expresión y n=36 con alta expresión; p=0.19; datos no mostrados). Por el contrario, el análisis de los datos de los pacientes con CEOv tipo seroso (n=482 con baja expresión y n=622 con alta expresión) reveló que el grupo con baja expresión del transcrito de *ADORA2B* tiene un menor tiempo de supervivencia (15.8 meses vs. 18.6 meses; $p \leq 0.05$; Figura 4B).

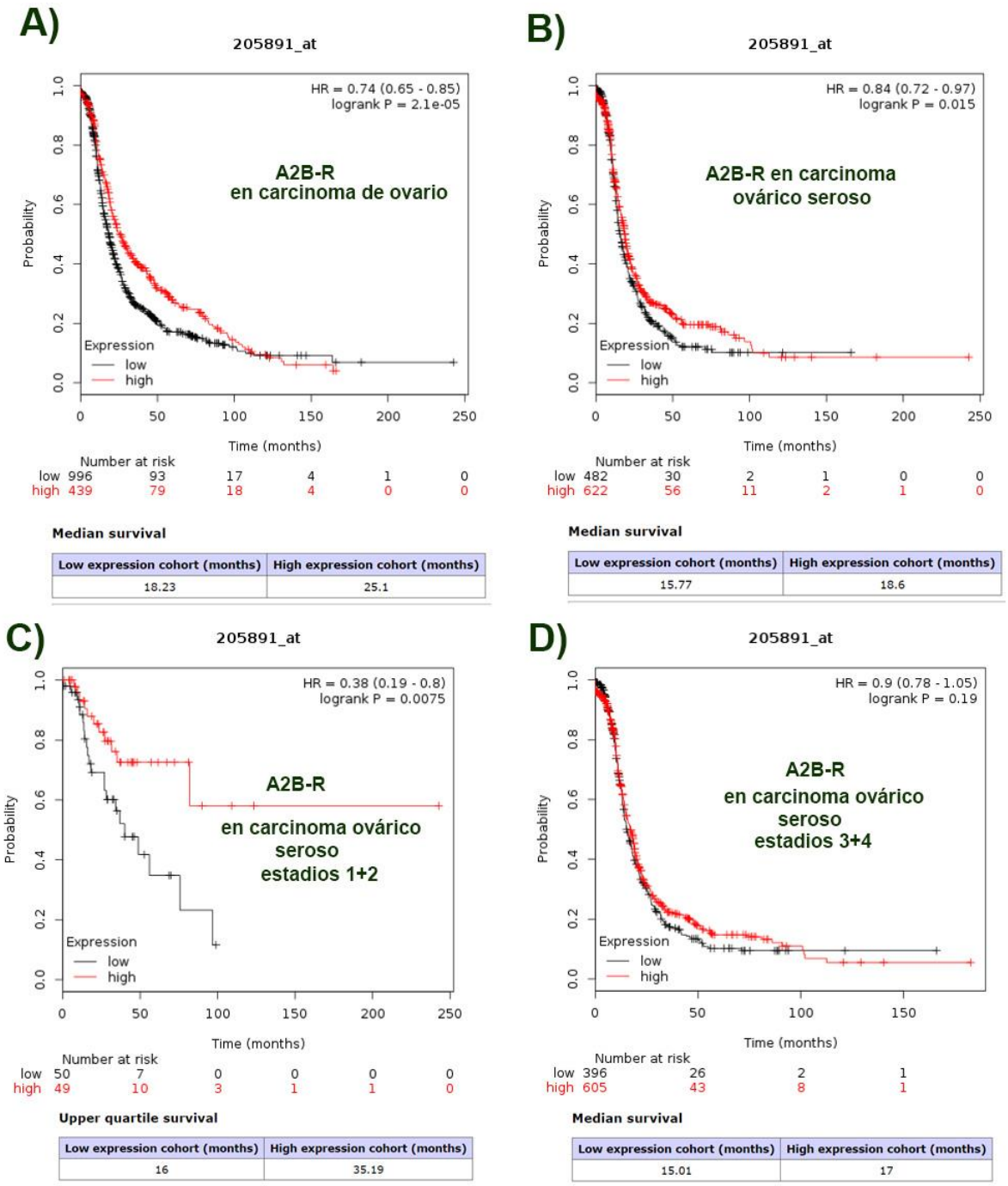

Figura 4. Análisis de Kaplan Meier para el transcrito de *ADORA2B*. Los gráficos muestran la supervivencia de los pacientes con expresión baja (línea negra) y alta (línea roja) del transcrito de *ADORA2B*. El gráfico muestra la probabilidad de supervivencia del paciente para un determinado intervalo tiempo. A) Todos los tipos histológicos y los estadios de diferenciación; ****p*≤0.001. B) Carcinoma ovárico seroso y todos los estadios de diferenciación; **p*≤0.05. C) Carcinoma seroso, estadios de diferenciación 1 y 2; ***p*≤0.01. D) Carcinoma ovárico seroso, estadios de diferenciación 3 y 4; *p*=0.14.

Con el objetivo de evaluar si la relación entre el nivel de expresión de *ADORA2B* y la supervivencia de los pacientes de CEOV tipo seroso se ve influenciada por la *etapa* (*stage*) de la enfermedad, se realizó el análisis restringiendo los datos a las etapas iniciales (1+2) y tardías (3+4). Utilizando los datos de los pacientes en los estadios 1 y 2 se encontró que la expresión alta (alta expresión n=49; baja expresión n=50) de *ADORA2B* se asocia con una mayor supervivencia (35.2 meses vs. 16 meses; $p \leq 0.01$; Figura 4C). No obstante, al evaluar la supervivencia en las etapas 3+4, en las cuales el tumor primario ha diseminado a órganos de la cavidad peritoneal y abdominal, no se encontraron diferencias entre los niveles de expresión (n=396 baja expresión y n=605 alta expresión) y la supervivencia (p=0.19; Figura 4D).

De igual modo, se realizó el análisis en la K-MPdb considerando el *grado* del tumor. En los grados 1 y 2, se encontró que el grupo de pacientes con una alta expresión de *ADORA2B* tiene una mayor probabilidad de supervivencia que aquellos con una baja expresión (p=0.018 y p=0.05 para el grado 1 y 2, respectivamente) (Figura 5A y B). Sin embargo, para el grado 3, no se encontraron diferencias significativas entre los grupos (p=0.061) (Figura 5C). Finalmente, no se pudo realizar el análisis en el grado 4 debido a que la base de datos no cuenta con el número adecuado de pacientes.

Estas observaciones resultan de gran significancia dado que 1) de manera general, la expresión alta de *ADORA2B* se asocia con una mayor supervivencia en el cáncer de ovario y 2) esta correlación se mantiene en las etapas tempranas (1+2) y en bajos grados de desdiferenciación (1 y 2) del carcinoma ovárico seroso. Esto sugiere un papel relevante para el receptor *ADORA2B* en la diseminación y/o migración del carcinoma ovárico dada la relación observada entre la alta expresión y la mayor supervivencia en las etapas 1 + 2. Asimismo, se puede sugerir que el bajo nivel de expresión de *ADORA2B* resulta en un mal pronóstico para el carcinoma ovárico seroso. Cabe mencionar que estos análisis se realizaron solamente en carcinomas de tipo seroso por ser el único que tiene el número necesario de pacientes en la base de datos para realizar el análisis.

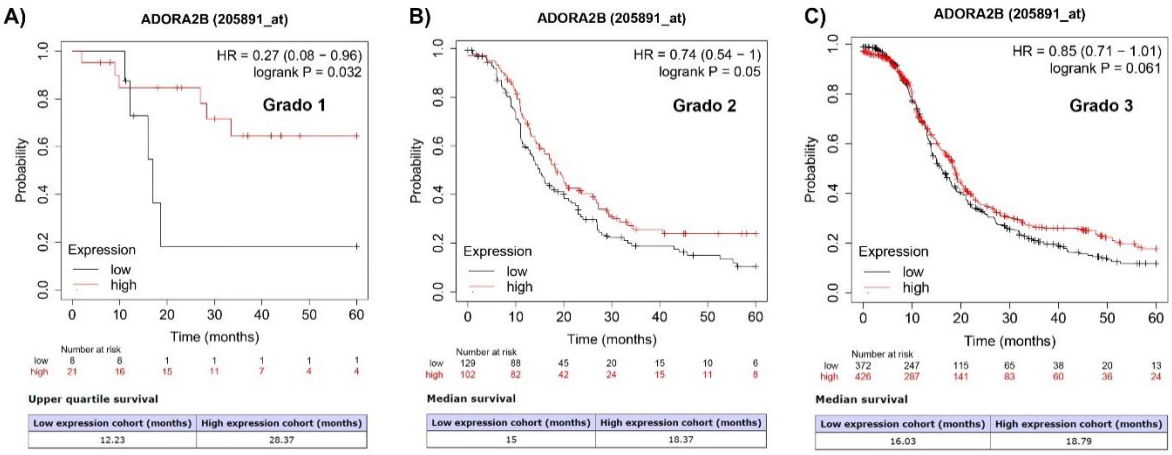

Figura 5. Análisis de Kaplan Meier para el transcrito de ADORA2B en pacientes de carcinoma ovárico seroso en los distintos grados de la patología. Los gráficos muestran la supervivencia de los pacientes con expresión baja (línea negra) y alta (línea roja) del transcrito. El gráfico muestra la probabilidad de supervivencia del paciente para un determinado intervalo tiempo. A) Grado 1; *$p \leq 0.05$ B) Grado 2; *$p \leq 0.05$ C) Grado 3; $p = 0.061$.

8.2. Evaluación de la expresión y funcionalidad del receptor A2B en las células SKOV-3

Con el fin de investigar los efectos celulares del receptor A2B en células de carcinoma ovárico se utilizó la línea celular SKOV-3. En estudios previos, de nuestro grupo de trabajo y otros, se ha reportado la expresión de A2B en esta línea celular (Hajiahmadi et al., 2015; Martínez-Ramírez, et al., 2017). En concordancia, en el presente trabajo hemos demostrado la expresión del receptor en las células por RT-PCR y *western blot* de proteínas de la membrana plasmática biotiniladas. La amplificación de *ADORA2B* de cDNA obtenido de células SKOV3 nos permitió obtener una banda de 161 pb (Figura 6A, izquierda). El amplicón fue purificado y secuenciado, posteriormente, la secuencia fue analizada en la plataforma BLAST (NIH, USA). La identidad del amplicón se identificó con el número de acceso NM_00676, que corresponde al transcrito de *ADORA2B* en *Homo sapiens*, el cual codifica para el receptor A2B. Como control constitutivo de la reacción se realizó la

amplificación del transcrito de la enzima gliceraldehído-3-fosfato-deshidrogenasa (GAPDH) (Figura 6A, panel central).

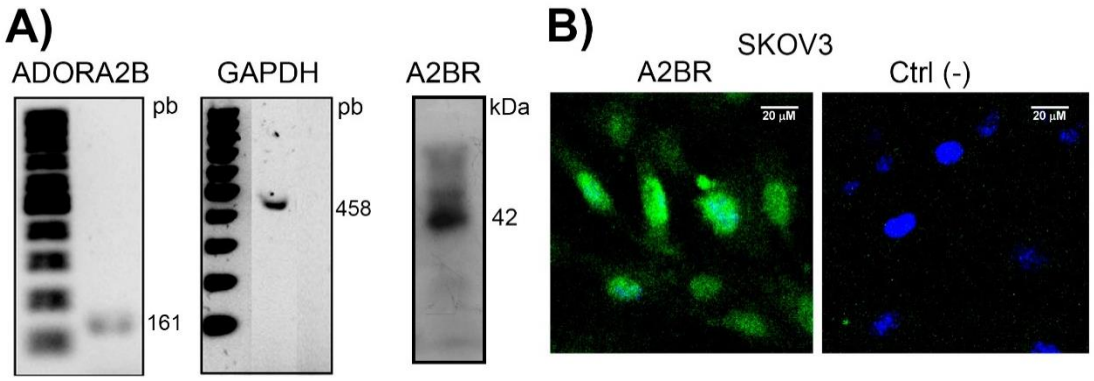

Figura 6. Expresión del receptor A2B en las células de carcinoma ovárico, SKOV-3. A) Expresión del transcrito de ADORA2B, se realizó RT-PCR utilizando oligonucleótidos específicos. Se detectó una banda de aproximadamente 161 pb. Se utilizó GAPDH como control constitutivo (panel izquierdo). Detección de A2B en proteínas de membrana biotiniladas, se detectó una banda de aproximadamente 42 kD (panel de la derecha). B) Inmunodetección del receptor A2B. La imunorreactividad se detectó mediante el uso de un anticuerpo secundario acoplado al colorante fluorescente Alexa-flúor 488 (señal verde). Los núcleos celulares fueron teñidos con DAPI (azul).

De igual modo, se identificó la expresión del receptor A2B en proteínas de membrana, las cuales fueron aisladas y biotiniladas para después ser aisladas con esferas conjugadas a estreptavidina. El precipitado se analizó por *western blot* y se utilizó un anticuerpo dirigido contra la epítope extracelular del receptor A2B. Se detectó una banda de aproximadamente 42 kDa (Figura 6A, panel de la derecha), en concordancia con la migración electroforética reportada por otros grupos (Sureechatchaiyan et al., 2018). Adicionalmente, se realizó una inmunofluorescencia contra el receptor A2B y se encontró que el receptor se encuentra distribuido a través de la superficie celular (Figura 6B). Estos resultados permiten confirmar que el receptor A2B es una proteína de membrana en las células SKOV-3.

Dado que las cinasas ERK1/2 son efectores del receptor A2B (Schulte y Fredholm 2000), se evaluó la inducción de su estado fosforilado, como un indicador de la actividad del receptor inducida por un agonista selectivo. Así, se incubaron

células SKOV-3 con el agonista BAY-606583 a una concentración 10 μM en distintos intervalos de tiempo entre 1 y 15 minutos. Como se puede observar en la Figura 7A, se encontró que la estimulación con BAY incrementó la fosforilación de ERK1/2, la cual alcanzó su máximo a los 5 minutos de estimulación (2.40 ± 0.14% sobre el basal). Con el propósito de confirmar que esta respuesta es inducida por la activación del receptor A2B, se evaluó la respuesta en células pre-incubadas durante 20 minutos con el antagonista selectivo PSB-603 a las concentraciones de 0.1 y 1 μM, previo a la estimulación con BAY-606583 10 μM. Se observó que el tratamiento con PSB603 inhibió la fosforilación inducida por el agonista a las dos concentraciones utilizadas en nuestro experimento (1.30±0.17% del basal a 0.1 μM y 0.90±0.22% del basal a 1 μM) (Figura 7B).

Dado que se ha reportado que el receptor A2B se acopla a las subunidades Gαs y Gαq, nos dimos a la tarea de explorar qué vía de señalización sería la responsable de la fosforilación de ERK1/2 en las células SKOV-3. Con este propósito, evaluamos el efecto de bloquear los efectores de las vías previamente mencionadas: PKA y PLC. La cinasa PKA fue inhibida mediante el uso de un fragmento permeable de la PKA 14-22 miristoilado (PKAi) mientras que para inhibir a la PLC se utilizó el inhibidor U73122. Ambos inhibidores fueron utilizados a las concentraciones 0.1 y 1 μM. El PKAi inhibió el efecto de fosforilación sobre ERK inducido por BAY (0.74±0.13% y 0.62±0.11% del basal a 0.1 o 1 μM del inhibidor, respectivamente; Figura 7C). De igual manera, U73122, también inhibió la fosforilación de ERK inducida por BAY (0.90±0.12% y 0.78±0.13% del basal a 0.1 o 1 μM del inhibidor, respectivamente; Figura 7D).

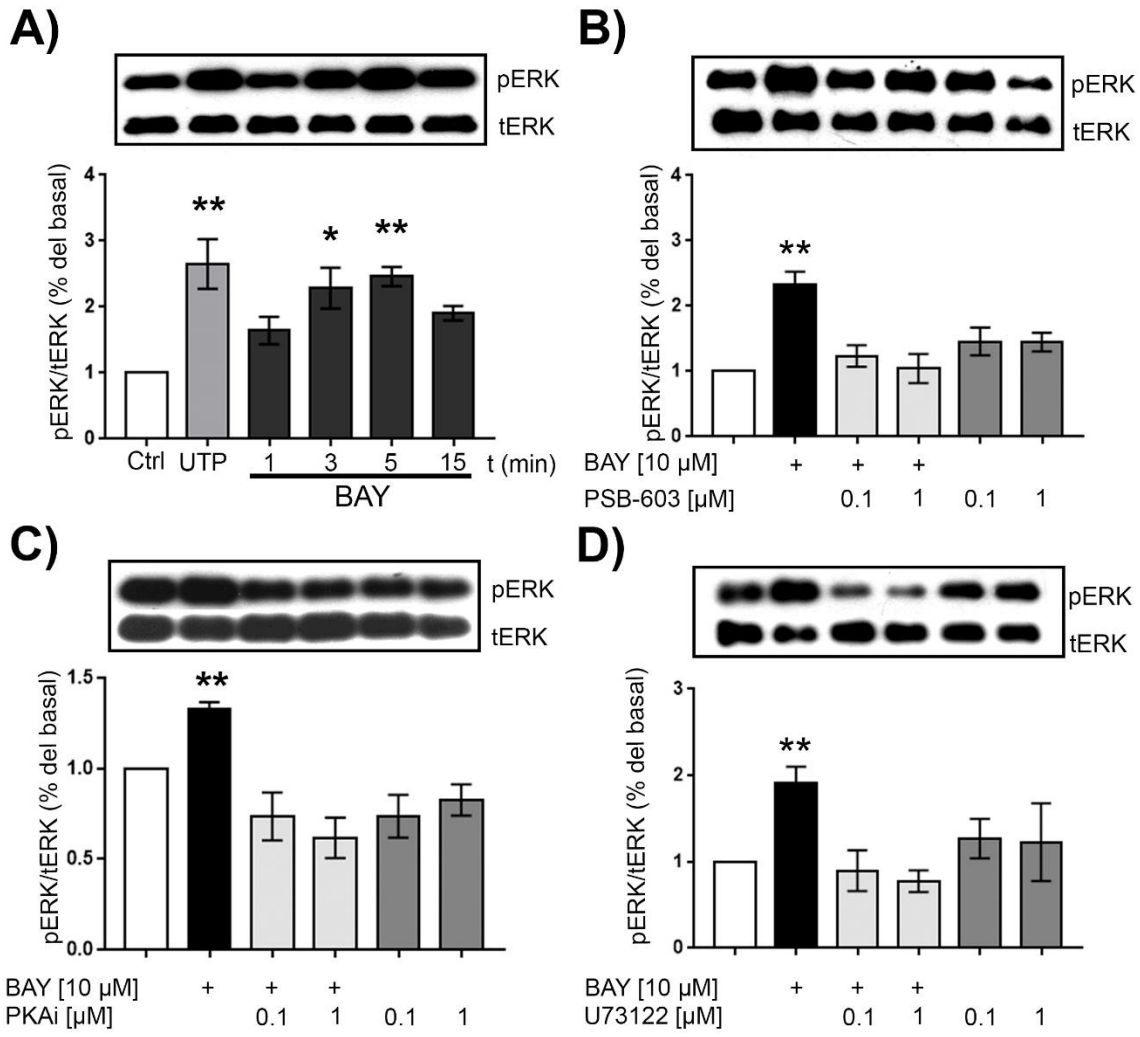

Figura 7. Evaluación de la funcionalidad del receptor A2B en las células SKOV3. A) Los cultivos celulares fueron incubados con el agonista BAY-606583 10 µM durante 1, 3, 5 y 15 min. Se analizó la ERK fosforilada (p-ERK) y ERK total (t-ERK) por western blot. Como control positivo del experimento se utilizó UTP 100 µM. Las células fueron estimuladas con BAY-603 10 µM durante 5 min y pre-incubadas 20 min con B) el antagonista del receptor A2B, PSB-603 0.1 y 1 µM; C) el inhibidor de PKA (PKAi) 0.1 y 1 µM y D) el inhibidor de la PLC, UT73122 0.1 y 1 µM. Los datos están normalizados con respecto a la fosforilación basal (control) y se expresan como la media ± error estándar de la media (E.E.M.). *$p \leq 0.05$; **$p \leq 0.01$; vs. el control. Se realizaron cuatro experimentos por duplicado.

8.3. La estimulación farmacológica del receptor A2B inhibe la migración celular

Una cuestión central del presente proyecto fue la evaluación del efecto de la actividad del receptor A2B sobre el fenotipo y por ende la migración de células derivadas de carcinoma ovárico. Con el propósito de discernir si los efectos encontrados sobre la migración celular están superpuestos con un aumento en la proliferación, se evaluó la viabilidad de las células SKOV-3 a la incubación con BAY 60-6583 (de 10 nM a 1 μM) en medio libre de suero por 24 h. Se utilizó SBF como control positivo del experimento (Figura 8A). El SBF provocó un aumento en la viabilidad celular (132.70±2.40% del control, $p \leq 0.001$), mientras que BAY 60-6583 no provocó cambios en ninguna de las concentraciones evaluadas (Figura 8A).

Posteriormente, para evaluar si el receptor A2B regula la migración celular se realizó el ensayo de cierre de la herida y se evaluó la respuesta a la estimulación farmacológica con distintas concentraciones de BAY (0.1, 1 y 10 μM). Las células se incubaron con los tratamientos farmacológicos durante 16 h. Como control positivo de la migración se utilizó UTP 100 μM y como control de la inhibición de la migración utilizamos la ectonucleótidasa apirasa (Apy) (10 U/ml) (Martínez-Ramírez, et al. 2017). El UTP promovió la migración de las células SKOV-3 (108.1±3.0 del control, $p \leq 0.05$) mientras que la Apy disminuyó este parámetro (71.5±1.6% del control, $p \leq 0.001$). El agonista de A2B redujo la migración a las concentraciones de 1 μM y 10 μM (88.7±3.5%, 83.3±2.0% y 75.7±1.6% respectivamente, $p \leq 0.01$ y $p \leq 0.001$) (Figura 8B).

Con el propósito de observar las fibras de estrés (FE), que sugieren un fenotipo mesenquimal, se marcó el citoesqueleto de actina con faloidina acoplada a rodamina. Como control positivo de nuestro experimento, se utilizó UTP 100 μM el cual aumentó las SF, mientas que el tratamiento con Apy (10 U/ml) redujo notablemente la presencia de estas estructuras. Finalmente, en las células tratadas con BAY 10 μM fue menos evidente la presencia de FE que en las células control (Figura 8C, señal roja).

