Universidad de Alcalá

Programa de Doctorado en Ciencias de la Salud

IMPLICACIÓN DE LA PROSTAGLANDINA E_2 INTRACELULAR EN PATOLOGÍA PROLIFERATIVA PROSTÁTICA

Tesis Doctoral presentada por

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Alcalá de Henares, 2020

"Nadie triunfa sin esfuerzo. Aquellos que triunfan deben su éxito a la perseverancia"

Ramana Maharshi

"Nada en este mundo debe ser temido… solo entendido. Ahora es el momento de comprender más, para que podamos temer menos"

Marie Curie

Agradecimientos

Cuando acabé la carrera de Farmacia pensé “he terminado y ya para siempre”, se acabaron las horas de estar estudiando delante de un libro, los fines de semana en la biblioteca, las noches de repaso antes de un examen, etc. Pasados unos días todos esos esfuerzos se olvidan, te das cuenta que la satisfacción personal por haberlo conseguido y el crecimiento personal están por encima de esas dificultades. Así que decidí continuar con mis estudios y me lancé a realizar el doctorado. Tras unos años duros científicamente y literariamente hablando, aquí estoy a punto de acabar la tesis y por eso quería dar las gracias a todas las personas que me han acompañado durante todos estos años.

En primer lugar, quería agradecer a mis directores de tesis **Javier** y **Ana**, por darme la oportunidad de poder realizar la tesis doctoral en vuestro laboratorio y por la gran formación que me habéis dado todos estos años. Cuando culminas tu tesis doctoral, comprendes que todos estos años han sido diferentes etapas de aprendizaje que te permiten ser un DOCTOR con letras mayúsculas. Gracias a vosotros he aprendido a manejarme dentro de un laboratorio, a preparar y exponer mis resultados en público, a redactar correctamente, y lo más importante he adquirido un pensamiento crítico. En cuanto a mi directora **Ana**, me quedo con los buenos momentos dentro y fuera del laboratorio (los partidos de pádel, las noches de risas en las ferias de Guadalajara y los “escapes room” literarios que me hacías desde la Autónoma, con los que he podido aprender a escribir no una tesis sino una obra literaria jaja). Respecto a mi director **Javier**, siempre recordaré tu buen humor en el laboratorio contando chistes y tu gran disponibilidad para resolver cualquier problema dentro del laboratorio.

En segundo lugar, quería dar la gracias en general a mis compañeros porque han hecho que mi estancia en el laboratorio sea una gran experiencia. Empezando con **Héctor** mi “tercer director de tesis”, por ayudarnos a entender algunos resultados obtenidos en el laboratorio y por prestarnos sus aparatos de laboratorio con los que he podido hacer tanto PCR como los Western. Además, quería darte las gracias por los buenos ratos que hemos tenido hablando de la NBA y de fútbol.

A continuación, quería agradecer a mis compañeras **Valdehita, Selma** y **Coral** por los buenos momentos que hemos tenido dentro del laboratorio, en las comidas, etc. No quería olvidarme de mis aprendices internacionales **Vera** y **Katy**. Os estoy muy agradecido por mostrar un gran interés en aprender y por tomaros en serio el trabajo encomendado, ha sido un placer enseñaros a trabajar en el laboratorio.

Como no, mencionar a mis vecinos **Eva, Bea y Sergio** que me alegraban todas las mañanas con un simple saludo “hola vecino”, y como buenos “vecinos científicos” nos prestábamos cualquier reactivo.

También quería dar las gracias a **Eva, Lauris, Ana bajo** y **María José** por acogerme en vuestro departamento para comer, y por supuesto por esos buenos ratos en las comidas. Particularmente, quería dar las gracias a **Lauris** por esos cuestionarios que me hacías acerca de mis "amores" en la hora de la comida, parecía que estaba con la Patiño en un plato de Telecinco jaja. Ahora en serio te agradezco el interés que has mostrado por mí, ayudándome en mis primeros pasos en el mundo de la investigación. Espero que te vaya muy bien en tu vida al lado de tu marido Felipe y tu perro Thor.

No me quería olvidar de **Ali, Nadia, Patri** y **Ceci**, que me lo han hecho pasar muy bien dentro y fuera del laboratorio, no se me olvidará las cenas que hemos tenido en el chino, mexicano, venezolano o colombiano. Especialmente, quería agradecer a **Ali** porque ha sido una gran compañera y amiga de laboratorio, y a su **padre** por interesarse por mi tesis.

También quería dar las gracias, al laboratorio de **Manolo Rodríguez Puyol** (Alicia, Sergio y Mercedes), porque siempre que me ha faltado un reactivo me lo habéis prestado. A **Patri Sosa, Ana** y **Agatha** por tener una sonrisa o una simple conversación que hacía más llevadera esas mañanas duras en el laboratorio.

En tercer lugar, quería agradecer a mis amigos de **"Alcarest"** por todo vuestro apoyo durante estos años. Especialmente, quería dar las gracias a **Nuria** por ayudarme a desconectar los findes de semana montándome planes deportivos por la naturaleza, y sobre todo porque siempre has estado cerca de mí apoyándome, intentando entender mis resultados del laboratorio y escuchándome cuando necesitaba desahogarme.

A continuación, quería agradecer al formidable equipo que tenemos en la farmacia, **Lourdes, Toñi, Laura, Ana** y mi **padre**, sin olvidarme de los alumnos en prácticas que he dirigido, por escucharme e interesaros por mi tesis, además de cubrirme en la farmacia cuando tenía congresos, reuniones, experimentos largos, etc.

Además, quería dar las gracias a mis tíos (**Magda, Felipe, Marilena, Pepe, Carmen** y **Andrés**), mis primas (**Lucia** y **María**) y primos (**Nacho** y **Edu**), simplemente por interesarse por mi doctorado e intentar comprender lo que estaba haciendo. A mi hermano (**Edu**) por la paciencia que ha tenido para explicarme los programas informáticos. A **Isabel Ríos**, que es como de la familia, por escucharme y animarme a seguir luchando durante todos estos años.

Por último, quería agradecer a mis **padres** la educación que me habéis dado siempre desde el cariño, y especialmente por darme todas las facilidades del mundo para que estudiara la carrera de Farmacia y luego continuase con mis estudios realizando el doctorado.

Abreviaturas

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Actinomicina D (Act.D)

Adenilato ciclasa (AC)

Adenosína monofosfato cíclica (AMPc)

Albúmina sérica bovina (BSA)

Antiinflamatorios no esteroideos (NSAIDs)

5-Bromo-2-desoxiuridina (BrdU)

Bromosulftaleína (Bs)

Bromuro de 3-(4,5-dimetiltiazol-2-il)-2,5-difenil-tetrazol (MTT)

Cáncer de próstata (CP)

Cicloheximida (CHX)

Ciclooxigenasa (COX)

Complejo Von Hippel-Lindau-E3 ubiquitina ligasa (VHL-E3)

Dihidrotestosterona (DHT)

Dimetilsulfóxido (DMSO)

Elemento respuesta a hipoxia (HRE)

Factor de crecimiento endotelial vascular (VEGF)

Factor de crecimiento epidérmico (EGF)

Factor de crecimiento transformante α/ β (TGF-α/TGF- β)

Factor inducible por hipoxia (HIF)

Factor de necrosis tumoral (TNF-α)

Factor de transcripción activador 6α (ATF6α)

Factor de transcripción nuclear kappa B (NF-κB)

Familia de transportadores de aniones orgánicos (OAT)

Familia de transportadores de aniones orgánicos polipeptídicos (OATP)

Fosfatidilinositol 3-quinasa (PI3K)

Fosfolipasa A_2 (PLA_2)

Fosfolipasa C (PLC)

15-hidroxi-prostaglandina deshidrogenasa (15-PGDH)

Hiperplasia benigna de próstata (HBP)

Interleuquina (IL)

2-oxoglutarato (OG)

Prolil-hidroxilasas (PHDs)

Prostaglandina (PG)

Prostaglandina E_2 (PGE_2)

Prostaglandina E_2 intracelular ($iPGE_2$)

Prostaglandina E_2 sintasa citosólica/asociada a membrana microsomal (cPGES/mPGES)

Proteína asociada a multirresistencia de fármacos (MRP-4)

Proteína quinasa A (PKA)

Proteína quinasa C (PKC)

Proteínas quinasas activadas por mitógenos (MAPK)

Receptor acoplado a proteínas G (GPCR)

Receptor del factor crecimiento epidérmico (EGFR)

Receptor de prostaglandina E_2 (EP)

Transportador de Prostaglandinas (PGT)

Verde de bromocresol (BG)

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Resumen

1. Resumen

La prostaglandina E_2 (PGE_2), cuya velocidad de síntesis está limitada por la actividad de las ciclooxigenasas-1 y 2 (COX-1/COX-2), contribuye en la progresión de la hiperplasia benigna de próstata (HBP) y del cáncer de próstata (CP). Una vez producida, PGE_2 es secretada al medio extracelular y se asume que ejerce exclusivamente sus efectos, de forma paracrina o autocrina, a través de receptores EPs localizados en la membrana plasmática. También se asume que el posterior transporte de PGE_2 al interior celular (mediante el transportador de prostaglandinas (PGT)) solo conduce a su inactivación. Sin embargo, un reducido número de trabajos han demostrado que PGE_2 intracelular ($iPGE_2$) también es biológicamente activa. Por otra parte, numerosos trabajos apoyan la importancia de PGE_2 (y de COXs), EGFR y HIF-1α en la progresión de las patologías proliferativas prostáticas. Dichos factores podrían estar relacionados, ya que $iPGE_2$, a través de la transactivación de EGFR por receptores EPs, regula la expresión de HIF-1α en células de CP*. Por lo tanto, en el presente trabajo nos plateamos como hipótesis que la vía COXs/$iPGE_2$/receptores EPs intracelulares/EGFR/HIF-1α puede contribuir a la estimulación de diversos fenotipos tumorales prostáticos. En relación con esta hipótesis, trabajaremos con la línea celular RWPE-1 de células epiteliales de próstata humana y la línea PC3 de células de CP resistente a castración para determinar, como objetivo general, el papel de $iPGE_2$ en la progresión de HBP y del CP, respectivamente. Este objetivo general se desglosa en los siguientes objetivos específicos: 1) Determinar el efecto de PGE_2 sobre diversos fenotipos tumorales benignos y/o malignos tales como proliferación, adhesión, migración, invasión celular y angiogénesis. 2) Determinar qué receptores EPs median la regulación por PGE_2 de los fenotipos tumorales mencionados, así como analizar la posible presencia y participación de receptores EPs intracelulares en los efectos de PGE_2. 3) Valorar el efecto de la inhibición de PGT, es decir, del bloqueo del transporte de PGE_2 al interior celular, sobre los fenotipos tumorales mencionados (bien sean constitutivos o bien sean estimulados por PGE_2 exógena). 4) Analizar el papel de la vía $iPGE_2$/receptores EPs intracelulares/EGFR/HIF-1α en la estimulación por PGE_2 de los fenotipos estudiados. 5) Valorar la posible inducción de COXs por $iPGE_2$ y, en su caso, analizar las vías de señalización implicadas y el papel de dicho efecto como regulador de los fenotipos tumorales estudiados (bien sean constitutivos o estimulados por PGE_2 exógena). Las principales conclusiones de nuestro trabajo se resumen así: 1) En células PC3 el tratamiento con PGE_2 potencia fenotipos tumorales malignos como proliferación celular, expresión transcripcional de HIF-1α,

* Cuando comenzamos esta tesis nuestro grupo había demostrado este mecanismo en células PC3, pero el resultado no fue publicado hasta el año 2015 (Fernández-Martínez y Lucio Cazaña, 2015a).

capacidad de migración y de invasión, angiogénesis y producción de VEGF (ambos efectos dependientes de HIF-1α), y pérdida de adhesión al colágeno. Para ejercer estos efectos, PGE_2 debe ingresar al interior celular mediante el transportador de prostaglandinas PGT y activar receptores EPs intracelulares (presumiblemente EP2-EP4). 2) El mecanismo por el que PGE_2 intracelular potencia los fenotipos tumorales anteriormente citados, depende de la transactivación de EGFR (vía Src) por receptores EPs y posterior aumento transcripcional de COX-2, de forma dependiente de quinasas, lo que da lugar a un incremento sostenido de la producción de PGE_2. Este hecho sugiere que, al menos durante las primeras horas posteriores a la exposición a PGE_2, puede existir una retroalimentación positiva entre $iPGE_2$ y COX-2 que contribuye a la potenciación de los fenotipos tumorales gracias al mantenimiento de un elevado contenido celular en PGE_2. 3) La producción endógena de $iPGE_2$ también contribuye probablemente a la agresividad intrínseca de las células PC3, ya que la inhibición de la actividad basal de COXs y del PGT atenúa los fenotipos tumorales mencionados anteriormente. 4) En células RWPE-1 el tratamiento con PGE_2, mediante mecanismos similares a los descritos para células PC3, provoca el mismo tipo de efectos que en células PC3. De esta manera, PGE_2 debe ingresar primero al interior celular mediante el transportador de prostaglandinas PGT y activar receptores EPs intracelulares, lo que provoca la transactivación de EGFR. Finalmente, se produce un incremento de la proliferación celular, de la producción de VEGF, de la angiogénesis, de la capacidad de migración y una pérdida de adhesión al colágeno que dependen del aumento de la expresión de HIF-1α inducido por EGFR. Teniendo en cuenta las conclusiones anteriores sobre el papel de $iPGE_2$ en las líneas celulares epiteliales prostáticas estudiadas, nuestros resultados sugieren que la activación de receptores EPs intracelulares por PGE_2 podría tener un papel fundamental en la progresión de HBP y del CP resistente a castración. En consecuencia, el bloqueo de la entrada de PGE_2 al interior celular, mediante la inhibición de PGT, debería ser evaluado como una nueva estrategia terapéutica para reducir la progresión de las citadas alteraciones proliferativas prostáticas.

Introducción

2. Introducción

2.1. La próstata

La próstata es una glándula que forma parte del aparato reproductor masculino y se ubica en la pelvis rodeando el cuello de la vejiga y la uretra. En la próstata se distinguen tres regiones anatómicas: central, transición y periférica. Histológicamente, está organizada en acinos y revestida por abundante estroma fibromuscular. La unidad acinar se compone de células neuroendocrinas y de células epiteliales (basales y secretoras cilíndricas). Su función es segregar un líquido ligeramente alcalino que forma parte del semen y que protege a los espermatozoides neutralizando el pH ácido de la vagina y aumentando su supervivencia (Epstein, 2015).

Tanto el desarrollo normal de la próstata como su mantenimiento en el adulto están regulados por diversas hormonas y mediadores paracrinos/autocrinos. Entre las hormonas que regulan la fisiología prostática, los andrógenos juegan un papel clave. La testosterona es la hormona androgénica más sintetizada por las células Leydig de los testículos y a través de la circulación sanguínea llega a las células de la próstata donde, por la acción de la 5α reductasa, se convierte en dihidrotestosterona (Epstein, 2015), un metabolito más activo que la testosterona que se une al receptor de andrógenos (localizado en el citoplasma de las células prostáticas) provocando la separación con las chaperonas que lo mantenían inactivo (Heinlein y Chang, 2002). A continuación, el receptor de andrógenos se fosforila y el complejo dihidrotestosterona-receptor se transloca al núcleo. Allí regula la transcripción de genes implicados en diversas funciones como la regulación del ciclo celular[*], la producción del antígeno prostático específico (PSA)[†] o la síntesis de proteínas transmembrana, como por ejemplo TMPRSS2 (cuya desregulación se asocia con el cáncer de próstata (CP)) (Tan y col., 2015). Por otro lado, parte de la testosterona se transforma en estradiol gracias a la acción de la aromatasa prostática. El papel de los estrógenos en la próstata no se conoce en profundidad. Su acción la realizan a través de la unión a sus receptores[‡] α y β, y parecen estar implicados en el desarrollo y función normal del tejido prostático, y también en diversas enfermedades proliferativas prostáticas (Prins y Korach,

[*] El complejo dihidrotestosterona-receptor activa genes como la ciclina A y la ciclina D1 que favorecen el avance del ciclo celular, y desactiva genes que frenan el ciclo celular como por ejemplo el de la proteína del retinoblastoma (Balk y Knudsen, 2008).
[†] PSA es una glucoproteína producida por la próstata que disuelve los coágulos seminales. Los niveles de PSA en sangre ayudan a determinar si hay patologías prostáticas considerándose como valores normales los que están por debajo de 4 ng/ml en sangre (NCI 2019).
[‡] El receptor de estrógenos α promueve la proliferación de las células prostáticas, en tanto que el receptor β limita su proliferación (Royuela y col., 2001).

2008). Las citoquinas como el factor de crecimiento epidérmico (EGF), los factores de crecimiento transformantes α y β (TGF-α y TGF-β), los factores de crecimiento similares a la insulina y múltiples mediadores inflamatorios, entre otros, también influyen en el crecimiento de la próstata, regulando su tasa de proliferación y apoptosis (Russell y col., 1998; Hamid y col., 2011 y Sfanos y col., 2018). Una alteración en el balance entre los diversos reguladores del crecimiento prostático, puede provocar que las señales que estimulan la proliferación de las células de la próstata sean predominantes, lo que constituye la base de las diferentes enfermedades proliferativas. Estas, junto a la prostatitis, constituyen las patologías más frecuentes de la próstata (Epstein, 2015).

2.2. Enfermedades proliferativas prostáticas

2.2.1. Hiperplasia benigna de próstata

Se define como hiperplasia benigna de próstata (HBP) al aumento del tamaño de la próstata caracterizado por un incremento no invasivo del número de células epiteliales y estromales que da lugar a nódulos grandes en la zona de transición de la próstata. Cuando los nódulos alcanzan un tamaño significativo, aparece un síndrome del tracto urinario inferior caracterizado por la aparición de disuria, polaquiuria y micción intermitente, consecuencia de la obstrucción e irritación de la zona. El hecho que las células de HBP no tengan la capacidad de invadir otros tejidos, confiere el carácter benigno a esta enfermedad (Epstein, 2015).

La incidencia de esta enfermedad se incrementa gradualmente con la edad: a los 60 años aproximadamente el 70% de los hombres presentan algún signo histológico de HBP, y a partir de los 80 años el valor aumenta hasta el 90% (Epstein, 2015). Los medicamentos más utilizados en el tratamiento de HBP son: i) Los bloqueantes alfa adrenérgicos (tamsulosina) que, al relajar los músculos en la base de la vejiga, mejoran la capacidad de micción. ii) Los inhibidores de 5α reductasa (finasterida o duasterida), que ayudan a reducir el tamaño de la próstata. iii) Los anticolinérgicos (solifenacina), que reducen la actividad de la vejiga hiperactiva (Kim y col., 2017 y 2018). Recientemente, la inyección de la toxina botulínica se está utilizando para el tratamiento de los síntomas relacionados con HBP dado que también relaja los músculos en la base de la vejiga (Chung y Woo, 2018). En casos moderados o graves, los tratamientos farmacológicos no son tan efectivos y hay que recurrir a procedimientos invasivos como la resección transuretral de la próstata con láser, la hidroablación prostática o la hipertermia, entre otros (Chung y Woo, 2018; Epstein, 2015), que permiten al paciente mejorar los síntomas del tracto urinario inferior y controlar el crecimiento anormal de la próstata.

2.2.2. Cáncer de próstata

El adenocarcinoma de próstata, es una patología proliferativa maligna (a diferencia de HBP, las células del CP invaden otros tejidos) multicausal y de progresión lenta. El inicio de la enfermedad se origina principalmente en la zona periférica (70%) y en menor medida en la zona de transición (20%) y en la zona central (10%) (Sociedad Española de Oncología Médica – SEOM-2017). Al igual que otros cánceres, CP se desarrolla por la acumulación de cambios genéticos y epigenéticos en las células de la próstata. Esto determina la aparición de características fenotípicas específicas en las células tumorales, tales como incremento de la proliferación, resistencia a la apoptosis, capacidad para inducir la formación nuevos vasos sanguíneos (neoangiogénesis), pérdida de la adhesión celular y aumento de la capacidad de migrar, invadir y metastatizar, fundamentalmente en hueso, hígado y pulmón (Laso y col., 2004). En la **Figura 1** se muestran los diferentes acontecimientos de la carcinogénesis.

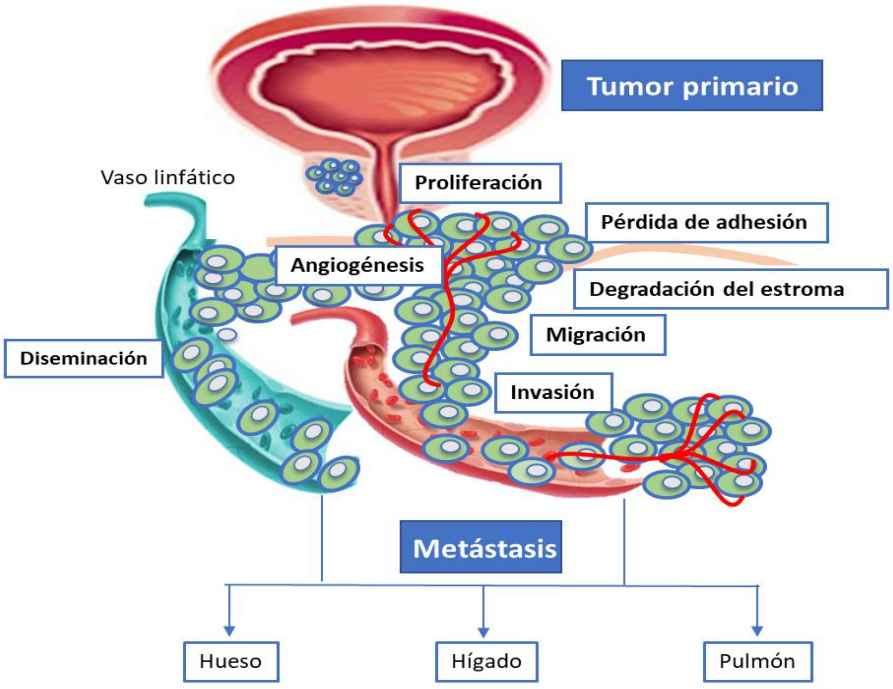

Figura 1. Esquema de la secuencia de los principales acontecimientos que suceden durante la progresión del CP (adaptado de Stricker y Kumar, 2015).

El tratamiento del CP depende del estadio en el que se encuentre. En estadios tempranos, donde el crecimiento de las células cancerígenas es dependiente de los niveles de andrógenos, los tratamientos se basan en la administración de fármacos como la leuprolida (Eligard), que inhibe la

síntesis de estas hormonas, o como la bicalutamida, que bloquea el receptor de andrógenos (Roisin y col., 2012). Sin embargo, en las etapas más avanzadas de la enfermedad, donde la proliferación de las células es independiente de hormonas, el estadio es denominado CP resistente a castración, y dichos tratamientos ya no son efectivos y suelen ir acompañados de tratamientos paliativos (Katzenwadel y Wolf, 2015). Por diversos mecanismos como mutaciones en el receptor de andrógenos (Cutress y col., 2008), activación constante de vías de señalización como por ejemplo RAS/MAPK (Bakin y col., 2003), etc., un 20% de los pacientes con CP progresarán a este estadio letal de la enfermedad (Chandrasekar y col., 2015). Las modificaciones en el receptor de andrógenos determinarán su activación mediante ligandos no androgénicos, la adquisición de hipersensibilidad a niveles bajos de andrógenos, o incluso la activación del receptor sin ligando (Nakazawa y col., 2017 y Banerjee y col., 2018).

En el año 2019, CP fue la segunda neoplasia diagnosticada más frecuente en hombres a nivel mundial después del cáncer de pulmón (Rawla, 2019). En el año 2017 se diagnosticaron en España 30.076 casos de CP, siendo el tipo más frecuente de cáncer en hombres y el segundo más diagnosticado en ambos sexos. Su incidencia aumenta del 20%, entre los 50-60 años, al 70% entre los 70-80 años (SEOM 2018). El riesgo de padecer CP no solo depende de la edad sino también de la herencia genética, de los niveles de testosterona, de las exposiciones medioambientales a carcinógenos y de la **inflamación,** entre otros factores. (Leitzmann y Rohrmann, 2012). Dado que cerca del 20% de todos los cánceres humanos pueden derivar de estados inflamatorios crónicos (Todoric y col., 2016), analizaremos, a continuación, la relación existente entre la inflamación y las patologías proliferativas prostáticas.

2.3. Inflamación y patología proliferativa prostática

La inflamación es una respuesta de los tejidos a las infecciones y al daño tisular que hace que las células y moléculas encargadas de la defensa se extravasen a las localizaciones en las que son precisas para eliminar el agente causante de la agresión. La inflamación se asocia a signos y síntomas como calor, rubor, dolor, hinchazón y pérdida de la función. La inflamación aguda es una respuesta protectora esencial para la supervivencia y desaparece una vez completado su objetivo. Sin embargo, si la respuesta inflamatoria persiste, deja de tener carácter beneficioso y provocará importantes daños tisulares (Kumar y col., 2015). Es entonces cuando hablamos de inflamación crónica, cuya presencia ha sido asociada a la patogenia de diferentes enfermedades como la aterosclerosis, artritis reumatoide, esclerosis múltiple, Alzheimer y cáncer (Sethi y col., 2012).

Rudolf Virchow fue el primero en relacionar la inflamación crónica con el cáncer, cuando observó la presencia de leucocitos en los tejidos neoplásicos y postuló que la inflamación podría promover un entorno celular que conduciría a la iniciación y desarrollo del tumor (Virchow, 1865). Desde entonces, la inflamación crónica ha sido reconocida como un factor de riesgo para el desarrollo de varios tipos de tumores tales como el de pulmón, colon, mama, ovario y próstata. (Todoric y col., 2016; Shrihari y col., 2017). Durante los procesos inflamatorios se producen diferentes moléculas que podrían contribuir a los procesos neoplásicos, al modificar el balance entre las señales de proliferación y muerte celular. Entre las sustancias producidas por las células inflamatorias, se encuentran especies reactivas de oxígeno y de nitrógeno, diferentes factores de crecimiento (EGF, TGF-α y β), distintos mediadores inflamatorios como interleuquinas (IL-8, IL-6, IL-17), quimioquinas (CCL-2, CXCL-12), eicosanoides (prostaglandinas, leucotrienos), etc. que mantienen un microambiente que favorece el inicio y posterior crecimiento del tumor. En dicho contexto, se ha demostrado que muchos mediadores inflamatorios -como por ejemplo la prostaglandina E_2 (PGE_2)- se producen no solo por las células inflamatorias sino también por las propias células tumorales (Landskron y col., 2014; Udensi y Tchounwou, 2016; Shrihari y col., 2017).

En una próstata adulta es frecuente la presencia de áreas inflamadas (Aaron y col., 2016). La inflamación aguda de la próstata o prostatitis puede originarse por diversos factores tales como la infección directa, el reflujo de la orina, una lesión traumática, dietas ricas en grasas saturadas y azúcares o por factores endocrinos (Epstein, 2015). Si la inflamación persiste, estaremos ante una inflamación crónica que puede contribuir a la iniciación y a la progresión de tumores prostáticos benignos o malignos. A continuación, desarrollaremos las evidencias existentes que relacionan la inflamación con HBP y CP.

2.3.1. Hiperplasia benigna de próstata e inflamación

La prevalencia de inflamación en biopsias de HBP se encuentra entre el 43-77% (Samarinas y col., 2018). Diferentes estudios han demostrado que existe una correlación entre la sintomatología del crecimiento prostático y la presencia de procesos inflamatorios (Gandaglia y col., 2013; Samarinas y col., 2018; Tyagi y col., 2018). De hecho, las dietas con propiedades antiinflamatorias y ricas en antioxidantes parece que tienen cierta capacidad para reducir el riesgo de padecer HBP porque disminuyen los altos niveles de especies reactivas de oxígeno asociados a la inflamación (Chung y col., 2015; Paterniti y col., 2018). Por otra parte, el contenido de la próstata en células inflamatorias como linfocitos T y macrófagos se incrementa en HBP (Steiner y col., 2003) y estas células, junto a las propias células de la próstata, producen diferentes citoquinas proinflamatorias

como IL- 2, 8, 15, 17 y el interferón-γ que estimulan directamente la proliferación de las células de la próstata. Otras citoquinas como IL-1β y TNF-α, inducen la expresión de la enzima ciclooxigenasa-2 (COX-2) (Jang y Schaeffer, 2003), clave en los procesos inflamatorios.

Todas estas evidencias no demuestran si la inflamación es causa o consecuencia de HBP, pero lo que sí avalan es que ambas entidades coexisten y sugieren que la inflamación puede contribuir a la progresión de las neoplasias prostáticas benignas.

2.3.2. Cáncer de próstata e inflamación

CP también ha sido relacionado con la inflamación (Bardan y col., 2014; Thapa y Ghosh, 2015; Sfanos y col., 2018). En biopsias de pacientes con CP se han encontrado lesiones inflamatorias en más del 92% de las muestras analizadas (Hamid y col., 2011) y en los pacientes en estadios más avanzados de CP, la presencia de inflamación fue más frecuente y se correlacionó con un peor pronóstico. También se ha observado que los hombres con inflamación prostática son 1,78 veces más propensos a desarrollar CP que los que no tienen evidencias histológicas de inflamación (Thapa y Ghosh, 2015). Además, se ha demostrado que los alimentos con propiedades antiinflamatorias como la soja y el té verde, pueden reducir el riesgo de padecer CP (Lin y col., 2015). Por otra parte, se ha postulado que la atrofia proliferativa prostática[†], frecuentemente acompañada de inflamación, puede ser una lesión precursora del CP (De Marzo y col., 1999). Es más, existen estudios que sugieren una transición directa entre la atrofia proliferativa prostática y CP (Wang y col., 2009; Benedetti y col., 2016) dado que se ha observado que ambas patologías comparten una serie de cambios moleculares y genéticos, incluyendo anomalías en el cromosoma 8, mutaciones de p53[‡] (Wang y col., 2009), incrementos de expresión de Bcl-2 y desregulación de genes supresores tumorales como *nkx3.1*, *cdkn1b*, *pten* (Sfanos y De Marzo, 2012; Felgueiras y col., 2014). La relación entre inflamación y CP, también se ha observado en un modelo *in vivo*, concretamente en ratones, en los cuales la infección de la próstata causada por cepas uropatógenas de *Escherichia coli,* incrementa la proliferación epitelial, el daño en el DNA y reduce la expresión de *nkx3.1* (Sfanos y De Marzo, 2012).

Sin embargo, dado que en algunos trabajos no se ha encontrado relación alguna entre inflamación y CP (De Marzo y col., 2004, De Nunzio y col., 2011 y Gurel y col., 2014), las estrategias

[†] De Marzo propuso dicho término para denominar una lesión localizada en la zona periférica de la glándula prostática, con células epiteliales altamente proliferativas (De Marzo y col., 1999).
[‡] Aproximadamente el 50% de todos los cánceres humanos presentan mutaciones de p53 (Hamzehloie y col., 2012). En concreto, en CP la mutación de p53 se correlaciona con un peor pronóstico de la enfermedad (Kluth y col., 2014).

actuales para establecer si existe o no relación entre ambos procesos se centran en determinar los mecanismos por los cuales la inflamación podría promover el desarrollo de neoplasias prostáticas. En este contexto, los mediadores inflamatorios producidos tanto por las células inflamatorias como por las células tumorales de la próstata, tienen una gran relevancia en la progresión tumoral (Sfanos y De Marzo, 2012; Thapa y Ghosh, 2015). Entre estos mediadores inflamatorios, nos ocuparemos a continuación de PGE_2, dado el papel central que tiene en la presente tesis.

2.4. Prostaglandina E_2 (PGE_2)

PGE_2, como los demás prostanoides (como por ejemplo prostaglandinas D_2 y F_2, prostaciclina y tromboxanos), pertenece a la familia de los eicosanoides, ácidos grasos oxigenados de 20 carbonos. La biosíntesis de los prostanoides implica cuatro reacciones enzimáticas secuenciales, tal y como se puede apreciar en la **Figura 2**. La primera reacción consiste en la liberación del ácido araquidónico de los fosfolípidos de membrana mediante la acción de fosfolipasas celulares, como la fosfolipasa A_2 (PLA_2) y la fosfolipasa C. Las dos siguientes reacciones enzimáticas se producen gracias a la acción de las ciclooxigenasas (COXs) o prostaglandina-endoperóxido H sintasas, que actúan de forma bifuncional: primero catalizan la oxidación de ácido araquidónico a un endoperóxido cíclico, introduciendo un átomo de O_2 en los carbonos C_{11} y C_{15} para formar prostaglandina G_2 (PGG_2), y a continuación, producen la reducción peroxidativa de PGG_2 a prostaglandina H_2 (PGH_2). Esta última se transforma en diferentes metabolitos biológicamente muy activos mediante la acción de las correspondientes sintasas, que son específicas de cada tipo tejido (Capra y col., 2015).

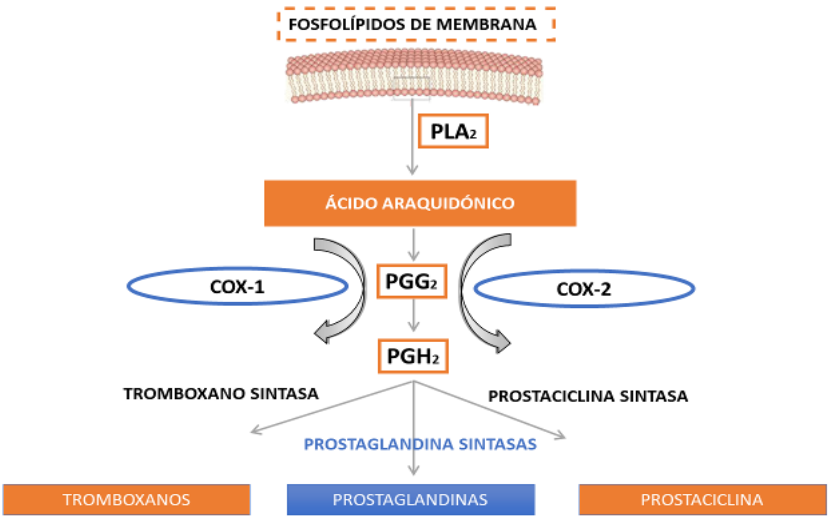

Figura 2. Biosíntesis de PGE_2 (adaptado de Legler y col., 2010).

Dentro de los prostanoides, PGE_2 es el más abundante en la próstata (Chaudry y col, 1994; Nithipatikom y Campbell, 2008). Es un mediador celular extremadamente potente y por ello tiene una vida media corta (Legler y col., 2010). Además de su papel en la próstata, del que hablaremos más adelante, PGE_2 regula diversas funciones fisiológicas en el organismo como la secreción gástrica, la presión intraocular, la presión arterial, la contracción uterina, el grado de constricción de las vías aéreas, la tasa de filtración glomerular, la formación del hueso, la temperatura corporal, el dolor, la vasodilatación, la permeabilidad endotelial, la angiogénesis y el reclutamiento de células inflamatorias (Norberg y col., 2013; Kawahara y col., 2015; Yang y Chen, 2016).

Estructuralmente, PGE_2 está formada por un anillo de ciclopentano y un ácido carboxílico insaturado. El subíndice 2 del término "PGE_2" hace referencia al número de dobles enlaces en las cadenas de carbono, tal y como se muestra en la **Figura 3** (Legler y col., 2010).

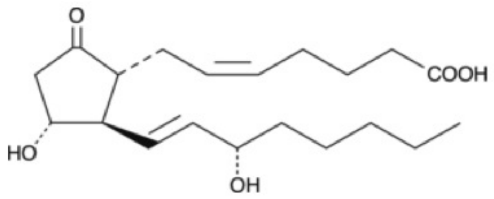

Figura 3. Estructura química de PGE_2 (11,15-Dihidroxi-9-oxo-prosta-5,13-dien-1-ácido oico) (Legler y col., 2010).

Como acabamos de comentar, la biosíntesis de PGE_2 requiere primero de la intervención de **COXs** y de las prostaglandinas E_2 sintasas (**PGES**) después. Referente a COXs se han identificado tres isoformas: COX-1, COX-2 y COX-3 (Legler y col., 2010). De estas tres isoformas, nos centraremos en COX-1 y COX-2 ya que son las más conocidas. El gen de **COX-1** se localiza en el cromosoma 9 (*q32-q33.3*) y su promotor presenta elementos de respuesta a factores de transcripción como AP-2, Sp1 y NF-IL6. La proteína COX-1 consta de 576 aminoácidos, tiene un peso molecular entre 72-74 kDa y se encuentra asociada tanto al retículo endoplásmico como a la membrana nuclear (Tanabe y Tohnai, 2002). La proteína COX-1 se expresa de forma constitutiva en prácticamente todos los tejidos, particularmente en el riñón, pulmón, estómago, intestino delgado y colon (De Long y Smith, 2005). El gen de **COX-2** se localiza en el cromosoma 1 (*q25.2-q25.3*) (Mohamadkhani y col., 2015) y su región promotora contiene diferentes elementos de respuesta para HIF, Ap-2, NF-κB, CREB, PEA3, NFAT, NF-IL6, SP1(Tanabe y Tohnai, 2002). La proteína COX-2 consta de 587 aminoácidos, tiene un peso molecular de 72-74 kDa y se localiza en la superficie interna de la

membrana nuclear, en el retículo endoplásmico y en la membrana del aparato de Golgi (Rumzhum y Ammit, 2016; Yuan y Smith, 2015). Esta enzima se induce rápidamente por la acción de diversos factores de crecimiento, promotores tumorales, hormonas, endotoxinas bacterianas y ciertas citoquinas relacionadas con la inflamación, como IL-1 y TNF-α (Rumzhum y Ammit, 2016). En condiciones basales sus niveles son prácticamente indetectables en la mayoría de los tejidos, excepto en el sistema nervioso, el riñón y la próstata (Tanabe y Tohnai, 2002).

En cuanto a **PGES**, se han identificado tres isoformas: la citosólica (cPGES) y las asociadas a membrana microsomal (mPGES-1 y mPGES-2). cPGES, está conectada funcionalmente con COX-1, se expresa de forma constitutiva en el citosol y puede translocarse a la membrana nuclear. En cambio, mPGES-2 se acopla funcionalmente a COX-1 y COX-2, y se localiza tanto en el aparato de Golgi como en el citosol. Por otra parte, mPGES-1 está conectada funcionalmente con COX-2, se localiza en la membrana nuclear y, dado que puede ser inducida por diferentes mediadores inflamatorios, se la relaciona con procesos como la propia inflamación, el dolor, la fiebre, la anorexia, la aterosclerosis, la tumorogénesis, etc. (Samuelsson y col., 2007; Hara, 2017).

Una vez sintetizada, PGE_2 se libera al medio extracelular mediante transportadores como la proteína asociada a multirresistencia de fármacos (MRP-4) (Kochel y col., 2017), por exocitosis (Kasai y col., 2016) y de manera menos eficiente, mediante difusión simple (Chan y col., 1998). A continuación, PGE_2 se une a sus receptores **EPs**, que están situados en la membrana plasmática de la propia célula o de las células vecinas (Markovič y col., 2017). Hasta la fecha se han descrito cuatro subtipos de receptores EPs: EP1, EP2, EP3 y EP4. Todos ellos pertenecen a la familia de receptores de siete dominios transmembrana acoplados a proteínas G (GPCRs). Tal y como podemos observar en la **Figura 4**, el receptor **EP1** está acoplado a la subunidad *Gq* y, a través de la fosfolipasa C (PLC), activa la proteína quinasa C (PKC) y produce un aumento del calcio citosólico. Los receptores **EP2** y **EP4** están acoplados a la subunidad G*αs* y activan la adenilato ciclasa (AC). Ello provoca un aumento de adenosína monofosfato cíclica (AMPc) y la consiguiente activación de la proteína quinasa A (PKA). Además, estos dos receptores permiten la acción de la proteína quinasa B/Akt, a través de la regulación de la vía fosfatidilinositol 3-quinasa (PI3K). Por último, el receptor **EP3** está acoplado a *Gi* y, en consecuencia, impide la activación de PKA mediante la inhibición de AC (O'Callaghan y Houston, 2015; Markovič y col., 2017).

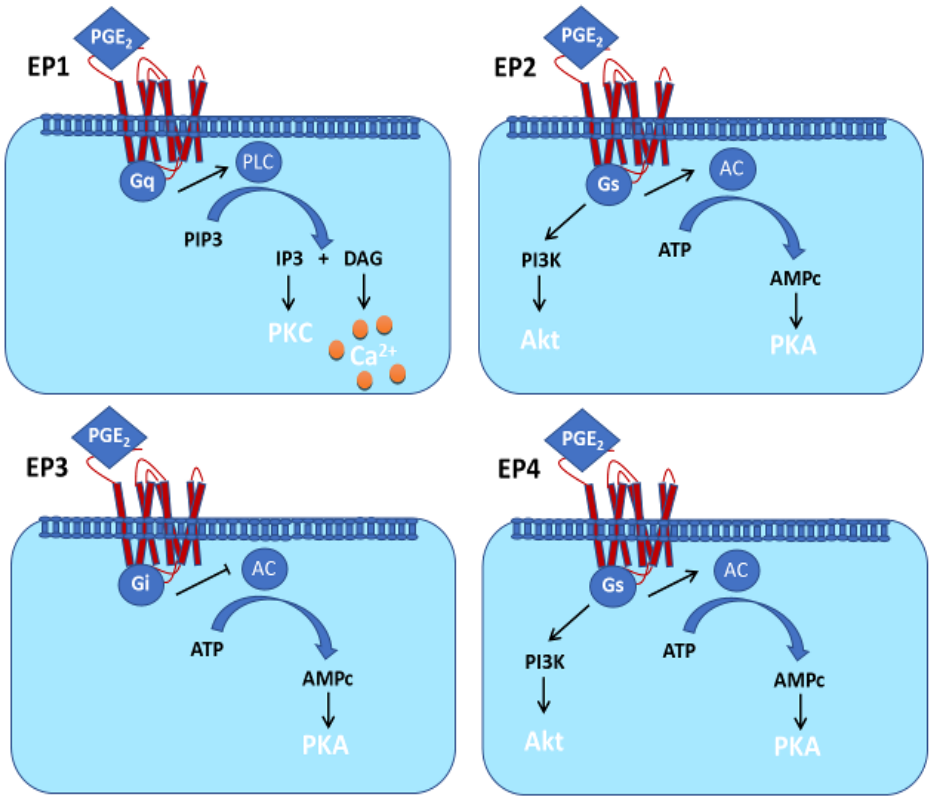

Figura 4. Vías de señalización canónicas activadas por los receptores EPs (adaptado de O'Callaghan y Houston, 2015).

La degradación de PGE_2 tras ser secretada al medio extracelular permite que su acción no se prolongue en el tiempo. El proceso de degradación consta de dos etapas: internalización de PGE_2 e inactivación intracelular. En primer lugar, PGE_2 es movilizada al interior celular por diversos transportadores, siendo el más relevante el denominado **transportador de prostaglandinas (PGT)**, que está situado en la membrana plasmática. PGT es un tipo de transportador de aniones orgánicos polipeptídicos (OATP), que se encuadra en una superfamilia de transportadores de solutos (SLC) según "The Human Genome Organization (HUGO) Gene Nomenclature Committee" (VanWert y col., 2010)[§]. PGT es una proteína con 12 dominios transmembrana con los extremos -NH_2 y -COOH terminales localizados intracelularmente y consta de 643-724 aminoácidos (Roth y col., 2012; Zhou

[§] Dentro de la superfamilia SLC, existe otro tipo de transportadores que también movilizan PGE_2 al interior celular, los transportadores de aniones orgánicos (OAT). En conjunto, los transportadores descritos hasta la fecha por su implicación en la recaptación de PGE_2 al interior celular son los siguientes: OATP1A2, OATP1B1, OATP2A1 (PGT, el que presenta mayor afinidad por PGE_2), OATP2B1 y OAT1, OAT2, OAT3, OAT4, OAT6, OAT9, OAT-PG (Roth y col., 2012).

y col., 2016). Actúa intercambiando PGE_2 extracelular por lactato, α-cetoglutarato, succinato o fumarato intracelular (Burckhardt, 2012). El sitio de unión de PGT con PGE_2 está formado por cuatro residuos (Ala_{526}, Ala_{529}, Cys_{530} y His_{533}) y se localiza en la hélice 10 (Chan y col., 1999). Además de PGE_2, PGT también transporta otros prostanoides como prostaglandina E_1 (PGE_1), prostaglandina $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandina D_2 (PGD_2), prostaglandina H_2 (PGH_2) y tromboxano A2, (Nakanishi y Tamai, 2017). El gen de PGT se encuentra localizado en el cromosoma 3 (*q21*), está formado por 14 exones y 2 intrones, y mediante *splicing* alternativo da lugar a distintos polimorfismos de la proteína final. (Chan y col., 1999). Aunque su expresión es constitutiva (Bao y col., 2002), se ha encontrado aumentada en líneas celulares de CP (Arakawa y col., 2012) y en tumores prostáticos (Wright y col., 2011).

Una vez, PGE_2 ha sido transportada al interior de la célula, comienza la segunda etapa del mecanismo de degradación: la inactivación enzimática. Para ello, PGE_2 sufre una reacción de oxidación catalizada por la enzima **15-hidroxi-prostaglandina deshidrogenasa (15-PGDH)**. El resultado es la producción de 15-ceto-PGE_2 que, a su vez, es catabolizada a 13,14 dihidro-15-ceto-PGE_2 por la 15-oxo- Δ^{13}-prostaglandina reductasa (PGR-2), tal y como podemos observar en la **figura 5** (Kochel y Fulton, 2015).

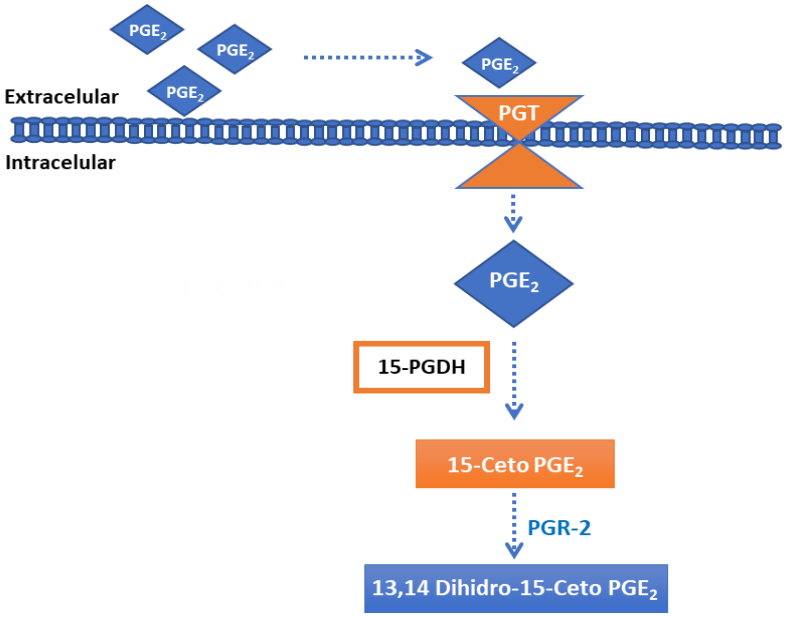

Figura 5. Internalización e inactivación enzimática de PGE_2 (adaptado de Kochel y Fulton, 2015).

Aunque se ha considerado que la internalización de PGE_2 solo tiene como objetivo finalizar su acción, algunos estudios (referenciados en la **Tabla 1**) han demostrado que determinados efectos de PGE_2 solo suceden cuando esta se encuentra en el interior de la célula, lo que sugiere que PGE_2 intracelular ($iPGE_2$) es también biológicamente activa. Es más, se ha demostrado que en la envuelta nuclear existen receptores EPs (**Tabla 1**) y las enzimas precisas para la síntesis de PGE_2: fosfolipasa A2, COXs y PGES (Helliwell y col., 2004). Ello sugiere que el núcleo de la célula podría ser una zona de síntesis de PGE_2 y de activación de receptores EPs nucleares. Los pocos estudios existentes hasta la fecha, han determinado que los receptores EPs nucleares también están acoplados a proteínas G, aunque la subunidad asociada puede ser diferente de la acoplada a los receptores EP de la membrana plasmática (Gobeil y col., 2002; O'Callaghan y Houston, 2015). Así, el receptor EP3 nuclear a modo de ejemplo, está acoplado a la subunidad G*αq*, a diferencia del receptor EP3 de membrana, que se acopla a la subunidad G*i*. Además, la unión de PGE_2 con el receptor EP3 nuclear, en lugar de disminuir AMPc (como es característico en los receptores EP3 de membrana), produce un aumento de calcio intracelular (Gobeil y col., 2002).

Tabla 1. Trabajos que demuestran efectos intracelulares de PGE_2.

Línea celular	Efectos	Cita
Células de la porción medular del túbulo colector de ratón (MCT)	Regula los niveles de NaCl	(Deane y col., 1990)
Fibroblastos de embrión de ratón (Swiss 3T3). Línea celular de riñón embrionario humano (HEK 293). Cultivo primario de células endoteliales de la microvasculatura del cerebro de cerdo	Aumenta mRNA de c-fos y el calcio	(Bhattacharya y col., 1998)
Línea celular de riñón embrionario humano (HEK 293). Cultivo primario de células endoteliales de la microvasculatura del cerebro de cerdo. Hepatocitos de rata adulta	Aumenta óxido nítrico sintasa y calcio intracelular	(Bhattacharya y col., 1999)
Cultivo primario de células endoteliales de la microvasculatura del cerebro de cerdo	Aumenta óxido nítrico sintasa, calcio, p-ERK1/2- p-AKT y NF-κB.	(Gobeil y col., 2002)
Cultivo primario de células endoteliales de la microvasculatura del cerebro de cerdo. Hepatocitos de rata adulta	Aumenta mRNA de c-fos, COX-2 y óxido nítrico	(Zhu y col., 2006)
Células de glioblastoma multiforme humano (BeGBM/ BdGBM)	Induce apoptosis vía Bax	(Lalier y col., 2007)
Línea celular de cáncer de colon humano (HCT-116 / SW1116)	Induce apoptosis vía Bax	(Lalier y col., 2011)
Línea celular del túbulo proximal renal humano (HK-2)	Aumenta la expresión de HIF-1α y VEGF	(Fernández-Martínez y col., 2012)
Células de la teca externa (TECs)	Induce proliferación	(Jia y col., 2013)
Línea celular del túbulo proximal renal humano (HK-2)	Aumenta la expresión de HIF-1α y VEGF de forma dependiente de EGFR	(Fernández-Martínez y Lucio-Cazaña, 2015b)
Línea celular de cáncer de próstata humano (PC3)	Activa fenotipos tumorales de manera dependiente de HIF-1α	(Fernández-Martínez y Lucio-Cazaña, 2015a)
Línea celular del túbulo proximal renal humano (HK-2)	Induce muerte celular por cisplatino	(Fernández-Martínez y col., 2016)
Fibroblastos dérmicos humanos (NHDFs)	Aumenta la expresión de ATF6α	(Cormenier y col., 2018)

Diferentes estudios han intentado vincular tanto a COX-2 como a PGE_2 con la patogénesis tumoral de la próstata. En relación a la patogenia de HBP se ha demostrado i) que la expresión de COX-2 se encuentra elevada (Bardan y col., 2014), ii) que la inhibición farmacológica de COX-2 puede prevenir la progresión de HBP (Ishiguro y Kawahara, 2014), iii) que la producción de PGE_2 aumenta en células de HBP (Wu y col., 2007), iv) que en células del estroma prostático PGE_2 inhibe la apoptosis, gracias al aumento de expresión de la proteína antiapoptótica Bcl-2 (Wang y col, 2004), e incrementa su proliferación (Xie y col., 2015) y contenido de estrógenos (Miao y col., 2010).

Referente al papel en CP de COX-2 (a través de la producción de PGE_2 y quizá otras PGs) se sabe que estimula la proliferación, invasión y angiogénesis. De hecho, se han detectado niveles altos de COX-2 en CP y también en otros tipos de cáncer como el de pulmón, mama y colon (Badawi, 2000; Harris, y col., 2007; Wang y Dubois, 2010). Además, se ha demostrado en modelos de CP en ratón y en pacientes con CP que la inhibición de COXs mediante NSAIDs induce la apoptosis e inhibe la proliferación y metástasis de las células cancerosas (Fernández-Martínez y col., 2009; Hussain y col., 2003). Del mismo modo, la inhibición específica de COX-2 también puede prevenir el desarrollo de CP (Bhindi y col., 2014; Lapi y col., 2016; Veitonmäki y col., 2016). Por otra parte, la expresión de COX-2 está aumentada durante todo el proceso tumorigénico, es decir, desde la fase inicial de hiperplasia prostática hasta CP metastático (Elkahwaji, 2012) y podría contribuir a la progresión del CP, ya que su expresión es más elevada en los estadios altos (T3-T4) que en los bajos (T1-T2) (Shao y col., 2012). De hecho, se ha descrito que la expresión de COX-2 se correlaciona con la malignidad del CP (Nithipatikom y col., 2002; Zhao y col., 2010) y se ha confirmado que el bloqueo de COX-2 disminuye el crecimiento del tumor prostático en modelos murinos (Liu y col., 2000; García y col., 2014; Kido y col., 2016).

Sin embargo, los estudios clínicos no han proporcionado evidencias concluyentes sobre el efecto protector de NSAIDs en CP: varios estudios no han demostrado protección (Antonarakis y col., 2009; James y col., 2012; Smith y col., 2006; Sooriakumaran y col., 2009; Vinogradova y col., 2011), en tanto que otros encontraron reducción del riesgo de CP (Doat y col., 2017; Harris y col., 2007). Más recientemente, un estudio de pre-prostatectomía sobre los efectos de celecoxib demostró que este no tuvo efecto sobre la apoptosis y los niveles de expresión del receptor de andrógenos en HBP y CP (Flamiatos y col., 2017). Sin embargo, celecoxib tampoco redujo los niveles de PGs en próstata, lo que podría explicar por qué el celecoxib (y quizá otros NSAIDs) no demuestran beneficios en la prevención o el tratamiento del CP.

Independientemente de las razones que expliquen la ausencia de evidencias concluyentes sobre los efectos beneficiosos del bloqueo de COX-2 con NSAIDs , habría que considerar otras líneas

de actuación que podrían ser más eficientes en el tratamiento del CP. Por ejemplo, las aproximaciones farmacológicas centradas en PGE_2 como principal prostanoide tumorigénico (Wang y Dubois, 2010), ya que pueden tener menos problemas de seguridad que las basadas en COX-2. Particularmente, en lo que se refiere a los inaceptables efectos adversos cardiovasculares derivados de la inhibición de COX-2, que dan lugar a la reducción de la producción y de los efectos fisiológicos de otros prostanoides (por ejemplo, prostaciclina y su acción antitrombótica) (Wang y Dubois, 2010). PGE_2 es considerada como un prostanoide prooncogénico porque puede facilitar el inicio del tumor (Chell y col., 2006) e inducir la secreción de factores de crecimiento, mediadores proinflamatorios y factores que estimulan la angiogénesis y la inflamación local (Wang y DuBois, 2013). Es más, PGE_2 puede autoperpetuar su propia producción ya que induce COX-2 en piel de ratón (Ansari y col., 2007) y en varios tipos de células cancerosas humanas como las de CP, mama y colon (Tjandrawinata y col., 1997; Yoshida y col., 2013). Finalmente, PGE_2 puede activar diferentes fenotipos tumorales en células de CP como proliferación, angiogénesis, migración, invasión y metástasis en hueso (Jain y col., 2008; Di Francesco y col., 2011; Misra y Pizzo, 2013; Vo y col., 2013; Jiang y Dingledine, 2013; Xu y col., 2014; Fernández-Martínez y Lucio-Cazaña, 2015a; Terzuoli y col., 2016; Panagiotopoulos y col., 2018; Xu y col., 2018).