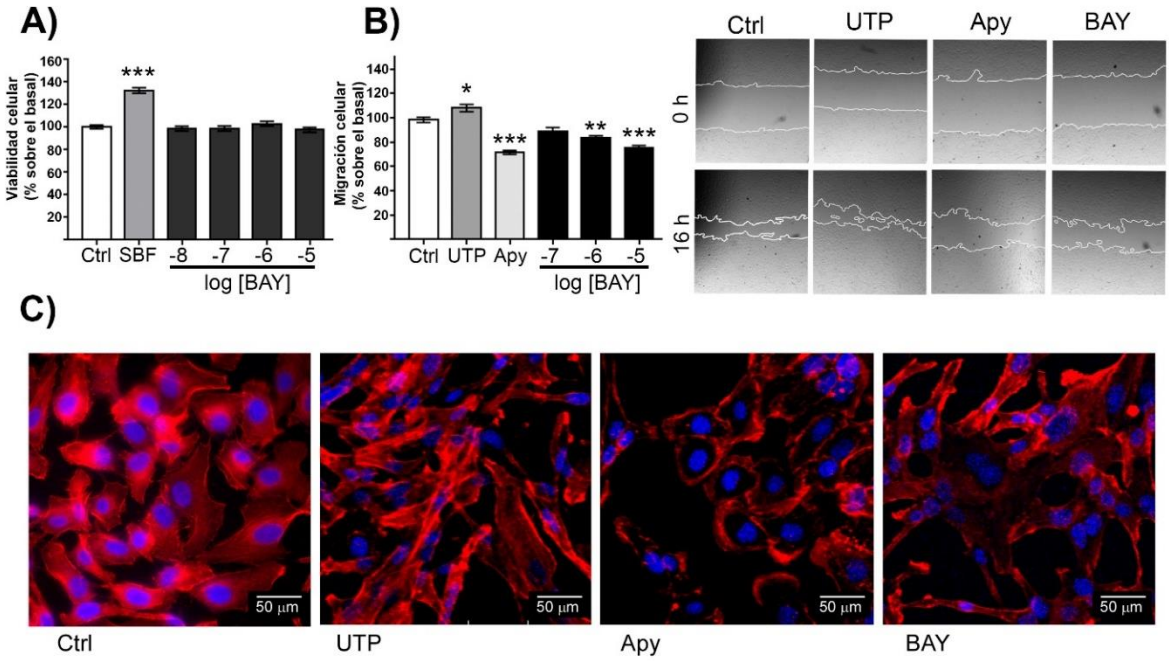

Figura 8. La estimulación del receptor A2B disminuye la migración de las células SKOV-3 pero no tiene un efecto sobre la viabilidad celular. A) Viabilidad celular en respuesta a la estimulación farmacológica con el agonista BAY-606883 de 10 nM a 10 µM durante 24 h. Control positivo del experimento: suero bovino fetal (SBF). *** $p \leq 0.001$ vs. SBF. B) Migración celular en respuesta a la incubación con BAY-606583 durante 16 h. A la derecha se muestran micrografías representativas de la migración celular analizada por el ensayo de cierre de la herida. Control positivo de la migración UTP 100 µM, control de la inhibición de la migración apirasa (Apy, 10 U/mL). Los datos están normalizados con respecto a la migración basal y se expresan como la media ± error estándar de la media (E.E.M.); *$p \leq 0.05$; **$p \leq 0.01$; *** $p \leq 0.001$; vs. control. Se realizaron 4 experimentos por sextuplicado para A y por triplicado para B. C) Tinción del citoesqueleto de actina en respuesta a los tratamientos. Los núcleos celulares fueron teñidos con DAPI (azul).

Adicionalmente, para investigar si en otra línea de carcinoma ovárico la activación del receptor A2B regula la migración celular, se utilizó la línea Caov-3, las cual proviene de un tumor primario de CEOv. Se evaluó la expresión del transcrito de *ADORA2B* mediante RT-PCR, de igual modo se corroboró la identidad del transcrito mediante secuenciación (Figura 9A). La migración se evaluó por el ensayo de cierre de la herida en respuesta a la incubación con BAY-606583 (0.1, 1 y 10 µM) durante 16 h. Se encontró que la activación del receptor A2B inhibe la migración celular (Figura 9B y C).

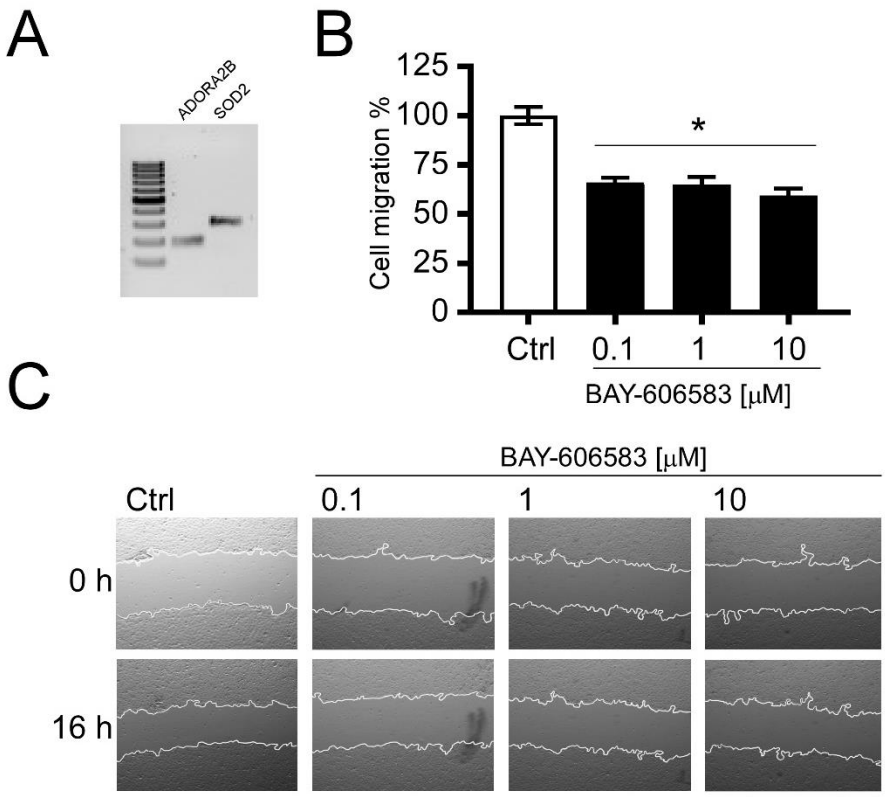

Figura 9. Las células CAOV-3 expresan el transcrito de *ADORA2B* y la activación del receptor reduce la migración celular. A) El transcrito de *ADORA2B* fue amplificado por RT-PCR. Como control constitutivo se amplifico la enzima superóxido dismutasa-2 (*SOD2*). B) La incubación de las células con el agonista BAY-60658 (0.1, 1 y 10 μM) durante 16 h disminuye la migración de las células. Los datos se expresan como la media ± error estándar de la media (E.E.M); *$p \leq 0.05$ vs. control. C). Se realizaron 3 experimentos por cuadruplicado. Micrografías representativas del ensayo de cierre de la herida.

Con la finalidad de reforzar nuestras observaciones sobre la atenuación de las características mesenquimales en las células SKOV3, se modificó la expresión del receptor de dos maneras: 1) mediante el sobre-expresión y 2) por medio de la disminución de la expresión por *shRNAs*, en ambas condiciones se evalúo el efecto sobre la migración celular. Se encontró que la sobre-expresión del receptor A2B disminuye la migración (41.24±5.96% del control, $p \leq 0.001$; Figura 10) mientras que la el *knock-down* del receptor con tres distintos shRNAs resultó en un incremento de la migración (135.80±3.72, 138.40±9.62 y 139.20±4.36% del control para los *shRNAs* 1, 2 y 3, respectivamente, $p \leq 0.05$; Figura 11A y B). La observación del citoesqueleto de actina en las células *sh-ADORA2B* reveló un notable incremento

en la presencia de FS (Figura 11C). En conjunto, los resultados sugieren que el receptor A2B no está involucrado en la proliferación celular, pero actúa como un regulador de la migración de LDCOv.

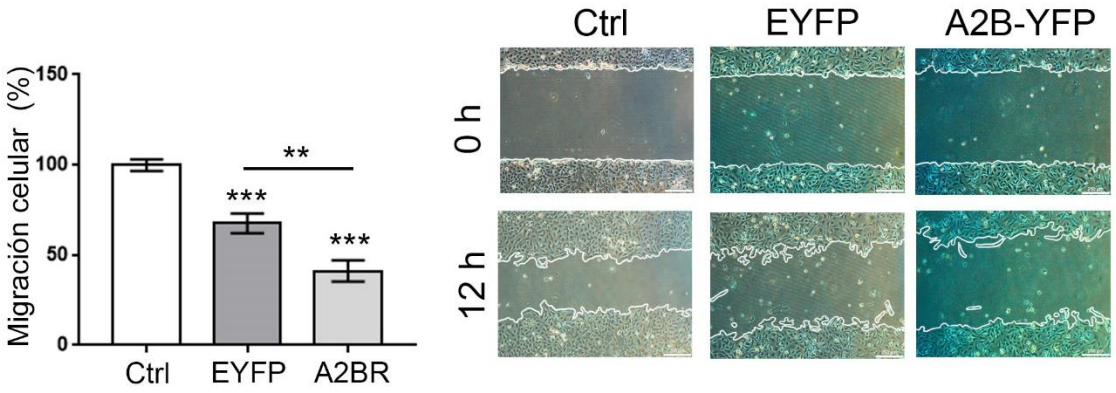

Figura 10. La sobre-expressión del receptor A2B inhibe la migración basal de las células SKOV-3. Las células fueron transfectadas con un plásmido que codifica para el receptor A2B fusionado con una proteína amarilla fluorescente (YFP) en el extremo carboxilo, se utilizó el plásmido con la YFP como control. Seguido de la transfección, las células fueron cultivadas y cuando fueron confluentes (48 h) se realizó el ensayo de cierre de la herida. Se realizaron 3 experimentos por triplicado. Los datos están normalizados con respecto a la migración basal y se expresan como la media ± error estándar de la media (E.E.M.); ** $p \leq 0.05$ vs. A2BR; *** $p \leq 0.01$ vs. control.

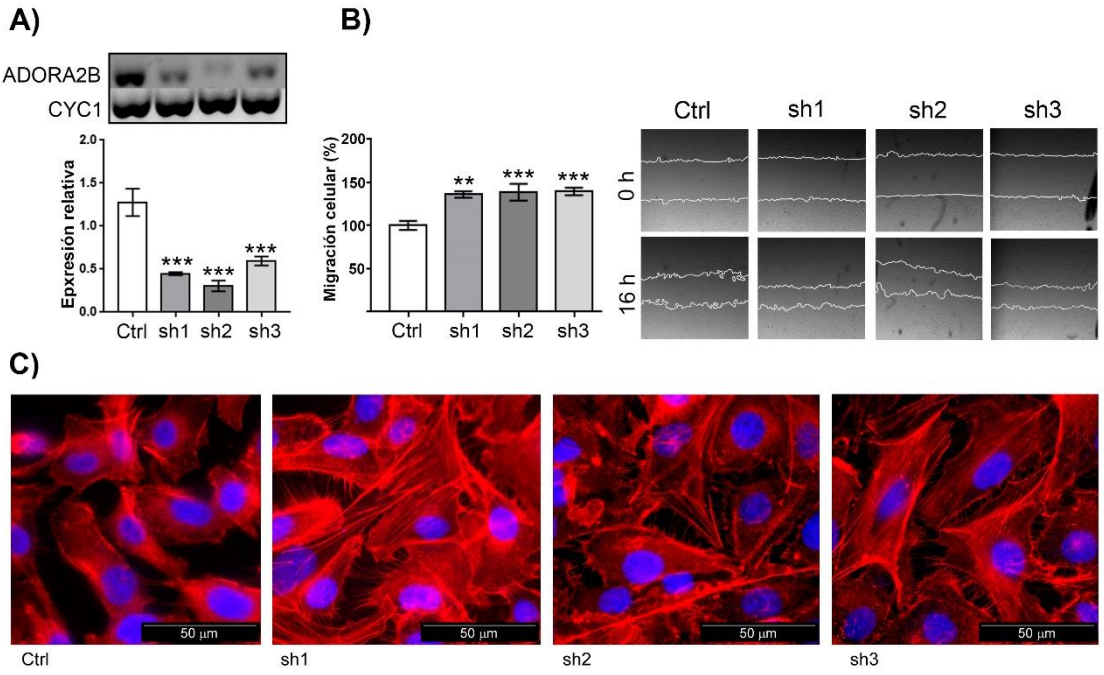

Figura 11. La disminución de la expresión del receptor *ADORA2B* promueve la migración de las células SKOV-3 y la formación de fibras de estrés. A) Las células fueron transducidas con partículas lentivirales con cargo de *shRNA* contra el transcrito de ADORA2B. El efecto de la disminución de la expresión se evaluó mediante RT-PCR. Se evaluó la expresión del transcrito constitutivo del complejo citocromo bc1 (*CYC-1*). ***$p \leq 0.001$ vs. control. B) Se evaluó la migración celular mediante el ensayo de cierre de la herida. **$p \leq 0.01$, ***$p \leq 0.001$ vs. control. Se muestran micrografías representativas de los experimentos (derecha). Los datos se expresan como la media ± error estándar de la media (E.E.M).C) Tinción del citoesqueleto de actina en células control o *sh-ADORA2B*. Los núcleos celulares fueron teñidos con DAPI (azul).

8.4. El receptor A2B modifica la expresión del marcador epitelial E-cadherina en las células SKOV-3

Con el objetivo de correlacionar la disminución de la migración celular inducida por la activación del receptor A2B con un cambio en el fenotipo de las células, se evaluó la localización y expresión del marcador epitelial E-cadherina (E-cadh) en respuesta al agonista BAY-606583. Las células SKOV-3 en la condición control, es decir la ausencia de estímulo, presentan una expresión discreta de E-cadh, la cual no está presente en las uniones celulares y muestra un patrón citoplásmico. En contraste,

cuando las células fueron incubadas durante 16 h con BAY 10 μM o ADO 100 μM, se observó un notable incremento de la expresión de la E-cadh, la cual se localizó en las uniones celulares. Como control negativo del experimento, se utilizó UTP 100 μM y no provocó cambios en la expresión y ubicación de la E-cadh (Figura 12A). Estas observaciones fueron reforzadas por el análisis de la expresión de la proteína por *western blot* (Figura 12B). Se encontró que BAY 10 μM provoco un aumento significativo en la expresión de la proteína E-cadh (1.29±0.03% veces el valor control). Por otra parte, el estímulo con ADO no provocó un aumento significativo en la expresión, sin embargo, se muestra una tendencia al incremento (Figura 12B).

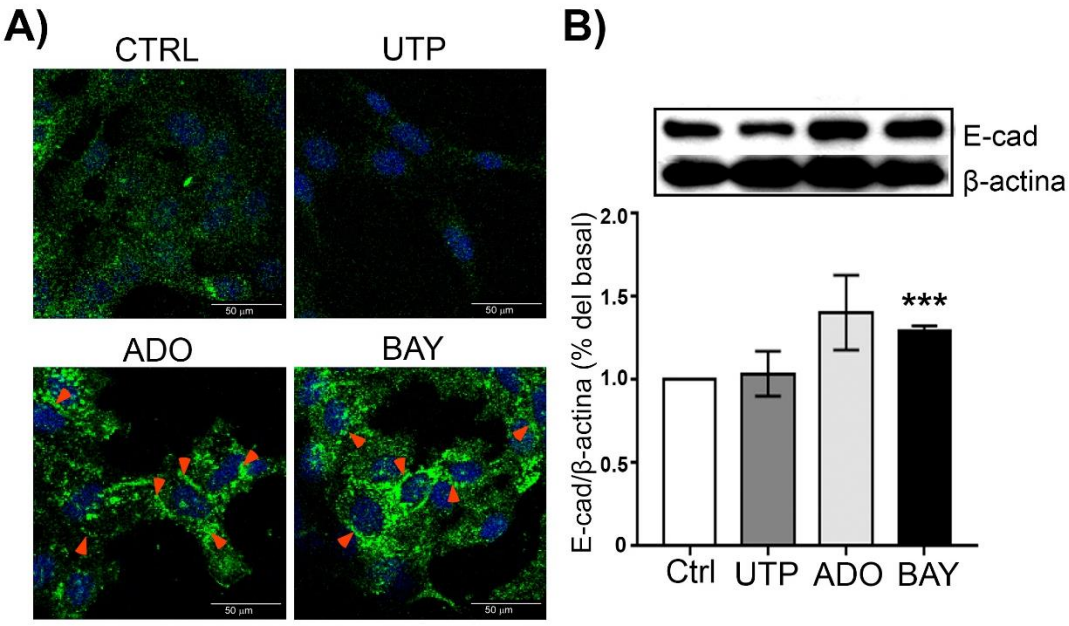

Figura 12. La estimulación del receptor A2B con BAY-606583 incrementa la expresión de la E-cadherina (E-cadh) e induce la relocalización a las uniones intercelulares. Las células fueron incubadas con los tratamientos farmacológicos durante 16 h. A) Inmunofluorescencia de la proteína E-cadh mediante el uso de un anticuerpo primario. Alexa Flúor 488 (señal verde). Los núcleos celulares fueron teñidos con DAPI (señal azul). B) Expresión de la proteína E-cadh en relación a la expresión de β-actina. Los datos fueron normalizados vs. La expresión basal (Ctrl) y se expresan como la media ± error estándar de la media (E.E.M.); se realizaron tres experimentos independientes; ***$p \leq 0.001$ vs. BAY. Las cabezas de flecha rojas señalan las uniones celulares,

8.5. Análisis de la expresión de genes en respuesta a la activación del receptor A2B

Dado que la adición de BAY-606583 al medio extracelular favorece el fenotipo epitelial e inhibe la migración, resulta de sumo interés evaluar los cambios en la expresión de genes que podrían estar asociados a los posibles mecanismos de regulación. Con este propósito, se realizó un análisis de microarreglos de cDNA de células SKOV3 que fueron incubadas con BAY-606583 10 μM durante 24 h. Para esto, se aíslo el RNA total, se marcó e hibridizó contra una librería de 35000 transcritos del genoma humano completo (los datos de este experimento fueron depositados en ArrayExpress, (# de acceso: E-MTAB-11130).

El análisis de los datos permitió identificar que la activación del receptor A2B provocó cambios sustanciales en el perfil de expresión génica de las células SKOV-3, se modificaron en total 884 transcritos, de los cuales 450 son regulados a la baja; y 434 trascritos regulados al alta (Figura 13A). Algunos de los transcritos modificados a la baja están relacionados con los siguientes procesos celulares*: Regulación de la migración celular*, *Proteólisis y organización de la matriz extracelular* y la *Fosforilación de peptidil-serina*. Dentro de estos grupos se encontraron los siguientes transcritos: *FGF9*, *AKT3*, *GSK3*β y las metaloproteínasas *ADAM12*, *MMP2* y *MMP6*, es sabido que estas proteínas tienen relación con la migración e invasión celular (Figura 13B, Tabla 3).

Por otra parte, el análisis ontológico (GO) permitió identificar que algunos de los transcritos regulados al alta se encuentran dentro de los procesos de *Regulación negativa de la migración celular*, *Organización del citoesqueleto* y la *Adhesión celular*. Algunos transcritos de relevancia son *C9orf16*, *PRKCBP1* (ZMYND8) y *PVRL2* (Nectina 2), los cuales tienen relación con la regulación negativa de la migración celular (Figura 13B, Tabla 4).

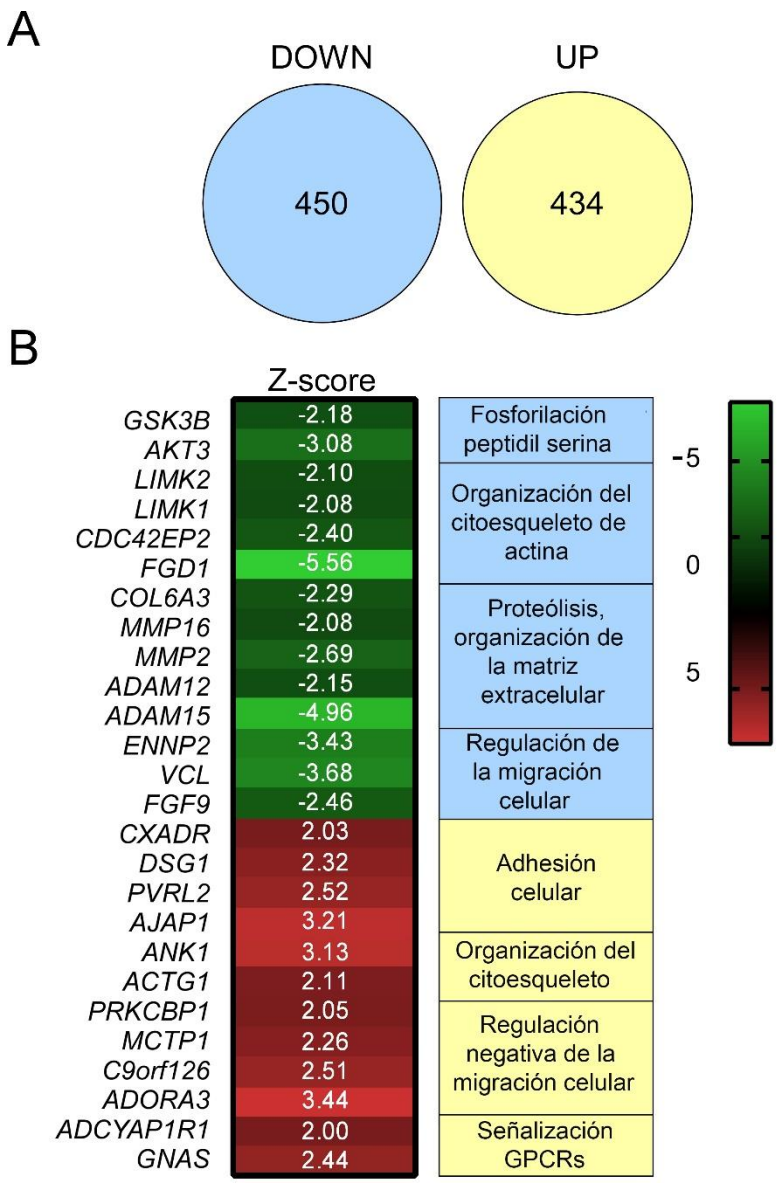

Figura 13. Análisis del patrón de expresión génica de microarreglos de cDNA obtenido de células SKOV-3 estimuladas con BAY-606583 10 μM durante 24 h. El RNA se obtuvo de cinco experimentos independientes y de la mezcla de estos se sintetizó el cDNA que se hibridizó con una librería de 30000 transcritos del genoma humano completo. A) Diagramas de Venn que indican el número de transcritos que fueron regulados a la alta (UP) o a la baja (DOWN). B) Mapa de calor que representa el Z-score de algunos transcritos de relevancia regulados a la alta (color rojo) y a la baja (color verde) por el tratamiento con BAY-606583. En la tabla se muestra el proceso celular determinado por el análisis ontológico (GO).