2.5. Receptor del factor de crecimiento epidérmico (EGFR)

Este receptor se encuadra dentro de la subfamilia de receptores tirosina quinasa de tipo HER o ErbB. En esta subfamilia, se han descrito cuatro tipos de receptores: HER1 (**EGFR** o ErbB-1), HER2 (neu o ErbB-2), HER3 (ErbB-3) y HER4 (ErbB-4). Todos ellos son glicoproteínas transmembrana constituidas por tres dominios: el extracelular, donde se une el ligando; el dominio transmembrana y el dominio citoplasmático, que posee secuencias susceptibles de ser autofosforiladas (excepto HER3) o bien, fosforiladas por otras tirosina quinasas (Roskoski y col., 2014). EGFR y HER2 están implicados en el crecimiento y diferenciación celular y han sido muy estudiados por su relevancia en la patología tumoral (Chen y col., 2016; Mitchell y col., 2018). EGFR está localizado en la membrana plasmática (Mitchell y col., 2018) y mitocondrial (Han y Lo, 2012), pero también en el núcleo (Lin y col, 2001; Fernández-Martínez y Lucio-Cazaña, 2015b). De hecho, nuestro grupo ha estudiado el papel de EGFR en relación con el mecanismo de acción de receptores EPs nucleares activados por $iPGE_2$ (Fernández-Martínez, y Lucio-Cazaña, 2015b).

EGFR puede formar un homodímero o bien, un heterodímero con HER2 y HER3, y se autofosforila en los residuos de tirosina quinasa, lo que provoca su activación (Roskoski y col., 2014; Chen y col., 2016). Se han descrito siete ligandos diferentes con capacidad de unirse a EGFR y activarlo: factor de crecimiento epidérmico (EGF), TGF-β, factor de crecimiento ligado a heparina, anfiregulina, epiregulina, β celulina y neuregulina. Todos ellos pueden encontrarse o bien de manera soluble en el medio extracelular y activar directamente EGFR, o como precursores anclados a la membrana plasmática y activar EGFR tras ser liberados por la metaloproteasa ADAM (transactivación dependiente de ligando) (**Figura 6**) (Pai y col., 2002; Tveteraas y col., 2012; Bazzani y col 2017; Sun y Li, 2018). La activación indirecta o *transactivación* de EGFR también puede suceder de forma independiente de ligando, como sucede cuando EGFR se activa por receptores EPs (Battersby y col., 2007; Donnini y col., 2007; Chun y col., 2010; Fernández-Martínez, y Lucio-Cazaña, 2015b) u otros receptores GPCRs activados por sus ligandos específicos. En el caso de receptores EPs, la transactivación de EGFR puede venir mediada por arrestinas, AMPc o Src (George y col., 2013) (**Figura 6**).

Tras su activación, EGFR activa diferentes rutas de señalización (**Figura 6**) como las que implican proteínas quinasas activadas por mitógenos (MAPK), fosfoinositol 3-quinasas (PI3K), miembros de la familia STAT de factores de transcripción y fosfolipasa Cγ1 (PLCγ1). Dichas rutas de señalización promueven la transcripción de genes implicados en la activación de fenotipos como

la angiogénesis, proliferación, migración, adhesión y diferenciación celular (George y col., 2013; Roskoski y col., 2014; Chen y col., 2016; Mitchell y col., 2018).

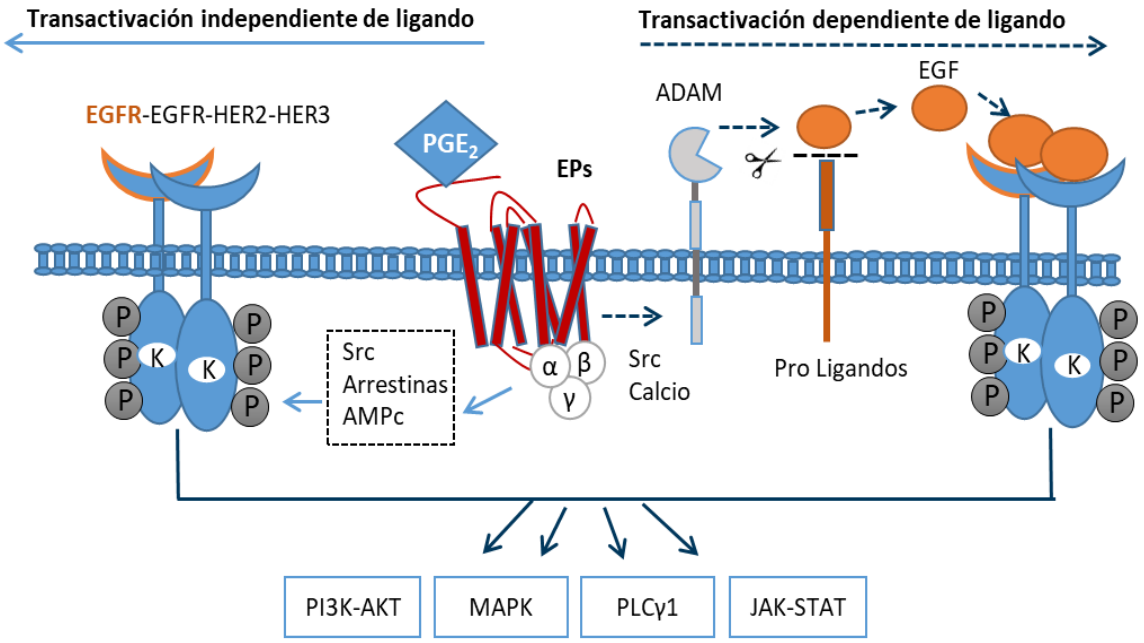

Figura 6. Esquema de la transactivación del receptor EGFR por receptores EPs (adaptado de George y col., 2013).

Aunque en la próstata humana sana se ha demostrado la presencia de los 4 tipos de receptores HER (Poncet y col., 2011), es EGFR quien parece tener un papel fundamental en el crecimiento y desarrollo de la próstata, así como en la patogenia de HBP (Hennenberg y col., 2015; Silva y col., 2016), y del CP (Day y col., 2017). Además, la sobreexpresión de EGFR en CP se ha asociado con una mayor resistencia a la quimioterapia (Hour y col., 2015), con una alta puntuación Gleason[††], con elevados niveles del PSA y con una mayor probabilidad de progresión tumoral hacia el estadio resistente a castración (Traish y Morgentaler, 2009). Estos efectos se han vinculado a la activación de diferentes vías de señalización (MAPK, PI3K, PLCγ1 y STAT) implicadas en la proliferación, migración, invasión celular y la metástasis de las células de CP (Xu y col., 2016; Day y col., 2017; Mandel y col., 2018). En este sentido, nuestro grupo de investigación ha demostrado que el factor

[††] La clasificación de Gleason es un sistema que determina el nivel de diferenciación histológica de las células del CP, con el fin de evaluar el grado de agresividad de la enfermedad (Gleason, 1992).

inducible por hipoxia 1α (HIF-1α) también tiene un papel relevante en los efectos de la transactivación de EGFR (inducida por receptores EPs activados por PGE_2) sobre los fenotipos tumorales mencionados (Fernández-Martínez y Lucio-Cazaña, 2015a).

2.6. Factor inducible por hipoxia (HIF)

La hipoxia es un descenso de los niveles de O_2 que puede producirse en todas las células del organismo (hipoxia sistémica) o bien únicamente en áreas localizadas (hipoxia local). Esta última sucede en diversos estados fisiológicos o patológicos (vasoconstricción arterial por el frio, focos inflamatorios, tumores, etc.) (Ward, 2008). Los niveles de O_2 en la próstata se reducen desde un 3,9% de O_2 en condiciones fisiológicas[‡‡] hasta un 0,3% en el caso del CP (Movsas y col., 2001), lo que aumenta la actividad del factor de transcripción HIF en las células tumorales. HIF estimula entonces diferentes mecanismos adaptativos de gran importancia para la biología del tumor, contribuyendo así a la progresión de las neoplasias prostáticas benignas y malignas (Wu y col., 2016; Huang y col., 2018).

HIF regula la expresión de genes implicados en el restablecimiento del aporte de O_2 y la adaptación al metabolismo anaerobio, lo que promociona la supervivencia celular en situaciones de hipoxia (Kietzmann y col., 2016). HIF es un heterodímero formado por una subunidad β (91-94 kDa) y otra α (120 kDa). La subunidad β se expresa de forma constitutiva y es independiente de la disponibilidad de O_2 en las células (Masoud y col., 2015). La subunidad α se regula por hipoxia y por otros estímulos, presenta tres isoformas: HIF-1α, HIF-2α y HIF-3α. HIF-1α se expresa de forma ubicua en los tejidos humanos (Masoud y col., 2015), a diferencia de HIF-2α, que se expresa únicamente en el cuerpo carotideo, pulmón, endotelio, riñón, cerebro, intestino, hígado, tracto gastrointestinal, páncreas y corazón (Mimeault y Batra, 2013). Ambas isoformas comparten la misma función, que es generar múltiples respuestas fisiológicas ante una situación de hipoxia. Por eso,

[‡‡] En una situación de normoxia, en cada respiración nos llega un 13,6% de O_2 (104 mmHg) a los alvéolos (frente al 20,84% de O_2 del aire atmosférico). Esta reducción de los niveles de O_2 se debe a cuatro razones: 1) El aire alveolar es sustituido solo de manera parcial por aire atmosférico en cada respiración. 2) El O_2 se absorbe constantemente hacia la sangre pulmonar. 3) El CO_2 está difundiendo desde la sangre pulmonar hacia los alvéolos y disminuye la presión parcial de O_2 atmosférica. 4) El aire atmosférico seco que entra en las vías aéreas es humidificado por vapor de agua, lo que también disminuye la presión parcial del O_2. Desde los alvéolos pulmonares el O_2 pasa a los capilares pulmonares por difusión. La sangre pulmonar contiene un 13,06-10,46% de O_2 (100-80 mmHg), pero dicha concentración se reduce cuando llega a los tejidos (Guyton y Hall, 2016). De esta manera, en la próstata hay solo 3,9% O_2 (Movsas y col., 2001).

algunos procesos como la inducción del factor de crecimiento endotelial vascular (VEGF), están regulados indistintamente por HIF-1α y HIF-2α (Hu y col., 2003). Pero otros procesos dependen específicamente de una de las isoformas: por ejemplo, la producción de eritropoyetina está regulada por HIF-2*α* (Rankin y col., 2007) y la transcripción de algunas enzimas del metabolismo de la glucosa depende únicamente de HIF-1α (Hu y col., 2003). Referente a la subunidad HIF-3α, se han descrito variantes de *splicing* alternativo y sus niveles también se regulan por hipoxia. Esta isoforma genera respuestas fisiológicas diferentes a las descritas para HIF-1α y HIF-2α. Una de las variantes más estudiada de HIF-3α es la proteína inhibitoria del dominio PAS (IPAS), que funciona como regulador negativo de la actividad de HIF-1α y HIF-2α, al impedir la interacción de estas isoformas con sus elementos de respuesta (Duan, 2016). De esta manera inhibe el efecto de HIF-1α y HIF-2α sobre la transcripción de los genes.

HIF-1α es la subunidad α más estudiada y la que mayor relevancia tiene en patología proliferativa prostática, por lo que nos centraremos en ella. Se produce de forma constitutiva y se degrada de forma independiente de O_2 -mediante las calpaínas citosólicas (dependiente de calcio) o las catepsinas lisosomales (Zhou y col., 2006; Olmos y col., 2009)- o bien, de forma dependiente de O_2 a través del proteasoma[§§] (Kietzmann y col., 2016). Esta última, es la vía canónica de degradación de HIF-1α en presencia de concentraciones fisiológicas de O_2. Dicha vía comienza con la acción de las prolil-hidroxilasas (PHD1, PHD2 y PHD3) que, en presencia de O_2, 2-oxoglutarato (OG) y hierro (Fe^{2+}), tienen la capacidad de hidroxilar dos residuos de prolina (en las posiciones 402 y 564) (Masoud y col., 2015 y Koyasu y col., 2018). A continuación se produce la unión entre HIF-1α hidroxilado y el complejo von Hippel-Lindau-E3 ubiquitina ligasa (VHL-E3), con lo que HIF-1α queda marcado para su posterior degradación en el proteasoma como se puede apreciar en la **Figura 7** (Masoud y col., 2015 y Koyasu y col., 2018).

[§§] HIF-1α también puede degradarse en el proteasoma en hipoxia si no logra estabilizarse mediante su unión a la chaperona HSP90 (Isaacs y col., 2002).

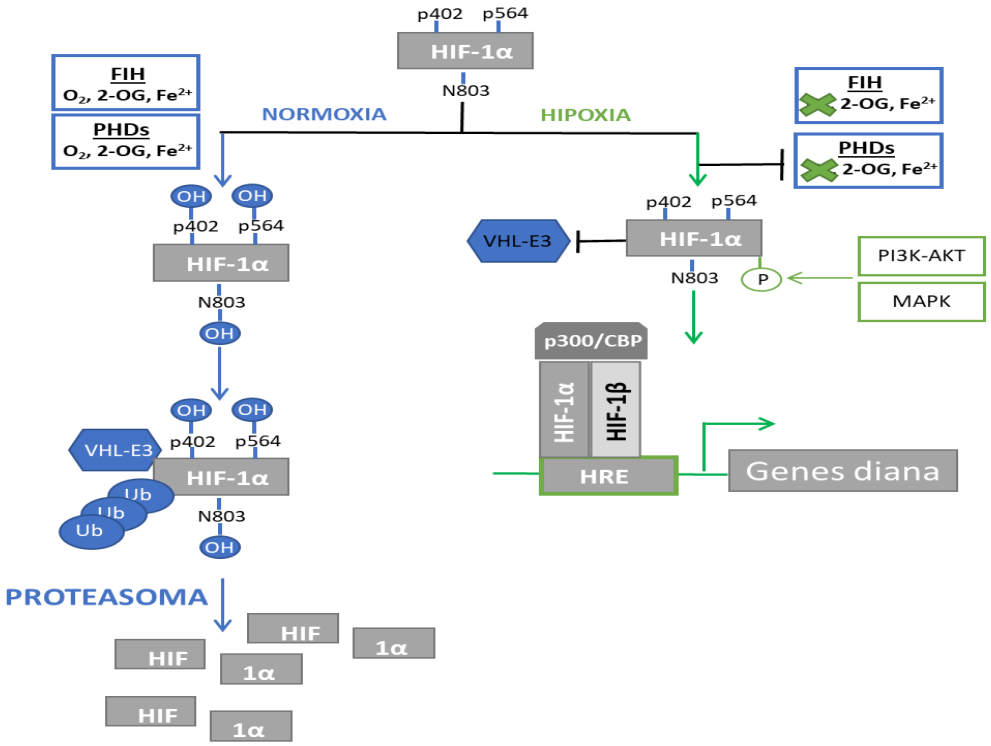

Figura 7. Ruta canónica de la regulación de HIF-1α (adaptado de Ke y Costa, 2006).

En una situación de hipoxia, la disponibilidad insuficiente de O_2 impide a PHDs hidroxilar HIF-1α, lo que provoca que se acumule en el citoplasma y que posteriormente se transloque al núcleo. La heterodimerización con la subunidad HIF-β da lugar al factor de transcripción completo, que se une a sus elementos de respuesta a hipoxia (HRE) (**Figura 7**) situados en los promotores de los genes diana. A continuación, se recluta el coactivador p300/CBP (gracias a que el factor inhibidor de HIF (FIH)*** tampoco puede hidroxilar a HIF-1α por la ausencia de O_2) y se produce la transcripción génica. Los genes diana de HIF están relacionados con angiogénesis, crecimiento, diferenciación y supervivencia celular, respuesta inmune, metabolismo de la glucosa, invasión y metástasis (Wigerup y col., 2016).

Aunque la síntesis de HIF-1α es constitutiva y relativamente independiente de la cantidad de O_2, sus niveles pueden aumentar a nivel transcripcional o traduccional por diferentes estímulos fisiológicos o patológicos, como factores de crecimiento (Richard y col., 2000), hormonas (Zhou y

*** FIH es una enzima dependiente de 2-oxoglutarato (OG), hierro y O_2 que impide la unión del coactivador p300/CBP con HIF-1α en una situación de normoxia mediante la hidroxilación del residuo de aspargina 803 en el dominio C-TAD de HIF-1α (Wigerup y col., 2016 y Koyasu y col., 2018).

Brüne, 2006), mediadores inflamatorios, como citoquinas y PGE_2 (Zhou y Brüne, 2006; Fernández-Martínez y Lucio-Cazaña, 2015a), etc. Así pues, dichos estímulos provocan que HIF-1α se acumule en normoxia pese a que su degradación proteasomal (dependiente de O_2) esté activa.

En tumores sólidos el insuficiente suministro de O_2 que llega a las células que proliferan sin control hace que la presencia de hipoxia sea relativamente frecuente (Huang y col., 2018). Los niveles de O_2 en CP pueden llegar a reducirse hasta un 0,3% (Movsas y col., 2001), lo que promueve el aumento de la expresión de HIF-1α (Huang y col., 2018). Además, en líneas celulares de CP y en biopsias de tejido prostático de pacientes con CP se ha observado un incremento de la expresión de HIF-1α (Ranasinghe y col., 2013; Huang y col., 2018). La relevancia patogénica en CP del aumento de HIF-1α queda de manifiesto por dos hechos: i) la hipoxia del tumor se correlaciona con un mal pronóstico (Stewart y col., 2010) y ii) la oxigenoterapia[†††] en líneas celulares de CP produce un descenso de la proliferación celular y un aumento de la sensibilidad a los tratamientos con determinados agentes antitumorales como el taxol (Moen y Stuhr, 2012). HIF-1α promueve la proliferación, la migración, la invasión y la supervivencia de las células de CP, así como estimula la angiogénesis[‡‡‡], (Ranasinghe y col., 2013; Huang y col., 2018). Estos efectos se deben a la acción reguladora de este factor de transcripción sobre la expresión de numerosos genes que contribuyen a la progresión de la enfermedad (Mimeault y Batra, 2013). De hecho, HIF-1α se ha llegado a considerar un biomarcador de lesiones premalignas de la próstata. De igual manera, se ha demostrado que HIF-1α participa en la supervivencia y la proliferación de las células prostáticas de pacientes con HBP y que se correlaciona con el peso de la próstata de dichos pacientes (Wu y col., 2016). Además, en un modelo *in vivo* de rata con HBP y en modelos *in vitro*, se ha observado que existe una correlación entre los niveles altos de expresión de HIF-1α con la proliferación celular y la transición epitelio-mesénquima. Dicho proceso es clave en el crecimiento anormal de la próstata (Kim y col., 2013; Chen y col., 2019).

[†††] La oxigenoterapia disminuye la proliferación celular mediante una degradación del DNA y una parada del ciclo celular de las células CP (Lu y col., 2019).

[‡‡‡] La angiogénesis o neovascularización se define como la formación de nuevos vasos sanguíneos a partir de la vasculatura preexistente. Está regulada por factores anti-angiogénicos (trombospondina-1, IL-12 y angiostatina, metaloproteinasas inhibidoras del tejido) y por factores pro-angiogénicos (EGF, PGE_2, IL-6, IL-8, IL-17, angiopoyetina, HIF-1α, VEGF) (Kazerounian y Lawler, 2018).

En relación con la presente tesis doctoral, nuestro grupo de investigación ha demostrado que: i) PGE_2, a través de receptores intracelulares, aumenta la expresión de HIF-1α en diferentes líneas celulares (células HK-2 del túbulo proximal renal, células A498 de carcinoma renal y células LNCaP de CP (Fernández-Martínez y col., 2012), ii) PGE_2, a través del receptor EP2 localizado en membrana nuclear, aumenta la expresión de HIF-1α en células HK-2 de forma dependiente de la transactivación del EGFR (Fernández-Martínez y Lucio-Cazaña, 2015b) y iii) este mecanismo es responsable en células PC3 de CP de la potenciación inducida por PGE_2 sobre los fenotipos tumorales como proliferación y capacidad de migración, invasión y angiogénesis (Fernández-Martínez y Lucio-Cazaña, 2015a).

2.7. Factor de crecimiento endotelial vascular (VEGF)

La familia del VEGF está constituida por varios miembros: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F y el factor de crecimiento placentario (PLGF) (Takahashi y Shibuya, 2005 y Kazerounian y Lawler, 2018). El miembro más estudiado de los VEGFs es **VEGF-A**, cuya expresión está regulada por factores de transcripción, como HIF-1α y NF-*kB* (Liao y Johnson, 2007), así como por diferentes estímulos angiogénicos, incluyendo EGF (Van Cruijsen y col., 2005) y diversos mediadores inflamatorios como el óxido nítrico, PGE_2, IL-17, etc., (Kimura y col., 2000; Fernández-Martínez y Lucio-Cazaña, 2015; Pan y col., 2015). Hasta la fecha, se han identificado 12 isoformas[§§§] por *splicing* alternativo (Canavese y col., 2017) cuya actividad biológica está mediada principalmente por dos receptores con actividad tirosina quinasa, VEGFR-1, y el VEGFR-2. Ambos se caracterizan por tener siete dominios extracelulares, una región transmembrana y otro dominio tirosina quinasa intracelular (Takahashi y Shibuya, 2005 y Canavese y col., 2017). Aunque VEGF-A tiene mayor afinidad por VEGFR-1, se considera que VEGFR-2 es el principal mediador de sus efectos, ya que tiene mayor capacidad para activar diferentes rutas de señalización como son MAPK, PI3K y PLCγ1 (Takahashi y Shibuya, 2005).

Las células de la próstata normal humana producen VEGF-A (Kollermann y Helpap, 2001), que contribuye a su crecimiento y supervivencia. En biopsias de tejido prostático inflamado de pacientes con HBP y de pacientes con CP se ha demostrado que hay un incremento en los niveles de VEGF-A (Kervancioglu y col., 2016). VEGF-A parece jugar un papel muy importante en el inicio y progresión del CP, ya que activa diferentes vías de señalización (MAPK, PI3K y PLCγ1) que inducen

[§§§] Las 12 isoformas del VEGF son $VEGF_{111}$, $VEGF_{121}$, $VEGF_{121b}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{162}$, $VEGF_{165}$, $VEGF_{165b}$, $VEGF_{183}$, $VEGF_{189}$, $VEGF_{189b}$ y $VEGF_{206}$ (Canavese y col., 2017).

la expresión de distintos factores de crecimiento, citoquinas y metaloproteasas. Estos mediadores biológicos están implicados tanto en la angiogénesis como en la proliferación, migración, invasión celular y la metástasis; es más, una vez colonizado el hueso, VEGF-A sigue estimulando el crecimiento del tumor, y es por ello que la sobreexpresión de VEGF-A se correlaciona con alta puntuación Gleason, supervivencia de las células del CP, metástasis y destrucción ósea (Roberts y col., 2013). Nuestro grupo ha demostrado que PGE_2, vía iEP2/EGFR/HIF-1α, aumenta la producción de VEGF en células de CP. En este contexto, no es sorprendente que inhibidores de VEGF-A como bevacizumab, sunitinib, sorafenib, se utilicen en el tratamiento del CP (Kluetz y col., 2010 y Small y Oh, 2012), ofreciendo resultados muy prometedores cuando se combinan con la terapia hormonal (Cereda y col., 2018).

Hipótesis y objetivos

3. Hipótesis y objetivos

3.1. Hipótesis

Como hemos visto en la introducción, se acepta que PGE_2 ejerce sus efectos activando receptores EPs localizados en membrana plasmática y que, a continuación, ingresa en las células mediante el transportador de prostaglandinas PGT, lo que da lugar a su degradación enzimática. Sin embargo, un reducido número de trabajos han demostrado que PGE_2 intracelular ($iPGE_2$) también es biológicamente activa. Por otra parte, también hemos citado numerosos trabajos que apoyan la importancia de PGE_2, COXs (que no solo median la síntesis de PGE_2, sino que podrían ser también inducidas por PGE_2), EGFR y HIF-1α en la progresión de las patologías proliferativas prostáticas. Todos los productos biológicos mencionados podrían estar relacionados, ya que $iPGE_2$, a través de la transactivación de EGFR por receptores EPs, regula la expresión de HIF-1α en células de CP[*].

Teniendo en cuenta lo anteriormente expuesto, en el presente trabajo planteamos como hipótesis que la vía COXs/$iPGE_2$/receptores EPs intracelulares puede contribuir a la estimulación por PGE_2 de diversos fenotipos tumorales prostáticos y que, por tanto, el transportador de prostaglandinas PGT, tiene un papel clave en este contexto. Así mismo, hipotetizamos que la transactivación de EGFR por receptores EPs y la activación de HIF-1α (dependiente de EGFR) son fenómenos clave en el mecanismo de acción protumoral de $iPGE_2$.

3.2. Objetivos

En relación con esta hipótesis, trabajaremos con la línea celular RWPE-1 de células epiteliales de próstata humana y la línea PC3 de células de CP resistente a castración para determinar, como objetivo general, el papel de PGE_2 intracelular en la progresión de la hiperplasia benigna de próstata y del cáncer de próstata, respectivamente. Este objetivo general se desglosa en los siguientes objetivos específicos:

1) Estudiar el efecto de PGE_2 sobre diversos fenotipos tumorales prostáticos benignos y/o malignos tales como proliferación, adhesión, migración, invasión celular y angiogénesis.

[*] En el momento del comienzo de la presente tesis nuestro grupo había demostrado este mecanismo en células PC3, pero no se publicó hasta 2015 (Fernández-Martínez y Lucio Cazaña, 2015a).

2) Determinar el papel de los receptores EPs como mediadores de los efectos de PGE_2 sobre los fenotipos tumorales mencionados, así como analizar la posible presencia y participación de receptores EPs intracelulares en dichos efectos.

3) Valorar el efecto de la inhibición de PGT (es decir, el bloqueo del transporte de PGE_2 al interior celular) sobre los fenotipos tumorales constitutivos o estimulados por PGE_2 exógena.

4) Analizar el papel de la vía $iPGE_2$/receptores EPs intracelulares/EGFR/HIF-1α en la estimulación por PGE_2 de los fenotipos tumorales benignos y malignos estudiados.

5) Valorar la posible inducción de COXs por $iPGE_2$ y, en su caso, analizar las vías de señalización implicadas y el papel de dicho efecto como regulador de los fenotipos tumorales malignos estudiados (bien sean constitutivos o estimulados por PGE_2 exógena).

Resultados

The International Journal of Biochemistry & Cell Biology 59 (2015) 52–61

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Role of intracellular prostaglandin E_2 in cancer-related phenotypes in PC3 cells

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ARTICLE INFO

Article history:
Received 12 May 2014
Received in revised form 20 October 2014
Accepted 3 November 2014
Available online 11 November 2014

Keywords:
Intracellular prostaglandin E2
Prostate cancer
EP receptor
PC3 cells
Tumor phenotypes

ABSTRACT

Prostaglandin E_2 (PGE_2) and hypoxia-inducible factor-1α (HIF-1α) affect many mechanisms that have been shown to play a role in prostate cancer. In PGE_2-treated LNCaP cells, up-regulation of HIF-1α requires the internalization of PGE_2, which is in sharp contrast with the generally accepted view that PGE_2 acts through EP receptors located at the cell membrane. Here we aimed to study in androgen-independent PC3 cells the role of intracellular PGE_2 in several events linked to prostate cancer progression. To this end, we used bromocresol green, an inhibitor of prostaglandin uptake that blocked the immediate rise in intracellular immunoreactive PGE_2 following treatment with 16,16-dimethyl-PGE_2. Bromocresol green prevented the stimulatory effect of 16,16-dimethyl-PGE on cell proliferation, adhesion, migration and invasion and on HIF-1α expression and activity, the latter assessed as the HIF-dependent activation of (i) a hypoxia response element-luciferase plasmid construct, (ii) production of angiogenic factor vascular endothelial growth factor-A and (iii) in vitro angiogenesis. The basal phenotype of PC3 cells was also affected by bromocresol green, that substantially lowered expression of HIF-1α, production of vascular endothelial growth factor-A and cell proliferation. These results, and the fact that we found functional intracellular EP receptors in PC3 cells, suggest that PGE_2-dependent intracrine mechanisms play a role in prostate cancer Therefore, inhibition of the prostaglandin uptake transporter might be a novel therapeutic approach for the treatment of prostate cancer.

1. Introduction

Prostate cancer (PC) is the second most common cancer in men worldwide and continues to impose a significant disease burden and a growing healthcare problem. However, our understanding of the mechanisms that contribute to the development of PC is still limited (Ellem and Risbridger, 2007). Epidemiological, histopathological and molecular pathological studies have been providing emerging evidence implicating inflammation in the pathogenesis of PC (Palapattu et al., 2005; De Marzo et al., 2004; Nelson et al., 2003; Platz and De Marzo, 2004; Lucia and Torkko, 2004).