Tabla 3. Transcritos regulados a la baja

Símbolo	Nombre del gen	Descripción	Z score
AKT3	AKT serina/treonina cinasa 3	Fosforilación peptidil-serina	-3.08
GSK3B	Glucógeno sintasa cinasa 3 beta		-2.18
FGF9	Factor de crecimiento de fibroblastos 9	Regulación de la migración celular	-2.46
VCL	Vinculina		-3.68
ENNP2	Ectonucleótido pirofosfatasa/Fosfodiesterasa 2		-3.43
ADAM15	ADAM Metalopeptidasa Dominio 15	Proteólisis, Reorganización de la matriz extracelular	-4.96
ADAM12	ADAM Metalopeptidasa Dominio 12		-2.15
MMP2	Metaloproteasa 2		-2.69
MMP16	Metaloproteasa 16		-2.08
COL6A3	Colágeno, Tipo VI, Alfa 3		-2.29
FGD1	FYVE, RhoGEF y dominio PH 1	Organización citoesqueleto de actina	-5.56
CDC42EP2	CDC42 Proteína efectora 2		-2.40
LIMK1	LIM Dominio cinasa 1		-2.08
LIMK2	LIM Dominio cinasa 2		-2.10
GNAI2	Nucleótido de guanina-Proteína de unión G(I) subunidad alfa-2	Señalización por receptores acoplados a proteínas G	-2.50
PDE3B	GMP cíclico-Fosfodiesterasa B		-4.49
PDE9A	Fosfodiesterasa 9A		-2.99

Tabla 4. Transcritos regulados al alta

Símbolo	Nombre del gen	Descripción	Z score
GNAS	Adenilato ciclasa- Proteína Gαs	Señalización mediada por GPCRs	2.44
ADCYAP1R1	Receptor tipo I ADCYAP		2.00
ARHGEF7	Factor intercambiador de nucleótidos de guanina Rho 7		2.20
PFN1	Profilina 1	Organización del citoesqueleto	6.10
CDC42BPA	CDC42 Proteína cinasa de unión alfa		2.31
ADORA3	Receptor de adenosina A3	Regulación negativa de la migración celular	3.44
C9orf126	Supresor de la invasión de células de cáncer		2.51
MCTP1	Múltiple C2 y Dominio transmembrana Containing 1		2.26
PRKCBP1	Proteína cinasa C beta		2.05
ACTG1	Actina Gamma 1	Organización del citoesqueleto	2.11
ANK1	Anquirina 1		3.13
AJAP1	Proteína 1 asociada a las uniones adherentes	Adhesión celular	3.21
PVRL2	Nectina 2 Molécula de adhesión celular		2.52
DSG1	Desmogleina 1		2.32
CXADR	CXADR Ig-Molécula de adhesión celular		2.03

9. DISCUSIÓN

El carcinoma ovárico (CEOv) es una patología con alto índice de mortalidad debido a que la mayoría de las pacientes son diagnosticadas en etapas avanzadas de la enfermedad cuando ya presentan metástasis. En gran medida, la capacidad invasiva de las células tumorales está determinada por el microambiente tumoral (TME). Se ha descrito que el ATP y la ADO son componentes importantes de este microambiente (Ohta et al., 2006; Pellegatti et al., 2008) puesto que, actuando sobre receptores específicos, pueden regular la proliferación celular, la migración y la EMT (Martínez-Ramírez, et al., 2017; Campos-Contreras et al., 2020). En trabajos previos de nuestro grupo de trabajo, demostramos que la incubación de células SKOV-3, con un fenotipo parcialmente mesenquimal (Rosso et al. 2017), con ADO favorece un fenotipo epitelial y disminuye la migración; se obtuvieron resultados similares cuando se utiliza NECA, un agonista selectivo para los receptores A2. Debido a que el receptor A2B mostró el mayor nivel de expresión en dichas células, el efecto de inhibición de la migración podría deberse a este receptor (Martínez-Ramírez et al. 2017). En el presente trabajo hemos estudiado el efecto de la estimulación farmacológica y la manipulación de la expresión del receptor A2B sobre la migración celular.

Con el propósito de entender si existe una correlación entre el nivel de expresión del transcrito de *ADORA2B* (que codifica para el receptor A2B) con el desarrollo y la letalidad del carcinoma ovárico, analizamos la supervivencia de los pacientes en relación con el nivel de expresión del transcrito (alto y bajo) en la plataforma de dominio público *Kaplan Meier Plotter* (*ovarian cancer*). El análisis de todos los datos de carcinoma ovárico (sin discriminar el tipo histopatológico), mostró una correlación positiva entre la expresión alta del receptor *ADORA2B* y una mejor tasa de supervivencia (Figura 4A). Esta correlación también fue observada analizando los datos disponibles para el carcinoma ovárico de tipo seroso (Figura 4B) y cuando los datos de carcinoma seroso se restringen a los estadios 1 y 2 (Figura 4C), en estos estadios el tumor se encuentra todavía confinado al ovario. Sin embargo, está correlación no se presenta al restringir los datos de carcinoma

seroso a los estadios 3 y 4, cuando el cáncer ha comenzado a diseminar hacia otros órganos (Figura 4D). En concordancia, se encontró el mismo efecto en los pacientes de CEOv seroso de los grados 1 y grado 2 (Figura 5A y B), el grado de un tumor describe el nivel de desdiferenciación de las células tumorales con respecto al tejido sano, cuando los tumores aún no están desdiferenciados. Este resultado no se reprodujo cuando se analizaron los tumores serosos de grado 3 (Figura 5C). Estas observaciones nos permiten sugerir que la alta expresión del transcrito de *ADORA2B* en estadios tempranos del carcinoma ovárico puede asociarse con un buen pronóstico de la enfermedad. Sin embargo, resulta pertinente mencionar que únicamente se realizó el análisis del CEOv seroso dado que es el único que cuenta con el número de datos suficientes para realizar el análisis.

Un análisis exploratorio de la expresión de *ADORA2B* en otros tipos de cáncer en la K-MPdb (datos no mostrados) demostró que en el carcinoma de pulmón la alta expresión del transcrito es asociada con una mala prognosis. Por el contrario, en los datos de carcinoma de mama y gástrico, no se encontraron diferencias significativas entre la alta y la baja expresión del transcrito. Estos resultados sugieren que la contribución del receptor A2B a la supervivencia es dependiente del tejido y del subtipo de cáncer. Dadas estas observaciones, realizamos el estudio y la caracterización del receptor A2B en la línea celular derivada de carcinoma ovárico, SKOV-3.

La expresión y funcionalidad del receptor A2B en las células SKOV-3 fue corroborada mediante las siguientes observaciones: 1) el transcrito de *ADORA2B* fue detectado mediante RT-PCR (Figura 6A) y la identidad del amplicón fue corroborada mediante secuenciación; 2) se evidenció la expresión de A2B en la membrana celular mediante *western blot* utilizando una preparación enriquecida en proteínas de membrana, por el método de biotinilación con un reactivo no permeable, seguido de inmunoprecipitación con esferas acopladas a avidina (Figura 6B); 3) el receptor es funcional dado que se observó una respuesta en la fosforilación de ERK1/2 mediada por BAY-606583, un agonista específico de A2B (Figura 7A). Adicionalmente, la respuesta inducida por BAY fue bloqueada cuando

las células fueron pre-incubadas con el antagonista PSB-603 (Figura 7B); 4) la fosforilación de ERK1/2 inducida por BAY también disminuyo cuando se pre-incubaron las células con a) un inhibidor de la PKA (PKAi) y b) U-73122, un inhibidor de la PLC (Figura 7C y D). Esta última observación sugiere que el receptor A2B en las células SKOV-3 está acoplado a las subunidades Gαs y Gαq. Por lo que los mecanismos de señalización del receptor A2B son complejos y debiesen ser evaluados en cada aproximación experimental. Por ejemplo, en células de cáncer de pulmón epitelial se ha demostrado que el receptor A2B se acopla a ambas subunidades y dependiendo de la vía de señalización activada se tienen respuestas fisiológicas distintas (Giacomelli et al., 2018). Asimismo, se ha reportado el acoplamiento de A2B a la subunidad Gαs en la línea MDAMB231 de cáncer de mama (Fernandez-Gallardo et al., 2016).

Posteriormente, nuestros experimentos se enfocaron en determinar el papel de la estimulación del receptor A2B en la migración celular, dado que este proceso es determinante para la metástasis del cáncer. La evidencia obtenida indicó que el receptor A2B es un regulador negativo de la migración celular y se basó en las siguientes observaciones: 1) La activación del receptor A2B con el agonista BAY-606583 (0.1 a 10 μM) disminuye la migración célular, la cual fue evaluada por el ensayo de cierre de la herida (Figura 8B); 2) Este resultado correlaciona con la presencia discreta de fibras de estrés (FE), las cuales son menos evidentes en las células que fueron incubadas con BAY o Apy, lo que indica una atenuación del fenotipo mesenquimal (Figura 8C); 3) La sobre-expresión del receptor A2B redujo la migración celular, este resultado fue similar al observado con la estimulación farmacológica del receptor (Figura 10); 4) la reducción de la expresión de *ADORA2B*, por medio de 3 shRNAs distintos, causó un incremento en la migración celular (Figura 11B), este efecto correlaciona con el incremento en la formación de FE en las células *sh-ADORA2B* (Figura 11C). En conjunto esta serie de observaciones señalan que la activación del receptor A2B o la actividad constitutiva disminuyen la migración de las células SKOV-3.

Para descartar que las observaciones previamente mencionadas fuesen afectadas por la proliferación celular, se evaluó el efecto de la activación del receptor A2B sobre la viabilidad celular. Con esta premisa, se incubaron las células SKOV-3 con BAY durante 24 h. Se encontró que el tratamiento con BAY-606583 (10 nM a 10 μM) no tiene efecto sobre la viabilidad celular (Figura 8A), lo que indica que los efectos observados sobre la migración no son influenciados por la proliferación celular.

Adicionalmente, el tratamiento con BAY provocó un aumento en la expresión de la E-cadh y se observa la localización de la proteína en las uniones celulares, lo que sugiere la adquisición de un fenotipo epitelial (Figura 12A y B) y la disminución del fenotipo mesenquimal. Otros grupos de trabajo han descrito observaciones similares en células de cáncer cervical en respuesta a la ADO (Gao et al., 2016).

En concordancia con los resultados obtenidos en la línea SKOV-3, el agonista de A2B, BAY-606583 también inhibió la migración de las células Caov-3, una línea de carcinoma ovárico, en la cual también se expresa el receptor A2B (Figura 9), lo que sugiere que la actividad del receptor A2B sobre la migración de células de carcinoma ovárico puede ser un mecanismo general.

No obstante, es importante considerar que los efectos observados de la activación del receptor A2B en células de carcinoma ovárico dependen del modelo celular bajo estudio. En este sentido, la activación del receptor A2B en células de cáncer de mama (Fernandez-Gallardo et al., 2016), de vejiga (Zhou et al., 2017) y de carcinoma de células claras escamosas de cuello y cabeza (Wilkat et al. 2020) incrementa la migración celular. Por el contrario y en concordancia con nuestro estudio, en células de cáncer cervical, la ADO extracelular inhibe la migración y reduce la capacidad invasiva (Gao et al., 2016). Asimismo, en células epiteliales no cancerosas de retina (Ou et al., 2016) la activación del receptor A2B inhibe la migración y en células epiteliales de cáncer de pulmón (Giacomelli et al., 2018) y en fibroblastos de mama de ratón (Vasiukov et al., 2021), la activación del receptor A2B contrarresta el efecto de TFG-β como inductor de la EMT.

El efecto de la reducción de la migración observada en el presente estudio, podría contribuir a la explicación de la correlación entre la alta expresión de

ADORA2B y la mayor probabilidad de supervivencia en los estadios iniciales de la enfermedad (Figura 4). Sin embargo, podría haber otra posibilidad; dado que las células SKOV-3 son metástasicas, la inhibición de la migración y el favorecimiento de un fenotipo epitelial podrían contribuir a la colonización del cáncer en los órganos secundarios, en la etapa avanzada de la metástasis donde las células activan el proceso de transición mesénquima a epitelio (MET) (Yousefi et al., 2020). Dado que las células cancerosas secretan exosomas que expresan CD39 y CD73, enzimas requeridas para la hidrólisis del ATP en ADO, es plausible proponer la hipótesis de que la ADO actuando a través del receptor A2B podría contribuir a la formación de tumores secundarios.

Finalmente, con el propósito de elucidar si la actividad del receptor A2B promueve cambios en el patrón de expresión de genes y si estos cambios se asocian con la regulación de la migración celular, se evaluó la actividad transcripcional de células SKOV-3 incubadas con BAY-606583 10 μM durante 24 h mediante el análisis de microarreglos de cDNA. El análisis de los datos permitió encontrar cambios importantes en el patrón de expresión de genes. Las categorías identificadas por el análisis GO de los transcritos regulados a la baja: *Proteólisis y organización de la matriz extracelular*, *Organización del citoesqueleto de actina* y *Regulación de la migración celular*. Mientras que en los transcritos regulados a la alta algunas categorías que se encontraron son: *Adhesión celular*, *Regulación negativa de la migración celular* y *Organización del citoesqueleto* (Figura 13B, Tablas 3 y 4).

De los transcritos regulados a la alta se encontró el factor de crecimiento 9 (*FGF9*), el cual ha mostrado ser un inductor de la invasión de células de carcinoma ovárico mediante la activación de la vía VEGF-A/VEGFR2 (Bhattacharya et al., 2018). Otros transcritos fueron *AKT3* y *GSK3β*, que codifican para la variable 3 de la proteína cinasa β y para la cinasa glucógeno sintetasa 3β, respectivamente; ambos factores inducen la proliferación y la migración de células de carcinoma ovárico (Hao et al., 2020; Yu & Zhao, 2016). Resultó de gran interés la regulación a la baja de los transcritos que codifican para las MMPs como *ADAM12*, un factor

asociado con un fenotipo agresivo en el HGSC (Cheon et al., 2015) y *MMP2* y *MMP16*, los cuales son proteínas reconocidas en la facilitación de la migración y la metástasis de las células de carcinoma ovárico (Li et al., 2021; Wang et al., 2021). Asimismo, el análisis mostró la disminución de los transcritos de *LIMK1*, *LIMK2* y *CDC42EP2*, los cuales están involucrados en la organización del citoesqueleto de actina y la regulación de la formación de fibras de estrés (Nobes y Hall 1995; Amano et al. 2001; Vallée et al. 2018).

De los transcritos regulados al alza destacan *C9orf126* (SCAI), *PRKCBP1* (ZMYND8), and *PVRL2* (Nectin 2). El transcrito *C9orf126* (SCAI) codifica para un supresor de la proteína de invasión (proteína SCAI), un componente de la vía de señalización de RhoA. Se ha demostrado en células de cáncer pulmón que *miR-317b-5p* induce proliferación celular, migración e invasión mediante la regulación a la baja de SCAI (Luo et al., 2020). El transcrito *PRKCBP1* (ZMYND8) codifica para un dedo de zinc tipo MYND que contiene un factor de transcripción y una proteína que interactúa con las histonas que regula el crecimiento celular. En células de cáncer, ZMYND8 modula la metilación y acetilación de histonas, de modo que regula la expresión de oncogenes y supresores de tumor (Chen et al., 2021). En cáncer de mama y nasofaringe, ZYMND8 está regulado a la baja y esto correlaciona con un incremento en la invasividad y en una prognosis desalentadora (Basu et al., 2017; Chen et al., 2019). Asimismo, resultó de interés la regulación a la alta del transcrito de *PVRL2* (Nectina 2), una proteína de adhesión que participa en la formación de las uniones célula-célula (Mandai et al., 1999; Takahashi et al., 1999) y que también interactúa con proteínas de andamiaje en la regulación de la migración celular y la diferenciación (Takai et al., 2003). Se ha propuesto que la nectina-2 es un blanco potencial para el cáncer de ovario y de mama (Oshima et al., 2013). Asimismo, se encontró la regulación al alta de los transcritos *ANK1* y *ACTG1*, los cuales están implicados en la organización del citoesqueleto. El transcrito *ANK1* codifica para una proteína adaptadora del citoesqueleto que brinda estabilidad (Sharma et al., 2020). Por otra parte, *ACTG1* codifica para la actina-γ,una isoforma

de actina no muscular; en estudios recientes se ha demostrado la participación de esta proteína en la formación de adhesiones focales (Malek et al. 2020). .

En conjunto nuestros resultados sugieren que los niveles altos del transcrito de *ADORA2B* podrían ser asociados con una buena prognosis en los estadios de diferenciación 1 y 2. Nuestras evidencias *in vitro* sugieren que la estimulación farmacológica y la sobre-expresión del receptor A2B disminuyen la migración celular, lo que está asociado con la adquisición de un fenotipo epitelial.

10. CONCLUSIONES

Los resultados del presente trabajo muestran que el receptor A2B contribuye al mantenimiento de un fenotipo epitelial en líneas derivadas de carcinoma ovárico (LDCOv) ya que su activación farmacológica favorece la relocalización de la E-cadherina a las uniones celulares y disminuye la migración celular y la presencia de fibras de estrés. Por otra parte, el análisis transcripcional por microarreglos, permitió identificar que se regulan a la baja los transcritos de genes asociados a la migración e invasión celular como *MMP2*, *MMP16* y *ADAM12*, las cuales codifican para metaloproteasas necesarias en la invasión celular. Entre los transcritos regulados a la alta, se encontraron algunos asociados con la adhesión celular por ejemplo: *DSG1* (desmogleina), una proteína que se asocia a los desmosomas y *PVRL2* (nectina), una proteína de adhesión que ha sido considerada un blanco en cáncer de mama y ovario. Los transcritos detectados, permitirán un estudio más detallado de los mecanismos de señalización del receptor A2B en el contexto del carcinoma ovárico.

De acuerdo con los resultados obtenidos en el presente estudio podemos afirmar que, en general el receptor A2B es un regulador de la migración celular, sin embargo también sugieren que los efectos podrían depender de la etapa de desarrollo tumoral. Así, en el CEOv en etapas tempranas de diseminación (*stages 1 y 2*), el receptor A2B podría ser considerado un blanco farmacológico potencial, cuya activación inhibiría el inicio de la metástasis; sin embargo, en atapas avanzadas del carcinoma (*stages* 3 y 4), el receptor A2B, podría contribuir al establecimiento de tumores secundarios en órganos blanco al favorecer un fenotipo epitelial.

El presente trabajo permite concluir que la activación del receptor A2B para adenosina, inhibe la migración en células derivadas de carcinoma ovárico.

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ANEXOS

1. **Artículo de revisión:**

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2. **Artículo de investigación original:**

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Review

Purinergic Signaling in the Hallmarks of Cancer

Anaí del Rocío Campos-Contreras, Mauricio Díaz-Muñoz and Francisco G. Vázquez-Cuevas *

Department of Cellular and Molecular Neurobiology, Instituto de Neurobiología,
Universidad Nacional Autónoma de México, Boulevard Juriquilla #3001, Juriquilla Querétaro 76230, Mexico;
anaicampos.c@gmail.com (A.d.R.C.-C.); mdiaz@comunidad.unam.mx (M.D.-M.)
* Correspondence: fvazquez@comunidad.unam.mx; Tel.: +52-(442)-238-1035

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Abstract: Cancer is a complex expression of an altered state of cellular differentiation associated with severe clinical repercussions. The effort to characterize this pathological entity to understand its underlying mechanisms and visualize potential therapeutic strategies has been constant. In this context, some cellular (enhanced duplication, immunological evasion), metabolic (aerobic glycolysis, failure in DNA repair mechanisms) and physiological (circadian disruption) parameters have been considered as cancer hallmarks. The list of these hallmarks has been growing in recent years, since it has been demonstrated that various physiological systems misfunction in well-characterized ways upon the onset and establishment of the carcinogenic process. This is the case with the purinergic system, a signaling pathway formed by nucleotides/nucleosides (mainly adenosine triphosphate (ATP), adenosine (ADO) and uridine triphosphate (UTP)) with their corresponding membrane receptors and defined transduction mechanisms. The dynamic equilibrium between ATP and ADO, which is accomplished by the presence and regulation of a set of ectonucleotidases, defines the pro-carcinogenic or anti-cancerous final outline in tumors and cancer cell lines. So far, the purinergic system has been recognized as a potential therapeutic target in cancerous and tumoral ailments.

Keywords: purinergic signaling; cancer; tumor microenvironment; immune evasion in cancer; purinergic receptors; ATP; adenosine; ectonucleotidase

1. Purinergic Signaling in Brief

In 1929, Drury and Szent-Györgi provided the first experimental evidence that adenine nucleotides function as signaling molecules. However, the term "purinergic", and ATP as a signaling molecule, was first proposed in 1972 by G. Burnstock [1]. Although his work was controversial, today it is well recognized that ATP, other nucleotides (adenosine diphosphate (ADP), UTP, uridine diphosphate (UDP)) and ADO are cellular messengers that modulate diverse signaling pathways and participate in physiological and pathological processes, mainly through specific membrane receptors (Figure 1).

Purinergic receptors have been classified into two families: P1, sensitive to ADO; and P2, sensitive to adenine and uridine nucleotides. P1 belongs to the G-protein coupled receptor (GPCR) superfamily, while P2 is divided in two subfamilies. The first is P2X, which are ligand-gated cation channels formed by homotrimeric or heterotrimeric complexes of known subunits (P2X1-P2X7). ATP is the natural ligand for P2X receptors. When activated, these receptors promote rapid depolarization associated with Ca^{+2} and Na^{+} influx, and K^{+} efflux [2]. The second subfamily is P2, and eight P2Y subtypes have been described in mammalian cells: P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11-14. These receptors can be activated by ATP (P2Y2 and P2Y11), ADP (P2Y1, P2Y12 and P2Y13), UTP (P2Y2 and P2Y4), UDP (P2Y6) and UDP-glucose (P2Y14). P2Y2, P2Y4 and P2Y6 are coupled to Gq proteins; thus, their activation leads to phospholipase C (PLC) activation, turnover of phosphoinositides and Ca^{+2} mobilization. P2Y12, P2Y13 and P2Y14 are coupled to Gi proteins producing adenylate

cyclase (AC) inhibition [3]. Once in the extracellular space, ATP can either activate P2R or be further dephosphorylated/hydrolyzed by a set of enzymes called ectonucleotidases (Figure 1). There are four families of these enzymes: ectonucleoside triphosphate diphosphohydrolases (NTPDases), ecto-59-nucleotidase (CD73), ectonucleotide pyrophosphatase/phosphodiesterase (ENPP) and alkaline phosphatases (AP) [4].

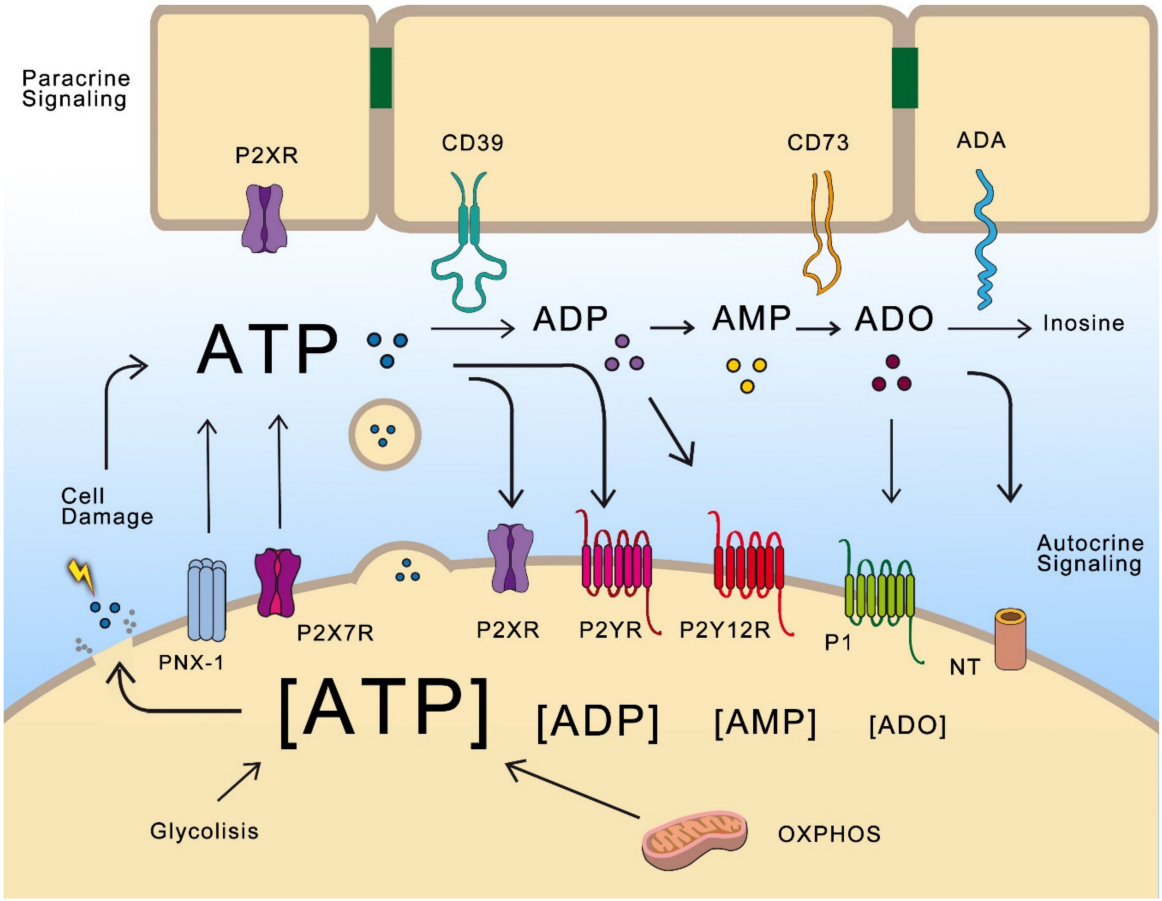

Figure 1. Nucleotides act as autocrine and paracrine messengers. ATP is produced by oxidative phosphorylation (OXPHOS) and glycolysis intracellularly reaching mM concentrations. It can be released to extracellular space by cellular lysis, exocytosis, transporters, hemichannels of pannexin-1 (PNX-1) and P2X7R. Once located at the extracellular space, ATP activates P2XR (ligand activated ion channels), P2YR receptors (belonging to GPCR superfamily), and it can be hydrolyzed by ectonucleotidases (here, CD39 and CD73 are illustrated by their relevance in cancer) to form ADP, AMP and adenosine (ADO). ADP is able to activate P2Y12R and ADO activate G-protein coupled receptor (GPCR) receptors of the P1 family named (A1R, A2AR, A2BR and A3R). ADO is hydrolyzed by adenosine deaminase (ADA) to inosine or it is transported into the cell by nucleoside transporters (NT).

These enzymes, besides limiting ATP signaling, produce additional ligands for P2Y receptors like ADP to P2Y12, and adenosine to A2-AR (A2-adenosine receptors). Extracellular adenosine (exADO) can activate P1 receptors which belong to a family of GPCRs. According to their sequence and signaling properties, P1 receptors are designated A1R, A2AR, A2BR and A3R. A1R and A3R are mainly coupled to the Gi/o subunit and thus inhibit AC and cAMP production; A2AR and A2BR are mainly coupled to the Gs subunit and stimulate cAMP synthesis through AC activation. Finally, exADO and its associated signaling are regulated by hydrolysis through adenosine deaminase (ADA) and transported into the cell by nucleoside transporters (NTs) [5].

When cells are damaged or stressed by changes in osmotic pressure and mechanic deformation, they respond by releasing ATP to the extracellular medium. Aside from this unspecific mechanism,

ATP can be released by controlled mechanisms in response to different stimuli. These mechanisms include efflux through membrane channels and transporters (e.g., connexins, pannexins, maxi-anion channels, volume-regulated channels, and ATP-binding cassette (ABC) transporters), purinergic receptors (e.g., P2X7R), and vesicle-mediated release [6]. Purinergic signaling is flexible and adaptable. Released ATP activates paracrine and autocrine communication and, as previously mentioned, its hydrolysis generates a cascade of additional signaling molecules. Almost every cell type expresses a dynamic set of purinergic receptors and ectonucleotidases; therefore, the final outcome depends on a variety of factors, including specific receptors and ectonucleotidases expressed by the cell, as well as the constant fluctuations in the proportion of extracellular and intracellular levels of ATP and ADO.

2. Purinergic Signaling and Cancer Hallmarks

2.1. Purines in Tumor Microenvironment

Intense efforts have been made to systematize the complex organization of cancer cells within the tumor and the interactions of these cells with the organism [7,8]. An essential concept to understand the principles of this organization is the tumor microenvironment (TME). The TME consists of all interactions between cancer cells and non-malignant cells, such as endothelial, fibroblast and immune cells. The structural, cellular and biochemical composition of this enclosed space modulates cancer cell metabolism, migration and proliferation. It also influences the host immune response [9]. Studying the cellular and molecular composition and interaction of these regions has become increasingly important in the field of pathology, because the downstream effects derived from these interactions could favor tumor growth, invasion and immune evasion.

Purinergic signaling in particular has gained attention in this context, because ATP and ADO are present in high concentrations in the TME [10,11]. Different tumor tissues and cancer cell lines express purinergic receptors and CD39/CD73 ectonucleotidases, generating diverse cellular responses that could depend directly on the cell context and the specific set of purinergic signaling components expressed by the tumor and host cells, known by some authors as the "purinome" [12]. In this section, we will review evidence about the presence of ATP and ADO in the TME.

Extracellular ATP (exATP) and exADO are accepted biochemical markers of cancer, due to their significant levels in the tumor interstitium. ATP release by cancer cells and the subsequent activation of purinergic receptors and intracellular pathways have been reported in various cancer models, such as pheochromocytoma PC-12 cells stimulated with maitotoxin [13]; Ehrlich ascites tumor cells, ATP release induced by mechanical stimulation [14]; A549 human lung cancer cells, by exocytosis triggered by TGF-β stimulation [15]; SKOV-3 ovarian carcinoma-derived cells released by a pipette generated flux [16]; I-10 testicular cancer cells through pannexin-1 [17]. A breakthrough was the monitoring of ATP in vivo in a tumor-bearing mouse with the use of reporter cells carrying an extracellular ATP sensor. ATP was within a low nM range in healthy pericellular space, but increased to high μM levels in the tumor stroma and vicinity [18]. This observation had significant relevance, because exATP is a putative direct source of exADO. Although ADO has not been measured within tumors using an in vivo approach, it was reported that exADO was more abundant in microdialysates from tumoral core regions [19]. However, some conditions in the tumor, such as hypoxia, favor ADO formation. It is well known that, in tumor growth, there is an oxygen gradient. The areas at the center of the cellular mass are hypoxic; in this condition, CD39 and CD73 expression is induced by hypoxia inducible factor 1α/β (HIF-1α/β), and ADO formation is promoted [20–23].

Furthermore, the ectonucleotidases CD39 and CD73 play a fundamental role in modulating ATP and ADO levels in the TME. These enzymes are expressed in cancer cell lines, immune cells and stromal cells, and they are considered immune checkpoints in cancer [24]. Practically all cell types can release ATP to the extracellular space; therefore, all cells in the tumor-host interface could contribute to the substantial amount of ATP in the tumor interstitium.

A mechanism that has gained attention in cancer is ATP release through pannexin-1 channel (PANX1), since a truncated PANX1 protein ($PANX1^{1-89}$) is significantly enriched in highly metastatic human cancer cell lines [25]. $PANX1^{1-89}$ in combination with wild type PANX-1 confers gain-of-function to channel activity, promoting a significant increase in ATP release. Moreover, this mechanism facilitated the resistance to mechanical deformation of cancer cells. This finding is relevant, because many cancer cells undergo apoptosis during metastasis through capillaries [25]. As previously mentioned, ATP can also be released through P2X7R channel, and the P2X7R has been associated with the regulation of NLRP3 inflammasome, which leads to the release of pro-inflammatory cytokines, specifically IL-1β and IL-18 [26–28]. Importantly, antitumor therapies like chemotherapy or radiotherapy induce tissue damage and cell death and, consequently, the corresponding release of damage-associated molecular patterns (DAMPs), mainly ATP [29]. exATP is quickly converted into ADO by the CD39/CD73 pathway, establishing a particular proportion of purines in the TME where both purines can potentially affect cancer and host cells; this equilibrium is decisive for the outcome of a given clinical treatment [30].

Nucleotides in the TME primarily serve as an interface of interaction with immune system cells since ATP can act as a "find me" signal for cells of the innate immune system [30]. However, in the extracellular space, ATP is modified by ectonucleotidases that generate ADO, whose fundamental antagonistic action is related to the evasion of an immune attack; this topic will be discussed later. On the other hand, nucleotides in the tumor stroma can function as paracrine-autocrine messengers, inducing specific cellular responses over all the cell types forming the tumor mass. Research has demonstrated that purines can regulate cell proliferation, epithelial to mesenchymal transition (EMT) and cellular migration in tumor cells. These actions are discussed below.

2.2. Purines in Proliferation and Tumor Growth

Nucleotides in the tumor microenvironment play a dual role; they function as lures to interact with immune system cells and as autocrine-paracrine signals directly affecting the physiology of cancerous cells.

Autocrine-paracrine actions of purines involve a feedback loop that joins the initial production and release of ATP by cancerous cells, with the subsequent activation of cell proliferation and tumor growth (Figure 2). Signaling actions of exATP depend on the presence and diversity of specific receptors in the own cell releasing the nucleotide and neighboring cells, as well as on the actions of ectonucleotidases, which will define the composition of ligands in the medium.

With respect to purinergic receptors, P2X7R is the best characterized in the cancer context, probably because this receptor was described as an apoptotic inducer [31], motivating the inquiry of a role in cancer. In addition, P2X7R has diverse signal transduction mechanisms. Unlike other P2XRs, it has a long intracellular COOH-end with putative protein-protein interaction domains, such as SH2, SH3 and dead domains [32], creating a potential signaling mechanism independently of ionic conductance.

Incremented expression of P2X7R has been demonstrated in cancerous tissue from organs including breast [33], thyroid [34], ovary [16], pancreas [35], colon [36,37] and liver [38]; in general, the increment in P2X7R expression was correlated with a high tumor grade. This observation suggests that P2X7R can be activated by autocrine-paracrine signaling and be a regulator of cancerous cell physiology.

The significance of the elevated expression of P2X7R was intriguing, because it was initially related to apoptosis induction; however, important observations supporting proliferation and/or survival roles for P2X7 were later reported; DiVirgilio's group proposed that P2X7R can act as a growth-promoting receptor based on the following evidence: 1) P2X7R exogenous expression in several cell lines incremented proliferation; 2) the TME contains high amounts of ATP (hundreds of μM) to activate P2X7R; 3) various cancerous tissues from different organs show high expression levels of P2X7R; and 4) P2X7R is a positive regulator of aerobic glycolysis [39]. This apparent antagonism between the ability of P2X7R to induce apoptosis and, in some conditions, support cell survival has been analyzed [40]. The most plausible explanation to explain this paradox could be related with the conformation of

P2X7R in activated state, P2X7R adopt structure conformations that specifically regulate the induction of apoptotic activity [41].

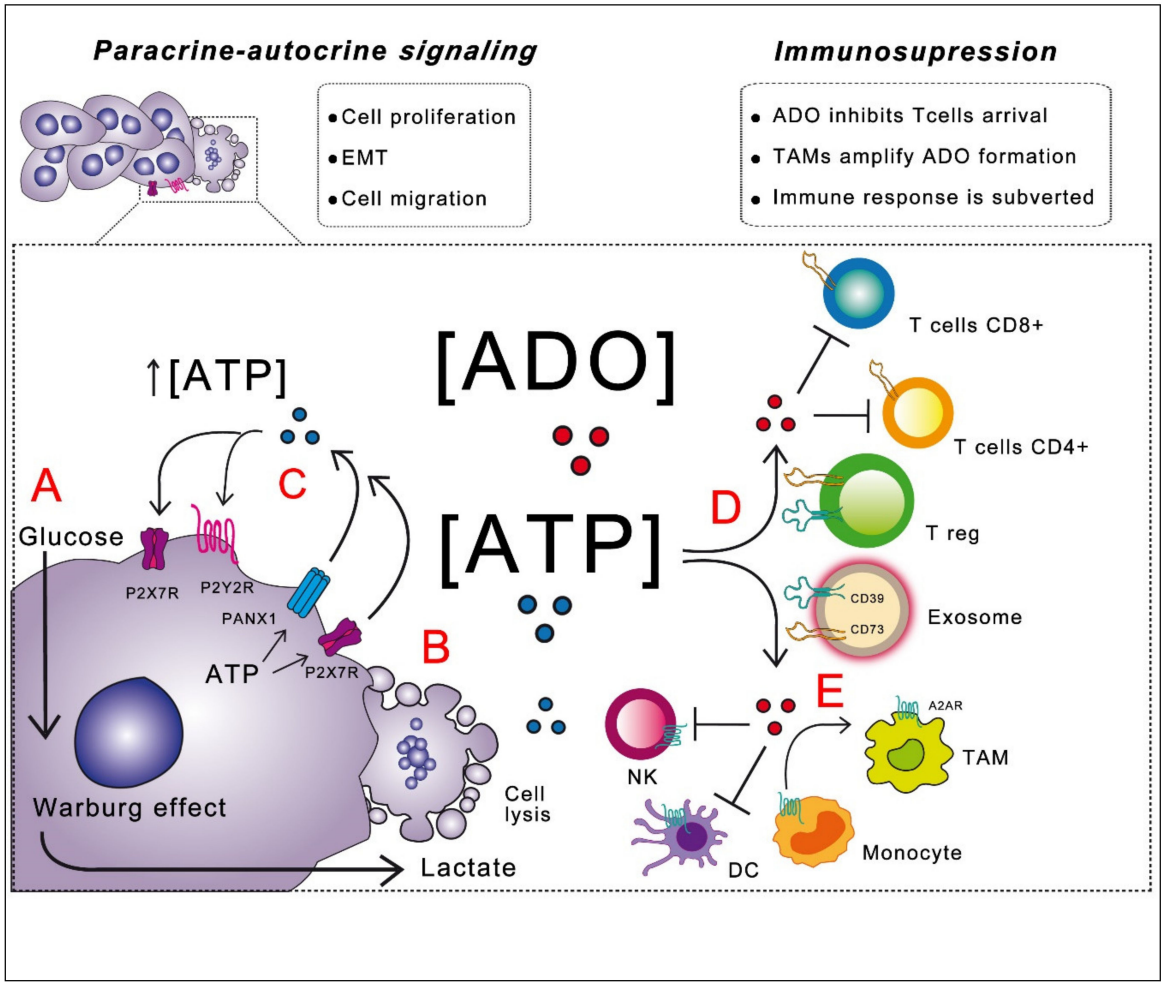

Figure 2. Purinergic signaling and tumor microenvironment (TME). (**A**) Cancer cells synthetize ATP rather from aerobic glycolysis (Warburg effect) which leads to lactate formation and subsequent extracellular acidification. (**B**) ATP is released from tumor cells (pannexin-1 hemichannels and P2X7R have an outstanding role) by general mechanisms or by cellular lysis as result of anticancer therapies and reach hundreds of mM levels, sufficient to activate any P2 receptor. (**C**) extracellular ATP in autocrine/paracrine way activate P2 receptors (mainly P2X7R and P2Y2R) to induce proliferation, migration and epithelial to mesenchymal transition (EMT) of cancer cells. (**D**) In TME, ATP is hydrolyzed to ADO by subsequent action of ectonucleotidases CD39 and CD73, which are expressed in the own tumor cells, exosomes and immune cells (i.e., CD4+, CD25+, Foxp3+ Treg); contributing to the significant increase of ADO, which in turn inhibits antitumor response of innate immune cells and T effector cells (CD4+ and CD8+). (**E**) ADO also contributes to monocyte differentiation into associated tumor macrophages 2s), which also amplify ADO formation.

The proliferative role of P2X7R has been documented in a variety of cancers, such as ovarian carcinoma cells [16], mesothelioma [42], pancreatic cancer cells [35,43] and osteosarcoma cells [44]. Although the molecular mechanisms of P2X7R are not completely understood, their transduction pathways involve ERK phosphorylation of both dependent and independent intracellular Ca^{2+} increments [45,46], the PI3K/AKT/GSK3β/β-catenin pathway and mTOR/HIF1α/VEGF signaling [16,43].

The overexpression of purinergic P2Y receptors belonging to the GPCR superfamily has also been observed in cancerous systems acting as promotors of cell proliferation; P2Y2R is the prototypical

and most analyzed receptor. It has been shown that P2Y2R is overexpressed in biopsies of basal cell and squamous cell carcinomas (non-melanoma skin cancers) [47]. One study also showed by cDNA microarray analysis that the P2RY2 transcript is highly expressed in fresh biopsies of gastric cancer tissue, compared to adjacent healthy tissue [48]. The incremented expression of P2Y2R was detected in primary cultured hepatocellular carcinoma cells and in the hepatocarcinoma-derived cell lines HepG2 and Bell-7404 compared with normal hepatocytes and the normal hepatocyte cell line LO2 [49].

Accordingly, it has been demonstrated that UTP activation of P2Y2R induced proliferation in C6 glioma cells [50], in human cutaneous squamous cell carcinoma lines (A431) [47]; in pancreatic duct epithelial cells PANC-1 [51]; in hepatocarcinoma cell lines HepG2 and Bell-7404 [49]; and in the gastric cancer lines AGS and MKN-74 [52]. In addition, P2Y2R-dependent cell proliferation involves the Ras/Raf/MEK-1 pathway, modulated by PLC/PKC and Ca^{2+} in C6 glioma cells [50]. In PANC-1 cells, UTP increased the phosphorylation level of AKT through PKC, PI3K, SRC and Ca^{2+}-calmodulin-dependent protein kinase II [51].

In addition, gastric cancer cell lines also express P2X4R, the activity of which exerts anti-proliferative effects contrary to P2Y2R activity [52]. In fact, P2X4R activity is able to revert the proliferative effects mediated by P2X7R in breast-derived cancers [53]. Both evidences reveal that functional interactions among subtypes of purinergic receptors are determinants for the final outcome of purinergic signaling in cancer; both observations highlight the anti-proliferative action of P2X4R.

Although the purinergic system has mainly been associated with the positive regulation of cell proliferation, diverse evidence supports that it is not a rule; for example, pharmacological activation of purinergic receptors induced apoptosis in cancerous cells. Thus, it was shown that P2X7R is downregulated in endometrial cancer. In endometrial epithelial carcinoma cells, P2X7R activation was able to induce apoptotic cell death [54]. Furthermore, activation of P2X7R inhibited the formation of virus-induced skin cancer in vivo [55]. As for P2YR, research has found that P2Y2R activity inhibits cell proliferation in endometrial carcinoma cells HEC-1A and Ishikawa cells [56], human colorectal carcinoma cell HT29 and Colo320 D [57], human esophageal cancer cells [58], and nasopharyngeal carcinoma cells [59]. Inhibition of cell proliferation was also related to the activity of P2Y6R through a pathway involving the store-operated Ca^{2+} entry (SOCE) and β-catenin [60]. These controversial observations must be analyzed considering the detailed characteristics of each cellular system.

The most relevant compound formed from exATP is ADO. The receptors for ADO are expressed in tumor tissues from various organs. It has been shown that A2BR, probably the best characterized in cancer, is overexpressed in tumor biopsies and cell lines derived from human hepatocellular carcinoma [61], colorectal carcinoma [62], oral squamous carcinoma [63] and bladder urothelial carcinoma [64]. To further support the role of A2BR in cancer progression, studies have shown that pharmacological or genetic inhibition of this receptor decreases cell proliferation [62–64]. Additionally, expression of A2BR has been described in cell lines derived from prostate cancer [65], breast cancer [66] and head and neck squamous cell carcinoma [67]. In all these systems, A2BR functioned as a cell proliferation promoter. Moreover, inhibition of A2BR expression in EJ and T24 cell lines, derived from bladder urothelial carcinoma, inhibited cell proliferation and arrested the cells in the G1 phase of the cell cycle [64].

Given that the equilibrium between nucleotides and nucleosides influences the autocrine-paracrine signals that regulate tumor growth, purinergic signaling elements, such as transporters, receptors and ectonucleotidases, emerge as potential pharmacological targets to modulate carcinogenesis.

2.3. Purines in Cancer Cell Migration, EMT and Metastasis

Cell migration involves a response to a chemical gradient and is required for physiological events including embryonic development and tissue repair; however, in cancer, it participates in the metastatic activity of cancerous cells to form secondary tumors. The stages of metastasis are loss of cell-cell adhesion in primary tumor, migration and invasion, anoikis evasion and implantation to form the secondary tumor [68]. These stages are initiated by epithelial-mesenchymal transition

(EMT), a process in which epithelial cells assume a mesenchymal phenotype, acquiring enhanced invasive and metastatic capacity. Extensive evidence indicates that purinergic signaling participates in the modulation of this phenomenon in different cancer types [69].

During EMT, cells lose their apical-basal polarity and epithelial cell-cell contacts including tight junctions, adherent junctions, and desmosomes. In addition, they acquire a spindle-shaped mesenchymal morphology, and gain motility by reorganizing their actin cytoskeleton. EMT is also accompanied by the loss of epithelial genes such as E-cadherin, keratins and zona occludens-1 (ZO-1). Conversely, the expression of metalloproteinases (MMPs), vimentin and N-cadherin is upregulated. Some classical EMT promoters are transforming growth factor β (TFG-β), epidermal growth factor (EGF) and wingless (WNT); these molecules elicit EMT through the activation of transcription factors such as SNAIL and TWIST [70]. ATP and purinergic signaling also modulate the EMT process, migration/invasion and metastasis in many different cancers.

It has been observed in different lung cancer cell lines that stimulation with high ATP concentrations, such as those found in the TME (0.5–1 mM), favors cell detachment, migration and invasion. These observations were associated with an increased expression of MMPs and the formation of filopodia and cell protrusions, as well as an increased expression of vimentin, SNAIL and SLUG. In parallel, there was a reduction of the epithelial proteins E-cadherin and ZO-1. These results were ingeniously related with the exATP micropinocytosis process, since the genetic deletion of SNX5 (a gene involved with cell micropinocytosis) caused a significant reduction in cancer cell proliferation, migration and invasion [71].

Moreover, evidence reveals the interaction between ATP and classical EMT inducers. For instance, it has been demonstrated that treatment with TGF-β1 elicits ATP release from lung cancer cells, thus activating P2 receptors. Actin remodeling and cell migration induced by TGF-β1 required the expression and autocrine stimulation of P2X7R, since these processes were suppressed after P2X7R knock-down or pharmacological inhibition [15]. In the PC9 human lung cancer cell line, which has a mutated EGFR, P2X7R was constitutively activated, promoting cell migration, even in the absence of TGF-β1. Cell motility and lamellipodium extension of PC9 cells were abolished by AG1478, an EGFR inhibitor. These data showed a cross-signaling between TGF-β1, P2X7R and EGFR in the regulation of cell migration [72].

P2X7R is associated with cancer cell migration and invasion. This receptor is expressed in cells from different types of cancer, such as pulmonary [15,70], prostatic [73], mammary [74], pancreatic [35], glioma [75], osteosarcoma [44] and glioblastoma stem cell cancer [76]. In the prostate, breast and osteosarcoma cell lines, it has been proven that P2X7R stimulation induces cell migration and up-regulation of EMT-related genes. At the same time, E-cadherin is down-regulated. These effects of P2X7R were mediated through PI3K/AKT phosphorylation and ERK1/2 signal transduction pathways [71,74].

Considering that nucleotides promote cell migration, evidence demonstrates the interaction between purinergic receptors and proteins involved in cell-to-cell and cell-to-extracellular matrix (ECM) junctions such as cell adhesion molecules (CAM) and integrins. For instance, P2Y2R interacts directly with αvβ3 and αvβ5 integrins in astrocytoma cells. These interactions are mediated through the integrin-binding domain arginine-glycine-aspartic acid (RGD) contained in P2Y2R. The RGD domain is necessary for UTP-induced chemotaxis through G_0 protein coupling; the mechanism elicited by P2Y2R stimulation involves Rac and Vav2 (a GEF for Rac) activation. Moreover, vitronectin, an ECM protein that binds to integrins αvβ3 and αvβ5, is up-regulated [77]. Additionally, P2Y2R activation through G_{12} coupling and integrin αvβ5 interaction mediates Rho activation, cofilin, myosin light chain (MLC-2) phosphorylation and stress fiber formation [78]. One study showed that P2Y2R activation increased intracellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression in a highly metastatic breast cancer cell line. This effect was also observed in endothelial cells incubated with cancer cell conditioned medium, leading to increased adhesion between cancer cells and ECs; this action could be associated with cancer cell metastasis [79].