Prostaglandins are a family of lipid mediators, which are involved in diverse physiological and pathological processes, including inflammation, pain, renal function, blood pressure and platelet function, as well as cancer progression (Smyth et al., 2009). Prostaglandins are produced from arachidonic acid after its liberation from membrane phospholipids by phospholipase A2. The rate limiting enzymes in the subsequent biosynthesis pathway are the cyclooxygenases COX-1 and COX-2, which are inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) (Funk, 2001). Among the prostaglandins, prostaglandin E_2 (PGE_2) is the most abundant, and it is the predominant tumor promoting prostaglandin, found at high levels in various tumor types including colon, lung, breast and prostate cancer (Badawi, 2000; Wang and Dubois, 2010). PGE_2 affects many mechanisms that have been shown to play a role in carcinogenesis such as proliferation, apoptosis, migration, invasion and angiogenesis (Greenhough et al., 2009). These PGE_2 effects have been also particularly documented in PC cells (Jiang and Dingledine, 2013; Jain et al., 2008; Vo et al., 2013), inhibition of cyclooxygenase (and thereby of PGE_2 production) with NSAIDs in experimental PC inhibits cell proliferation, induce apoptosis and decreases metastasis (Hussain et al., 2003; Fernández-Martínez et al., 2009).

PGE_2 mediates its effects via four distinct receptors EP1–EP4, which belong to the family of G-protein coupled receptors. EP2, EP3 and EP4 receptors have been demonstrated to play a role in human PC (Huang et al., 2013) as well as in the proliferation, migration and angiogenic ability of PC cells (Jiang and Dingledine, 2013; Jain et al., 2008; Huang et al., 2013). We have previously shown that

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http://dx.doi.org/10.1016/j.biocel.2014.11.004
1357-2725/

PGE_2 increases the expression of hypoxia-inducible factor-1α (HIF-1α) in androgen-dependent human prostate cancer cells LNCaP (Fernández-Martínez et al., 2012a). HIF-1 is a heterodimer transcription factor consisting of a constitutively expressed β subunit and a α subunit. HIF-1α plays a relevant role among the mechanisms through which PGE_2 contributes to the development of PC: the expression and activity of HIF-1 (which has been shown to contribute to metastasis and chemo-resistance of castrate-resistant PC and to promote in PC cells migration, invasion, angiogenesis and chemoresistance (Dai et al., 2011; Ranasinghe et al., 2013)) increases upon treatment with PGE_2 in PC cells (Liu et al., 2002a). Furthermore, treatment with NSAIDs reduces the levels of HIF-1α in PC cells (Palayoor et al., 2003). As a result, there is a decrease in the levels of vascular endothelial growth factor (VEGF) (Palayoor et al., 2003), which is a HIF-regulated protein and the major growth factor that regulates angiogenesis and tumor growth (Liao and Johnson, 2007).

As stated above, we have found that PGE_2 up-regulates HIF-1α in LNCaP PC cells, but also in human renal cell carcinoma A498 cells and human renal proximal tubular HK-2 cells (Fernández-Martínez et al., 2012a). Strikingly, the inhibition of PGE_2 uptake with the inhibitor of prostaglandin transporter bromocresol green (BG) abolished PGE_2-induced HIF-1α up-regulation (Fernández-Martínez et al., 2012a), which indicated that only PGE_2 located inside the cell (i.e. intracellular PGE_2 or $iPGE_2$) was able to increase the expression of HIF-1α. This effect was probably mediated by a set of EP receptors located intracellularly, since further studies in HK-2 cells indicated that PGE_2-induced HIF-1α up-regulation was blocked by antagonists of EP receptors (Fernández-Martínez et al., 2012a).

Taking into account this background, in the present work we aimed to study in androgen-independent PC3 cells (a model of lethal castrate-resistant PC) the role of $iPGE_2$ in the expression and activity of HIF-1α and in cell proliferation and migration/invasion under both basal conditions and after stimulation with PGE_2 analogue 16,16-dimethyl-PGE_2 (16,16-PGE_2)

2. Materials and methods

2.1. Reagents and antibodies

16,16-PGE_2 and diclofenac were acquired from Cayman Chemical (Ann Arbor, MI). Bromocresol green (BG), bromosulfophthalein (BS), YC-1, type I-collagen and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) were purchased from Sigma (St. Louis, MO). Antibodies were from obtained from the following sources: anti-PGE_2 antibody from Abcam (Cambridge, UK), anti-HIF-1α antibody from BD Biosciences (Palo Alto, CA), anti-EP2 and anti-EP4 antibodies from Cayman Chemical (Ann Arbor, MI), anti-β-actin and rabbit anti-mouse IgG peroxidasa conjugate from Sigma (St. Louis, MO), α-mouse-Alexa-Fluor® 633 and α-rabbit-Alexa-Fluor® 488 Invitrogen (Eugene, Oregon USA).

2.2. Cell culture

The human prostate cancer cell line PC3 was from purchased to American Type Culture Collection (Manassas, VA). Cells were grown in sterile conditions and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/amphoterycin B from Life Technologies (Barcelona, Spain). The human umbilical vein endothelial cells (HUVECs) were purchased from ATCC and cultured in endothelial basal medium supplemented with VEGF, EGF, FGF basic, IGF-1, L-glutamine, heparin sulfate, hydrocortisone and fetal bovine serum (ATCC). The culture was performed in a humidified 5% CO_2 environment at 37 °C. The culture medium was changed every 3 days.

2.3. Western blot analysis

PC3 cells were split into six-well plates at a density of 3×10^5 cells/well and incubated for 24 h before treatment. Afterwards immunoblotting was performed essentially as described previously (Fernández-Martínez et al., 2011). In short, cell lysates were prepared and measured for protein content using the Bradford assay. Approximately 30 μg of protein was electrophoresed 10% SDS-polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes. Membranes were incubated overnight at 4 °C with anti-HIF-1α antibody (1:1000 dilution) and then for 1 h at room temperature with the horseradish peroxidase-conjugated secondary antibody (1:4000 dilution). To ensure equal loading of proteins, the membranes were stripped and re-probed with anti-β-actin antibody. The signals were detected with enhanced chemiluminescence reagent (Amersham Healthcare, Buckinghamshire, England).

2.4. Hypoxia response element (HRE) reporter gene assay

Cells were split into six-well plates at a density of 3×10^5 cells/well. 24 h before later, the cells were cotransfected with 1 μg/well of p9HIF1-Luc firefly luciferase reporter plasmid (it contains nine copies of the human HIF binding sequence located between positions −985 and −951 of the 5′ human VEGF gene promoter (Aragonés et al., 2001) and was generously gifted by Dr. Manuel Ortiz de Landázuri, (Service of Immunology, Hospital Princesa, Madrid, Spain)) and 0.5 μg/well of the *R. reniformis* luciferase reporter pRL-CMV using lipofectamine (Invitrogen, CA). Transfected cells were next incubated with complete growth medium for 24 h, and then they were treated as indicated in the results section. Afterwards cells were harvested and luciferase activity was measured using the dual-luciferase reporter assay system (Promega, Madison, WI). The data were normalized against the *R. reniformis* luciferase activity.

2.5. Determination of VEGF secretion

PC3 cells were seeded in 24 well plates (5×10^4 cells/well), grown for 24 h and treated as indicated in the Results section. Then, the medium containing the secreted VEGF was removed and kept at −80 °C until analyzed. Afterwards VEGF was measured essentially as described previously (Fernández-Martínez et al., 2011) using the human VEGF DuoSet (R&D Systems, Minneapolis, MN, USA) following the manufacturer's protocol

2.6. In vitro vascular endothelial cell tube formation assay

PC3 cells were seeded in 24 well plates (5×10^4 cells/well), grown for 24 h and treated in triplicate as indicated in the results section. Then, the medium containing the secreted VEGF was used for the assay. To this end, a 96-well-plate was coated with 40 μl/well Matrigel (BD Bioscience) and allowed to polymerize for 2 h at 37 °C and 5% CO_2 a humidified atmosphere. Then human umbilical vein endothelial cells were resuspended in the medium that had been previously collected from PC3 cells (see above). Afterwards, endothelial cells were seeded on top of the gel (25×10^3 cells/well) and incubated for 3 h to allow the formation of tubes. Microphotographs were then taken and tube formation was quantified by counting the number of meshes using ImageJ software (NIH, Bethesda).

2.7. Immunofluorescence analysis

PC3 cells (10^4 cells/glass coverslip) were treated as indicated in the results section. Then, they were fixed with 2% paraformaldehyde for 10 min, permeabilized with 0.1% (v/v) Triton X-100 in PBS for 10 min, washed with PBS, blocked with 4% bovine serum albumin (BSA) for 1 h at room temperature and incubated overnight at 4 °C with anti-HIF-1α (1:100 dilution), anti-PGE_2 (1:50 dilution) and anti-EP 2 or anti-EP 4 (1:500 dilution) antibodies. Cells were then incubated at 37 °C with α-mouse-Alexa-Fluor® 633 (1:2000) and α-rabbit-Alexa-Fluor® 488 (1:2000) for 1 h in the darkness. Coverslips were then washed and mounted with ProLong Gold antifade reagent with DAPI (Invitrogen Eugene, Oregon). Detection was performed by confocal laser scan microscopy LEICA TCS-SL (Heidelberg, Germany).

2.8. MTT assay

Cell proliferation was determined using a colorimetric assay with MTT. The MTT assay measures the conversion of MTT to insoluble formazan by dehydrogenase enzymes of the intact mitochondria of living cells. Cells were plated in 96-well plates (10^4 cells/well) and maintained in serum free RPMI-1640 for 24 h before being treated as indicated in the results section. Afterwards cell proliferation was evaluated by measuring the conversion of the tetrazolium salt MTT to formazan crystals. Briefly, 0.1 mg/ml MTT was added to the cells and incubated for 3 h at 37 °C. The medium was removed and 100 μl of dimethyl sulfoxide was added to dissolve the formazan precipitates. The amount of formazan crystals formed correlates directly with the number of viable cells. The reaction product was quantified by measuring the absorbance at 570–630 nm using an ELISA plate reader. Results were expressed as percentage of the control (control equals 100%)

2.9. Cell adhesion assay

The cell adhesion to collagen was evaluated as follows: cultured cells were detached by trypsinization, resuspended in serum-free RPMI-1640 medium (2.5×10^5 cells/ml) and treated as stated in the results section. Then cells were plated in 96-well plates precoated with 0.1 ml of 0.0167 g/100 ml type-I collagen and incubated at 37 °C for up to 2 h. The non-adherent cells were washed out with PBS, and the number of cells that adhered to collagen was assessed by MTT assay. The independent experiments were run in three times.

2.10. Cell migration and invasion assays

Cell migration was determined using both wound-healing and transwell assay. For the wound-healing migration assay, PC3 cells were seeded in 24-well plates (3×10^5 cells per well). At 24 h after seeding, the monolayer cells were manually scratched with a pipette yellow tip to create extended and definite scratches in the center of the dishes with a bright and clear field (~2 mm). Cells were treated as indicated in the results section and the narrowing of the wound by migrating cells was monitored by measuring the width in microphotographs. Three representative fields of each monolayer wound were captured using a Nikon Diaphot 300 inverted microscopy camera (20×) up to 48 h. Monolayer wound areas of untreated samples were averaged and assigned a value of 100.

For the transwell migration and invasion assays, we used transwell polycarbonate filters (8-μm pore size, Corning Costar, Cambridge, UK), which were coated with 50 μl of Matrigel Basement Membrane Matrix (BD Biosciences Bedford, MA, 1:10 dilution with serum free media) only for the invasion assay. Cells were harvested and resuspended in serum-free RPMI-1640 medium at a concentration of 5×10^5 cells/ml. Cells in suspension were treated as indicated in the results section and then 0.2 ml of cell suspension and 0.6 ml medium were added to the upper and lower chamber, respectively. Cells were incubated for 48 h to allow them to colonize the lower chamber. Migrated cells were fixed with methanol, stained with Giemsa and counted in four different fields in a Nikon inverted microscope.

2.11. Statistical analysis

Each experiment was repeated at least three times. The results are expressed as the mean ± SD. They were subjected to one way analysis of variance (ANOVA) following by the Bonferroni's test for multiple comparisons. The level of significance was set at $P < 0.05$.

3. Results

3.1. The inhibitor of prostaglandin uptake transport bromocresol green (BG) blocks 16,16-dimethyl-PGE_2 (16,16-PGE_2)-induced expression and activity of HIF-1α in PC3 cells

We have previously shown in LNCaP cells that PGE_2 increases the expression of HIF-1α, which is abolished by blocking its uptake by LNCaP cells with BG (Fernández-Martínez et al., 2012a). This indicated that the effect of PGE_2 on HIF-1α expression was due to an intracrine mechanism since it had to reach the inside the cells to induce HIF-1α up-regulation. In the present study, we first analyzed the effect of 16,16-dimethyl-PGE_2 (16,16-PGE_2), a synthetic analogue of PGE_2, on HIF-1α expression. To this end, PC3 cells were incubated with 1 μM 16,16-PGE_2 for up to 8 h and HIF-1α expression was assessed by Western blot. Our results (Fig. 1a) confirmed that 16,16-PGE_2 increases HIF-1α expression in PC3 cells. Therefore we next investigated the role of iPGE_2 in this effect by incubating cells with prostaglandin uptake transporter inhibitor BG (50 μM/1 h) before treating them with 16,16-PGE_2. Fig. 1b shows that BG prevented the increase in HIF-1α expression induced by 6 h incubation with 16,16-PGE_2. These results suggested that 16,16-PGE_2 has to reach the intracellular compartment as a prerequisite for triggering HIF-1α up-regulation in PC3 cells.

In order to confirm that iPGE_2-induced HIF-1α was biologically active in PC3 cells, we studied the effect of 16,16-PGE_2 on three different HIF-regulated targets and its prevention by preincubation with prostaglandin uptake transporter inhibitor BG or HIF-1 inhibitor YC-1: (i) Activation of HIF-1α-dependent transcription. It results from the binding of HIF-1α (together with HIF-1β and transcriptional coactivators) to hypoxia-responsive elements (HRE) in target genes (Lando et al., 2002). HIF-1α-dependent transcription was assessed as the luciferase activity in PC3 cells that were transfected with p9HIF1-Luc (a plasmid which contained nine copies of the human HIF binding sequence located between positions −985 and −951 of the 5′ human VEGF gene promoter (Aragonés et al., 2001). 16,16-PGE_2 induced a BG- and YC-1-sensitive increase in luciferase activity (Fig. 1c), (ii) Production of HIF-regulated-VEGF-A. Treatment with 16,16-PGE_2 resulted in enhanced release of VEGF-A to the medium, which was prevented by preincubation with BG or with HIF inhibitor YC-1 (Fig. 1d) and (iii) Angiogenic ability. It was assessed by measuring endothelial tube formation induced by PC3 culture medium (i.e. medium containing the secreted VEGF-A from 16,16-PGE_2-treated cells). As shown in Fig. 1e, treatment of PC3 cells with 16,16-PGE_2 resulted in a BG- and YC-1-sensitive increase in endothelial tube formation. Worth to mentioning, COX inhibitor diclofenac, but not BG, inhibited the basal activities analyzed in Fig. 1c–e. This suggests that they are regulated by prostaglandins but not in an intracrine fashion.

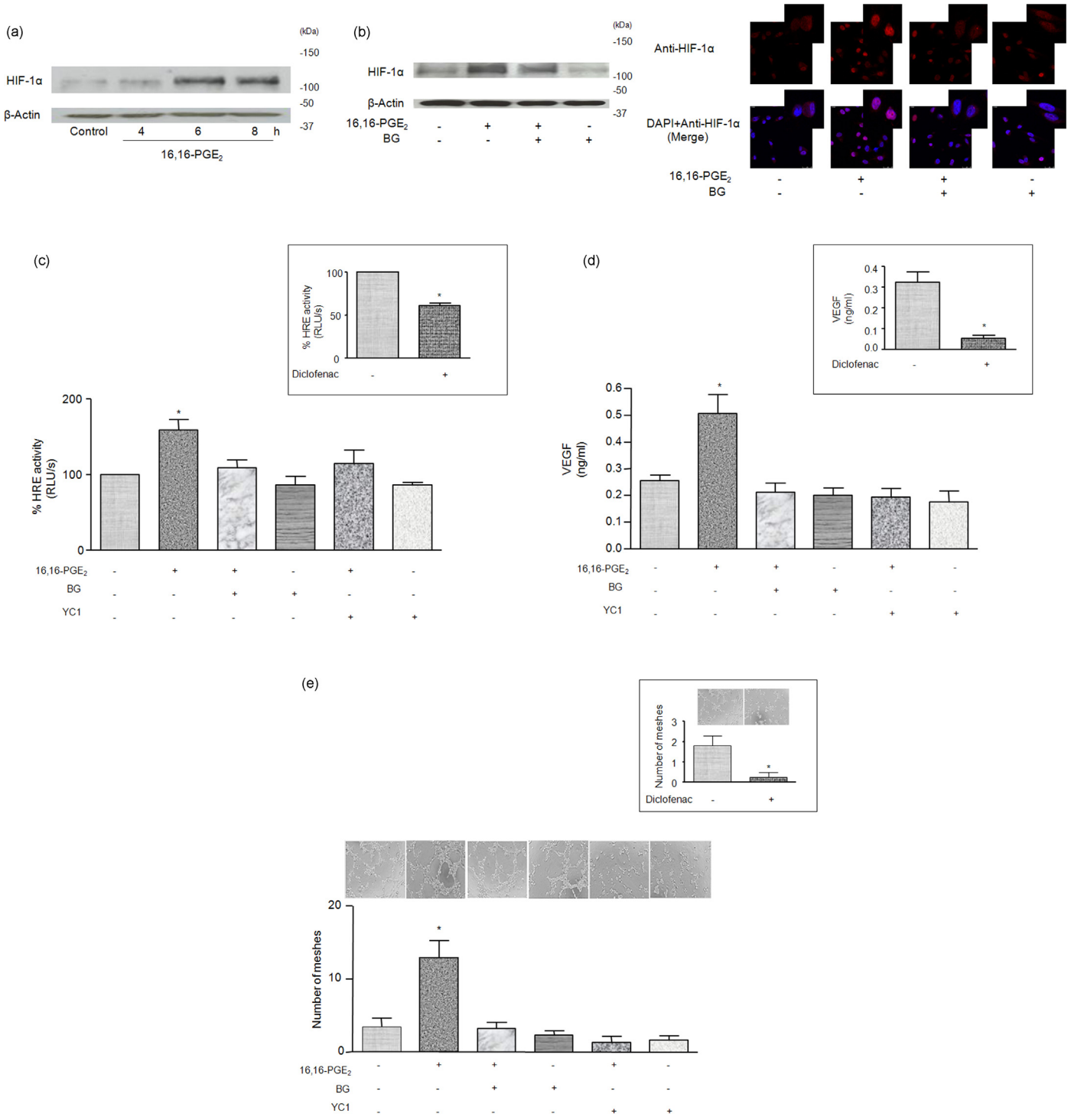

Fig. 1. The inhibitor of prostaglandin uptake transport bromocresol green (BG) blocks 16,16-dimethyl-PGE_2 (16,16-PGE_2)-induced expression and activity of HIF-1α in PC3 cells. (a) *16,16-PGE_2 up-regulates HIF-1α.* Cells were incubated with 1 μM 16,16-PGE_2. Expression of HIF-1α was assessed by Western blot analysis. Equal protein loading was confirmed by probing with an anti-β-actin antibody. Normalized density ratio of HIF-1α over β-actin is indicated for each band. (b) *BG inhibits basal and 16,16-PGE_2 induced HIF-1α up-regulation.* Cells were pre-incubated for 1 h with 50 μM BG and then for 6 h with 1 μM 16,16-PGE_2. (c) *16,16-PGE_2 increases the activity of a hypoxia-responsive element (HRE)-driven reporter construct in a BG-sensitive manner.* PC3 cells were transiently transfected with p9HIF-1-Luc (a plasmid which contained nine copies of the human HIF binding sequence located between positions −985 and −951 of the 5′ human VEGF gene promoter); then they were pre-incubated for 1 h with 50 μM BG or 10 μM YC-1 (a HIF inhibitor) and incubated with 1 μM 16,16-PGE_2 for 2 h. Afterwards luciferase activity in lysates of cells was measured and expressed as relative luminescence units (RLU) Each bar represents the mean ± SD of the luciferase activity normalized by Renilla luciferase activity *Inset*: Treatment with 0.3 mM diclofenac for 7 h inhibits the basal activity of the HRE-driven reporter construct. (d) *16,16-PGE_2 increases the production of VEGF-A in a BG-, YC1-sensitive manner.* PC3 cells were pre-incubated with either 50 μM BG or 10 μM YC-1 for 1 h and treated for 48 h with 1 μM 16,16-PGE_2. Then production of VEGF-A was quantified by ELISA. *Inset*: Treatment with 0.3 mM diclofenac for 48 h inhibits the basal production of VEGF-A. (e) *16,16-PGE_2 increases endothelial tube formation induced by PC3 culture medium.* PC3 cells were treated as in (d). Then culture medium was collected and used to assess its effect on tube formation by human umbilical vein endothelial cells cultured on Matrigel. All images shown are from a representative experiment. Photographs are taken at a 20x magnification. All experiments were repeated three times. Statistical analysis: * $P < 0.01$ vs control.

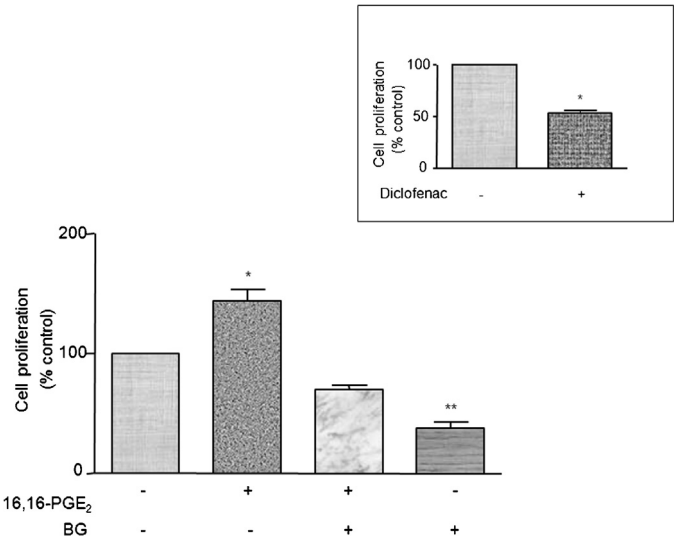

Fig. 2. BG inhibits both basal and 16,16-PGE_2-induced proliferation of PC3 cells. Cells were plated in 96-well plates (10^4 cells per well) for 24 h and maintained in serum free medium. Then they were pretreated with 50 μM BG for 1 h and incubated in the absence or presence of 1 μM 16,16-PGE_2 for 24 h. Cell proliferation was assessed by the MTT assay as described in Materials and Methods. Results were analyzed and expressed as percentage of the control (control equals 100%) *Inset*: Treatment with 0.3 mM diclofenac for 24 h All experiments were repeated three times Statistical analysis: * $P < 0.01$ vs control; ** $P < 0.01$ vs control and 16,16-PGE_2.

Overall, the results shown in Fig. 1c–e confirm that 16,16-PGE_2-induced HIF-1α is biologically active and that 16,16-PGE_2 has to reach the inside of the cell to promote HIF-1α expression and the consequent HIF-regulated angiogenic activity.

3.2. BG inhibits both basal and 16,16-PGE2-induced proliferation of PC3 cells

Unchecked proliferation is a hallmark of cancer cells that commonly exhibit increased proliferation when compared to normal cells. Treatment with PGE_2 enhances PC3 growth in a concentration-dependent manner, with a maximal response at 1 μM (Jiang and Dingledine, 2013) which facilitates PC progression. To investigate the involvement of $iPGE_2$ in PGE_2-induced proliferation of PC3 cells, we measured the mitochondrial reduction of MTT in cells which were pre-treated with BG before treating them for 24 h with 1 μM, 16,16-PGE_2. As shown in Fig. 2, 16,16-PGE_2-induced cell proliferation was prevented by prostaglandin transporter inhibitor BG. Interestingly, the basal proliferation of PC3 cells was substantially inhibited by COX inhibitor diclofenac (Fig. 2, inset) and by BG, which suggested that intracellular prostaglandins are a major determinant of the proliferation of PC3 cells in control conditions. In summary, the results shown in Fig. 2 indicate that $iPGE_2$ (and, perhaps, other intracellular prostaglandins) influence the proliferation of PC3 cells.

3.3. BG inhibits 16,16-PGE2-induced PC3 cell adhesion to collagen

Tumor cells often show a decrease in cell–cell and/or cell–matrix adhesion. An increasing body of evidence indicates that this reduction in cell adhesion correlates with tumor invasion and metastasis. Therefore, subsequent experiments evaluated the impact of 16,16-PGE_2 on the attachment of PC3 cells to an extracellular matrix of collagen. To this end, cells in suspension were treated with 1 μM 16,16-PGE_2 for 60 min, seeded onto 96-well plates coated with collagen type I and incubated for up to 120 min. Afterwards, wells were washed and adherent cells were assessed by the MTT assay. As shown in Fig. 3a, the values for optical density in the MTT assay

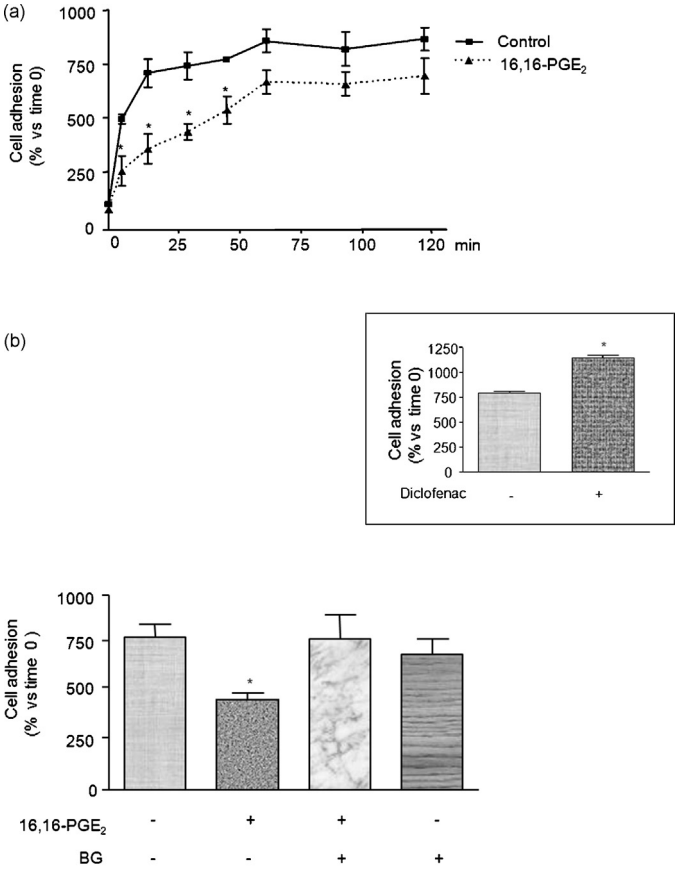

Fig. 3. PC3 cell adhesion to collagen type I is inhibited by 16,16-PGE_2 in a BG-sensitive way. (a) *PC3 cell adhesion to collagen type I is inhibited by 16,16-PGE_2* Cells in suspension were incubated for 1 h with or without 1 μM 16,16-PGE_2. Then cells were plated on collagen coated dishes (25×10^3 cells per dish) and incubated for up to 2 h. The number of adherent cells was assessed by the MTT assay. (b) *BG prevents the loss of cell adhesion induced by16,16-PGE_2* Experiments were carried out as in (a), except that cells were first pre-incubated for 1 h with 50 μM BG and then incubated with 16,16-PGE_2 for 30 min. *Inset:* PC3 cells were incubated for 24 h with or without 0.3 mM diclofenac. Then cell adhesion to collagen type I was assessed after 30 min incubation. All experiments were repeated three times. Statistical analysis: * $P < 0.01$ vs control.

increased steadily over the time in control cells until reaching a plateau after the first 60 min. Treatment with 16,16-PGE_2 resulted in loss of cell adhesion along the time interval previous to the plateau (Fig. 3a). In order to investigate the role of $iPGE_2$ in these results, PC3 cells in suspension were preincubated with inhibitor of prostaglandin uptake transporter BG prior being treated with 16,16-PGE_2. Cells were then plated and the effect of BG on 16,16-PGE_2-dependent inhibition of PC3 adhesion was assessed after a 30 min incubation. Our results (Fig. 3b) indicated that BG prevented 16,16-PGE_2-induced loss of cell adhesion. Worth to mentioning, diclofenac but not BG increased the attachment of unstimulated PC3 cells to collagen (Fig. 3b). Overall, these results indicate that $iPGE_2$ is responsible for PGE_2-induced increase in PC3 cell adhesion but not for PC3 cell adhesion under basal conditions.