P2Y2R is a purinergic receptor that seems crucial to mediate ATP pro-metastatic effects. For instance, ATP in prostate cancer cells promotes Cdc42 and Rac1 activation and MMP expression through P2YR activation [80,81]. This effect is mediated through P2Y2R activation [82]. P2Y2R is also expressed in diverse breast cancer cell lines: MCF-7, Hs578T, MDAMB-231 and T43D [83–85]. In breast tumor tissue, P2Y2R expression is higher at the invasive edge of the tumor, in infiltrating cells in adipose mammary tissue and in the tumor embolus in lymphatic sinuses, suggesting the participation of P2Y2R in metastasis [84]. It has been proven that highly metastatic breast cancer cell lines release more ATP to the extracellular medium and, thus, exhibit a greater ability to migrate and invade [86], the effects are mediated through the activation MEK/ERK1/2-dependent signaling pathway [80,83,87]. Another pathway involved in cell invasion of breast cancer cells is ATP-P2Y2R-β-catenin [85]. Studies in prostate [87] and ovarian cancer cells [88] found that P2Y2R activation also promoted the expression of EMT-related genes, and demonstrated a transactivation pathway between P2Y2R and EGFR.

Conversely, CD73 over-expression using pcDNA-NT5E has shown to increase cancer cell invasion, migration and adhesion in the breast cancer cell lines T-47D and MDAMB231 [89,90]. Following the same experimental strategy, increased cell migration was observed in human cervical cancer cell lines. However, the effect did not depend on CD73 activity [91]. In contrast to the general assumption that CD73 is pro-tumorigenic, it was reported that CD73 promotes epithelial integrity through an increase in membrane E-cadherin, β-catenin and Na^+-K^+ ATPase in endometrial cancer; also, in vitro experiments showed increased migration and invasion after pharmacological CD73 inhibition [92].

Analysis of CD73 expression in human tissue from head and neck squamous cell carcinoma (HNSCC) samples showed a higher CD73 expression in samples from patients with lymph node metastasis. This finding correlated with in vitro experiments, in which, after CD73 knock-down, cancer cell migration and expression of EMT-genes were reduced and A3R activation promoted HNSCC cell migration and presumably involving the EGFR signaling pathway [93]. In ovarian cancer cells, CD73 confers stemness and the expression of EMT-associated genes [94].

CD73 expression in hepatocellular carcinoma is correlated with a mesenchymal phenotype. CD73 activity was required for inducing mesenchymal characteristics. A2AR activation could restore the effect of knocking down CD73. These data suggest a synergist treatment with A2AR and CD73 inhibitors [95].

Activity of CD73 produces ADO and the potential activation of P1 receptors. Virtanen et al. in 2014 [96] demonstrated that ADO at low μM inhibited cell migration and invasion in prostate and breast cancer cell lines. However, the authors suggested that these effects were not mediated by the activation of P1 receptors, but by one intrinsic receptor-independent mechanism. However, the inhibitory effect of ADO in cell migration and invasion has also been proven in human cervical and ovarian cancer cell lines [89,97].

Despite the opposite effects regarding ADO modulation in cancer cell migration and invasion, it is necessary to consider the receptor involved and the type of cancer under study. For instance, pharmacological A1R inhibition reduces cell migration in renal cancer cell lines [98]. On the contrary, gastric cancer cell incubation with ADO enhances the expression of stemness and EMT genes, which is attributed to A2AR activation and the AKT-mTor pathway [99]. The effect of A2BR on EMT has been evaluated in human epithelial lung cancer cells; interestingly, two modulatory roles were described. The first consisted in a partial EMT induction through A2BR activation that involved the cAMP/PKA and MAPK/ERK transduction pathways. The second consisted in the ability of the selective A2BR agonist, BAY-606583, to counteract TFG-β–induced EMT [100]. These roles suggested that EMT maintenance/inhibition is based on the balance of extracellular environment signals. In agreement, in MDAMB231 cancer cells, ADO increased cells migration through the A2BR/AC/PKA/cAMP axis [66] and A2BR pharmacological inhibition decreased cell migration in human epithelial lung cancer cells and renal cancer cell lines [67,101]. Finally, A3R modulation in cell migration has been evaluated in AT6.1 rat prostate [102], MDA-MB-23 human breast [103], HepG2 hepatocellular and Caco3 colorectal

cancer cell lines [104]. These reports demonstrated that A3R stimulation arrested cancer cell motility, migration and invasiveness by hindering AC/PKA and reducing NADPH oxidase activity [102]. On the other hand, in primary cultures of glioblastoma (GBM) stem-like cells obtained from GBM patients, and also in a GBM cell line, A3R blockade promoted a reduction in cell migration and invasion associated with the expression of EMT genes [105].

Evaluating ATP cellular regulation and consumption during metastatic cell migration is essential, considering that ATP is the cell's biological energy currency. This has been elegantly achieved by Zanotelli and colleagues, who used genetically encoded fluorescent biomarkers to evaluate cancer cell migration in 3D matrices. They found that the ATP:ADP ratio was modulated in response to collagen architecture. This ratio increased in denser matrices where migration is impaired and decreased in aligned matrices where migration is facilitated. Therefore, the cellular energy requirement changes in response to the adhesion environment. It can be suggested, however, that increases of ATP in the TME could facilitate cancer metastasis [106]. Additionally, striking evidence has shown that ATP is required locally in invadopodia formation. It participates in F-actin network growth, even in the absence of MMPs [107]. These data indicate that ATP per se engages in the physiology of cell migration and metastasis.

2.4. Energy Metabolism in Cancerous Cells

Cancer is a pathology with multifaceted expression. Neoplastic conditions that cause tumor growth involve a variety of molecular, cellular and metabolic adequacies. Foremost among them are biochemical reactions, considered hallmarks of cancer, especially in the form in which cancerous cells display energy transformations in cytoplasmic and mitochondrial compartments. Almost 100 years ago, Nobel Laurate Otto H. Warburg described that carcinogenic cells obtain ATP preferably from glycolysis, regardless of the availability of oxygen and the suitability of mitochondrial oxidative phosphorylation [108]. This "aerobic glycolysis" or "Warburg effect," as it was rapidly known, was a widely spread metabolic feature in many tumor-derived cells and cancerous cell lines [109,110].

However, there is an accepted rationale for the Warburg effect in the biology of cancer. It has been more difficult to reach consensus regarding the metabolic mechanisms that sustain this neoplastic energy adaptation. Indeed, cancerous cells are systems specialized in cellular growth and duplication. The more undifferentiated and aggressive the cancer cells, the more prone they are to activate their cellular cycle and the metabolic pathways to synthesize biomolecules and build new genetic material and phospholipidic membranes [111]. Therefore, growing tumors and carcinogenic cells face a "metabolic dilemma"; that is, deciding what is more important in a replicating system: 1) cellular energy like ATP to enable the biosynthetic processes, or 2) the availability of biomolecules, such as reductive power (NADPH), fatty acids, amino acids, glycerol and sugars, to be used as structural elements for the synthesis of membrane and genetic material. In this context, understanding the implications of the complex metabolic adaptations associated with cancer is necessary to visualize successful therapeutic approaches [112].

Metabolic reprogramming in cancerous cells does is not just an imbalance between cytoplasmic glycolysis and mitochondrial oxidative metabolism. Genetic activation of glycolytic-promoting factors, such as c-Myc and HIF-1α (transcriptional factors), glucose transporters and glycolytic enzymes and regulators (hexokinase 2, pyruvate kinase M2, pyruvate dehydrogenase kinase isozyme 1 and lactate dehydrogenase A) underlie an enhanced glycolytic flux associated with aerobic glycolysis [113]. The immediate consequences of this enhanced glycolytic flux include increased glucose uptake with concomitant glycogen formation, as well as extra-cellular acidification connected to prominent lactate production.

Another metabolic flux that is activated in cancerous cells is the pentose phosphate pathway (PPP). When glucose is metabolized by the PPP, it promotes the synthesis of 5-carbon sugars used in the polymerization of nucleic acids, but most importantly, it favors the formation of the redox coenzyme NADPH. This cofactor is key for various anabolic pathways such as lipogenesis (β-reduction)

and isoprenoid/sterol synthesis; in addition, NADPH is necessary to maintain functional levels of the antioxidant glutathione in its reduced form (GSH) [114].

The increased glutamine metabolism that is characteristic of neoplastic cells is also part of the adaptations associated with the Warburg effect. In this case, glutaminase catalyzes the conversion of glutamine into glutamate. Glutamate, by action of the glutamate dehydrogenase located within the mitochondria, loses ammonium molecules and forms α-ketoglutarate. α-Ketoglutarate is an intermediate of the Krebs cycle, which acts as a redox substrate, to form NADH and supply oxaloacetate. Overall, glutamine is used by cancerous cells as an anaplerotic substrate by the coordinated action of cytoplasmic and mitochondrial enzymes [115].

Mitochondrial citrate is also crucial in cancer metabolism. Citrate is constantly leaving mitochondria to enter the cytoplasm and be converted into the lipogenic substrate acetyl-CoA by the activity of ATP-citrate lyase. Acetyl-CoA acts as a substrate for the formation of fatty acids, which are incorporated into phospholipids and triacylglycerols. Citrate exits the mitochondria, so the mitochondrial role of glutamine metabolism is relevant for aerobic glycolysis: the amino acid contributes as a carbon skeleton to supply the carbons lost by the exit of the mitochondrial citrate [116]. It has been reported that mitochondrial activities during carcinogenesis, such as ATP production, glutamine metabolism, fusion/fission balance and calcium dynamics, are regulated by the metabolic master regulator mTORC1 [117].

Some cellular populations display a Warburg-like effect in the metabolic adaptation, without being cancerous. For example, the functional unit glia-neuron in the nervous system. It has been reported that astrocytes are primarily glycolytic and effective lactate producers. Eventually, the lactate formed by the glia is taken up by the neuron, where it is oxidized as energy substrate. The glycolytic activity in astrocytes occurs whether they possess functional mitochondria and adequate oxygen availability or not [118,119].

Warburg Effect and Purinergic Signaling

Signal transduction by purinergic receptors has been little explored in the characterization of the Warburg effect and other metabolic adaptations in cancerous cells. Until March 24, 2020, from the total of entries in PubMed focused on the Warburg effect (2693), only 0.7% were related to purinergic signaling (20).

Among the purinergic signaling elements, P2X7R has been the most studied, in relation to the metabolic adaptations that occur in cancerous cells. More than 20 years ago, it was recognized that P2X7R promoted proliferative actions in lymphoid cells [120], in contrast to the pro-apoptotic and necrotic role previously designated to this cationic channel receptor [121]. Growth-promoting effects associated with elevated levels of extracellular ATP and P2X7R activation also involved MAPK/ERK kinases, by inducing de novo synthesis of pyrimidine nucleotides [122]. The pro-mitotic role of P2X7R was also recognized in B-cell chronic lymphocytic leukemia, one of the most common neoplastic diseases in the Western world. P2X7R expression was higher in patients suffering from an aggressive form of this cancer [123]. Tumor progression has also been related to the expression of P2X7R in prostate and breast cancer [33].

A seminal article demonstrated a direct role of P2X7R in the metabolic adaptations that underline the Warburg effect [124]. This group showed that in P2X7R-transfected HEK293 cells and the neuroblastoma cell line ACN, there was an increased lactate output associated with cell proliferation in the absence of serum, a hallmark of aerobic glycolysis. P2X7R action was accompanied by the upregulation of the following glycolytic promoters: glucose transporter Glut1, glyceraldehyde 3-phosphate dehydrogenase (G3PDH), phosphofructokinase (PFK), pyruvate kinase M2 (PKM2) and pyruvate dehydrogenase kinase 1 (PDHK1). Furthermore, P2X7R expression inhibited pyruvate dehydrogenase (PDH) activity, increased phosphorylated Akt/PKB and hypoxia-inducible factor 1a (HIF-1α) expression, and enhanced intracellular glycogen stores. These are all metabolic adjustments to avoid aerobic adaptations.

To accomplish the promotion of the Warburg effect and the proliferative effect independent of serum, P2X7R must reach higher levels of activation to function not just as an ion channel, but as a large conductance non-selective pore. Acting in this way, P2X7R is capable of mitochondrial stimulation by increasing the resting mitochondrial potential ($\Delta\Psi$) and the basal mitochondrial calcium [125].

The authors of [126] reported that human non-small cell lung cancer A549 showed the capacity to internalize the highly concentrated extracellular ATP by clathrin- and caveolae-mediated endocytosis, but mainly by macropinocytosis. The internalized ATP favored elevation of intracellular energy charge and promoted cancer growth, survival, and drug resistance, as well as the induction of EMT. More recently, these observations were extended to other neoplastic cell lines [71]. In the context of the Warburg effect, an interesting interpretation is that the metabolic role played by the internalized ATP serves as an energy supplement for the glycolytic ATP in cancerous cells. This phenomenon resulted only partially dependent on P2X7R [71].

It was demonstrated in prostate cancer cell lines that activation of the pro-inflammatory Toll-like receptor 3 stimulated the Warburg effect (glucose utilization and lactate production). This effect involved the intracellular participation of HIF-1α, and was synergized by the extracellular activation of A2BR [127].

The high extracellular ATP concentration characteristic of neoplastic cells is also related to the elevated presence of exADO, according to the expression and activity of various ectonucleotidases. In this context, the nucleoside ADO has been recognized as a pro-tumoral factor [128]. For example, in non-small cell cancer tissues and cancer-associated fibroblast, antagonists for A2AR (ZM241385 and SCH5826) inhibited cellular proliferation and the human tumor xenograft in mice [129].

P2XR is also involved in the regulation of metabolic responses and the Warburg effect. P2X1R and P2X7Rs were studied in leukemia T cells (Jurkat) showing that basal activation of both receptors increases the levels of intracellular calcium. Upon pharmacological inhibition of these receptors, Jurkat, THP-1, U-937 and HL-60 cells decrease mitochondrial activity, calcium signaling and cell proliferation. The authors concluded that the coordination of cytoplasmic and mitochondrial energy responses promotes autocrine purinergic signaling and the uncontrolled proliferation of leukemia cells [130].

2.5. Purines and Evasion of Immune Attack

Signaling through extracellular nucleotides by tumor cells is relevant for interactions with a host immune system. While exATP elicits a "find me" signal that promotes an innate and adaptive immune response by attracting immune cells. In the tumor context, this response is subverted, mainly by the sequential processing of exATP into ADO by action of the CD39 and CD73 ectonucleotidase pathway; ADO acts as an immunosuppressive molecule directing the phenotype of infiltrated immune cells in the TME dismantling the antitumor immune attack [24,30,131]. Thus, the purinergic molecular code defines the significance and outcome of the interaction between the tumor and the host immune system cells.

In the tissue damage context, cells release DAMPs in response to conditions as hypoxia, inflammation and necrosis. ATP is recognized as a DAMP, since exATP recruits neutrophils, macrophages and dendritic cells (DCs) to contribute to damage resolution [132–134]. In cancer, it has been described that the ATP released by dying cells because of anticancer therapies circulates through the TME to activate receptors in the membrane of tumor-infiltrated cells. When P2X7R is activated in DCs, IL-1β is secreted through the P2X7R-dependent assembly of the NLRP-3 inflammasome. IL-1β, a proinflammatory cytokine, induces the immunogenic response associated with $CD8^+$ T cells. Thus, anticancer therapy with oxaliplatin and anthracyclines, in $p2rx7^{-/-}$, $casp1^{-/-}$ or $nlrp3^{-/-}$ genetic background was inefficient; these evidences link NLRP-3 inflammasome activity with anticancer therapy treatment efficacy [135,136].

Moreover, the relevance of P2X7R expression in tumor-host interactions, specifically immune cell diversity of the TME, has been analyzed, by comparing the identity of immune cells and cytokine expression in the TME of tumors induced by xenotransplantation of murine B16 melanoma cells (a cell

line expressing high levels of P2X7R), in both null mice for P2X7R ($p2rx7^{-/-}$) and wild-type animals treated with a P2X7R antagonist (wt_{AT}). Tumor growth was accelerated in the $p2rx7^{-/-}$ background. The cells infiltrated in tumor-bearing $p2rx7^{-/-}$ contained an immunosuppressive microenvironment, compared to those growing in the wt_{AT} background, with fewer effector T cells (T_{eff}) ($CD8^+$ and $CD4^+$), increased T_{reg} cells ($CD4^+$, $CD25^+$, $Foxp3^+$) and a decline in cytotoxic effector $CD8^+$ T cells (T_{cyt}). Additionally, the TME was enriched in pro-inflammatory cytokines, such as IL-1β, IL-18 and IFN-γ. Regarding ectonucleotidases, CD73 was highly expressed, not only in immunosuppressive T_{reg}, but also in $CD8^+$ T_{eff} and macrophages; CD39 was also elevated in T_{eff}. These changes in CD39/CD73 expression produced a reduction in exATP levels in the TME and an increment in ADO production, causing a general immunosuppressive effect. Conversely, the pharmacological blockade of P2X7R in wt_{AT} mice, besides reducing tumor growth, promoted an anti-tumor immune infiltrated with incremented IFN-γ and reduced IL-1β, but without affecting the TME exATP [137]. These results showed that P2X7R expression in the host tissue contributed with an anti-tumor immune response, and confirmed that the deficiency of host P2X7R induces immune failure, suggesting that P2X7R plays a relevant role in the establishment of the immune response in the TME, thus integrating tumor-host interaction.

In contrast, it has been suggested that exATP contributes to immunosuppression in the TME. A study in acute myeloid leukemia showed that exATP, induced by chemotherapeutic agents, promoted the up-regulation of T_{reg} cells [138].

Taken together, this evidence indicates that ATP mediates the interaction of tumor cells with components of the immune system and modulates the inflammatory state in the TME. On the other hand, a common mechanism of purine actions in TME is the formation of ADO, which will be discussed next.

It has been established that hypoxia, aside from generating a protective environment for tumor cells, is the main cellular condition favoring ADO accumulation in the TME. Hypoxia increments the expression level of the ectonucleotidases CD39 and CD73 in a way that depends on HIF-1 transcription factor activity [20–23]. ADO inhibits T cell arrival in the tumor through the activation of its receptors, thus preventing these cells from producing their cytotoxic activity against cancer cells. It has been proven that the main P1 involved in immunosuppression is A2AR, since its genetic deletion facilitates tumor rejection by T cells [19]. These observations are supported by findings in which supplemental oxygenation (hyperoxia) facilitated tumor regression, enhanced tumor infiltration of $CD8^+$ T cells, reduced immunosuppression executed by regulatory T_{reg} and increased levels or pro-inflammatory cytokines and chemokines. These effects are accomplished through action on the hypoxia/adenosine/A2AR immunosuppressive pathway, because they were not replicated in $A2AR^{-/-}$ mice [22].

A2AR has been considered a target in anticancer immunotherapy. In a pharmacological intervention assay, the efficiency of reverting the immunosuppression of induced tumors with PD-1 antibodies (responsible for the immunological checkpoint [139]) improved, if an A2AR antagonist was co-administrated [140]. Another approach has consisted in evaluating the effect of A2AR deletion in cultured-activated tumor-draining lymph node (TDLN) T cells. In tissue lacking A2AR, tumor rejection improved, immunosuppression was diminished and the secretion of IFN-γ by T cells was enhanced [141]. Additionally, the ablation of ADO signaling promoted natural killer cell (NK) maturation and reduced tumor growth [142]. It has also been demonstrated in colorectal cancer cells that A2BR working synergically with A2AR, expressed in tumor-associated fibroblast, participated in the immune checkpoint dependent on NT5E/ADO to establish the immunosuppressive response characteristic of tumor cells [143].

Recently, it was reported that A1R deletion suppressed melanoma-derived cell growth and induced the inhibition of T cells in co-culture, antagonizing the anti-tumor immune response depending on another A2AR receptor, through a pathway involving overexpression of PD-L1 driven by the transcription factor ATF3 [144]. Taken together, these observations lead us to conclude that

actions of ARs are too complex and could be opposite in diverse physiological events. Therefore, they need to be considered specifically.

The immunosuppressive actions of ADO are orchestrated by tumor cells, but it has been proposed that this signaling can be amplified by influencing myeloid cell constituents of the TME, such as tumor-associated macrophages (TAMs). An interesting work has demonstrated that ADO generation by ovarian cancer cell lines attracts myeloid cells, inducing their differentiation in M2-TAM (macrophages with a non-inflammatory phenotype). Moreover, TAMs display an incremented expression of CD39 and stromal fibroblast (SF) for CD73; thus, TAMs and SF collaborate to amplify ADO formation and, consequently, the immunosuppressive effect [145].

In general, the actions of ADO inhibiting the anti-tumor immune response have been demonstrated in a broad group of host immune cells in the TME. An overview of these actions is presented in Table 1.

Table 1. Summary of ADO's actions on immune cells in the TME context.

Cell Type	Observations	References
T regulatory cells	CD39 and CD73 are markers of Foxp3$^+$ T_{reg} which express A2AR. A2AR activity induces cell proliferation and PD-1 expression, promoting an anergic state. ADO stimulation creates a feedback loop that maintain a constant number of CD4$^+$Foxp3$^+$ T_{reg} in tumors to inhibit antitumor response. Blocking of A2AR increases CD8$^+$ cells.	[146–148]
T effector cells	T_{eff} cells express CD73; its pharmacological inhibition with APCP induces increment of NFkB activity and IFNγ released by CD4$^+$ T-cells. Stimulation of A2AR induces: 1) a marked reduction in IL-1, 2, 3, 4, 12 and 13, TNFα, IFNγ, GM-CSF, CCL3 and CCL4. 2) a reduction of CD8$^+$ and CD4$^+$ expansion by inhibition of cell proliferation. 3) a decrement of cytotoxic activity of CD8$^+$ cells, and 4) T-cell apoptosis;	[149–153]
NK cells	ADO acting through A2AR, limits maturation of NK cells by suppressing cytotoxic activity and cytokine production. In NK cells positive to CD73, the expression of proteins related with immune check points as: LAG-3, VISTA, PD-1, and PD-L1 have higher expression; IL-10 is also up-regulated, producing inhibition of CD4$^+$ T cells proliferation and IFNγ production. ADO acting through A2AR inhibits the cytotoxicity of activated NK cells.	[142,154–156]
Myeloid cells	In macrophages ADO acting by A2AR inhibits M-CSF dependent proliferation and suppresses IL-12 and TNF-α production. By A2BR induces IL-10 synthesis. CD14$^+$ CD163$^+$ -TAM, from ovarian cancer, express incremented levels of CD39 that modulates their immunosupresive functions; ectoenzyme expression is modulated by IL-27. In TME ADO attract myeloid cells, induces their differentiation in M2 macrophages to favor immune evasion. In hematopoietic cells, A2BR induce accumulation of immunosupressive MDSC. A2BR activity alters DC differentiation and induces generation of cells. expressing suppressors of immune antitumor response.	[145,157–164]

Since ADO accumulation in the TME has deleterious effects on immune surveillance, the ectonucleotidases (CD39/CD73) involved in their synthesis are an obvious target to unleash the immune inhibition executed by ADO. Recently developed antibodies targeting these enzymes were used to promote antitumor immunity, by targeting ectonucleotidase expression in DCs, macrophages and T cells [165]. Previous research has demonstrated the effect of antibodies against CD39 and CD73 in the immune response against ovarian cancer cell lines; it was found that NK and T cell cytotoxicity was improved and the proliferation of CD4$^+$ T cells were uninhibited. These effects were achieved by a reduction in ADO synthesis [166]. Moreover, since focal radiotherapy induces overexpression of CD73 and, thus, an increment in ADO in the TME, blocking of CD73 has been assayed in combination with focal radiotherapy and immune checkpoint blockade (directed to cytotoxic T-lymphocyte-associated

protein 4, PDL-1 and PD-1 in breast cancer cells); in these assays, CD73 blocks improved DC infiltration and the induction of anti-tumor T cell-dependent responses [167].

An important mechanism of interaction between tumor cells and TME and host is exosome-dependent signaling. Exosomes are signalosomes assembled in small vesicles (30-100 μm) that are released by exocytosis and induce cellular responses in the target. In cancer, exosomes have been characterized as entities carrying elements to induce EMT and metastasis, such as TGF-β and HIF-1α, as well as immunosuppressive elements [168].

The expression of CD39 and CD73 in cancer exosomes (CSE) was demonstrated in bladder cancer cells [169]. The ability to dephosphorylate ATP to form ADO was documented in exosomes from bladder (HT1376 line), colon cancer (CaCo line) and in malignant effusions of mesothelioma patients. ADO promoted a negative regulation of T cells in the TME [170]. It was shown that exosomes from the prostate carcinoma cell line DU145, expressing CD39 and CD73, inhibited DC activities that resulted in an immunosuppressive environment [171]. Thus, exosomes represent an important piece in the interaction and modification of the environment by purines in tumor cells.

Research is increasingly aimed at understanding the mechanism that regulates CD39 and CD73 expression. CD73 expression is regulated by a net of cellular messengers, transcription factors (TF) and miRNA [172]. Recently, it was shown that the P30 isoform—but not the wild-type version of CEPBA TF, a protein frequently mutated in acute myeloid leukemia (AML)—interacts with the promoter region of the NT5E gene in AML, to induce its expression and mediate AML progression via the NT5E-A2AR pathway [173]. In hepatic stellate cells, it has been shown that SMAD2, SMAD3, SMAD4 and SMAD5 and SP1 TF bind the CD73 gene promoter [174], demonstrating that TGF-β is a regulator of CD73 expression [175]. Additionally, it has been observed that HIF-1 binds the NT5E gene promoter, confirming that hypoxia is a strong regulator of immune checkpoints dependent on ADO [20]. In a model of induction and reversion of EMT in hepatocellular carcinoma, TNF-⟨ induced overexpression of the NT5E gene and reversion of EMT downregulation [176]. In agreement, a bioinformatics analysis using the gene Signature Finder Algorithm (gSFA) found in colorectal cancer that NT5E belongs to the gene signature of this disease, and that it is a transcriptional target of TNF-⟨ [177]. In Th17 cells, differentiated in vitro by a combination of IL-6 and TGF-®, IL-6 through Stat3 positively regulated NT5E expression, while TGF-β through Gfi-1 repressed its expression, but the cells displayed an immunosuppressive phenotype [178]. Moreover, the processing of RNAs coding for NT5E is regulated by a miRNA group, the presence of this miRNA contributes to the role of CD73 in cancer [170].

3. Concluding Remarks

Cancer is a complex disease; intense efforts have been made to understand and systematize the general principles underlying cancer cells identity and tumor-host interactions, to decipher how tumor cells self-regulate their differentiation, growth and expansion. For that, cancer hallmarks involve an important frame of reference, encompassing those characteristics that made cancer cells biologically successful [7,8]. In this review, we organized existing evidence showing that purinergic signaling is an important modulator in the acquisition and maintenance of cancer cell phenotypes, the establishment of their social interactions and bidirectional relationship with the environment.

From the accumulate data, it is deduced that purinergic system in TME impacts tumor biology in two main ways: (1) exerting autocrine-paracrine actions over the own tumor cells, to establish a feedback loop that integrate energy metabolism with cellular tasks, such as cell proliferation, migration and metastatic induction; and (2) regulating the cellular interactions with the host, mainly by mediating a dialog with the immune system, to avoid a correct immunological response. Thus, purinergic signaling could be considered a master regulator of tumor cells identity and collective cellular properties.

A detailed knowledge of purinergic signaling elements and its mechanistic processes in the distinct level of cellular interactions in cancerous cells will be necessary to open new avenues in the search of therapeutic targets against carcinogenesis.

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Adenosine Receptor A2B Negatively Regulates Cell Migration in Ovarian Carcinoma Cells

Anaí del Rocío Campos-Contreras, Adriana González-Gallardo, Mauricio Díaz-Muñoz and Francisco G. Vázquez-Cuevas *

Departamento de Neurobiología Celular y Molecular, Instituto de Neurobiología, Campus UNAM-Juriquilla, Querétaro 76230, CP, Mexico; anaicampos.c@gmail.com (A.d.R.C.-C.); gallardog@unam.mx (A.G.-G.); mdiaz@comunidad.unam.mx (M.D.-M.)
* Correspondence: fvazquez@comunidad.unam.mx

Abstract: The purinergic system is fundamental in the tumor microenvironment, since it regulates tumor cell interactions with the immune system, as well as growth and differentiation in autocrine-paracrine responses. Here, we investigated the role of the adenosine A2B receptor (A2BR) in ovarian carcinoma-derived cells' (OCDC) properties. From public databases, we documented that high A2BR expression is associated with a better prognostic outcome in ovarian cancer patients. In vitro experiments were performed on SKOV-3 cell line to understand how A2BR regulates the carcinoma cell phenotype associated with cell migration. RT-PCR and Western blotting revealed that the *ADORA2B* transcript (coding for A2BR) and A2BR were expressed in SKOV-3 cells. Stimulation with BAY-606583, an A2BR agonist, induced ERK1/2 phosphorylation, which was abolished by the antagonist PSB-603. Pharmacological activation of A2BR reduced cell migration and actin stress fibers; in agreement, A2BR knockdown increased migration and enhanced actin stress fiber expression. Furthermore, the expression of E-cadherin, an epithelial marker, increased in BAY-606583-treated cells. Finally, cDNA microarrays revealed the pathways mediating the effects of A2BR activation on SKOV-3 cells. Our results showed that A2BR contributed to maintaining an epithelial-like phenotype in OCDC and highlighted this purinergic receptor as a potential biomarker.

Keywords: purinergic signaling; A2B receptor; ovarian cancer; cancer cell migration; SKOV-3 cells

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1. Introduction

Extracellular adenosine (ADO) is originated from the ectonucleotidase-mediated hydrolysis of extracellular ATP released by tumor cells into the tumor microenvironment (TME), mainly by the CD39-CD73 pathway [1]. It is well established that ATP concentration in the TME is significantly higher than in healthy tissue [2]; moreover, in some anticancer therapies, ATP released from dying tumor cells contributes to maintaining an elevated concentration of this nucleotide in the TME [3]. Correspondingly, ADO increases in cancer conditions [4]. In the TME, ADO acts as a mediator in the interaction among host immunological cells to induce tumor-immune evasion [5,6] and displays autocrine-paracrine actions to regulate cellular processes such as proliferation, migration and differentiation in tumor cells [7–11].

ADO exerts its actions through P1 receptors, which belong to the G protein-coupled receptor (GPCR) superfamily. The P1 receptor family has four members: A1R, A2AR, A2BR and A3R. In general, A1R and A3R receptors are coupled mainly to Gi proteins, whereas A2AR and A2BR are coupled to Gs proteins [12]. In addition, A2BR regulates various effectors through different pathways; that is, mitogen-activated protein kinases p38 and ERK [13], and is also coupled to Gq proteins to promote PLC-IP_3-Ca^{2+} pathway activity [14].

The present work sought to characterize the autocrine–paracrine actions mediated by A2BR in SKOV-3 OCDC. In addition, A2BR expression has been demonstrated in biopsies

from hepatocellular [15], colorectal [16] and bladder urothelial [17] carcinoma. A2BR activity promoted cell proliferation in breast cancer cells [18].

An important feature of cancer cells is their ability to migrate and invade secondary organs. To dissociate from primary tumors and survive in peritoneal fluid, cells go through epithelial–mesenchymal transition (EMT), switching from an epithelial to a mesenchymal phenotype [19]. Purines are active modulators of EMT induction in cancer [20].

ADO/A2BR, specifically, has been demonstrated to regulate cell migration in several tissues; thus, A2BR activation increases cell migration in MDA-MB-231 breast cancer line [18]. Accordingly, pharmacological inhibition of A2BR decreases cell migration in urothelial and renal cancer cell lines [17,21]. On the other hand, in human cervical cancer cells, extracellular ADO inhibits migration and invasion [22], and in epithelial lung cancer cells, A2BR activity counteracts the TFG-β–dependent EMT induction [23]. In SKOV-3 cells, addition of apyrase (Apy), which hydrolyzes extracellular ATP generating ADO, was shown to decrease cell migration and favor an epithelial-like phenotype due to the relocation of E-cadherin to cellular junctions, probably through A2BR [24]. In the present study, we analyzed this possibility.

2. Results

2.1. ADORA2B Expression Is Related to the Surveillance Probability of Ovarian Carcinoma Patients and Is Specific to the Tumor Type

We analyzed if the expression level of the *ADORA2B* transcript (coding for A2BR) correlated with the surveillance probability of ovarian carcinoma patients using the Kaplan–Meier Plotter database (K-MPdb) [25,26]. K-MPdb contains the transcript expression level data collected from different transcriptomic studies, in correlation with the surveillance of 1435 ovarian cancer patients.

The first analysis correlated the expression level of the *ADORA2B* transcript with the survival rate of ovarian cancer patients (996 of low expression and 439 of high expression) (Figure 1A). We observed that the survival was different between both groups of patients: the reduced expression of the transcript showed a lower probability of survival (18.23 months), whereas the higher expression showed an increased survival (25.10 months, $p = 0.00002$). Then, we made the K-MPdb analysis according to the type of carcinoma: for the endometrioid subtype, no differences were detected between 15 patients with low expression and 36 patients with high expression of *ADORA2B* ($p = 0.19$, data not shown). In contrast, for serous carcinoma, the surveillance of both cohorts (482 with low expression vs. 622 with high expression) revealed that the group with reduced *ADORA2B* had a worse prognosis than the group with high *ADORA2B* levels (15.80 months vs. 18.60 months, respectively) ($p = 0.015$) (Figure 1B).

To know if the tumor stage in serous carcinoma was relevant for the direct relation between *ADORA2B* expression level and patient survival, K-MPdb analysis was also performed considering tumor stages (1 + 2 or 3 + 4). Interestingly, for patients in stages 1 + 2 (50 with low expression vs. 49 with high expression levels) notable differences were observed (16.0 months of average surveillance for the low expression cohort vs. 35.20 months for the high expression cohort, $p = 0.0075$) (Figure 1C). For patients in stages 3 + 4, no differences were detected (396 showing low expression vs. 605 showing high expression, $p = 0.19$) (Figure 1D). Accordingly, K-MPdb analysis was performed considering the grade of tumor. For grade 1 and 2, the group of patients with high expression of *ADORA2B* shown a major survival probability than those with lower expression level ($p = 0.03$ and $p = 0.05$ for grades 1 and 2, respectively); for grade 3, differences were not observed ($p = 0.06$); for grade 4, it was not possible to perform the analysis due to an insufficient number of patients (Supplementary Figure S2).

These data strongly suggested that low *ADORA2B* expression is a bad prognosis factor for serous ovarian carcinoma.

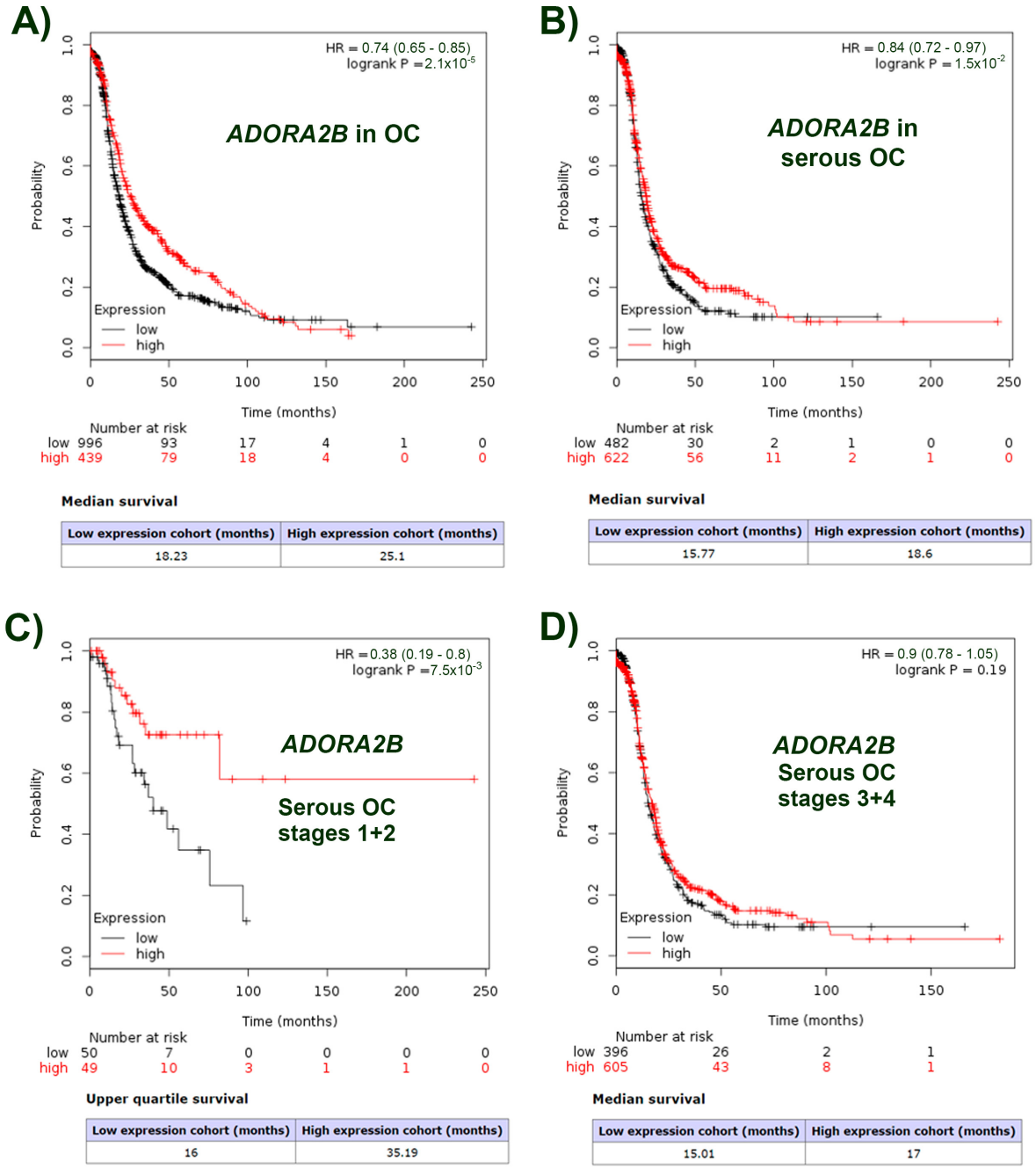


Figure 1. Relation between *ADORA2B* transcript (coding for A2BR) and survival time for patients with OC. Kaplan–Meier plots were constructed for the *ADORA2B* transcript using the Kaplan–Meier plotter database (KMPdb) for (**A**) Ovarian carcinoma in general carcinoma patients (996 low expression and 439 high expression); (**B**) Serous ovarian carcinoma patients (482 low expression and 622 high expression); (**C**) Serous ovarian carcinoma patients in stages 1 + 2 (50 low expression and 49 high expression) and (**D**) Serous ovarian carcinoma patients in stages 3 + 4 (396 low expression and 605 high expression).

2.2. SKOV-3 Cell Line Expresses a Functional A2BR

To analyze the cellular effects elicited by A2BR activity in ovarian carcinoma cells, we used the SKOV-3 cell line. Although, in previous work, our research group and others reported A2BR expression in this cell line, we confirmed this observation by RT-PCR and Western blotting of biotinylated membrane proteins. For RT-PCR, an expected band of 161 bp was obtained (Figure 2A, left panel). The amplicon was purified and sequenced; then,

the sequence was analyzed in the BLAST platform (NIH-USA). The amplicon was identified with the entry NM_000676, corresponding to the *Homo sapiens ADORA2B* transcript (coding for A2BR). Glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) transcript was used as a constitutive control (Figure 2A, central panel).

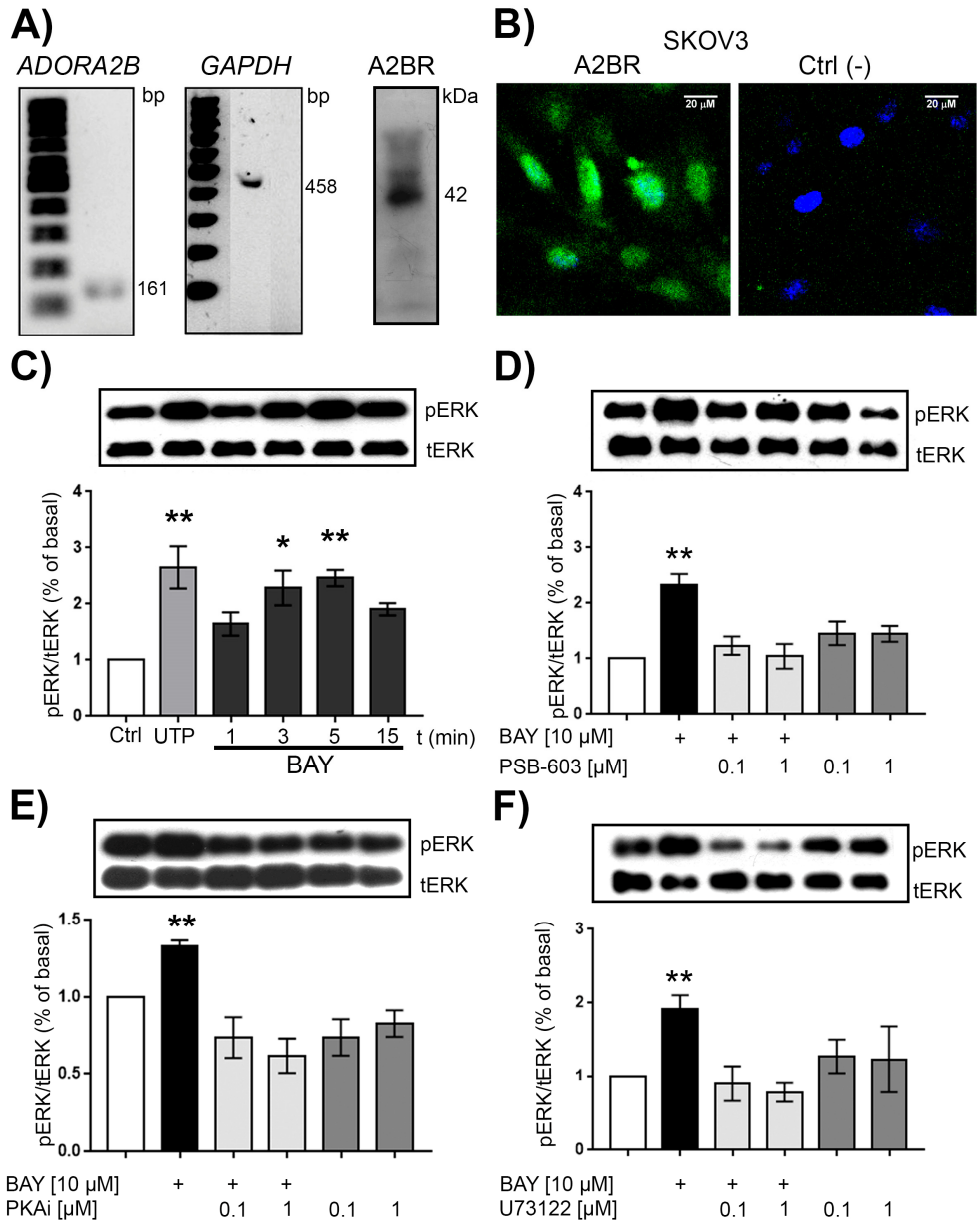


Figure 2. Ovarian carcinoma-derived SKOV-3 cells express functional A2BR. (**A**) A fragment of the *ADORA2B* transcript was amplified by RT and PCR using specific oligonucleotides. An amplicon of 161 bp was obtained. *GAPDH* was used as a constitutive transcript (**left panels**). A2BR was detected by Western blot and immunoprecipitation of biotinylated membrane proteins from SKOV-3 cells; a main band of around 42 kDa was detected (**right panel**). (**B**) A2BR was labeled by immunofluorescence. The immunoreactivity was detected with a secondary antibody conjugated with AlexaFluor 488 (green signal). Nuclei were counterstained with DAPI (blue signal). (**C**) Cell cultures were stimulated with 10 µM of BAY-606583 for 1, 3, 5 or 15 min. Phosphorylated ERK (p-ERK) and total ERK (t-ERK) were detected by Western blot in the same membrane. As a positive control, UTP 100 µM was utilized. (**D**) ERK phosphorylation induced after 5 min of stimulation with BAY-606583 was prevented by the A2BR antagonist PSB-603; this effect was also inhibited by a PKA inhibitor (**E**) or by U73122, a PLC inhibitor (**F**). In the graphs, bars represent the mean ± S.E.M. of four independent experiments in duplicate. * $p < 0.05$, ** $p < 0.01$.

Membrane proteins from SKOV-3 cells were biotinylated and isolated by immunoprecipitation with streptavidin-conjugated beads. The precipitates were analyzed by Western blot using an antibody directed against an extracellular epitope of A2BR as described in Methods. A main band of around 42 kDa was identified (Figure 2A, right panel) in accordance with the electrophoretic migration described by others [27]. Furthermore, A2BR immunostaining depicted that the receptor was distributed across the cell surface (Figure 2B). These results confirmed that A2BR is a membrane protein expressed in SKOV-3 cells.

It is known that extracellular signal-regulated kinases (ERK) are A2BR effectors [28]. To test A2BR functionality, SKOV-3 cells were stimulated with 10 μM of BAY-606583 for different time intervals between 1 and 15 min, and ERK phosphorylation was evaluated by Western blot. The stimulus induced an increment in ERK phosphorylation that reached its maximum level after 5 min (2.40 ± 0.14% of basal) (Figure 2C). To support that this response was mediated by A2BR, cultures were preincubated (20 min) with 100 nM and 1 μM of the A2BR antagonist PSB-603 (PSB) before applying the BAY-606583 stimulus. PSB inhibited ERK phosphorylation induced by the agonist at both concentrations (1.30 ± 0.17% of basal at 100 nM and 0.90 ± 0.22% of basal at 1 μM) (Figure 2D).

It has been described that *ADORA2B* can couple both Gαs and Gαq proteins; to explore the pathway driving the A2BR-dependent ERK phosphorylation in SKOV-3 cells, we analyzed the effects of blocking the effectors of each pathway (PKA for Gαs and PLC for Gαq pathways). PKA was inhibited with the PKA inhibitor fragment 14–22 myristoylated (PKAi), whereas U73122 inhibited PLC. Both inhibitors were used at 100 nM and 1 μM. PKAi abolished the BAY-606583 (10 μM)-induced ERK phosphorylation (0.74 ± 0.13% and 0.62 ± 0.11% of basal at 100 nM or 1 μM of the inhibitor, respectively; Figure 2E). U73122 also inhibited ERK phosphorylation induced by BAY-606583 (0.90 ± 0.12% and 0.78 ± 0.13% of basal at 100 nM or 1 μM of the inhibitor, respectively, Figure 2F).

2.3. A2BR Stimulation Inhibited Cell Migration without Modifying Cell Proliferation

A central aim of this project was to evaluate the effects of A2BR activity on the phenotype of ovarian carcinoma-derived cells; thus, cell viability and cell migration were analyzed after pharmacological stimulation of this receptor. SKOV-3 cells were stimulated for 24 h with increased concentrations (from 10 nM to 10 μM) of BAY-606583 in serum-free media. We used 10% fetal bovine serum (FBS) as a positive control for the experiment; after the stimulus, cell viability was evaluated applying the MTS method. FBS induced an increment in cell viability (132.70 ± 2.40% of control, $p < 0.05$), whereas BAY-606583 did not induce changes at any of the evaluated concentrations (Figure 3A).

To test if A2BR regulates cell migration, SKOV-3 cells were stimulated with 100 nM, 1 μM and 10 μM of BAY-606583 and migration was estimated by scratch assay after 16 h. Based on a previous report, we used UTP as a positive control for cell migration and Apy to inhibit cell migration [29]. UTP promoted SKOV-3 cell migration (108.1 ± 3.0) and Apy reduced this parameter (71.5 ± 1.6% of control, $p < 0.05$; Figure 3B). Interestingly, BAY-606583 100 nM, 1 μM and 10 μM also reduced cell migration (88.7 ± 3.5%, 83.3 ± 2.0% and 75.7 ± 1.6%, respectively, $p < 0.05$) (Figure 3B).