3.4. BG inhibits 16,16-PGE2-induced PC3 cell migration

These results shown in Fig. 3 suggested that 16,16-PGE_2, through inhibiting PC3 cell adhesion to the extracellular matrix in an $iPGE_2$-dependent manner, might contribute to PC invasion and metastasis. The migratory activity of PC cells also contributes to both processes and it has been recently reported that PGE_2 promotes PC3 cell migration in transwell assay (Vo et al., 2013).

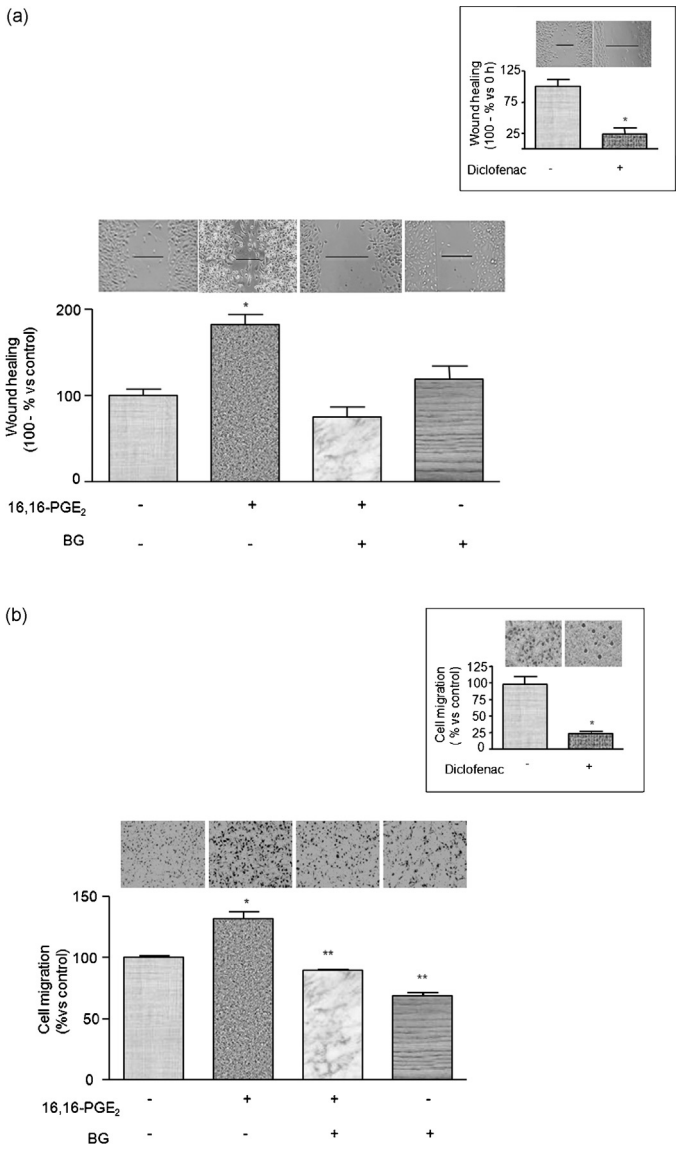

Fig. 4. BG inhibits 16,16-PGE_2-induced cell migration. (a) *PC3 cell migration in the wound healing assay is inhibited by 16,16-PGE_2 in a BG-sensitive way*. PC3 cells were cultured until confluent. The cell layer was then carefully wounded using sterile tips. Afterwards cells were treated with 50 μM BG for 1 h and then with 1 μM 16,16-PGE_2 for 48 h. *Inset*: Cells were incubated with 0.3 mM diclofenac for 24 h. The wounded cell layer was photographed and measured at 0 to 48 h time points and a representative photograph is shown at 24 h (20× amplification). (b) *PC3 cell migration in transwell assay is inhibited by 16,16-PGE_2 in a BG-sensitive way.* Cells in suspension (5×10^5 cells/ml) were treated for 1 h with 50 μM BG, which was followed by an 1 h incubation with 1 μM 16,16-PGE_2. Then cells were seeded into a transwell insert and migration to the lower chamber was assessed as indicated in Materials and Methods. *Inset*: Cells were incubated with 0.3 mM diclofenac for 1 h and then they were seeded and allowed to migrate for 48 h, a representative photograph (10x magnification) is shown. All experiments were repeated three times. Statistical analysis: * $P < 0.01$ vs control; ** $P < 0.01$ vs control and 16,16-PGE_2.

Therefore, we studied the role of $iPGE_2$ on PC3 cell migration. To analyze the migratory phenotype, we performed a scratch wound healing assay and a transwell assay. Our results in both assays (Fig. 4) in cells untreated with 16,16-PGE_2 showed that diclofenac – but not BG – fully prevented wound healing (Fig. 4a inset) and diminished dramatically the migration in the transwell assay (Fig. 4b inset), This suggested that autologous prostaglandins, acting through non-intracrine mechanisms, play a relevant role in PC3 cell migration under basal conditions. As regards 16,16-PGE_2, it potentiated the migratory activity in PC3 cells since it

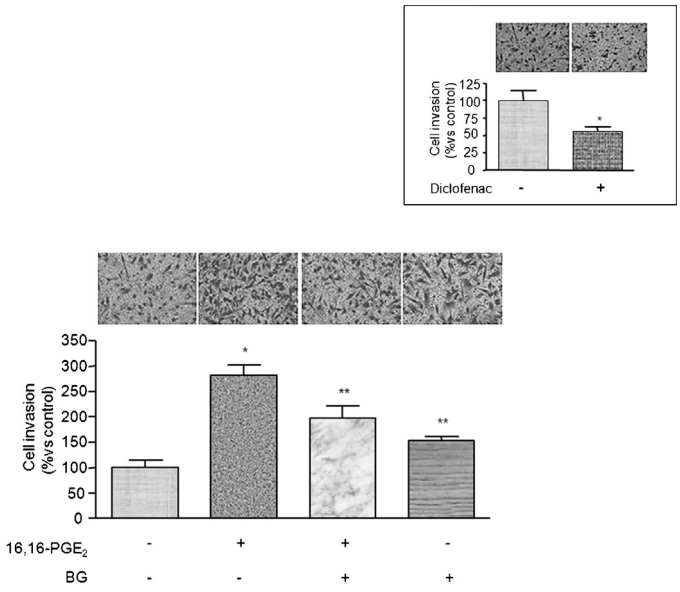

Fig. 5. BG inhibits 16,16-PGE_2-induced PC3 cell invasion. Cells in suspension (5×10^5 cells/ml) were treated fo1 h with 50 μM BG, which was followed by an 1 h incubation with 1 μM 16,16-PGE_2. Then cells were seeded into transwell inserts which had been previously coated with Matrigel basement membrane matrix and invasion was assessed as indicated in Materials and Methods. *Inset*: Cells were incubated with 0.3 mM diclofenac for 1 h and then they were seeded and allowed to migrate for 48 h. All experiments were repeated three times and a representative photograph (20× magnification) is shown. Statistical analysis: * $P < 0.01$ vs control; ** $P < 0.01$ vs control and 16,16-PGE_2.

accelerated both wound healing (Fig. 4a) and transwell migration capacity (Fig. 4b). These effects were prevented by the inhibitor of prostaglandin uptake transporter BG.

In summary, the results shown in Fig. 4 support the view that the basal migratory phenotype of PC3 cells is regulated by prostaglandins and that PGE_2, through $iPGE_2$, increases the migration of these cells.

3.5. BG inhibits 16,16-PGE2-induced PC3 cell invasion

The most threatening feature of malignancy in cancer is the potential for invasion and metastases. PGE_2 has been recently found to enhance cell invasion in PC cell lines LNCaP, DU145 and PC3 cells (Vo et al., 2013). To investigate the role of $iPGE_2$ on the invasive potential of PC3 cells, we examined the effect of prostaglandin uptake transport inhibitor BG on both basal- and 16,16-PGE_2-induced cell invasion using a transwell insert pre-loaded with Matrigel. BG did not have any effect on the ability of untreated PC3 cells to invade through the Matrigel layer, which contrasts with the inhibitory effect of diclofenac (Fig. 5, inset). This suggests that prostaglandins released by PC3 cells, but not $iPGE_2$, play a relevant role in PC3 cell invasion under basal conditions. On the other hand, pre-incubation with BG partially prevented 16,16-PGE_2-induced cell invasion, which does agree with a potential role of $iPGE_2$ in this effect (Fig. 5).

3.6. PC3 cells have functional intracellular EP receptors

The biological actions of PGE_2 have been generally attributed to result from its interaction with four different plasma membrane G protein-coupled receptors, namely EP1, EP2, EP3 and EP4. However, it has been also documented in a variety of cell types and tissues the intracellular presence of a subset of functional EP receptors (Fernández-Martínez et al., 2012b; Bhattacharya et al., 1998; Bhattacharya et al., 1999; Gobeil et al., 2002; Zhu et al., 2006).

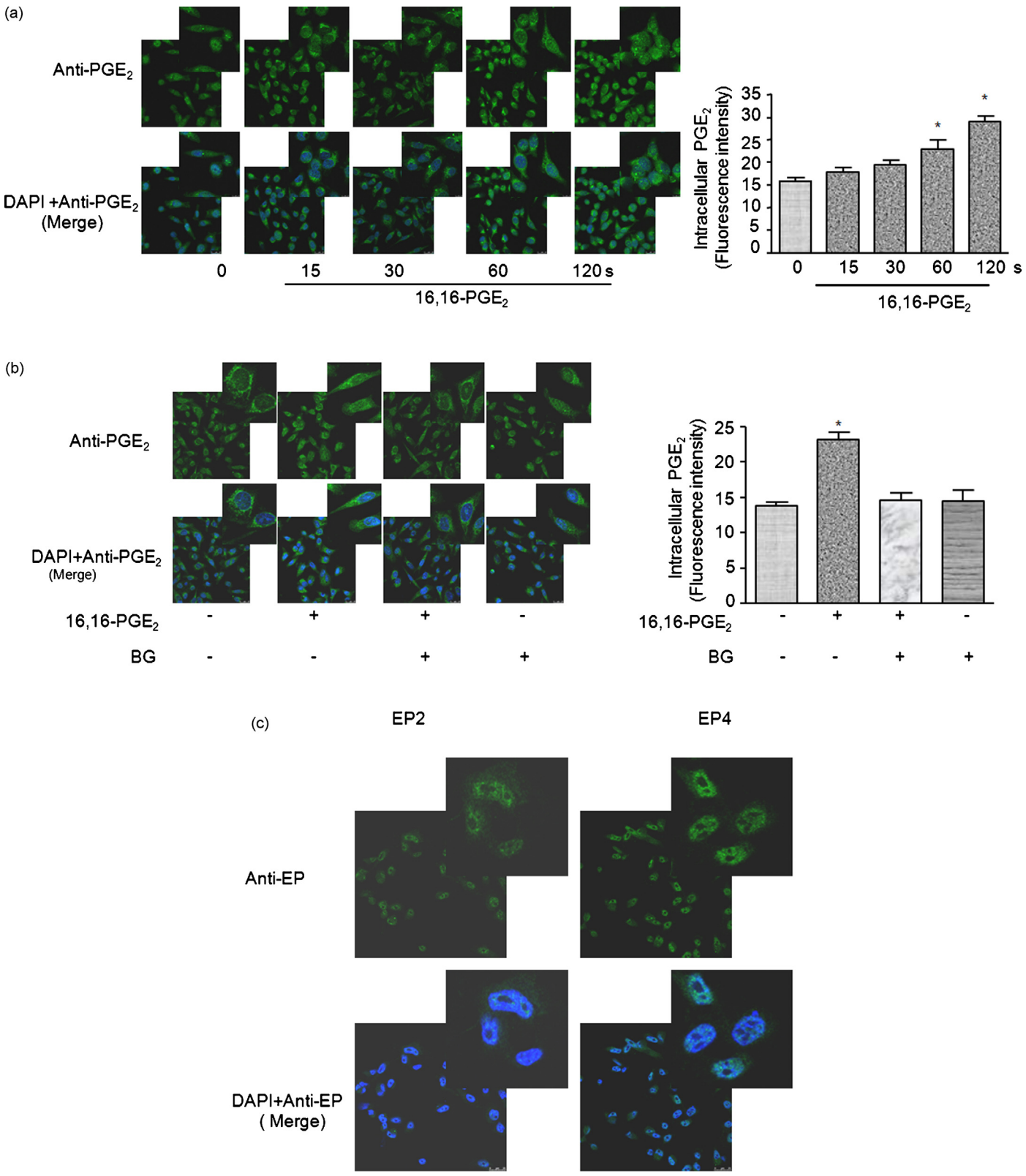

Fig. 6. PC3 cells have functional intracellular EP receptors. (a) *Internalization of 16,16-PGE$_2$* Cell were incubated with 1 μM 16,16-PGE$_2$ for up to 120 s and they were fixed, permeabilized and subjected to immunofluorescence analysis after incubation with anti-PGE$_2$ antibody. Note that control PC3 cells had low, but detectable expression of iPGE$_2$. (b) *BG prevents the internalization of 16,16-PGE$_2$* PC3 cells were first incubated for 1 h with 50 μM BG and then with 1 μM 16,16-PGE$_2$ and intracellular immunoreactive PGE$_2$ was assessed by immunofluorescence analysis as in (a). (c) *Cellular location of EP2 and EP4 receptors.* Cells were fixed, permeabilized and subjected to immunofluorescence analysis after incubation with anti-EP receptors antibodies as indicated in Material and Methods. Nuclei were stained with DAPI. (d) *AH6809, an antagonist of EP receptors 1-3, inhibits 16,16-PGE$_2$ induced HIF-1α up-regulation and cell proliferation.* Cells were pre-incubated for 1 h with 10 μM AH6809 and then for 6 h (HIF studies) or 24 h (cell proliferation studies) with 1 μM 16,16-PGE$_2$. All experiments were repeated three times. The images shown (20x) are from a representative experiment. Statistical analysis of the quantification of immunofluorescence: * $P < 0.01$ vs control.

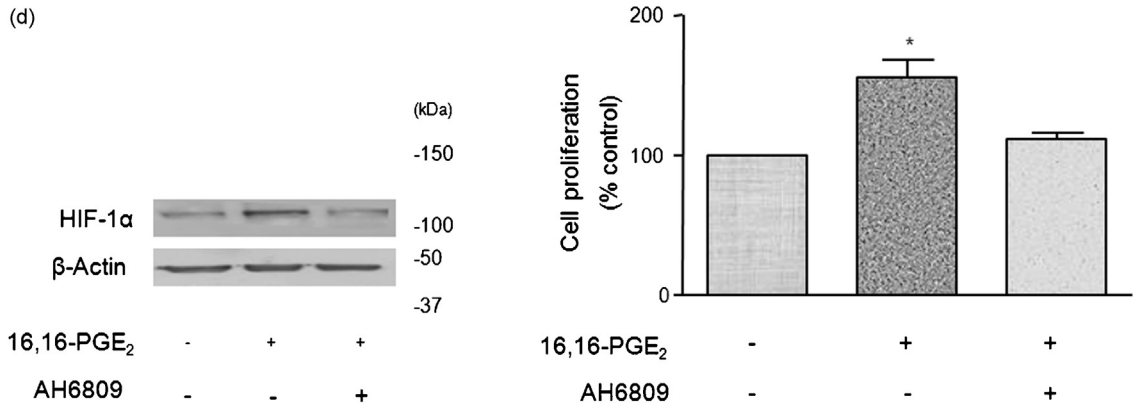

Fig. 6. (*Continued*)

Since $iPGE_2$ is likely involved in the regulation of the expression and activity of HIF-1α in PC3 cells (Fig. 1), we first studied the effect of treatment with 16,16-PGE_2 on the intracellular levels of immunoreactive PGE_2. Immunofluorescence analysis in PC3 cells (Fig. 6a) revealed intracellular expression of immunoreactive PGE_2 (i.e. $iPGE_2$), which increased in less than 2 min upon treatment with 16,16-PGE_2. This increase was most likely due to the reactivity of intracellular 16,16-PGE_2 with the antibody anti-PGE_2, since it was prevented by the inhibitor of prostaglandin uptake transport BG (Fig. 6b).

Taking into account that PC3 cells only express mRNA for EP2 and EP4 receptors (Vo et al., 2013; Wang and Klein, 2007), we asked whether both receptors could be also located inside PC3 cells. The results of a preliminary immunofluorescence analysis (Fig. 6c) confirmed this possibility. We finally studied whether AH6809, an antagonist of EP receptors 1,2,3, prevented the effect of 16,16-PGE_2, on a sample of two cancer-related phenotypes. Our results (Fig. 6d) indicated that AH6809 prevented 16,16-PGE_2-induced increase in cell proliferation and HIF-1α expression. Taking into account that BG also prevented these actions of 16,16-PGE_2, our results strongly suggest that PC3 cells have functional intracellular EP receptors that mediate at least two of the effects of exogenously imported 16,16-PGE_2.

The results shown in Fig. 6 gave further support to the view that, besides the cell membrane, the intracellular compartment may be a scenario in which PGE_2 regulates pathological phenotypes in PC3 cells.

4. Discussion

The current study in PC3 cells shows that BG, an inhibitor of the prostaglandin uptake transporter, prevents the stimulatory effects of 16,16-PGE_2 on HIF-1α expression, in vitro angiogenesis and cell proliferation, adhesion, migration and invasion. This suggests that PGE_2 has to gain access to the intracellular compartment as a prerequisite for stimulating important PC-related features. Therefore, the prostaglandin uptake transporter might be an important player in PC.

Prostaglandins such as PGE_2 are autocrine and paracrine lipid mediators that trigger a wide variety of signals. It is accepted that, once prostanoids are formed, they are quickly released to the outside of cells by simple diffusion and act as autocrine or paracrine mediators in the vicinity of their sites of production to maintain local homeostasis (Sugimoto and Narumiya, 2007; Tsuboi et al., 2002; Chan et al., 1998; Schuster, 2002).Therefore, it has generally been assumed that PGE_2 exerts its actions via plasma membrane spanning G-protein coupled EP receptors (EP1, EP2, EP3 and EP4), each EP activating a distinct G protein-coupled signaling pathway (Sugimoto and Narumiya, 2007; Tsuboi et al., 2002). Furthermore, the current view is that these signals are terminated by the metabolism of PGE_2: PGE_2 has to be translocated from the outside to the inside of cells for it to be inactivated by oxidation by 15-hydroxy-prostaglandin dehydrogenase (Nomura et al., 2004). Thus, prostaglandin uptake transporter-mediated PGE_2 influx is required and is rate-limiting for PGE_2 metabolism (Nomura et al., 2004). The prostaglandin transporter, a member of the organic anion transporter family (Schuster, 2002), mediates the energetically active influx of PGE_2, accounting for almost 100% of PGE_2 internalization (Schuster, 2002; Nomura et al., 2004; Chi et al., 2006; Chi and Schuster, 2010). However, the biological role of this transporter is still the subject of speculation and an interesting alternative possibility exits: given that it is now apparent that prostaglandins also activate intracellular prostaglandin receptors (Fernández-Martínez et al., 2012b; Bhattacharya et al., 1998; Bhattacharya et al., 1999; Gobeil et al., 2002; Zhu et al., 2006), the prostaglandin uptake transporter appears to provide at least one mechanism through which PGE_2 and other prostaglandins can be delivered to an intracellular site of action (Helliwell et al., 2004). In our work, the stimulatory effects of 16,16-PGE_2 on cancer related phenotypes were prevented by inhibitors of the prostaglandin transporter BG (Figs. 1–5) and bromosulphophthalein (it was used for cross-examination for specificity of BG on PGT (Fernández-Martínez et al., 2012b) on a sample of cancer-related phenotypes: cell proliferation and cell adhesion (Suppl. Fig. 1)). These results and the presence of functional intracellular EP receptors in PC3 cells (Fig. 6) indicate that intracrine mechanisms exist whereby $iPGE_2$ may promote the progression of PC.

Several studies have disclosed that functional EP receptors can be localized at the nuclear envelope of a variety of cell types and tissues (Fernández-Martínez et al., 2012b; Bhattacharya et al., 1998; Bhattacharya et al., 1999; Gobeil et al., 2002; Zhu et al., 2006). The cell nucleus has thus been considered as a possible intracellular location for the initiation of signal transduction cascades dependent on EP receptors. Nuclear G-protein-coupled EP3-dependent signaling in porcine brain microvascular endothelial cells has been linked to regulation of gene transcription through a signaling sequence of calcium-mitogen activated protein kinases-nuclear factor-kB (Fernández-Martínez et al., 2012b) (to date, this is the only report on the signaling activated by nuclear EP receptors). Given that PC3 cells only express EP2 and EP4 mRNA (Vo et al., 2013; Wang and Klein, 2007), it does not seem likely that the signaling through their nuclear EP receptors is the same described for nuclear EP3 receptors. On the other hand, our immunofluorescence studies (Fig. 6) indicate that intracellular EP receptors are not only located in the nucleus but across the intracellular compartment. Therefore, identifying the signaling activated by the interaction of 16,16-PGE_2

with intracellular EP receptors is not a straightforward task and further studies are needed to address this relevant issue.

Recent interest has focused on the role that inflammation may play in the development of PC and whether use of NSAIDs affects risk. The majority of these studies have focused on COX inhibitors in regulating prostate carcinogenesis. However, the direct role of PGE_2 in PC development and progression has received limited attention. Available data suggest that PGE_2 may mediate signaling pathways that promote cell proliferation (Liu et al., 2002b; Chen and Hughes-Fulford, 2000), invasion (Nithipatikom et al., 2002) and angiogenesis (Jain et al., 2008) in PC cells. On the other hand, the use of NSAIDs has been associated with a reduced risk of PC in some, but not all, studies (Salinas et al., 2010; Mahmud et al., 2004; Singer et al., 2008). Moreover, COX inhibitors are criticized for causing unwanted side effects, which is a major obstacle for widespread use in the prevention of PC.

Therefore, targeting downstream signaling pathways of PGE_2 may represent an attractive new strategy for PC prevention and therapy. Since our results suggest that PGE_2 has to gain access to the intracellular compartment as a prerequisite for stimulating important functions for cancer progression, we propose to test inhibitors of the prostaglandin uptake transporter as a novel therapeutic modality to treat PC.

Conflicts of interest

There are no conflicts of interest to disclose.

Acknowledgments

This work was supported by grant SAF2011-26838 from the Spanish Ministerio de Ciencia e Innovación. Ana Belén Fernández Martínez is the recipient of a post-doctoral fellowship from the Junta de Comunidades de Castilla-La Mancha.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2014.11.004.

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Received: 29 June 2017 | Accepted: 29 September 2017
DOI: 10.1002/jcp.26220

ORIGINAL RESEARCH ARTICLE

WILEY Journal of Cellular Physiology

Intracrine prostaglandin E_2 pro-tumoral actions in prostate epithelial cells originate from non-canonical pathways

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Funding information
Spanish Ministerio de Ciencia e Innovación, Grant numbers: grants SAF2011-26838, SAF2014-53218-R

Prostaglandin E_2 (PGE_2) increases cell proliferation and stimulates migratory and angiogenic abilities in prostate cancer cells. However, the effects of PGE_2 on non-transformed prostate epithelial cells are unknown, despite the fact that PGE_2 overproduction has been found in benign hyperplastic prostates. In the present work we studied the effects of PGE_2 in immortalized, non-malignant prostate epithelial RWPE-1 cells and found that PGE_2 increased cell proliferation, cell migration, and production of vascular endothelial growth factor-A, and activated in vitro angiogenesis. These actions involved a non-canonic intracrine mechanism in which the actual effector was intracellular PGE_2 ($iPGE_2$) instead of extracellular PGE_2: inhibition of the prostaglandin uptake transporter (PGT) or antagonism of EP receptors prevented the effects of PGE_2, which indicated that PGE_2 activity depended on its carrier-mediated translocation from the outside to the inside of cells and that EP receptors located intracellularly (iEP) mediated the effects of PGE_2. $iPGE_2$ acted through transactivation of epidermal growth factor-receptor (EGFR) by iEP, leading to increased expression and activity of hypoxia-inducible factor-1α (HIF-1α). Interestingly, $iPGE_2$ also mediates the effects of PGE_2 on prostate cancer PC3 cells through the axis $iPGE_2$-iEP receptors-EGFR-HIF-1α. Thus, this axis might be responsible for the growth-stimulating effects of PGE_2 on prostate epithelial cells, thereby contributing to prostate proliferative diseases associated with chronic inflammation. Since this PGT-dependent non-canonic intracrine mechanism of PGE_2 action operates in both benign and malignant prostate epithelial cells, PGT inhibitors should be tested as a novel therapeutic modality to treat prostate proliferative disease.

KEYWORDS
bromocresol green, HIF-1α, intracellular prostaglandin E2, prostate proliferative disease, RWPE-1 cells

1. INTRODUCTION

Prostaglandin E_2 (PGE_2) is among the inflammatory mediators that play a role in the pathogenesis of prostatic neoplasms: PGE_2, a well-known regulator of cell growth, is the only major prostaglandin that is produced in significant amounts by the prostate in benign prostatic hyperplasia (BPH) and prostate cancer (PC) (Chaudry, Wahle, McClinton, & Moffat, 1994; Nithipatikom & Campbell, 2008). PGE_2 promotes tumor progression via several mechanisms. PGE_2 can directly bind to its receptors on tumor cells to regulate cell proliferation, apoptosis, migration and invasion as well as to induce tumor cells to secrete growth factors, pro-inflammatory mediators,

and angiogenic factors that stimulate angiogenesis and local inflammation (Wang & DuBois, 2013). Most of these PGE_2 effects have also been specifically documented in PC cells (Huang et al., 2013; Jain, Chakraborty, Raja, Kale, & Kundu, 2008; Jiang & Dingledine, 2013; Madrigal-Martínez, Lucio-Cazaña, & Fernández-Martínez, 2015; Vo et al., 2013; Wang & Klein, 2007).

It is generally accepted that the biological actions of PGE_2 are mediated by plasma membrane G protein-coupled EP receptors which are located at the cell membrane. However, we have found that PGE_2 has to reach the intracellular compartment and activate a subset of EP receptors, which is located intracellularly, as a prerequisite for being effective in PC3 cells (Fernández-Martínez & Lucio-Cazaña, 2015; Madrigal-Martínez et al., 2015). This is a non-canonical intracrine mechanism of PGE_2 action for which the energetically active influx of PGE_2 (accounting for almost 100% of PGE_2 internalization), is mediated by the prostaglandin transporter (PGT), a member of the organic anion transporter family (Nomura, Lu, Pucci, & Schuster, 2004; Schuster, 2002). Once PGE_2 is internalized in PC3 cells and becomes intracellular PGE_2 ($iPGE_2$), it inhibits cell adhesion and stimulates cell proliferation, migration and in vitro angiogenesis (Fernández-Martínez & Lucio-Cazaña, 2015; Madrigal-Martínez et al., 2015). The mechanism involves transactivation of epidermal growth factor-receptor (EGFR) by iEP, leading to increased expression and activity of hypoxia-inducible factor-1α (HIF-1α) (Fernández-Martínez & Lucio-Cazaña, 2015).

The effects of PGE_2 on non-transformed prostate epithelial cells are currently unknown, despite they may be relevant in non-malignant proliferative diseases of the prostate associated with chronic inflammation of the prostate such are BPH or proliferative inflammatory atrophy (Bardan, Dumache, Dema, Cumpanas, & Bucuras, 2014; Thapa & Ghosh, 2015). Here we found in immortalized, non-malignant prostate epithelial RWPE-1 cells that PGE_2, again through the axis $iPGE_2$-iEP receptors-EGFR-HIF-1α, inhibits cell adhesion and stimulates cell proliferation, migration and in vitro angiogenesis (i.e., the same effects than in PC3 cells (Fernández-Martínez & Lucio-Cazaña, 2015; Madrigal-Martínez et al., 2015). Since $iPGE_2$ mediates the growth-stimulating effects of PGE_2 on prostate epithelial cells, regardless they are benign or malign ones, the pharmacological inhibition of PGT could be a novel therapeutic approach to treat prostate proliferative diseases associated with chronic inflammation.

2 | MATERIALS AND METHODS

2.1 | Reagents and antibodies

PGE_2, actinomycin D (Act.D), AG1478, bromocresol green (BG), 5′-Br-2′-deoxyuridine *(BrdU)*, cycloheximide (CHX), 3-(5′-Hydroxymethyl-2′-furyl)-1-benzyl indazole (YC1), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT), and type I-collagen were purchased from Sigma (St. Louis, MO). AH6809 and GW627368X were purchased from Cayman Chemical (Ann Arbor, MI). Antibodies were from obtained from the following sources: anti-PGE_2 antibody from Abcam (Cambridge, UK), anti-HIF-1α and anti-BrdU antibodies from BD Biosciences (Palo Alto, CA), anti-phospho EGFR was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-EP2, EP4 antibodies from Cayman Chemical, anti-β-actin and anti-mouse IgG peroxidasa conjugate from Sigma, anti-rabbit IgG peroxidasa conjugate from Calbiochem Millipore (Darmstadt, Alemania), α-mouse-Alexa-Fluor® 488 and α-rabbit-Alexa-Fluor® 488 Invitrogen (Eugene, OR). All other reactive were pursached from Sigma.

2.2 | Cell culture

The human non-malignant prostate epithelial cell line RWPE-1 and the human umbilical vein endothelial cells (HUVECs) were purchased to American Type Culture Collection (ATCC) (Manassas, VA). Both cell lines were grown in sterile conditions and were performed in a humidified 5% CO_2 environment at 37°C. The culture medium was changed every 3 days. RWPE-1 cells were maintained in keratinocyte-serum free medium (K-SFM), medium supplemented with 50 µg/ml bovine pituitary extract, 5 ng/ml human recombinant EGF and 1% penicillin /streptomycin/amphoterycin B from Gibco (Carlsbad, CA). HUVECs cells were cultured in endothelial basal medium supplemented with 5 ng/ml VEGF, 5 ng/ml EGF, 5 ng/ml FGF basic, 15 ng/ml IGF-1, 10 mM L-glutamine, 0.75 U/ml heparin sulfate, 1 µg/ml hydrocortisone and 2% fetal bovine serum (ATCC).

HUVEC were isolated from human umbilical cord veins and they express factor VIII. RWPE-1 cells were originally derived from normal human prostate epithelial cells and are immortalized but nontumorigenic (Bello, Webber, Kleinman, Wartinger, & Rhim, 1997; Webber, Bello, Kleinman, & Hoffman, 1997). RWPE-1 cells have retained the Y chromosome, normal cytokeratin expression and a normal epithelial morphology and the cells mimic normal epithelium in their response to growth factors as well as androgens by the expression of PSA and up-regulation of androgen receptor. They can also undergo acinar morphogenesis while tumorigenic cells have lost this ability (Bello et al., 1997; Webber et al., 1997). It is for these reasons that RWPE-1 cell line is considered to be invaluable for studies on growth regulation at the cell and molecular level.

2.3. MTT assay

Cell proliferation was determined using a colorimetric assay with MTT. The MTT assay measures the conversion of MTT to insoluble formazan by dehydrogenase enzymes of the intact mitochondria of living cells. Cells were plated in 96-well plates (10^4 cells/well) and they were grown for 24 hr. Then, they were cultured for 24 hr in medium without EGF/bovine pituitary extract and treated as described later. Afterwards cell proliferation was evaluated by measuring the conversion of the tetrazolium salt MTT to formazan crystals. Briefly, 0.1 mg/ml MTT was added to the cells and incubated for 3 hr at 37°C. The medium was removed and 100 µl of dimethyl sulfoxide were added to dissolve the formazan precipitates. The amount of formazan crystals formed correlates directly with the number of viable cells. The reaction product was quantified by measuring the absorbance at 570–630 nm

using a plate reader (ELX 800, Bio-Tek Instruments, Winooski, VT). Results were expressed as percentage of the control.

2.4 | Cell proliferation assay with BrdU

DNA synthesis was assessed by BrdU uptake. RWPE-1 cells were placed in 24-well plates (6×10^4 cells/glass coverslip) and they were grown for 24 hr. Then, they were cultured for 24 hr in medium without EGF/bovine pituitary extract and treated as described later. Afterwards, the cells were pulsed with 10 μM BrdU during the last 16 hr of incubation. Then, they were fixed with 2% paraformaldehyde for 15 min, DNA was partially denatured by incubation with 2 M HCl for 20 min, after which the cells were incubated with 0.1 M sodium borate for 2 min at room temperature. Then, the cells were permeabilized with PBS containing 0.2% Triton X-100 (pH 7,4) for 5 min and 3% bovine serum albumin and were washed three times with PBS. Cells were incubated at 37°C with anti-BrdU monoclonal antibody (BD Bioscience) (1:50) for 1 hr in the darkness. Then, they were then incubated at 37°C with α-mouse-Alexa-Fluor® 488 (1:2000) for 1 hr in the darkness. Cell nuclei were counterstained with Prolong Gold antifade Reagent with DAPI (Invitrogen). Detection was performed by confocal laser scan microscopy LEICA TCS-SL (Heidelberg, Germany). To estimate DNA synthesis, the percentage of BrdU-positive nuclei was determined through manual count in five fields in a blind manner. Quantitative analysis of BrdU-positive cells among the sorted cells was performed by counting the positive nuclei in total cells per field (total cells were visualized by DAPI-positive nuclei).

2.5 | Cell migration assay

RWPE-1 cells were seeded in 24-well plates (3×10^5 cells per well). Cells were treated as described in the legends to figures. The experiments were done in medium without EGF/bovine pituitary extract. At 24 hr after seeding, the monolayer cells were manually scratched with a pipette yellow tip to create extended and definite scratches in the center of the dishes with a bright and clear field (~2 mm). Cells were treated as indicated in the results section and the narrowing of the wound by migrating cells was monitored by measuring the width in microphotographs. Three representative fields of each monolayer wound were captured using a Nikon Diaphot 300 inverted microscopy camera (10×) up to 30 hr. Monolayer wound areas of untreated samples were averaged and assigned a value of 100.

2.6 | Cell adhesion assay

The cell adhesion to collagen was evaluated as follows: cultured cells were detached by trypsinization, resuspended in medium with 0.1% BSA and without EGF/bovine pituitary extract (2.5×10^5 cells/ml) treated as stated in the results section. Then cells were plated in 96-well plates which were pre-coated with concentrated type-I collagen solution diluted in 10 mM glacial acetic acid and incubated at 37°C for up to 2 hr. The non-adherent cells were removed by aspiration, and the number of cells that adhered to collagen was assessed by MTT assay. The independent experiments were run in three times.

2.7. In vitro vascular endothelial cell tube formation assay

RWPE-1 cells were seeded in 24-well plates (8×10^4 cells/well), grown for 24 hr and cells were treated as described in the legends to figures. The experiments were done in medium without EGF/bovine pituitary extract. Then, the medium containing the secreted VEGF was used for the assay. To this end, a 96-well-plate was coated with 40 μl/well Matrigel (BD Bioscience) and allowed to polymerize for 2 hr at 37°C and 5% CO_2 a humidified atmosphere. HUVEC cells were resuspended in the medium that had been previously collected from RWPE-1 cells. Afterwards, endothelial cells were seeded on top of the gel (25×10^3 cells/well) and incubated for 1–2 hr to allow the formation of tubes. Microphotographs were taken using a Nikon Diaphot 300 inverted microscopy camera (10×) and tube formation was quantified by counting the number of meshes using ImageJ software (NIH, Bethesda).

2.8 | Determination of VEGF secretion

RWPE-1 cells were seeded in 24-well plates (8×10^4 cells/well), grown for 24 hr and cells were treated as described in the legends to figures. The experiments were done in medium without EGF/bovine pituitary extract. Then, the medium containing the secreted VEGF was removed and kept at −80°C until analyzed. Afterwards VEGF was measured essentially as described previously (Fernández-Martínez et al., 2011) using the human VEGF DuoSet (R&D Systems, Minneapolis, MN) following the manufacturer's protocol.

2.9 | Isolation RNA and RT-PCR

RWPE-1 cells were seeded in 6-well plates (5×10^5 cells/well) grown for 24 hr and they were cultured for 24 hr in medium without EGF/bovine pituitary extract and treated as described in the legends to figures. Total cell RNA was isolated with TriReagent reagent from Sigma according to the instructions of the manufacturer. One microgram of total RNA was reverse-transcribed using 50 pmol of hexamer random primer and 0.5 μl PrimeScript RT Enzyme supplemented with 25 pmol of Oligo dT Primers from Takara (Shiga, Japon). RT conditions were: denaturation at 94°C for 10 min, followed by the reaction of RT at 37° for 1 hr and then a final reaction of 5 min at 95°C. Two microliters of RT reaction were then PCR-amplified with specific primers for VEGF-A: sense 5′-ATC TTC AAG CCA TCC TGT GTG C-3′, and antisense 5′-TCA CCG CCT CGG CTT GTC ACA T-3′. HIF-1α: sense 5′-GAA AGC GCA AGT CCT CAA AG-3′; antisense, 5′-TGG GTA GGA GAT GGA GAT GC-3′. PCR conditions were: denaturation at 94°C for 5 min, followed by 37–40 cycles of 95°C 1 min, 54°C 1 min and then a final cycle of 1 min at 72°C. The signals were normalized with the β-actin and GAPDH gene expression level; the primers for β-actin were: sense 5′-AGA AGG ATT CCT ATG TGG GCG-3′ and antisense 5′-CAT GTC GTC CCA GTT GGT GAC-3′; the primers for GAPDH were: sense 5′-CAA GGG CAT CCT GGG CTA C-3′ and antisense 5′-TTG AAG TCA GAG GAG ACC ACC TG-3′. The PCR products were separated by electrophoresis and visualized in 2% agarose gels.

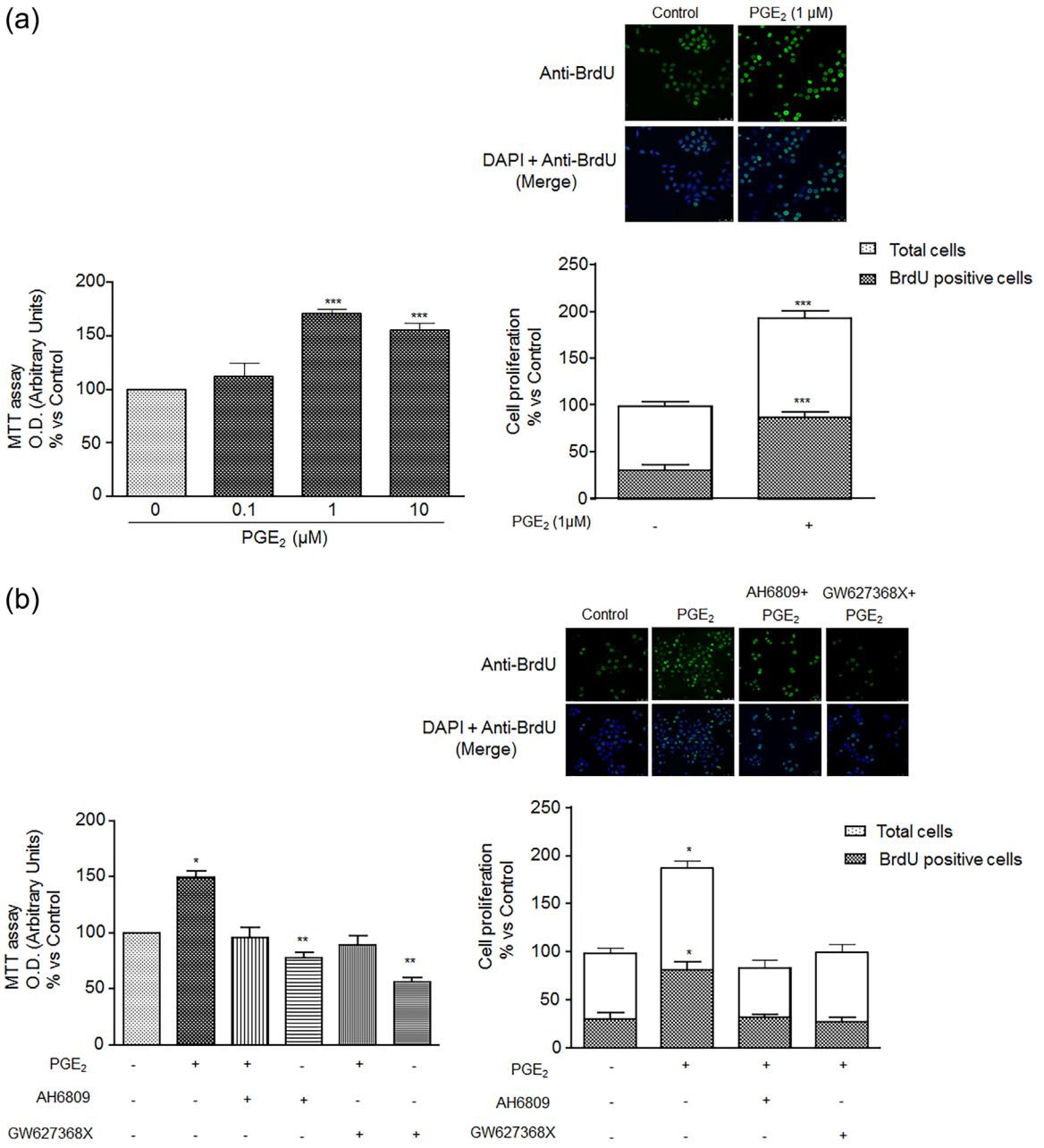

FIGURE 1 PGE_2 increases RWPE-1 cell proliferation in an EP receptor antagonist-sensitive manner. (a) PGE_2 increases cell proliferation. Cells were incubated for 24 hr in medium without EGF/bovine pituitary extract and treated with PGE_2 for 24 hr. Cell proliferation was assessed by the MTT or BrdU assays as described in section 2. Results were analyzed and expressed as percentage of the control. (b) PGE_2-induced cell proliferation is prevented by antagonists of EP receptors. Cells were preincubated for 1 hr with 10 μM AH6809 (EP1-3 receptor antagonist) or 10 μM GW627368X (EP4 receptor antagonist) before being treated with 1 μM PGE_2 for 24 hr. General information: All experiments were repeated three times. Bars are mean ± SD. For BrdU experiments, cell nuclei were counterstained with DAPI so that white bars represent total nuclei and gray bars represent the fraction of BrdU positive nuclei. Statistical analysis: $^{*}p < 0.01$ versus other groups; $^{**}p < 0.01$ versus control and PGE_2. $^{***}p < 0.01$ versus control

2.10 | Hypoxia response element (HRE) reporter gene assay

Cells were split into 6-well plates at a density of 5×10^5 cells/well. Twenty-four hours later, the cells were cotransfected with 1 μg/well of p9HIF1-Luc firefly luciferase reporter plasmid (it contains nine copies of the human HIF binding sequence located between positions −985 and −951 of the 5′human VEGF gene promoter and was generously gifted by Dr. Manuel Ortiz de Landázuri, (Service of Immunology, Hospital Princesa, Madrid, Spain) and 0.5 μg/well of the *R. reniformis* luciferase reporter pRL-CMV using lipofectamine (Invitrogen, CA). Transfected cells were next incubated with complete growth medium without antibiotic for 24 hr, and cells were treated as described in the legends to figures. Treatments were done in medium

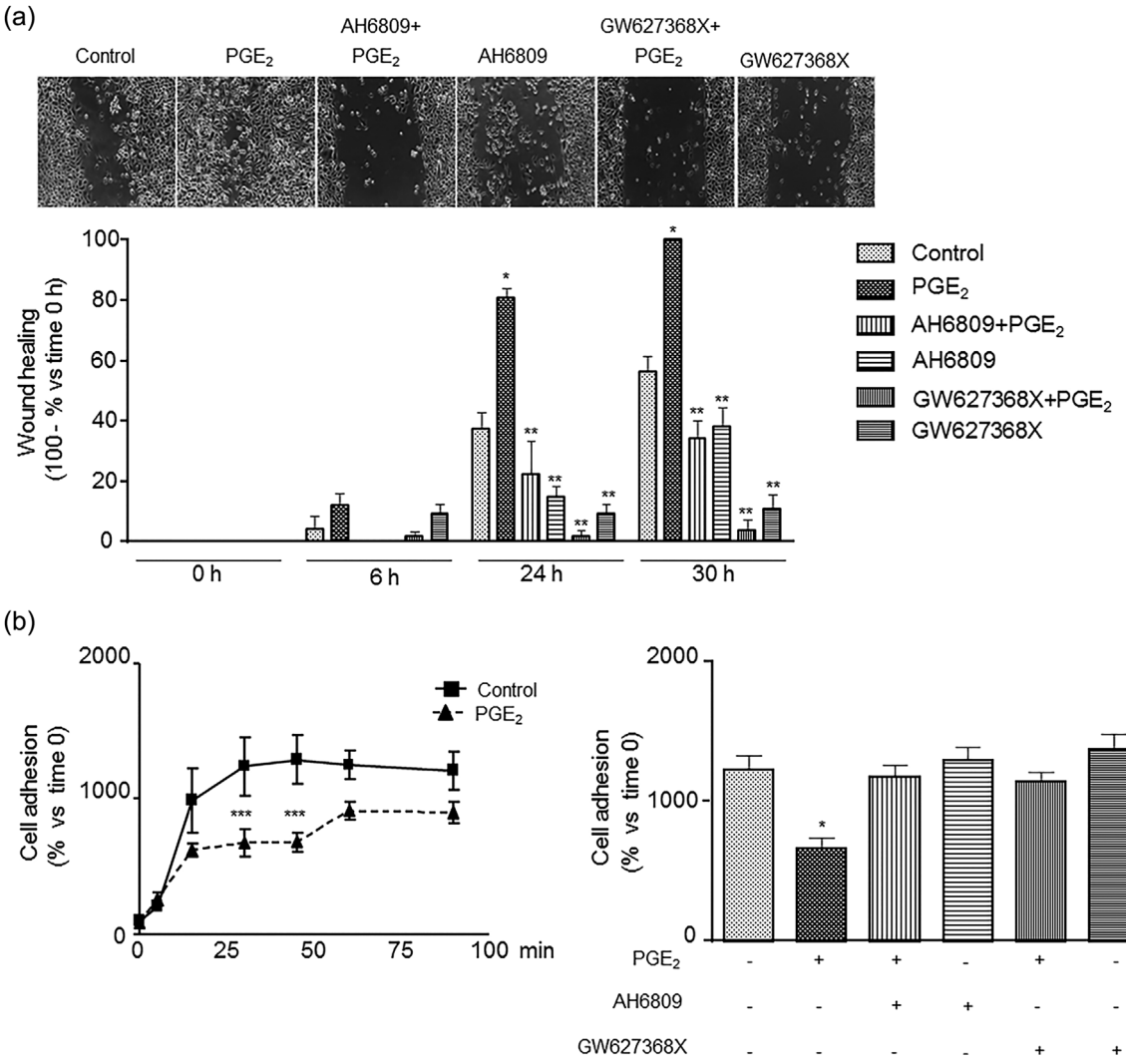

FIGURE 2 PGE_2 increases RWPE-1 cell migration and decreases RWPE-1 cell adhesion to collagen I in an EP receptor antagonist-sensitive manner. (a) PGE_2 increases RWPE-1 cell migration in the wound healing assay in an EP receptor antagonist-sensitive manner. Cells were cultured until confluent. Then, cell layer was then carefully wounded using sterile tips. Afterwards cells were treated for 1 hr with 10 μM AH6809 (EP1-3 receptor antagonist) or 10 μM GW627368X (EP4 receptor antagonist) and then with 1 μM PGE_2. The wounded cell layer was photographed and measured at 0–30 hr time points and a representative photograph is shown at 24 hr (10× amplification). (b) PGE_2 decreases RWPE-1 cell adhesion to collagen I in an EP receptor antagonist-sensitive manner. Left: Cells in suspension were incubated for 1 hr with or without 1 μM PGE_2. Then cells were plated on collagen I coated wells and incubated for up to 90 min. The number of adherent cells was assessed by the MTT assay. Right: Cells were preincubated for 1 hr with 10 μM AH6809 or 10 μM GW627368X before being treated with 1 μM PGE_2 for 30 min. General information: All experiments were repeated three times. Bars are mean ± SD. Statistical analysis: $^*p < 0.01$ versus other groups; $^{**}p < 0.01$ versus control and PGE_2. $^{***}p < 0.01$ versus control

without EGF/bovine pituitary extract. Afterwards cells were harvested and luciferase activity was measured using the dual-luciferase reporter assay system (Promega, Madison, WI). The data were normalized against the *R. reniformis* luciferase activity.

2.11 | Western blot analysis

RWPE-1 cells were split into 6-well plates at a density of 4×10^5 cells/well. Then, they were cultured for 24 hr in medium without EGF/bovine pituitary extract and treated as described in the legends to figures. Afterwards immunoblotting was performed essentially as described previously (Fernández-Martínez et al., 2011). In short, cell lysates were prepared and measured for protein content using the Bradford assay. Approximately 35 μg of protein was electrophoresed 10% SDS–polyacrylamide electrophoresis gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% powdered skimmed milk/0.2% Tween 20 in PBS and were incubated overnight at 4°C with mouse anti-HIF-1α antibody (1:1000 dilution) and rabbit anti-phospho-EGFR (1:500). After, membranes were incubated at room temperature for 1 hr, with the corresponding secondary antiserum (1:4000). To ensure equal loading of proteins, the membranes were stripped and re-probed with anti-β-actin antibody. The signals were detected with enhanced chemiluminescence reagent (Amersham Healthcare, Buckinghamshire, England). Quantification of

band densities was performed using Quantitive One Program (Bio-Rad, Alcobendas, Spain).

2.12 | Immunofluorescence analysis

RWPE-1 cells were placed in 24-well plates (10^4 cells/glass coverslip) were treated as indicated in the results section. Then, they were fixed with 2% paraformaldehyde for 10 min, permeabilized with 0.1% (v/v) Triton X-100 in PBS for 10 min, washed with PBS, blocked with 4% bovine serum albumin for 1 hr at room temperature and incubated overnight at 4°C with anti-PGE_2 (1:50 dilution) and anti-EP2-EP4 (1:500 dilution) antibodies. Cells were then incubated at 37°C with α-rabbit-Alexa-Fluor® 488 (1:2000) for 1 hr in the darkness. Coverslips were then washed and mounted with ProLong Gold antifade Reagent

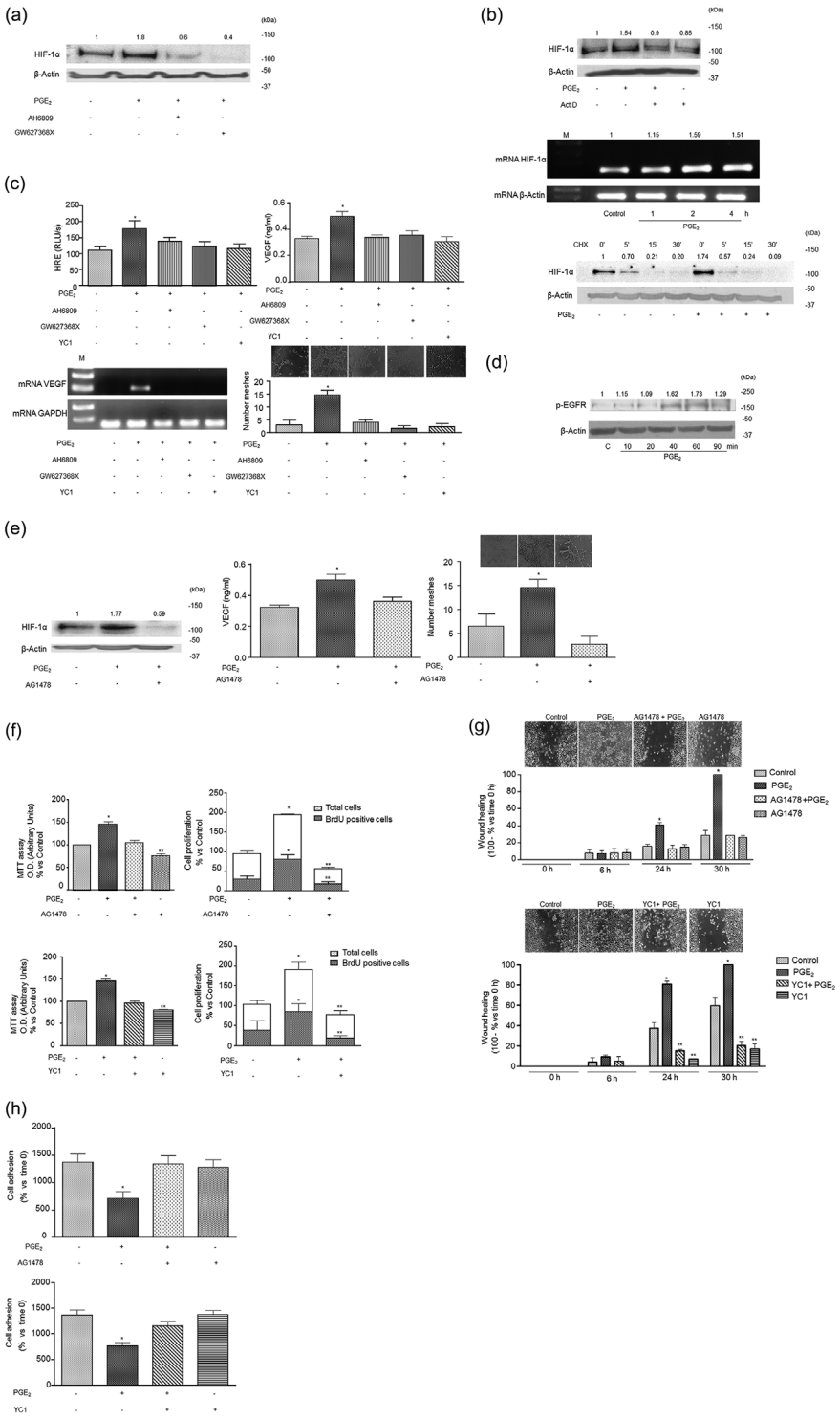

FIGURE 3 Continued.

with DAPI (Invitrogen). Detection was performed by confocal laser scan microscopy LEICA TCS-SL (Heidelberg, Germany).

2.13 | Statistical analysis

Each experiment was repeated at least three times. The results are expressed as the mean ± standard deviation (SD). Statistical significance was assessed by Student's *t*-test or one way analysis of variance (ANOVA) following by the Bonferroni's test for multiple comparisons. The level of significance was set at $p < 0.05$.

3 | RESULTS

3.1 | PGE_2 increases RWPE-1 cell proliferation in an EP receptor antagonist-sensitive manner

PGE_2 is a major downstream mediator of COX-2 that promotes cellular proliferation in PC cells (Jain et al., 2008; Jiang & Dingledine, 2013; Huang et al., 2013; Madrigal-Martínez et al., 2015; Vo et al., 2013; Wang & Klein, 2007) but its effect on the proliferation of human non-malignant prostate epithelial cells is unknown. We addressed this subject through measuring the mitochondrial reduction of MTT in RWPE-1 cells incubated with 1 μM and 10 μM PGE_2 for 24 hr. Our results indicated that cell proliferation increased upon treatment with PGE_2 (Figure 1a, left). In order to confirm the proliferative effect of PGE_2, we assessed DNA synthesis by BrdU uptake in RWPE-1 cells. PGE_2 significantly increased BrdU uptake by cells (Figure 1a, right), which reflects its enhancing effect on cell proliferation.

PGE_2 acts through four different receptor subtypes, EP1 to EP4, and we have previously found that EP receptors mediate the increase in the proliferation of prostate cancer PC3 cells induced by PGE_2 (Madrigal-Martínez et al., 2015). In order to study the role of EP receptors in PGE_2-induced increase in RWPE-1 cell proliferation, MTT and BrdU assays were performed in RWPE-1 cells which were incubated for 1 hr with AH6809 or GW627368 (respective antagonists of EP1-3 receptors and EP4 receptors) before being treated with PGE_2. As shown in Figure 1b, the pre-incubation treatments resulted in the inhibition of PGE_2-induced RWPE-1 cell proliferation, this suggesting that it is mediated by EP receptors.