Furthermore, to observe stress fibers (SF), which indicate a mesenchymal-like phenotype, the actin cytoskeleton was labeled with phalloidin coupled to rhodamine. As controls, UTP increased the SF, whereas Apy treatment notably reduced them. In addition, the presence of SF in SKOV-3 cells treated with BAY was less evident than in control cells (Figure 3C, red signal).

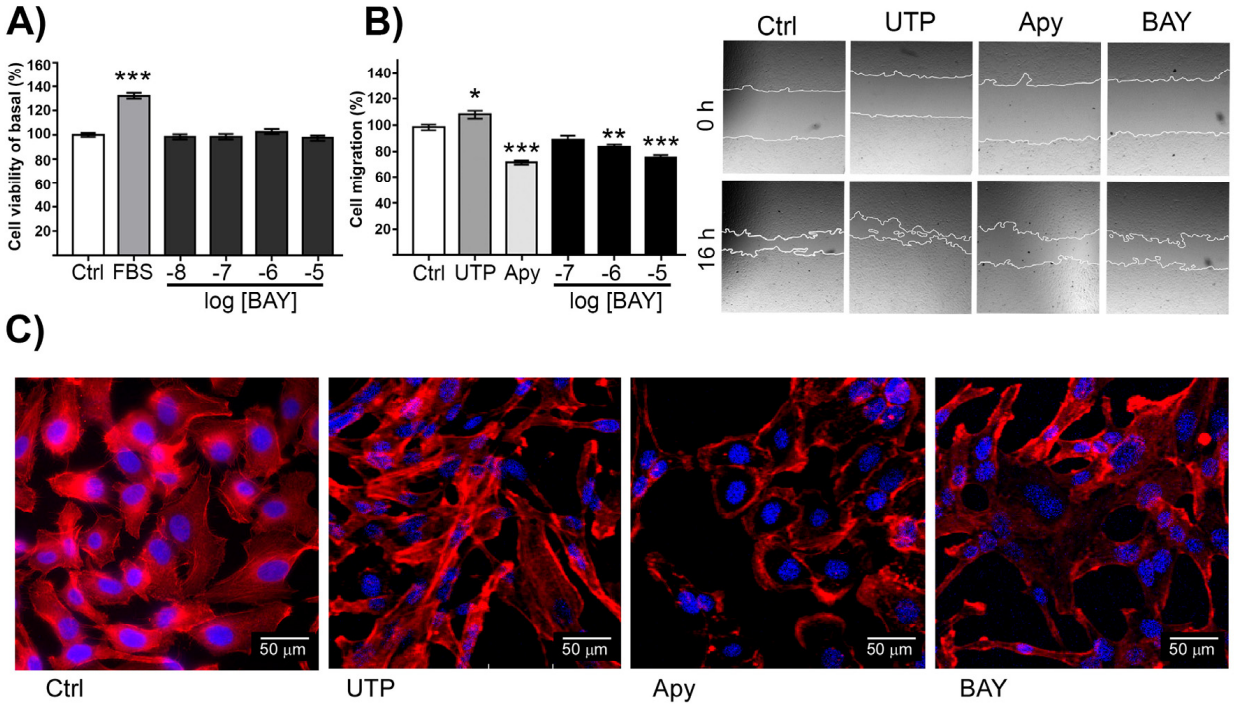
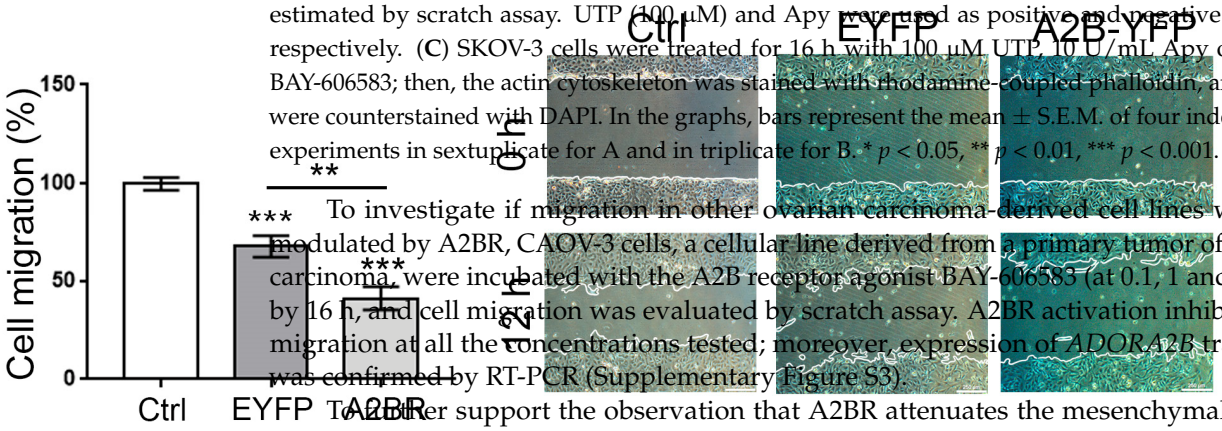


Figure 3. Stimulation of A2BR inhibits basal SKOV-3 cell migration but has no effect on cell viability. (**A**) Cultures of SKOV-3 cells at 50% confluence were stimulated for 24 h with BAY-606583 from 10 nM to 10 µM; then, cell viability was estimated by MTS assay. (**B**) Confluent cultures of SKOV-3 cells were stimulated for 16 h with BAY-606583 from 100 nM to 10 µM, and cell migration was estimated by scratch assay. UTP (100 µM) and Apy were used as positive and negative controls, respectively. (**C**) SKOV-3 cells were treated for 16 h with 100 µM UTP, 10 U/mL Apy or 10 µM BAY-606583; then, the actin cytoskeleton was stained with rhodamine-coupled phalloidin, and nuclei were counterstained with DAPI. In the graphs, bars represent the mean ± S.E.M. of four independent experiments in sextuplicate for A and in triplicate for B. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



To further support the observation that A2BR attenuates the mesenchymal characteristics of SKOV-3 cells and their concomitant cell migration capacity, its expression was modified in two ways: (1) by overexpression and (2) by knockdown with shRNAs. A2BR overexpression induced an inhibition of cell migration (41.24 ± 5.96%, Figure 4), whereas A2BR knockdown with three different shRNAs resulted in enhanced cell migration (135.80 ± 3.72, 138.40 ± 9.62 and 139.20 ± 4.36 % of control for shRNAs 1, 2 and 3, respectively, $p < 0.05$%) (Figure 5A,B). Visualization of the actin cytoskeleton by phalloidin-rhodamine staining revealed that the knockdown of the *ADORA2B* transcript induced an increment in the presence of SF (Figure 5C). Taken together, the evidence suggests that A2BR was not involved in cell proliferation, but it negatively regulated cell migration.

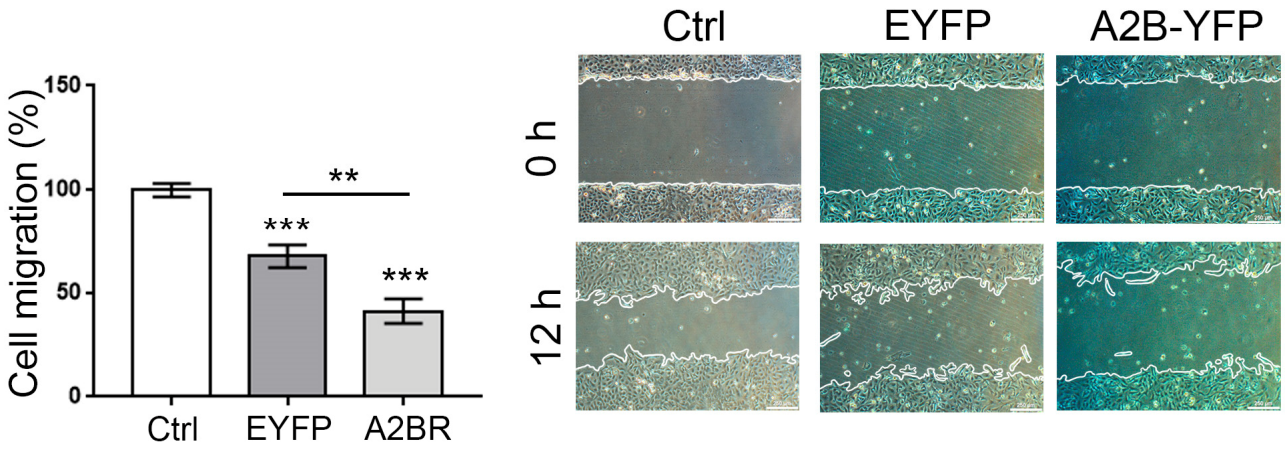


Figure 4. Overexpression of A2BR inhibits basal migration of SKOV-3 cells. SKOV-3 cells were transfected with a plasmid coding for A2BR fused with yellow fluorescent protein (YFP) at the carboxy-end; another plasmid coding only for YFP was used as a control. After transfection, cells were cultured to reach confluence (48 h) and a scratch assay was performed. Pictures show the wound at the time it was made and 16 h after. In the graphs, bars represent the mean ± S.E.M. of three independent experiments. ** $p < 0.05$, *** $p < 0.01$. Scale bar = 250 µm.

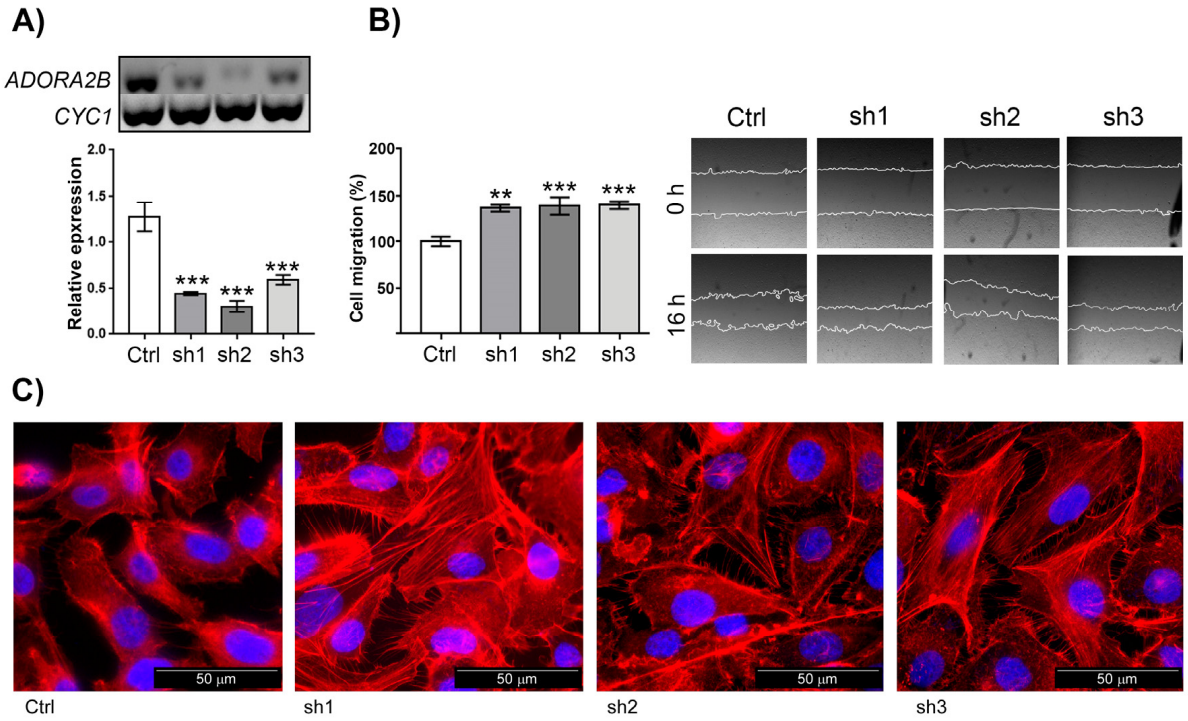
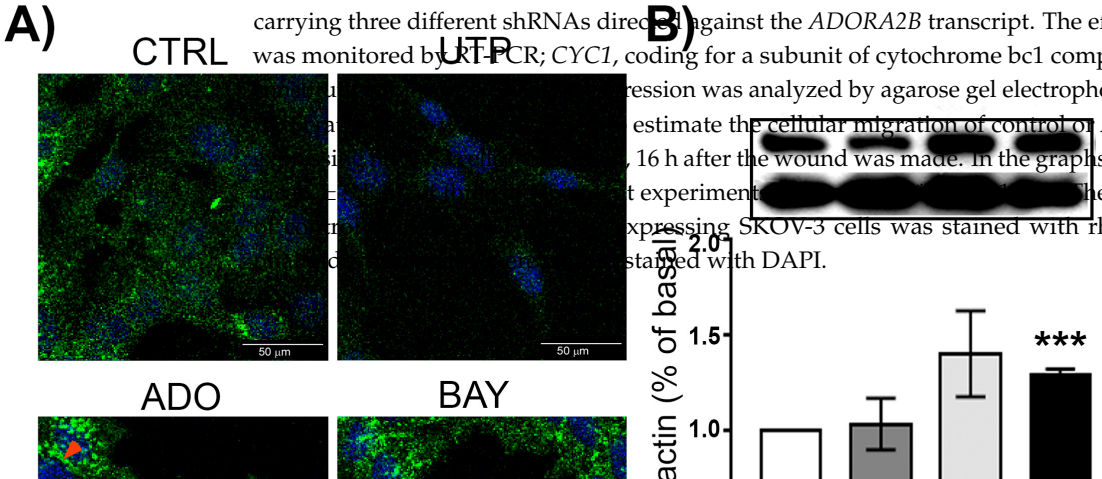
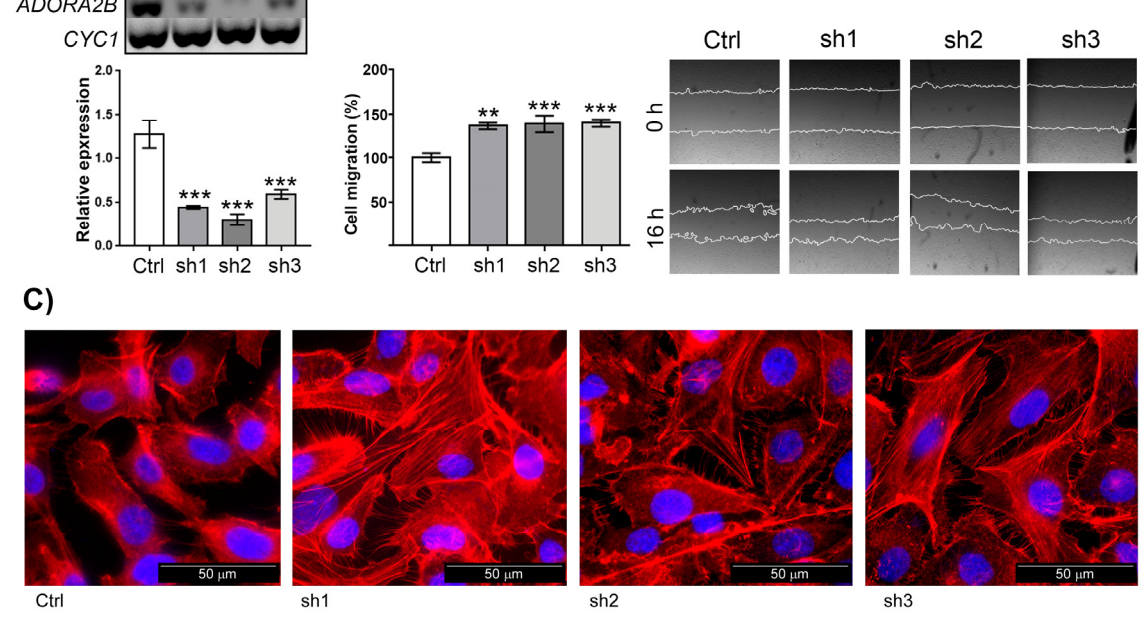


Figure 5. Knockdown of the *ADORA2B* transcript induced an increment in basal cell migration and in stress fiber formation. (**A**) Cultures of SKOV-3 cells were transduced with lentiviral particles carrying three different shRNAs direc[illegible] against the *ADORA2B* transcript. The effect of knockdown was monitored by RT-PCR; *CYC1*, coding for a subunit of cytochrome bc1 complex, was used as a [illegible]ression was analyzed by agarose gel electrophoresis densitometry. [illegible] estimate the cellular migration of control or *ADORA2B*-shRNA [illegible], 16 h after the wound was made. In the graphs, bars represent the [illegible]t experiment[illegible]. The actin cytoskeleton [illegible]xpressing SKOV-3 cells was stained with rhodamine-coupled [illegible] stained with DAPI.





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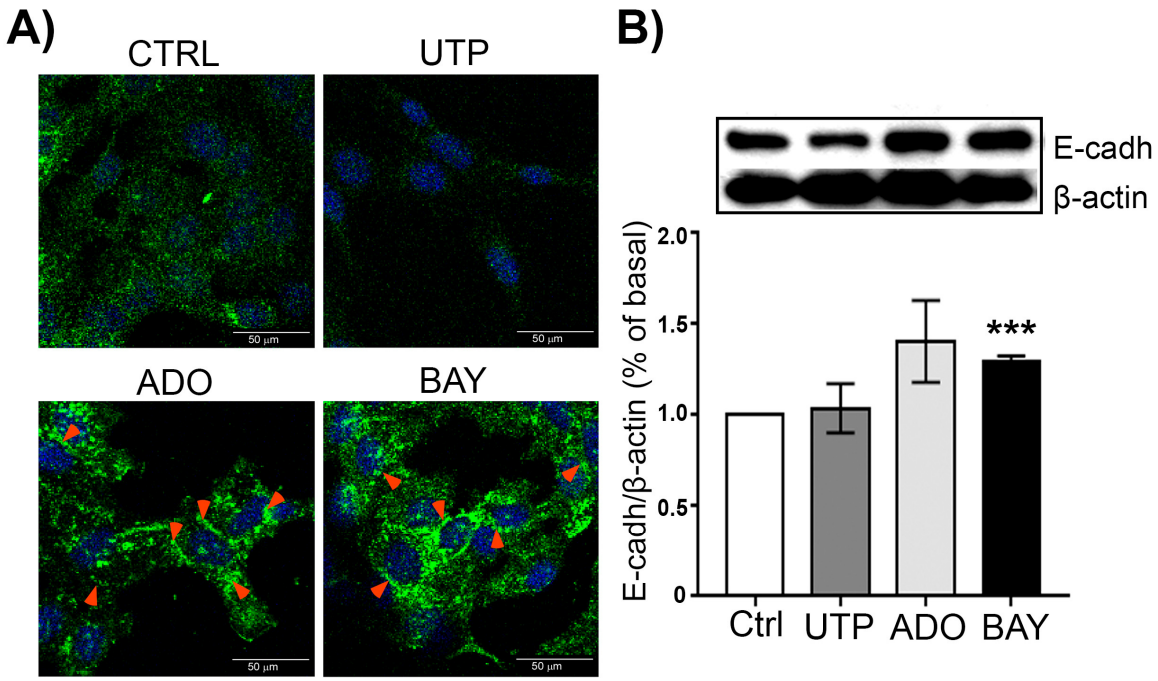

Figure 6. Stimulation of A2BR with BAY-606583 incremented the expression and induced the relocation of E-cadherin. SKOV-3 cell cultures were stimulated for 16 h with 10 µM BAY-606583, 10 µM ADO or 100 µM UTP. E-cadherin was detected by immunofluorescence (**A**) using a specific primary antibody and a secondary one coupled to AlexaFluor 488 (green signal). Nuclei were counterstained with DAPI (blue signal) and Western blot (**B**), where the abundance of E-cadherin was expressed in relation with against β-actin as housekeeping protein. In the graph, bars represent the mean ± S.E.M. of three independent experiments, *** $p < 0.01$ vs. Ctrl.

2.5. Gene Expression Mediated by A2BR Activation in SKOV-3 Cells

To analyze the transcriptional regulation promoted by A2BR activation, and its relationship with cell migration, we stimulated SKOV-3 cells with BAY-606583 10 µM for 24 h; then, the total RNA was isolated, labeled and hybridized with a 35k library from the whole human genome.

We observed that A2BR activity induced substantial changes in the gene expression profile of SKOV-3 cells: from the 884 modified transcripts, 450 were down-regulated and 434 up-regulated (Figure 7A).

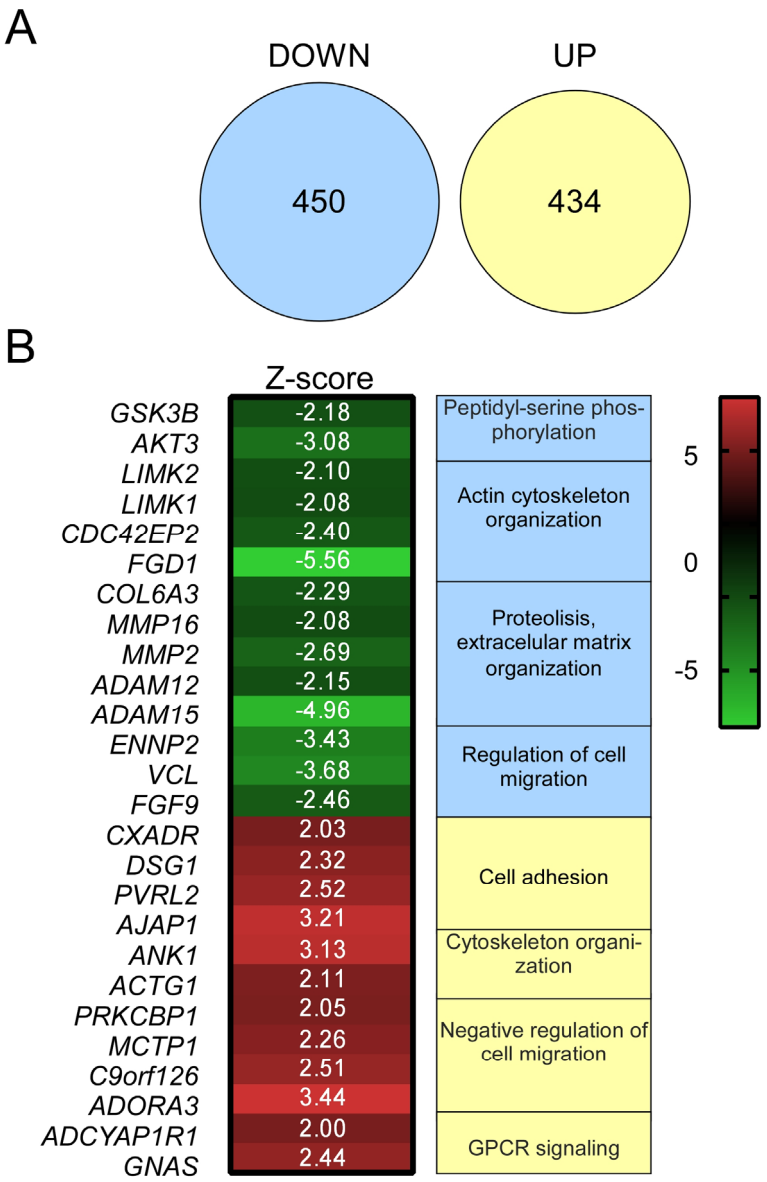

Figure 7. Regulation of gene expression patterns by A2BR activation with BAY-606583 in SKOV-3 cells was analyzed by cDNA microarrays. SKOV-3 cell cultures were stimulated with 10 μM BAY-606583 for 16 h and total RNA was isolated; a pool of five independent cultures was hybridized against a library of 30 K genes of the complete human genome. (**A**) BAY-606583 induced broad changes in gene expression; the Venn diagram represents the set of transcripts up- or down-regulated by the agonist. (**B**) A heat-map representation of the Z-score of outstanding transcripts up- or down-regulated by BAY-606583. The cellular process determined by GO analysis is shown.