3.2 | PGE_2 increases RWPE-1 cell migration and diminishes RWPE-1 cell adhesion to collagen in an EP receptor antagonist-sensitive manner

RWPE-1 cells have a remarkable migration potential. For instance, using a migration insert chamber, it has been found that RWPE-1 cells migrated more than PC cell lines such as PC3 cells and LNCaP cells (Moroz et al., 2013). To assess the effect of PGE_2 on RWPE-1 cell migration we used a scratch wound healing assay. Treatment with PGE_2 increased the migration of RWPE-1 cells, which was prevented by pre-incubation with antagonists of EP receptors (Figure 2a).

FIGURE 3 PGE_2 increases the expression and activity of HIF-1α in an EP receptor antagonist-sensitive manner: the role of EGFR-dependent increase in HIF-1α expression in the effects of PGE_2 on RWPE-1 cells. (a) PGE_2 up-regulates HIF-1α in an EP receptor antagonist sensitive manner. Cells were preincubated for 1 hr with 10 μM AH6809 or 10 μM GW627368X before being treated with 1 μM PGE_2 for 7 hr. Expression of HIF-1α was assessed by Western blot analysis. (b) PGE_2 up-regulates HIF-1α at the transcriptional level. Upper panel: Transcriptional inhibitor actinomycin D (Act. D) prevents PGE_2-induced HIF-1α up-regulation. Cells were pre-treated for 1 hr with 1 μg/ml Act. D before being treated with 1 μM PGE_2 for 7 hr. Expression of HIF-1α was assessed by Western blot analysis. Middle panel. PGE_2 increases mRNA expression. Cells were treated with 1 μM PGE_2 for up to 4h and expression of HIF-1α mRNA was assessed by semi-quantitative RT-PCR. Lower panel: PGE_2 does not modify HIF-1α protein half-life. Cells were first treated with 1 μM PGE_2 for 7 hr and then incubated with translation inhibitor cycloheximide (50 μg/ml). (c) PGE_2 increases the activity of HIF-1α in an EP receptor antagonist-sensitive manner. Upper panel, left. Activity of a hypoxia-responsive element (HRE)-driven reporter construct. RWPE-1 cells were transiently transfected with p9HIF-1-Luc (a plasmid which contained nine copies of the human HIF binding sequence located between positions −985 and −951 of the 5′human VEGF gene promoter). Luciferase activity in lysates of cells treated with PGE_2 for 4 hr was measured and expressed as relative luminescence units (RLU). Each bar represents the luciferase activity normalized by Renilla luciferase activity. Lower panel, left. VEGF-A mRNA expression. Cells were treated with 1 μM PGE_2 for 8 hr and expression of mRNA VEGF-A mRNA was assessed by semi-quantitative RT-PCR. Upper panel, right. VEGF-A production. Cells were treated with 1 μM PGE_2 for 24 hr and VEGF-A production was quantified by ELISA. Lower panel, right. Endothelial tube formation induced by RWPE-1 culture medium. RWPE-1 cells were treated with 1 μM PGE_2 for 24 hr. Then culture medium was collected and tube formation by endothelial cells incubated in this medium was quantified by counting the number of meshes. Photographs are taken at a 10× magnification. All panels: The prevention of PGE_2 effects by HIF inhibitor YC-1 demonstrates that these effects are HIF-dependent effects. (d) Treatment with PGE_2 results in increased EGFR tyrosine phosphorylation. RWPE-1 cells were incubated for 24 hr in medium without EGF/bovine pituitary extract and treated with 1 μM PGE_2 for up to 90 min. Then, tyrosine phosphorylation of EGFR (EGFR-P) was assessed by Western blot analysis. (e) The inhibitor of EGFR activation AG1478 prevents PGE_2-induced increase in expression and activity of HIF-1α. These experiments were done as above. Left. Effect on HIF-1α expression. Middle. Effect on VEGF production. Right. Effect on endothelial tube formation induced by RWPE-1 culture medium. (f–h) AG1478 and YC-1, respective inhibitors of the activation of EGFR and HIF, prevent PGE_2-induced increase in RWPE-1 cell proliferation, migration, adhesion. Treatments with PGE_2 and assessment of cell proliferation, migration and adhesion were as indicated in Figures 1 and 2, except that here AG1478 and YC-1 were the inhibitors used. General information. RWPE-1 cells were pre-incubated for 1 hr with 10 μM AH6809 (EP1-3 receptor antagonist), 10 μM GW627368X (EP4 receptor antagonist), 10 μM YC-1 (HIF inhibitor), or 1 μM AG1478 (inhibitor of EGFR activation) and incubated with 1 μM PGE_2. Equal protein or mRNA loading were confirmed, respectively, by probing with an anti-β-actin antibody or by assessing the β-actin or GAPDH gene expression level. Each experiment was repeated three times. Photographs are representative of the results obtained. Bars are mean ± SD. Statistical analysis $^*p < 0.01$ versus other groups $^{**}p < 0.01$ versus control and PGE_2

Cell adhesion has a fundamental role in the motility of normal cells. When cells adhere too little, they are unable to develop the traction force necessary for motility; but if they adhere too tightly to their matrix they fail to move. Therefore we studied the effect of PGE_2 on cell adhesion to collagen I as described in Material and Methods. As shown in Figure 2b, left panel, treatment with PGE_2 resulted in loss of RWPE-1 cell adhesion. We chose an incubation time of 30 min with PGE_2 to assess the role of EP receptors in PGE_2-induced loss of RWPE-1 cell adhesion to collagen type I and found that it was prevented by pre-incubation for 1 hr with antagonists of receptors (Figure 2b, right panel).

These results indicated that PGE_2-induced increase in RWPE-1 cell motility and loss of cell adhesion to collagen type I are mediated by EP receptors.

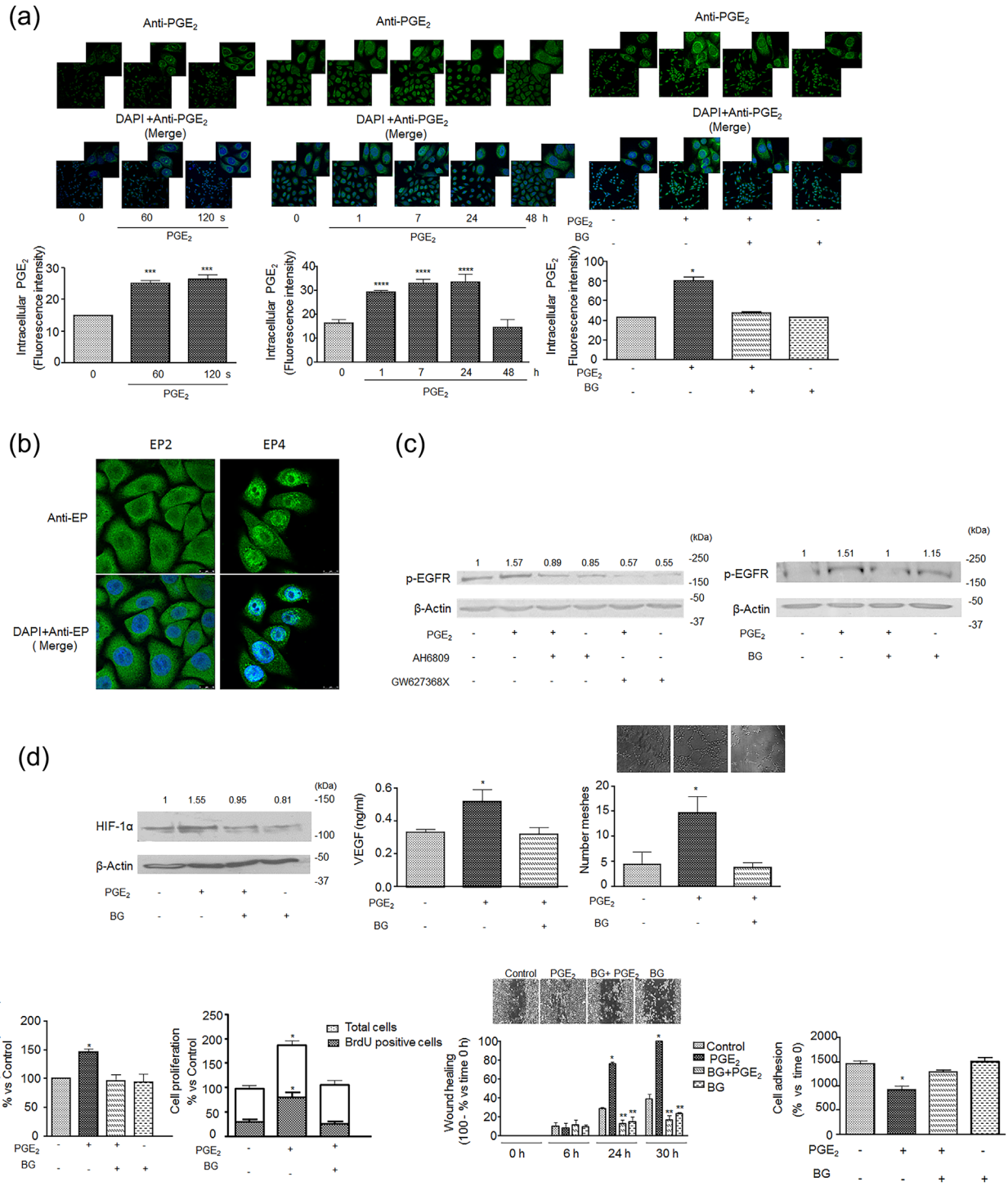

FIGURE 4 Continued.

3.3 | PGE_2 increases the expression and activity of HIF-1α in an EP receptor antagonist-sensitive manner: The role of EGFR-dependent increase in HIF-1α expression in the effects of PGE_2 on RWPE-1 cells

HIF-1α participates in the survival and proliferation of prostate cells (Li et al., 2010; Yun, Li, Cho, Park, & Chun, 2010). HIF-1α has also been found to mediate testosterone-induced hyperplasia in the rat prostate (Li et al., 2010) and elevated expression of HIF-1α in actively growing prostatic tissues has been associated with clinical features of BPH (Wu et al., 2016). Furthermore, we have previously found in PC-3 and LNCaP cells (Fernández-Martínez, Jiménez, Manzano, & Lucio-Cazaña, 2012; Madrigal-Martínez et al., 2015) that PGE_2 increases the expression of HIF-1α in an EP receptor-dependent manner. Here, we conformed that this is also the case in RWPE-1 cells (Figure 3a).

Earlier studies from our group on the mechanism involved in the up-regulation of HIF-1α by PGE_2 in human renal proximal tubular HK2 cells indicated that PGE_2 did not increase the half-life of HIF-1α protein (a crucial difference with the canonical, pvHL-proteasome pathway of accumulation of HIF-1α under hypoxia (Wigerup, Påhlman, & Bexell, 2016). Instead, transcriptional mechanisms were found to be responsible for the increase in HIF-1α expression induced by PGE_2 in HK-2 cells (Fernández-Martínez, Arenas, & Lucio-Cazaña, 2012). To examine the possibility that transcriptional mechanisms were also responsible for PGE_2-induced HIF-1α up-regulation in RWPE-1 cells, we asked whether pre-incubation with the inhibitor of transcription actinomycin D prevented the effect of PGE_2, which was confirmed by our results (Figure 3b, upper panel). Furthermore, semi-quantitative PCR showed that the levels of HIF-1α mRNA increased upon treatment with PGE_2 (Figure 3b, middle panel). While these results indicate the intervention of transcriptional mechanisms, they do not exclude a hypothetical contribution of stabilization of HIF-1 protein by PGE_2. This possibility was ruled out by analysing the effect of PGE_2 on the turnover of HIF-1 in RWPE-1 cells which were first incubated for 8 hr under control conditions or with PGE_2 and then treated for 0–30 min with translation inhibitor cycloheximide (Figure 3b, lower panel).

We next asked whether PGE_2-induced HIF-1α was biologically active, through studying the effect of PGE_2 on three different HIF-dependent events: activation of hypoxia-responsive elements (HRE), production of VEGF-A and activation of in vitro angiogenesis

i) Activation of HRE. HIF-1α-dependent transcription results from the binding of HIF-1α (together with HIF-1β and transcriptional coactivators) to HRE in target genes (Lando, Peet, Whelan, Gorman, & Whitelaw, 2002). We assessed the luciferase activity in RWPE-1 cells that were transfected with p9HIF1-Luc (a plasmid which contained nine copies of the human HIF binding sequence located between positions −985 and −951 of the 5′human VEGF gene promoter) (Aragonés et al., 2001). PGE_2 induced an YC-1 (HIF-inhibitor)-sensitive increase in luciferase activity (Figure 3c, upper left panel). Increased activity of HRE in PGE2-treated cells was also prevented by the antagonists of EP receptors AH6809 and GW627368X.

ii) Production of VEGF-A. Treatment with PGE_2 resulted in enhanced expression of VEGF-A mRNA and in increased release of VEGF-A to the medium, which was prevented by preincubation with AH6809 and GW627368X or with HIF inhibitor YC-1 (Figure 3c, lower left and upper right panels).

iii) Angiogenic ability. It was assessed by measuring endothelial tube formation induced by RWPE-1 culture medium (i.e., medium containing the secreted VEGF-A from PGE_2-treated cells). As shown in Figure 3c, right panel, treatment of RWPE-1 cells with PGE_2 resulted in a AH6809, GW627368X, and YC-1-sensitive increase in endothelial tube formation by conditioned medium from RWPE-1 cells. Overall, the results shown in Figure 3c–e confirm that PGE_2-induced HIF-1α is biologically active and that it promotes angiogenic activity. EGFR-dependent increase in HIF-1α mediates PGE_2-induced PC3 cell proliferation, migration and loss of PC3 cell adhesion to collagen type I (Fernández-Martínez & Lucio-Cazaña, 2015). Since it is possible that this mechanism also mediates these actions of PGE_2 in RWPE-1 cells, we first asked whether treatment with PGE_2 resulted in activation of EGFR. To

FIGURE 4 Transactivation of EGFR by intracellular EP receptors plays a critical role in mediating the effects of PGE_2 on RWPE-1 cells. (a) Internalization of PGE_2 and its prevention by the inhibitor of prostaglandin transporter bromocresol green (BG). Cells were incubated with 1 μM PGE_2 and they were fixed, permeabilized and subjected to immunofluorescence analysis after incubation with anti-PGE_2 antibody. For BG experiments (left panel), cells were first incubated for 1 h with 50 μM BG and then for 120 s with 1 μM PGE_2. Note that control RWPE-1 cells had low, but detectable expression of $iPGE_2$. (b) Cellular location of EP receptors. Cells were fixed, permeabilized and subjected to immunofluorescence analysis after incubation with anti EP2 or anti-EP4 receptor antibodies as indicated in Material and Methods. Nuclei were stained with DAPI. (c) Transactivation of EGFR by intracellular EP receptors: the antagonists of EP1-3 receptors AH6809 and EP4 receptor GW627368X and the inhibitor of prostaglandin transporter BG prevent PGE2-induced increase in phosphorylated EGFR (EGFR-P). RWPE-1 cells were incubated for 24 hr in medium without EGF/bovine pituitary extract, then they were incubated for 1 hr with 10 μM AH6809, 10 μM GW627368X or 50 μM BG, and treated with 1 μM PGE_2 for 1 hr. Afterwards, tyrosine phosphorylation of EGFR (EGFR-P) was assessed by Western blot analysis. (d) The effects of PGE_2 on RWPE-1 cells are dependent on intracellular PGE_2: prevention by BG of PGE_2 effects. Treatments with PGE_2 and assessment of HIF-1α expression and activity, cell proliferation, migration, adhesion, production of vascular endothelial growth factor-A, and activated in vitro angiogenesis, were as indicated in Figures 1 to 3, except that here 50 μM BG was the inhibitor used. General information. Equal protein loading was confirmed by probing with an anti-β-actin. Each experiment was repeated three times. Photographs are representative of the results obtained. Bars are mean ± SD. Statistical analysis $^{*}p < 0.01$ versus other groups $^{**}p < 0.01$ versus control and PGE_2 $^{***}p < 0.01$ versus control $^{****}p < 0.01$ versus control and 48 hr

this purpose, we studied by Western blot analysis the time course of the phosphorylation of EGFR in RWPE-1 cells incubated with PGE_2 for up to 90 minutes. As seen in Figure 3d, EGFR tyrosine phosphorylation increased from the very first minutes and remained in high values after 90 min incubation. We then analyzed in PGE_2-treated cells the effect of the inhibitor of EGFR activation AG1478 on the expression of HIF-1α, the production of HIF-regulated-VEGF-A and in vitro. As shown in Figure 3e, AG1478 prevented these PGE_2 effects, which suggests that EGFR activation plays a relevant role in the increase of HIF-1α expression and biological activity induced by PGE_2 in RWPE-1 cells.

As indicated above EGFR-dependent increase in HIF-1α mediates PGE_2-induced PC3 cell proliferation, migration and loss of PC3 cell adhesion to collagen type I (Fernández-Martínez & Lucio-Cazaña, 2015). Since PGE_2 has the same effects on RWPE-1 cells we studied if they were prevented by either the inhibitor of EGFR activation AG1478 or the inhibitor of HIF-1α YC-1, which was confirmed by our results (Figure 3f–h).

In summary, these results shown in Figure 3 indicate that activation of EGFR leading to increased expression of HIF-1α plays a determinant role in the effects of PGE_2.

3.4 | Transactivation of EGFR by intracellular EP receptors plays a critical role in mediating the effects of PGE_2 on RWPE-1 cells

We have previously found that PGE_2 has to reach the intracellular compartment and activate a subset of EP receptors, which is located intracellularly, as a prerequisite for being effective in PC3 cells (Madrigal-Martínez et al., 2015). In order to determine whether intracellular PGE_2 ($iPGE_2$) and intracellular EP receptors (iEP) have a role in the effects of PGE_2 in RWPE-1 cells, we first asked whether treatment with PGE_2 resulted in increased intracellular levels of immunoreactive PGE_2, which was confirmed by immunofluorescence analysis (Figure 4a). Furthermore, this increase in $iPGE_2$ was prevented by inhibitor of prostaglandin uptake transport bromocresol green (BG) (Figure 4a, right).

The biological actions of PGE_2 have been generally attributed to result from its interaction with four different plasma membrane G protein-coupled EP receptors. However, it has been also documented in a variety of cell types and tissues the intracellular presence of a subset of functional EP receptors receptors (Bhattacharya et al., 1998, 1999; Fernández-Martínez, Arenas, et al., 2012; Gobeil et al., 2002; Zhu et al., 2006). Since we have previously found EP2 and EP4 receptors inside PC3 cells (Madrigal-Martínez et al., 2015), we next asked whether these receptors could be also located inside RWPE-1 cells. The results of a preliminary immunofluorescence analysis (Figure 4b) confirmed this possibility.

Activation of EGFR through a molecule that does not, itself, bind EGFR is called transactivation. Earlier studies from our group have shown that transactivation of EGFR by intracellular EP2 receptors plays a critical role in the stimulating effect of PGE_2 on cancer-related phenotypes in PC3 cells (Fernández-Martínez & Lucio-Cazaña, 2015). Since our results indicate that activation of EGFR is also critical for the effects of PGE_2 on RWPE-1 cells, we sought to confirm that EGFR was transactivated by EP receptors in PGE_2-treated RWPE-1 cells: since antagonists of EP receptors prevented PGE_2-induced EGFR phosphorylation (Figure 4c, left), it follows that this is most likely an EP receptor-dependent transactivation event. Next, to ascertain whether PGE_2-induced EGFR phosphorylation involved $iPGE_2$, we analyzed the phosphorylation of EGFR in RWPE-1 cells which were treated with the inhibitor of prostaglandin transport BG prior to treatment with PGE_2. Figure 4c, right, shows that BG prevented PGE_2-induced EGFR phosphorylation. Taken together, the results shown in Figure 4a–c indicated that transactivation of EGFR by PGE_2 in RWPE-1 cells is mediated by intracellular EP receptors activated by $iPGE_2$.

We have shown above that EGFR activation is responsible for the up-regulation of HIF-1α in PGE_2-treated RWPE-1 cells. Given that activation of EGFR is due to $iPGE_2$ rather than to extracellular PGE_2, one might expect that the inhibition of the transport of PGE_2 to the intracellular compartment will result in loss of the up-regulation of HIF-1α induced by PGE_2. This hypothesis was confirmed through analysing the expression of HIF-1α in RWPE-1 cells which were pre-incubated with the inhibitor of prostaglandin transport BG before being treated with PGE_2 (Figure 4d, upper panel, left). This is a key result since EGFR-dependent HIF-1α up-regulation mediates the effects of PGE_2 in RWPE-1 cells. In fact, all these effects were prevented by BG (Figure 4d, upper panels middle and right and lower panels). Collectively, the results shown in Figure 4d indicate that PGE_2 has to reach the inside of RWPE-1cells to exert its effects.

4 | DISCUSSION

Chronic inflammation is a common finding in the cancerous and non-cancerous prostate gland. A growing body of evidence suggests the important role of inflammatory infiltrates and their mediators in the development of chronic prostatic diseases. Therefore, chronic prostatic inflammation could be considered a possible link between prostate proliferative diseases such as BPH and PC (Elkahwaji, 2012). In the present work we show in human prostate epithelial RWPE-1 cells that the inflammatory mediator PGE_2, through a non-canonical intracrine mechanism involving the axis $iPGE_2$/iEP receptors/EGFR/HIF-1α, diminishes cell adhesion and increases cell proliferation and migration and activates in vitro angiogenesis. These changes are not likely malignant ones: acquisition of invasive characteristics by epithelial cells requires epithelial to mesenchymal transition, which is characterized by dramatic molecular changes with corresponding changes in cellular morphology (Savagner, 2001). However PGE_2 did not induce any changes in cell morphology nor in the expression of E- and N-cadherin (these results are not shown), which are molecular markers of epithelial to mesenchymal transition. Therefore, the effects of PGE_2 on RWPE-1 cells are more likely related to non-malignant prostate diseases associated with inflammation, such are BPH or proliferative inflammatory atrophy. Importantly, we have previously

shown that PGE_2 has the same effects in PC3 cells (i.e., diminished cell adhesion and enhanced cell proliferation and migration and activates in vitro angiogenesis) and the same non-canonical intracrine mechanism (i.e., the axis $iPGE_2$/iEP receptors/EGFR/HIF-1α) (Fernández-Martínez & Lucio-Cazaña, 2015). Therefore, our data suggest that this PGT-dependent mechanism may be a possible link between the stimulating effects of PGE_2 in cancerous and non-cancerous prostate proliferative diseases associated with chronic inflammation and that PGT inhibitors should be tested as a novel therapeutic modality to treat prostate proliferative disease (Figure 5).

HIF-1α is a master transcription factor that orchestrates the expressions of about 80 genes, which include erythropoietin, angiogenic factors, glycolytic enzymes, and cell growth/survival factors (Wigerup et al., 2016). HIF-1α has been shown to contribute to metastasis and chemo-resistance of castrate-resistant prostate cancer and to promote migration, invasion, angiogenesis, and chemo-resistance in prostate cancer cells (Dai, Bae, & Siemann, 2011; Ranasinghe et al., 2013). HIF-1α is upregulated by testosterone in the hyperplasic prostates of rats and in cultured human prostate cells (Li et al., 2010) and it has been proposed that HIF-1α mediates prostate enlargement under inflammatory conditions (Kim et al., 2013). Although evidence accumulated over the last decade supports the notion that inflammation plays a role in the pathogenesis of prostate cancer (Sfanos & De Marzo, 2012) and benign prostate diseases such as BPH or proliferative inflammatory atrophy (Bardan et al., 2014; Thapa & Ghosh, 2015), the mechanisms of how prolonged inflammation promotes these proliferative prostate diseases remain poorly understood. Our results suggest that PGE_2, through up-regulating HIF-1α, might be one of the key inflammatory mediators in the pathogenesis of these diseases. Further specific studies should be performed to confirm this hypothesis.

The prostaglandin uptake transporter PGT (or SLCO2A1), a member of the organic anion transporter family,is positioned at the center of the effects of PGE_2 on RWPE-1 and PC3 cells because all of them are prevented by its inhibition (Figure 3 and Fernández-Martínez & Lucio-Cazaña, 2015, respectively). Therefore, PGE_2 has to be translocated from the outside to the inside of cells for exerting its effects as $iPGE_2$. Interestingly, PGT is likely involved in the metabolic termination of PGE_2 signalling because it allows PGE_2 to gain access to cytoplasmic 15-hydroxy-prostaglandin dehydrogenase, which inactivates PGE_2 through its oxidation (Nomura et al., 2004). However, given that it is now apparent that prostaglandins also activate intracellular prostaglandin receptors (Bhattacharya et al., 1998; Fernández-Martínez, Arenas, et al., 2012; Gobeil et al., 2002; Zhu et al., 2006), carrier-mediated uptake of PGE_2 across the plasma membrane appears to provide at least one mechanism through which PGE_2 and other prostaglandins can be delivered to an intracellular site of action (Helliwell, Berry, O'Carroll, & Mitchell, 2004). Despite the general assumption that PGE_2 is an autocrine or paracrine mediator which exerts its actions via plasma membrane spanning G-protein coupled EP receptors (Legler, Bruckner, Uetz-von Allmen, & Krause, 2010) our results indicate that an intracrine mechanism mediate the effects of PGE_2 on RWPE-1 cells. Intracrine signaling refers to a process whereby

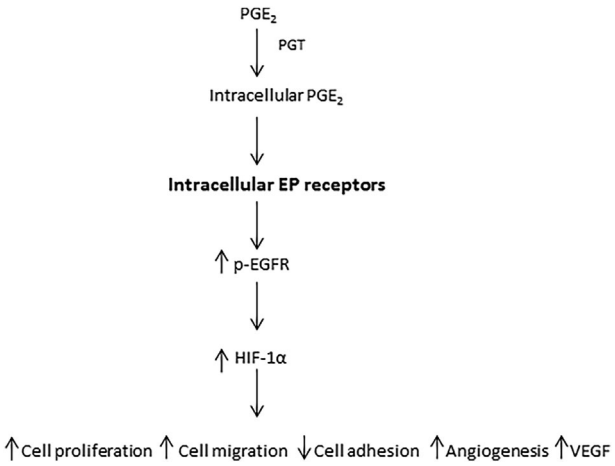

FIGURE 5 Proposed pathway by which intracellular PGE_2 mediates the protumoral actions of PGE_2

a ligand, originating within a target cell or taken up from the extracellular milieu, acts upon intracellular receptors. In our case, this intracrine mechanism involves the transport of PGE_2 to the inside of the cell and the transactivation of EGFR by iEP receptors (the term transactivation refers to this nonclassical mode of signaling system cross-talk, in distinction to receptor activation induced by cognate ligands, e.g the activation of EGFR by EGF). In recent years it has been reported transactivation of plasma membrane EGFR by PGE_2-activated EP receptors (Liebmann, 2011). These findings are just a particular case of activation of EGFR by plasma membrane G-protein coupled receptors (GPCR). We do not know whether iEP receptors are coupled to G-proteins in RWPE-1 cells but it is not unlikely because it has been previously described the existence of G-protein EP receptors in the nuclear envelope of cell types and tissues the intracellular presence of a subset of functional EP receptors (Bhattacharya et al., 1998; Fernández-Martínez, Arenas, et al., 2012; Gobeil et al., 2002; Zhu et al., 2006). Interestingly, the diverse functions exerted by agonists and hormones acting at intracellular GPCRs, although still poorly understood, suggest that intracrine signaling may play important roles distinct from those of the same receptors activated at the cell surface (Tadevosyan, Vaniotis, Allen, Hébert, & Nattel, 2012). In fact, our results indicate that only iEP receptors (and not the plasma membrane ones) are able to transactivate EGFR and trigger all the HIF-1α-dependent effects in PGE_2-treated RWPE-1 cells.

Of note, we have previously found that PGE_2 induced EP2 antagonist-dependent DNA synthesis in nuclei isolated from PC3 cells, which indicates that the cell nucleus contains functional EP2 receptors (Fernández-Martínez & Lucio-Cazaña, 2015). These results and our current findings open the possibility that signal cascades that proceed entirely in the cell nucleus or other intracellular locations might be responsible for several PGE_2 effects on prostate cells that are assumed to be due to cell membrane EP receptors.