Analysis of the down-regulated genes revealed transcripts grouped in the following categories: *Regulation of Cell Migration*, *Proteolysis and Extracellular Matrix Organization* and *Peptidyl-Serine Phosphorylation* (Figure 7B). Among the most interesting down-regulated transcripts, it was observed *FGF9*, *AKT3*, *GSK3b* and the metalloproteinases *ADAM12*, *MMP2* and *MMP16*, all related with cell invasiveness (Table 1). On the other hand, GO analysis showed up-regulation of transcripts in the categories *Negative Regulation of Cell Migration*, *Cytoskeleton Organization* and *Cell Adhesion* (Figure 7B). Some relevant transcripts up-regulated were *C9orf126* (*SCAI*), *PRKCBP1* (*ZMYND8*) and *PVRL2* (Nectin 2), related with negative regulation of cell migration (Table 1). More detail is developed in the Discussion section.

Table 1. Transcripts regulated by A2BR stimulation with 10 mM of BAY for 24 h.

Down-Regulated Transcripts			
Gene symbol	Gene name	Description	Z score
AKT3	AKT serine/threonine kinase 3	Peptidyl-serine phosphorylation	−3.08
GSK3B	Glycogen synthase kinase 3 beta		−2.18
FGF9	Fibroblast Growth Factor 9	Regulation of cell migration	−2.46
VCL	Vinculin		−3.68
ENNP2	Ectonucleotide pyrophosphatase/phosphodiesterase 2		−3.43
ADAM15	ADAM Metallopeptidase Domain 15	Proteolysis, extracellular matrix reorganization	−4.96
ADAM12	ADAM Metallopeptidase Domain 12		−2.15
MMP2	Matrix Metallopeptidase 2		−2.69
MMP16	Matrix Metallopeptidase 16		−2.08
COL6A3	Collagen, Type VI, Alpha 3		−2.29
FGD1	FYVE, RhoGEF and PH domain containing 1	Actin cytoskeleton organization	−5.56
CDC42EP2	CDC42 effector protein 2		−2.40
LIMK1	LIM domain kinase 1		−2.08
LIMK2	LIM domain kinase 2		−2.10
GNAI2	Guanine Nucleotide-Binding Protein G(I) Subunit Alpha-2	G protein-coupled receptor signaling pathway	−2.50
PDE3B	Cyclic GMP-Inhibited Phosphodiesterase B		−4.49
PDE9A	Phosphodiesterase 9A		−2.99
Up-Regulated Transcripts			
Gene symbol	Gene name	Description	Z score
GNAS	Adenylate Cyclase-Stimulating G Alpha Protein	G protein-coupled receptor signaling pathway	2.44
ADCYAP1R1	ADCYAP receptor type I		2.00
ARHGEF7	Rho Guanine Nucleotide Exchange Factor 7		2.20
PFN1	Profilin 1	Actin cytoskeleton organization	6.10
CDC42BPA	CDC42 Binding Protein Kinase Alpha		2.31
ADORA3	Adenosine A3 Receptor	Negative regulation of cell migration	3.44
C9orf126	Suppressor Of Cancer Cell Invasion		2.51
MCTP1	Multiple C2 And Transmembrane Domain Containing 1		2.26
PRKCBP1	Protein Kinase C Beta	Cytoskeleton organization	2.05
ACTG1	Actin Gamma 1		2.11
ANK1	Ankyrin 1		3.13
AJAP1	Adherents Junctions Associated Protein 1	Cell adhesion	3.21
PVRL2	Nectin Cell Adhesion Molecule 2		2.52
DSG1	Desmoglein 1		2.32
CXADR	CXADR Ig-Like Cell Adhesion Molecule		2.03

3. Discussion

Ovarian carcinoma is one of the most lethal gynecologic malignancies. Most patients are diagnosed in advanced stages, explaining in part its high mortality rate. Therefore, it is important to study the mechanisms that enable ovarian cancer cells to become metastatic. ATP and ADO are major components of the TME [2,30]. Both molecules have proved to modulate cancer cell migration and EMT by acting through purinergic receptors [20]. In previous works, we have shown that ADO and NECA, an agonist of A2 receptor with preference to A2BR, favors an epithelial phenotype and decreases SKOV-3 cell migration [24]. Moreover, A2BR is the most expressed ADO receptor [24], suggesting that it plays a key role in the modulation of ovarian cancer cell migration.

By using public K-MPdb (ovarian cancer) we detected a positive correlation between higher expression of A2BR and the survival rate of patients (Figure 1A and Supplementary Figure S2). This correlation was observed in serous ovarian carcinoma (Figure 1B); in stages 1 + 2, when the tumor is yet confined to the primary tumor, but not in stages 3 + 4, when cancer showed dissemination (Figure 1C,D); in agreement, the same correlation was observed in patients with serous ovarian carcinoma grade 1 and 2, when tumor cells are not yet

undifferentiated, but not in grade 3 (Supplementary Figure S2). Thus, high expression of *ADORA2B* at early stages of ovarian cancer could be associated with good prognosis. However, it is pertinent to mention that we were only able to perform the analysis for serous subtype of OvCa because this was the only pathological subtype with the necessary number of patients in the database.

An explorative analysis of K-MPdb in other cancers (supplementary Figure S1), showed that for lung carcinoma a high expression level of *ADORA2B* transcript is associated with bad prognosis, in opposition with ovarian carcinoma; while for breast and gastric carcinoma, no differences between low or high level of the transcript were observed, suggesting that the contribution of A2B receptor to patient survival is tissue-specific and cancer subtype-specific. These observations justify the characterization of A2B receptor in particular cell lines. Hence, to understand if the autocrine-paracrine activation of A2BR contributes to ovarian cancer-derived cell physiology, we used SKOV-3 cells.

To analyze the possible role of A2B receptor in cell migration, first, A2BR expression and functionality was confirmed by the following evidence: (1) The transcript of the *ADORA2B* gene was detected by RT-PCR (Figure 2A), amplicon identity was confirmed by sequencing; (2) A2BR expression in the plasma membrane of SKOV-3 cells was shown by Western blot analysis of proteins labeled by biotinylation with a non-permeant reactive and isolated by immunoprecipitation with avidin-coupled beads (Figure 2A); (3) concentration-dependent ERK phosphorylation in response to BAY-606583, a specific A2BR agonist, was blocked by the selective antagonist PSB-603 (Figure 2C,D); (4) BAY-606583-induced ERK phosphorylation was inhibited by PKAi fragment 14–22 myristoylated and by U-73122, a PLC inhibitor, strongly suggesting that A2BR is coupled to both Gαs and Gαq subunits in SKOV-3 cells (Figure 2E,F). This latter observation is interesting because A2BR signaling mechanisms are complex and must be delineated in each experimental model. For instance, in human epithelial lung cancer A2BR can couple to both subunits and, depending on the activated cell signaling pathway, can elicit different physiological processes [23]. Moreover, Gαs coupling has been demonstrated in breast cancer cells (MDA-MB-231) [18].

Then, we aimed our studies to evaluate the influence A2B receptor has on cell migration given that this process is determinant for cancer outcome. From our studies we conclude that A2BR activation in SKOV-3 cells inhibited cell migration. The evidence was as follows: (1) activation of A2BR with BAY-606583 (100 nM to 10 μM) induced a reduction in cell migration evaluated by the scratch assay (Figure 3B); (2) this result correlated with less-marked SF when cells were treated with A2BR agonist or Apy (Figure 3C), indicating an attenuated mesenchymal phenotype; (3) A2BR overexpression reduced cell migration (this result was similar to the one observed with pharmacological stimulation of the receptor (Figure 4)); (4) the reduction in A2BR expression by three different shRNAs caused an increase in cellular migration (Figure 5B) (this effect was in parallel with an increment in SF (Figure 5C)). Altogether, our data suggests that A2BR activation or constitutive activity inhibits SKOV-3 cell migration. To discard the idea that migration experiments were interfered with by cell proliferation, we evaluated if A2BR regulated cell viability, and found that treatment with BAY-606583 for 24 h had no effect on cell viability (Figure 3A), indicating that migration experiments are not influenced by proliferation. Furthermore, analysis of E-cadherin expression and localization pattern in response to BAY-606583 (Figure 6), indicated that A2BR stimulation favored mesenchymal phenotype. Similar observations were described when cervical cancer cells were treated with ADO [22].

In agreement with these observations, BAY-606583 also inhibited migration of CAOV-3 cells, another line derived from ovarian carcinoma, where A2BR is also expressed (Supplementary Figure S2). These data are suggestive that the actions of A2BR on ovarian carcinoma cell migration could be a more general mechanism.

To consider the implications of our observations in ovarian cancer, it is necessary to take into account that the role of A2BR on cell migration depends on the cellular model studied. Thus, it was shown that A2BR activation in breast [18] urothelial; [17] and head and neck squamous cell carcinoma [31] increased cell migration. On the other hand, in human

cervical cancer cells, extracellular ADO inhibited migration and reduced invasion [22] and, in noncancerous human retinal epithelial pigment cells, A2BR activation also inhibited cell migration [32]. Moreover, in epithelial lung cancer cells [23] and in mouse mammary fibroblast [33], A2B activity negatively modulated cell migration and the induction of markers of the epithelium to mesenchymal transition, counteracting the actions of TGF-β [23,33].

Thus, the inhibition of cell migration observed in the present study could contribute to explain the correlation between a high expression level of *ADORA2B* and a higher probability of patient survival at initial stages of the disease (Figure 1). However, there is at least another possibility; because Skov-3 cells are metastatic, the inhibition of migration and favoring of epithelial phenotype could contribute to cancer colonization of secondary organs, where cells display mesenchymal to epithelial transition (MET) [34]. Since cancer cells secretes exosomes expressing CD39 and CD73, enzymes necessary to convert ATP in ADO [35,36], is plausible to hypothesize that ADO acting through A2BR could be contributors to secondary tumor formation.

Finally, to understand if A2BR activity promoted changes in gene expression patterns and if these changes regulated cell migration, transcriptional activity of stimulated SKOV-3 cells was analyzed by cDNA microarrays. Indeed, pharmacological A2BR activation induced important changes in gene expression patterns. The main categories identified by GO analysis in down-regulated transcripts were Proteolysis and Extracellular Matrix Organization, Actin Cytoskeleton Organization and Regulation of Cell Migration, while in the up-regulated transcripts, the principal categories were Cell Adhesion, Negative Regulation of Cell Migration and Cytoskeleton Organization (Figure 7, Table 1). Fibroblast growth factor 9 (*FGF9*) stands out from the down-regulated transcripts. It has been reported that FGF9 is able to induce ovarian cancer cell invasion by activating the VEGF-A/VEGFR2 pathway [37]. Other transcripts included *AKT3* and *GSK3β*, coding for the variable 3 of protein kinase b and for Glycogen synthase kinase 3 beta, respectively; both factors induce cell proliferation and migration in ovarian carcinoma cells [38,39]. Of notable interest was the down-regulation of transcripts coding for metalloproteinases including *ADAM12*, a factor associated with an aggressive phenotype in high-grade serous ovarian carcinoma [40]. In addition, *MMP2* and *MMP16* transcripts, which are recognized proteins mediating enhanced migration and metastasis of ovarian carcinoma cells, were also reduced [41,42].

Some outstanding up-regulated transcripts were *C9orf126*(*SCAI*), *PRKCBP1* (*ZMYND8*), and *PVRL2* (Nectin 2). *C9orf126*(*SCAI*) codes for suppressor of cancer cell invasion protein (SCAI protein), a component of the RhoA signal transduction pathway. It has been shown in non-small lung cancer cells that miR-371b-5p induces cell proliferation, migration and invasion by negatively regulating SCAI expression [43]. *PRKCBP1* (*ZMYND8*) codes for zinc finger MYND-type containing 8, a transcription factor and histone-interacting protein that regulates cellular growth. In cancer cells, ZMYND8 modulates histone methylation and acetylation, regulating the expression of oncogenes and tumor suppressors [44]. In breast and nasopharyngeal cancers, ZMYND8 is down-regulated and its low expression correlates with increased invasiveness and poor prognosis [45,46]. Another interesting up-regulated transcript was *PVRL2* (Nectin 2), an adhesion protein participating in the initial step of cell-to-cell adherens junctions [47,48] that can also interact with scaffold proteins to regulate cell movement and differentiation [49]. It has been proposed that Nectin-2 is a potential target for breast and ovarian cancers [50].

Taken together, our results suggest that elevated levels of A2BR could be associated with a good prognosis in early ovarian cancer stages (1 + 2). Our in vitro results indicate that A2BR pharmacological stimulation and overexpression decreased ovarian cancer cell migration, which is associated with the acquisition of an epithelial phenotype.

4. Materials and Methods

4.1. Cell Culture

SKOV-3 (HTB-77) cells were acquired from the American Type Culture Collection (ATCC, Manassas, VI, USA). Cells were maintained in RPMI medium supplemented with

10% fetal bovine serum (FBS) and 1X antibiotic–antimycotic solution (for each mL: penicillin 100 U, streptomycin 100 µg and fungizone 0.25 µg) (Thermo Scientific, Waltham, MA, USA) at 37 °C in a humidified 5% CO_2 atmosphere. All reagents were obtained from Gibco-Thermo Fisher Scientific, USA.

4.2. Reverse Transcription (RT) and Polymerase Chain Reaction (PCR)

Total RNA was isolated following the guanidine isothiocyanate method [51]. RNA integrity was evaluated by electrophoresis and its concentration was determined by spectrophotometric analysis (NanoDrop 1000, Wilmington, DE, USA). cDNA was synthesized with 1 µg of total RNA treated with DNAse activity-free RNAse and used for reverse transcription reaction. The mixture contained oligodT and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA). Amplification of the transcripts coding for A2BR (*ADORA2B*), glyceraldehyde 3-phophate dehydrogenase (*GAPDH*) and cytochrome C1 (*CYC1*) was performed by end-point PCR. The oligonucleotide sequences were as follows: A2BR-forward 5′-TCC ATC TTC AGC CTT CTG GC-3′, A2BR-reverse 5′-AAA GGC AAG GAC CCA GAG GA-3′; GAPDH-forward 5′-CAA GGT CAT CCA TGA CAA CTT TG-3′, GAPDH-reverse 5′-GTC CAC CAC CCT GTT GCT GTA G-3′ and CYC-1-forward 5′-CTC CTG CCA CAG CAT GGA C-3′, CYC1-reverse 5′-CAT GCC TAG CTC GCA CGA T-3′. Reactions were performed in a final volume of 20 µL. All primers were synthesized by Sigma-Aldrich, USA. End-point PCR reactions were performed in a BioRad thermocycler. Amplicon identity was corroborated by sequences and BLAST (NIH) analysis.

4.3. Biotinylation of Plasma Membrane Proteins

For biotinylation of proteins located in the plasma membrane of SKOV-3 cells, cultures at 80–90% confluence were incubated with 300 µM of Ez-link Sulfo NHS-LC-LC-Biotin reagent (Thermo Scientific, Waltham, MA, USA) diluted in phosphate buffer (PBS, in mM:136 NaCl, 2.7 KCl, 10 Na_2HPO4, 1.8 KH_2PO4, pH 7.4) for 20 min. Then, biotinylation solution was withdrawn and cells were washed twice with PBS to be solubilized in TNTE buffer (containing in mM: 50 Tris–HCl pH 7.4, 150 NaCl, 1 EDTA, and 0.1% Triton X-100) for 20 min on ice. Cellular homogenate was collected and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was recovered, and the pellet discarded. Protein concentration of the cellular extract was estimated by the Lowry method and 1 mg of protein was incubated with 50 µL of sepharose–streptavidin-conjugated beads (Cell Signaling Technology, Danvers, MA, USA) for 90 min at room temperature. After, the beads were washed 3 times with PBS and resuspended in 100 µL of Laemmli buffer, boiled for 5 min and analyzed by Western blot as described below. For A2BR detection an antibody against an extracellular epitope (KDSATNNSTEPWDGTTNESC) of the receptor was employed (Allomone Labs, Jerusalem, Israel; #AAR-003) at a 1:1000 dilution.

4.4. Western Blot

Equal numbers of cells were seeded in 12-well plates. When the cells reached 80% confluency, they were serum-starved overnight. Then, pharmacological treatment was performed at the concentration and time indicated in each experiment. Cell lysates were obtained using Laemmli solution. Electrophoresis was made in 10% or 8% SDS-polyacrylamide gels and cells were transferred to PVDF membranes. For detection, membranes were incubated overnight at 4 °C with the following primary antibodies in 1:1000 dilution: anti-phospho p44/42 MAPK, anti-total p42/p44 MAPK, anti-E-cadherin (Cell signaling Technologies, Danvers, MA, EUA) or anti-A2BR (Alomone Labs, Jerusalem, Israel). Antibodies were raised in rabbit and anti-E-cadherin in mouse. After primary antibody incubation, membranes were incubated for 1 h with conjugated horseradish peroxidase goat anti-rabbit or donkey anti-mouse antibodies at a 1:5000 dilution. Signal was revealed by chemiluminescence and autoradiography. Densitometry analysis was performed using ImageJ software.

4.5. Cell Viability

MTS (3-(4-5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-H- tetrazolium) salt reduction assay (Cell Titter, Promega, USA) was used to evaluate cell viability. SKOV-3 cells (3.5×10^3) were cultured in 48-well plates in RPMI medium supplemented with 10% FBS to reach 50% confluence, then they were starved and incubated for 12 h. A2BR was stimulated by the addition of BAY-606583 from 10 nM to 10 µM and incubated for an additional 24 h. MTS reduction was determined according to the manufacturer's protocol. Briefly, MTS reactive was added to the cell culture at a final concentration of 16.6%, incubated for 2 h under standard culture conditions, recollected in a clean 96-well dish and read in a spectrophotometer at 490 nM. Data were normalized against control (non-stimulated condition). The assay was repeated four times in septuplicates.

4.6. Lentiviral Infection

Lentiviral particles were generated by transfecting HEK293-T cells with 10 µg of pLKO.1 plasmid carrying one of the anti-*ADORA2B* shRNA: sh1 5′-CCG GGC AGA TGT CAA GAG TGG GAA TCT CGA GAT TCC CAC TCT TGA CAT CTG CTT TTT G-3′ (SIGMA #TRCN0000065335); sh2 5′-CCG GGC AAT GAA TAT GGC CAT TCT TCT CGA GAA GAA TGG CCA TAT TCA TTG CTT TTT G-3′ (SIGMA # TRCN0000065337) or sh3 5′-CCG GGC TGG TGA TCT ACA TTA AGA TCT CGA GAT CTT AAT GTA GAT CAC CAG CTT TTT G-3′, (SIGMA #TRCN0000065334) (SHCLNG_NM000676; Sigma-Aldrich) and the packaging vectors pRSV-Rev, pMD2.G and pMDLg/pRRE, by the calcium phosphate precipitation method. Supernatant HEK293-T medium was collected and used to transduce SKOV-3 cells. Transduced cells were selected by puromycin (1.5 µg/mL) resistance for 5–7 days. Monitoring of *ADORA2B* knockdown was made by RT-PCR as described above.

4.7. Immunofluorescence

Immunostaining was performed as previously described [52]. Briefly, SKOV-3 cells were cultured on coverslips and pharmacological treatments were applied if necessary. For immunodetection, cells were washed with PBS, fixed for 20 min in a PBS solution containing 4% paraformaldehyde (PFA), washed with PBS for 5 min, permeabilized with 0.01% Triton X-100 in PBS and blocked with 5% fat-free milk in PBS for 1 h. Then, cells were incubated overnight with primary antibodies against E-cadherin (1:100; mouse; Cell Signaling) or A2BR (1:100; rabbit; Allomone). The next day, samples were washed twice with PBS and incubated for 1 h with secondary antibodies, anti-rabbit IgG coupled to Alexa 488 or anti-mouse IgG coupled to Alexa 488 (Thermo Scientific). Finally, the samples were mounted with VectaShield containing 4′, 6′-diamidino-2-phenylindole (DAPI) (Vector, Burlingame, CA, USA). The samples were analyzed by confocal microscopy (LSM-780 Carl Zeiss).

4.8. Actin Cytoskeleton Labeling

Wild-type or *ADORA2B* knockdown SKOV-3 cells were cultured on coverslips. After the indicated pharmacological treatment, they were fixed and permeabilized as described for immunostaining. Then, cells were incubated for 10 min in a PBS-DAPI solution (dilution 1:1000), washed, and mounted in VectaShield containing phalloidin coupled to rhodamine (Vector, CA, USA). The samples were analyzed by fluorescence microscopy (Apotome, Carl Zeiss, Jena, Germany).

4.9. Wound Closure Assay

Cells were cultured on 12-well plates. When the cultures reached 90% confluence, they were starved for 16 h. Then, a wound was made along the culture well using a 200 µL pipette tip, and cells were incubated with the indicated pharmacological treatment for another 16 h. Pictures were taken just after pharmacological stimulation (t = 0) and after 16 h. Analysis of the wound closure area was performed using microphotographs

and Image J software. All wound closure experiments were repeated at least three times in triplicate.

4.10. Cancer Database Analysis

The Kaplan–Meier plotter database (https://kmplot.com/analysis/; accessed on 1 January 2022) was used to generate survival curves from ovarian cancer patients [26]. Log rank P, hazard ratio and median survival or upper quartile survival were calculated and displayed on the webtool.

4.11. cDNA Microarray Analysis

The cDNA microarray experiment was performed in the Microarray Unit at the Institute of Cellular Physiology (Universidad Nacional Autónoma de México, CDMX, Mexico). SKOV-3 cells were cultured in 100 mm Petri dishes to achieve a confluence of 70–80%. Then, cells were stimulated with BAY-606583 for 24 h or kept under control conditions. Later, total RNA was isolated by the Trizol method (Thermo Fisher Scientific, Waltham, MA, USA) and used for cDNA synthesis with a commercial kit using 10 mg of total mRNA (First-Strand cDNA labeling kit, Thermo Fisher Scientific, Waltham, MA, USA); dUTP-Alexa555 or dUTP-Alexa647 probes were incorporated at this stage. Fluorescence emission was analyzed at 555 nm for Alexa555 and 650 nm for Alexa647.

Labeled cDNA was utilized to hybridize a 35 K library of the whole human genome containing 70-mer oligos (from OPERON) manufactured by the Microarray Unit of the Institute of Cellular Physiology at UNAM. The acquisition and quantification of the array images was performed with GenePix 4100A software (OMICtools, RRID:SCR_002250; Molecular Devices, San José, CA, USA). Mean density values for the fluorescent probes and mean background were calculated. Analysis of the microarray data was performed with genArise (RRID:SCR_001346; http://www.ifc.unam.mx/genarise/; accessed on 1 August 2021) developed by the Computing Unit of the Institute of Cellular Physiology (UNAM, Mexico). This freeware calculates the intensity-dependent Z-score from the images to identify different gene expression patterns. Elements with a Z-score > 1.5 standard deviations were defined as differentially expressed transcript genes. Data were deposited in ArrayExpress-EMBL-EBI (accession number: E-MTAB-11130). Bioinformatic analysis was performed with GeneCodis and KEGG tools and focused on identifying genes regulated by BAY-606583 with a gene ontology focus.

4.12. Statistics Analysis

The results are expressed as the mean standard error of the mean (S.E.M.). The statistical differences between the groups were evaluated with a Student's t-test and marked with * for $p \leq 0.05$, ** for $p \leq 0.01$ * and *** for $p \leq 0.001$.

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Data Availability Statement: Microarray data were deposited in ArrayExpress-EMBL-EBI (accession number: E-MTAB-11130). Other data supporting the results of the current study are available from the corresponding author on reasonable request.

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