COX inhibitors are criticized for causing unwanted side effects, which is a major obstacle for widespread use in the prevention of

inflammation associated prostate proliferative diseases. Therefore, targeting downstream signaling pathways of PGE_2 may represent an attractive new strategy for prevention and therapy of these diseases. Taking into account the relevant role of $iPGE_2$ in the effects of PGE_2 on RWPE-1 cells and PC3 cells, we propose to test inhibitors of PGT as a novel therapeutic modality to treat prostate proliferative disease.

ACKNOWLEDGMENT

This work was supported by grants SAF2011-26838 from the Spanish Ministerio de Ciencia e Innovación.

CONFLICTS OF INTEREST

There are no conflicts of interest to disclose

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How to cite this article: Madrigal-Martínez A, Fernández-Martínez AB, Lucio Cazaña FJ. Intracrine prostaglandin E_2 pro-tumoral actions in prostate epithelial cells originate from non-canonical pathways. *J Cell Physiol*. 2018;233: 3590–3602. https://doi.org/10.1002/jcp.26220

Received: 12 December 2017 | Accepted: 10 September 2018

DOI: 10.1002/jcp.27515

ORIGINAL RESEARCH ARTICLE

WILEY Journal of Cellular Physiology

PROSTAGLANDIN E_2 stimulates cancer-related phenotypes in prostate cancer PC3 cells through cyclooxygenase-2

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Funding information
Spanish Ministerio de Ciencia e Innovación, Grant/Award Number: SAF2011-26838

Abstract

Cyclooxygenase (COX)-derived prostaglandin E2 (PGE_2) affects many mechanisms that have been shown to play roles in carcinogenesis. Recently, we found that, in androgen-independent prostate cancer PC3 cells, PGE_2 acts through an intracrine mechanism by which its uptake by the prostaglandin transporter (PGT) results in increased intracellular PGE_2 ($iPGE_2$), leading to enhanced cell proliferation, migration, invasion, angiogenesis, and loss of cell adhesion to collagen I. These $iPGE_2$-mediated effects were dependent on hypoxia-inducible factor 1-α (HIF-1α), whose expression increased upon epidermal growth factor receptor (EGFR) transactivation by a subset of intracellular PGE_2 receptors. Here, we aimed to study the role of COX in PGE_2 protumoral effects in PC3 cells and found that the effects were prevented by inhibition of COX-2, which highlights its crucial role amplifying the levels of $iPGE_2$. Treatment with exogenous PGE_2 determined a transcriptional increase in COX-2 expression, which was abolished by genetic or pharmacologic inhibition of PGT. PGE_2-induced increase in COX-2 expression and, thereby, in transcriptional increase in HIF-1α expression was due to EGFR activation, leading to the activation of Phosphoinositide 3-kinase/Akt, Extracellular signal -regulated kinases 1/2, p38 and Mitogen- and stress-activated protein kinase-1 (PI3K/Akt, Erk1/2, p38 and MSK-1). Collectively, the data suggest that EGFR-dependent COX-2 upregulation by a novel positive feedback loop triggered by $iPGE_2$ underlies the intracrine pro-tumoral effects of PGE_2 in PC3 cells. Therefore, this feedback loop may be relevant in prostate cancer for the maintenance of PGE_2-dependent cancer cell growth through amplifying the activity of the COX-2 pathway.

KEYWORDS
cyclooxygenase-2 (COX-2), hypoxia-inducible factor 1-α (HIF-1α), intracellular prostaglandin E2 ($iPGE_2$), PC3 cells, prostate cancer (PC)

1 | INTRODUCTION

Chronic inflammation is a risk factor for the development and progression of prostate cancer (PC; reviewed in Thapa & Ghosh, 2015). In PC, cyclooxygenase-2 (COX-2) and inflammatory mediators, such are cytokines and chemokines, have been found to be over-expressed (Fernández-Martínez et al., 2012; Gupta, Srivastava, Ahmad, Bostwick, & Mukhtar, 2000; Kirschenbaum et al., 2000). COX-1 and COX-2 are isoforms of COX that convert arachidonic acid to prostaglandins (PGs). COX-1 is constitutively expressed, whereas inflammation is associated to the induction of COX-2. Prostaglandin E2 (PGE_2) has been found at increased levels in PC as well as in other

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malignant neoplasms, such as lung cancer, breast cancer, and colon cancer (Badawi, 2000; Wang & Dubois, 2010). Proliferation, migration, invasion, apoptosis, and angiogenesis are targets of the carcinogenetic effects of PGE_2 in PC and other types of cancer (Greenhough et al., 2009; Hanaka et al., 2009; Jain, Chakraborty, Kale, & Kundu, 2008; Jiang & Dingledine, 2013; Vo et al., 2013). It has been shown that, in experimental PC, treatment with nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit COX (and thereby the production of PGE_2), induces apoptosis, inhibits cell proliferation, and decreases metastasis (Fernández-Martínez et al., 2009; Hussain, Gupta, & Mukhtar, 2003). We have found that several protumoral actions of PGE_2 in PC3 cells depend on its transport to the inside the of cells and further activation by intracellular PGE_2 (iPGE2) of a subset of intracellular PGE2 receptors (EP receptors) (Fernández-Martínez & Lucio-Cazaña, 2015a; Madrigal-Martínez, Lucio-Cazaña, & Fernández-Martínez, 2015). This is a noncanonical intracrine mechanism of PGE_2 action in which the prostaglandin transporter (PGT, responsible for most PGE_2 internalization) mediates the influx of PGE_2 (Nomura, Lu, Pucci, & Schuster, 2004; Schuster, 2002). In PC3 cells, $iPGE_2$ inhibits cell adhesion and stimulates cell proliferation, migration, invasion, and in vitro angiogenesis (Fernández-Martínez & Lucio-Cazaña, 2015a; Madrigal-Martínez et al., 2015), and these effects are dependent on hypoxia-inducible factor-1α (HIF-1α). The expression and activity of HIF-1α increased upon epidermal growth factor receptor (EGFR) transactivation by intracellular EP receptors (Fernández-Martínez & Lucio-Cazaña, 2015a). It has been previously described in human cancer cells from the prostate, breast, and colon, that PGE_2 induces COX-2 (Tjandrawinata, Dahiya, & Hughes-Fulford, 1997; Yoshida et al., 2013), which suggests that the intracrine effects of PGE_2 in PC3 cells might be mediated by the induction of COX-2. In the current study, we show that this is indeed the case because inhibition of COX-2 resulted in prevention of HIF-1α upregulation and of all the protumorigenic intracrine effects of PGE_2 in PC3 cells. We also show that PGE_2 triggers an EGFR-dependent positive feedback loop of COX-2 upregulation, which might contribute to the upregulation of COX-2 during PC as well and, thereby, to the maintenance of PGE_2-dependent cancer cell growth through the continuous synthesis of PGE_2.

2 | MATERIALS AND METHODS

2.1 | Reagents and antibodies

PGE_2, bromosulfophthalein, AG1478, 5′-Br-2′-deoxyuridine (BrdU), actinomycin D (Act. D), cycloheximide (CHX), 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC1), nimesulide, LY294002, PD98059, H89, PF04419948, SB203598, and type I-collagen were purchased from Sigma (St. Louis, MO). Celecoxib and diclofenac were acquired from Cayman Chemical (Ann Arbor, MI), and Protein phosphatase 2 (PP2) was purchased from Calbiochem Millipore (Darmstadt, Germany). PGT small interfering RNA (siRNA) or MSK-1 siRNA, and scramble were purchased from Santa Cruz Biotechnology (Temecula, CA) and Applied Biosystem (Foster City, CA), respectively.

Antibodies were from the following sources: anti-COX-2 and anti-PGE_2 were acquired from Abcam (Cambridge, UK); anti-β-actin was purchased from antibodies Sigma. Anti-PGT, α-mouse-Alexa-Fluor®488, and α-rabbit-Alexa-Fluor®488 antibodies were from Invitrogen (Eugene, OR). Anti-HIF-1α antibody and anti-BrdU antibodies were acquired from BD Biosciences (Palo Alto, CA). Anti-COX-1, anti-phospho EGFR, and anti-phospho MSK-1 antibodies were purchased from Santa Cruz Biotechnology. Anti-phospho Src and anti-rabbit IgG peroxidase conjugate antibodies were from Calbiochem Millipore. Anti-phospho Akt, anti-phospho Erk1/2, and anti-phospho p38 antibodies were acquired from Cell Signaling (Danvers, MA). All other reactives were purchased from Sigma.

2.2 | Cell culture

The human PC cell line PC3 was from purchased to American Type Culture Collection (Manassas, VA). Cells were grown in sterile conditions and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin/amphotericin B from Life Technologies (Barcelona, Spain). The culture was performed in a humidified 5% CO_2 environment at 37°C. After the cells reached 70–80% confluence, they were washed with phosphate-buffered saline (PBS), detached with 0.25% trypsin/0.2% ethylene-diaminetetraacetic acid (EDTA), and seeded at 30,000–40,000 cells/cm^2. The culture medium was changed every 3 days.

2.3 | Cell proliferation assay with BrdU

DNA synthesis was assessed by BrdU uptake. PC3 cells were placed in 24-well plates (6×10^4 cells/glass coverslip), and they were grown for 24 hr. Then, they were maintained for 24 hr in serum free RPMI-1640 and treated as described later. Afterward, the cells were pulsed with 10 μM BrdU during the last 16 hr of incubation. Then, they were fixed with 2% paraformaldehyde for 15 min, DNA was partially denatured by incubation with 2 M HCl for 20 min, after which the cells were incubated with 0.1 M sodium borate for 2 min at room temperature. Then, the cells were permeabilized with PBS containing 0.2% Triton x-100 (pH 7.4) for 5 min and 3% bovine serum albumin and were washed three times with PBS. The cells were incubated at 37°C with anti-BrdU monoclonal antibody (1:50) for 1 hr. Then, they were then incubated at 37°C with α-mouse-Alexa-Fluor® 488 (1:2,000) for 1 hr in the darkness. Cell nuclei were counterstained with Prolong Gold antifade Reagent with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Detection was performed by confocal laser scan microscopy LEICA TCS-SL (Heidelberg, Germany). To estimate DNA synthesis, the percentage of BrdU-positive nuclei was determined through manual counting in five fields in a blind manner. Quantitative analysis of BrdU-positive cells among the sorted cells was performed by counting the positive nuclei in total cells per field (total cells were visualized by DAPI-positive nuclei).

2.4 | Cell adhesion assay

The cell adhesion to collagen was evaluated as follows: cultured cells were detached by trypsinization, resuspended in serum-free RPMI-1640 medium (2.5×10^5 cells/ml), and treated as stated in the results section.

Then cells were plated in 96-well plates precoated with 0.1 ml of 0.0167 g/100 ml acetic acid and type-I collagen from Sigma and incubated at 37°C for 30 min. The nonadherent cells were washed out with PBS, and the number of cells that adhered to collagen was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The independent experiments were run three times.

2.5 | Cell migration assay

PC3 cells were seeded in 24-well plates (3×10^5 cells per well). At 24 hr after seeding, the monolayer cells were manually scratched with a pipette yellow tip to create extended and definite scratches in the center of the dishes with a bright and clear field (~2 mm). The cells were treated as indicated in the results section, and the narrowing of the wound by migrating cells was monitored by measuring the width in microphotographs. Three representative fields of each monolayer wound were captured using a Nikon Diaphot 300 inverted microscopy camera (10×) up to 30 hr. Monolayer wound areas of untreated samples were averaged and assigned a value of 100.

2.6 | Cell invasion assays

For the invasion assays, we used transwell polycarbonate filters (8-μm pore size; Corning Costar, Cambridge, UK), which were coated with 50 μl of Matrigel Basement Membrane Matrix (BD Biosciences, Bedford, MA; 1:10 dilution with serum free media). Cells were harvested and resuspended in serum-free RPMI-1640 medium at a concentration of 5×10^5 cells/ml. The cells in suspension were treated as indicated in the results section, and then 0.2 ml of cell suspension and 0.6 ml complete medium were added to the upper and lower chamber, respectively. The cells were incubated for 48 hr to allow them to colonize the lower chamber. Invasive cells were fixed with methanol, stained with Giemsa, and counted in four different fields in a Nikon inverted microscope camera (20×).

2.7 | Western blot analysis

PC3 cells were split into six-well plates at a density of 3×10^5 cells/well and incubated for 24 hr before treatment. Afterward, immunoblotting was performed essentially as described previously (Fernández-Martínez et al., 2011). In short, cell lysates were prepared and measured for protein content using the Bradford assay. Approximately 35 μg of protein was electrophoresed on 10% sodium dodecyl sulfate–polyacrylamide electrophoresis gels and transferred to nitrocellulose membranes. The membranes were incubated overnight at 4°C with: anti-COX-2 (1:1,000), anti-COX-1 (1:1,000), anti-HIF-1α (1:1,000), anti-phospho EGFR (1:500), anti-PGT (1:1,000), anti-phospho- c-Src (1:1,000), anti-phospho-Erk1/2 (1:1,000), anti-phospho-MSK-1 (1:1,000), anti-phospho-Akt (1:1,000), anti-phospho-p38 (1:1,000). Afterward, the membranes were incubated at room temperature for 1 hr, with the corresponding secondary antiserum (1:4,000). To ensure equal loading of proteins, the membranes were stripped and reprobed with anti-β-actin antibody. The signals were detected with enhanced chemiluminescence reagent (Amersham Healthcare, Buckinghamshire, England). Quantification of band densities was performed using Quantitative One Program (Bio-Rad, Alcobendas, Spain).

2.8 | Promoter COX-2 reporter gene assay

The cells were split into six-well plates at a density of 3×10^5 cells/well. Twenty-four hours later, the cells were cotransfected with 0.5 μg/well luciferase plasmid COX-2-Luc. The human COX-2 luciferase reporter construct phPES2 containing the promoter fragments −327 to +59 was a gift from Dr. Hiroyasu Inoue (Nara Women's University, Nara, Japan), and 0.5 μg/well of the *Renilla reniformis* luciferase reporter pRL-CMV using lipofectamine (Invitrogen, CA). Transfected cells were next incubated with complete growth medium for 24 hr, and then they were treated as indicated in the results section. Afterward, the cells were harvested and luciferase activity was measured using the dual-luciferase reporter assay system (Promega, Madison, WI). The data were normalized against the *R. reniformis* luciferase activity.

2.9 | Transient transfection with siRNA

For PGT and MSK-1 inhibition, we used either PGT siRNA sc-78211 or MSK-1 siRNA sc-35977 (Santa Cruz Biotechnologies) respectively, and scramble siRNA AM4637 (Applied Biosystems) as a control. PC3 cells at 80% confluence were transfected with 100 nM siRNA PGT or 100 nM siRNA scramble. According to the manufacturer's protocol, we used Lipofectamine 2000 (Invitrogen) to get the transfection. The transfected cells were next incubated with complete growth medium for 24 hr, and then they were treated as indicated in the results section. Afterward, the cells were harvested and protein expression was measured by western blot analysis.

2.10 | RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

PC3 cells were seeded in 6-well plates (3×10^5 cells/well), grown for 24 hr, and treated as described later. Total cellular RNA was isolated with TriReagent reagent from Sigma according to the instructions of the manufacturer. One microgram of total RNA was reverse-transcribed using 50 pmol of hexamer random primer and 0.5 μl PrimeScript reverse transcription (RT) enzyme supplemented with 25 pmol of oligo dT primers from Takara (Shiga, Japan). The RT conditions were denaturation at 94°C for 10 min, followed by the reaction of RT at 37°C for 1 hr, and then a final reaction of 5 min at 95°C. Two microliters of RT reaction were then polymerase chain reaction (PCR)-amplified with specific primers for COX-2 (sense 5′-GAT ACT CAG GCA GAG ATG ATC TAC CC-3′; antisense 5′-AGA CCA GGC ACC AGA CCA AAG A-3′), HIF-1α (sense 5′-GAA AGC GCA AGT CCT CAA AG-3′; antisense 5′-TGG GTA GGA GAT GGA GAT GC-3′) or GAPDH (sense 5′-CAA GGG CAT CCT GGG CTA C-3′; antisense 5′-TTG AAG TCA GAG GAG ACC ACC TG-3′). PCR conditions were as follows: 94°C for 5 min (denaturation), followed

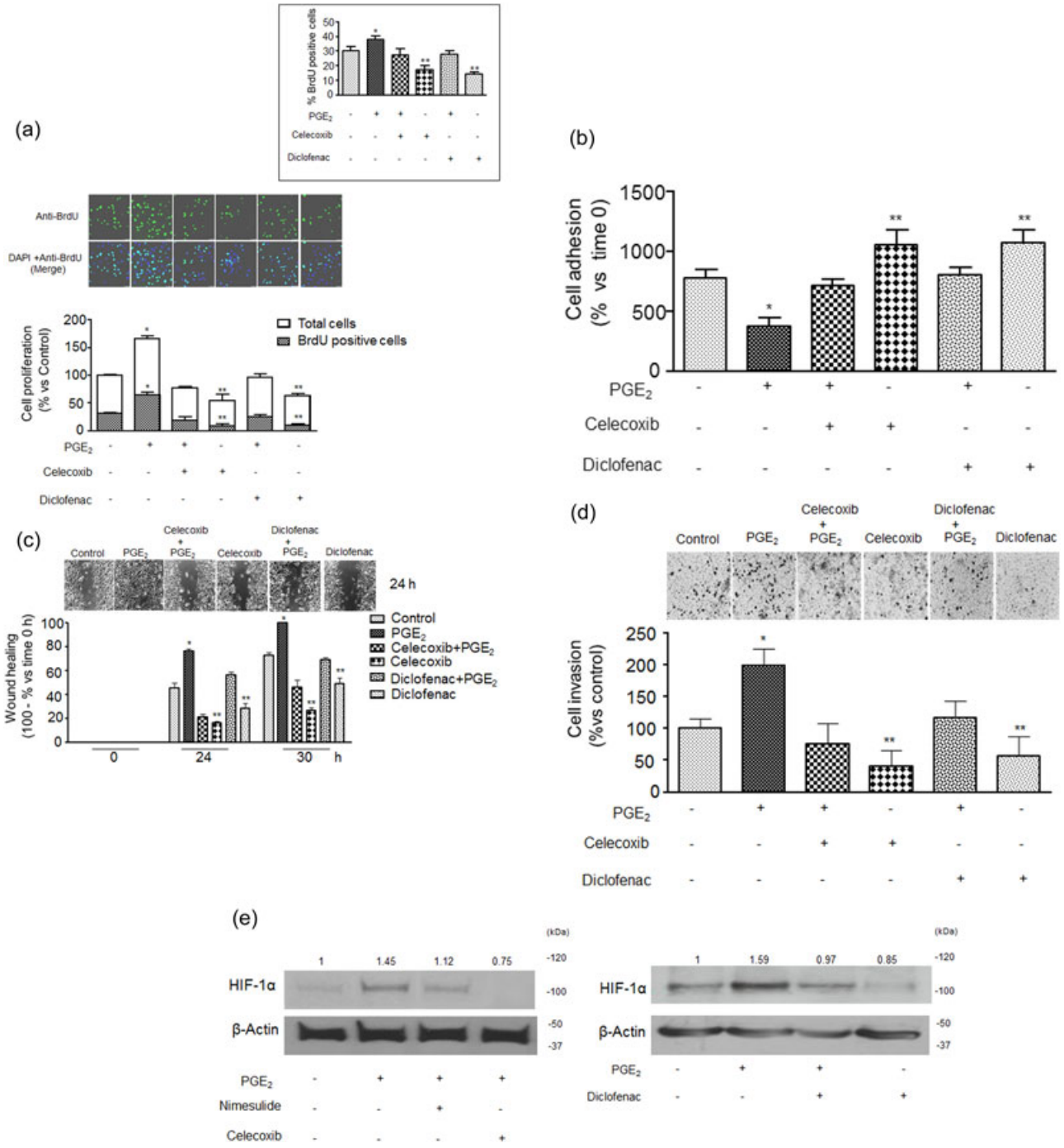

FIGURE 1 PGE_2-induced increase in PC3 cell proliferation, migration, invasion and HIF-1α expression, and PGE_2-induced decrease in PC3 cell adhesion to collagen I is prevented by COX inhibitors. (a) Cell proliferation (BrdU assay). PC3 cells in serum free medium (24 hr) were treated with COX inhibitors and then with PGE_2 for 1 hr. (b) Cell adhesion to collagen I. Cell suspensions in serum free medium were treated COX inhibitors and then with PGE_2 for 30 min. PC3 cells were then plated on collagen I coated wells for 30 min. (c) Cell migration. The layer of confluent PC3 cells was wounded using sterile tips and treated with COX inhibitors and then with PGE_2. (d) Cell invasion. PC3 cells in suspension were treated with COX inhibitors, then with PGE_2 and seeded into transwell inserts coated with Matrigel Basement Membrane matrix. Cell invasion was assessed after 24 hr. (e) HIF-1α expression. Cells were treated with COX inhibitors and then with PGE_2 for 6 hr. Normalized density ratio of HIF-1α over β-actin is indicated for each band. General information: Treatments: 1 hr with 20 μM diclofenac or 2 μM celecoxib or 3 μM nimesulide and then with 1 μM PGE_2 (unless otherwise indicated). All experiments were repeated three times. Photographs are representative of the results obtained. Bars are mean ± *SD*. Statistical analysis: $^*p < 0.01$ versus other groups. $^{**}p < 0.01$ versus control and PGE_2. BrdU: 5′-Br-2′-deoxyuridine; COX: cyclooxygenase; HIF-1α: hypoxia-inducible factor 1-α; PGE_2: prostaglandin E2; SD: standard deviation [Color figure can be viewed at wileyonlinelibrary.com]

by 37–40 cycles of 95°C 1 min, 54°C 1 min, and then a final cycle of 1 min at 72°C. The signals were normalized with the GAPDH gene expression level. The PCR products were separated by electrophoresis and visualized in 2% agarose gels.

2.11 | Immunofluorescence analysis

PC3 cells were placed in 24-well plates (104 cells/glass coverslip) and treated as indicated in the results section. Then, they were fixed with 2% paraformaldehyde for 10 min, permeabilized with 0.1% (v/v) Triton X-100 in PBS for 10 min, washed with PBS, blocked with 4% bovine serum albumin for 1 hr at room temperature, and incubated overnight at 4°C with anti-PGE_2 antibody (1:50 dilution). The cells were then incubated at 37°C with α-rabbit-Alexa-Fluor® 488 (1:2,000) for 1 hr in the dark. Coverslips were then washed and mounted with ProLong Gold antifade Reagent with DAPI (Invitrogen). Detection was performed by confocal laser scan microscopy LEICA TCS-SL. Images were analyzed using ImageJ software (NIH).

2.12 | Statistical analysis

Each experiment was repeated at least three times. The results are expressed as the mean ± standard deviation. They were subjected to one-way analysis of variance followed by Bonferroni's test for multiple comparisons. The level of significance was set at $p < 0.05$.

3 | RESULTS

3.1 | PGE_2 increases PC3 cell proliferation, migration, invasion and HIF-1α expression, and decreases PC3 cell adhesion to collagen I in a COX-2-dependent manner

We have previously found that PGE_2 increases PC3 cell proliferation, migration, invasion and decreases PC3 cell adhesion to collagen I. These effects are very relevant in the pathogenesis of cancer. First, unchecked proliferation is a hallmark of cancer cells that commonly

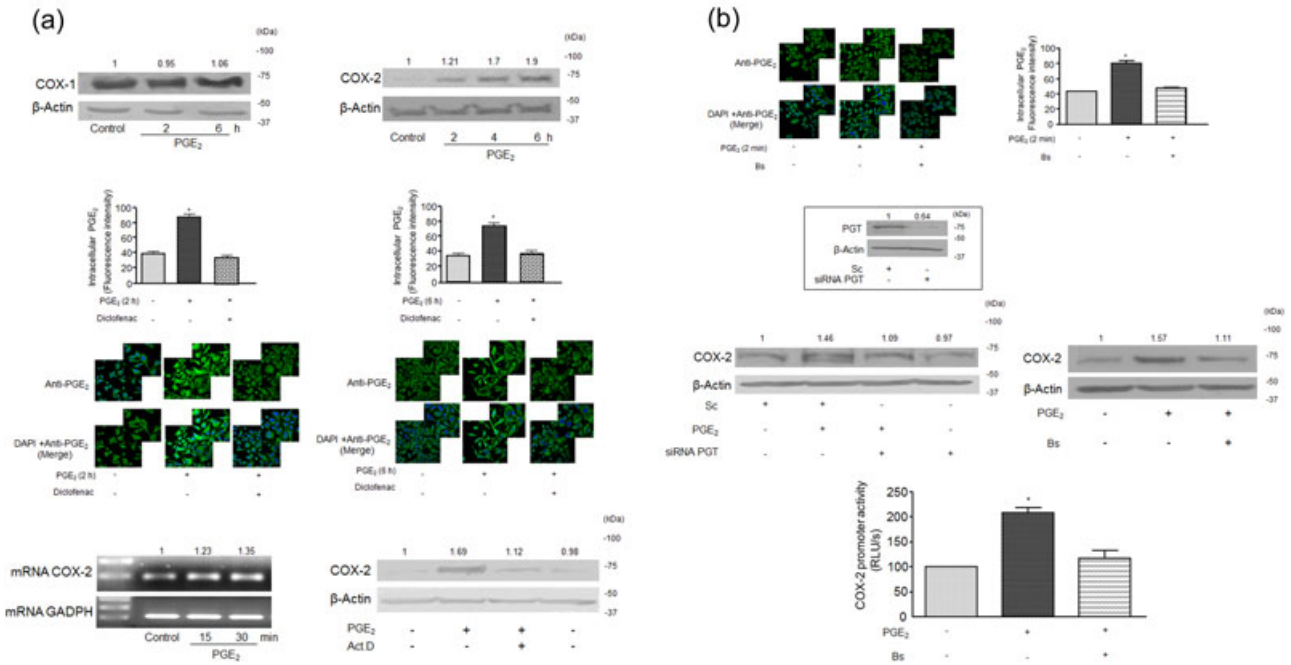

FIGURE 2 PGE_2 upregulates transcriptionally the expression of its own synthesizing enzyme COX-2 in a PGT-dependent manner. (a) PGE_2 upregulates COX-2. PGE_2 upregulates but not COX-1 (upper panel). Diclofenac-sensitive rise in the levels of iPGE2 (a, middle panel). PGE_2 upregulates transcriptionally COX-2 (lower panel). Left: Semi-quantitative RT-PCR. Right: PC3 cells were treated for 1 hr with 1 μg/ml Act. D and then with PGE_2 for 2 hr. (b) PGE_2 upregulates COX-2 in a PGT-dependent manner. Upper panel: Internalization of PGE2 in a PGT inhibitor-sensitive manner. Cells were preincubated for 1 hr with the inhibitor of PGT Bs (10 μM) and then with PGE_2 for 2 min. Afterwards, cells were fixed, permeabilized and subjected to immunofluorescence analysis after incubation with anti-PGE_2 antibody Images were analyzed using ImageJ software (NIH). Middle panel, left: siRNA PGT prevents PGE_2-induced COX-2 upregulation. Transfected cells were treated with PGE_2 for 2 hr. Inset: siRNA PGT blunts the constitutive expression of PGT. Middle panel, right: Bs prevents PGE_2-induced COX-2 upregulation. Treatments: 10 μM Bs/1 hr and then PGE_2/2 hr. Lower panel: Bs prevents PGE_2-induced increase in the activity of a human COX-2 gene promoter. Treatments: 10 μM Bs/1 hr and then PGE_2/30 min. Each bar represents the mean ± *SD* of the luciferase activity (RLU) normalized by Renilla luciferase activity. Statistical analysis: $^*p < 0.01$ versus other groups. General information: PC3 cells were treated with 1 μM PGE_2. Western blot analysis: Normalized density ratio of COX-1 or COX-2 over β-actin is indicated for each band. RT-PCR: Equal mRNA loading was confirmed by assessing the expression of GADPH mRNA. Normalized density ratio of COX-2 over GAPDH is indicated for each band. All experiments were repeated three times. Act. D: actinomycin D; Bs: bromosulfophtalein; COX: cyclooxygenase; $iPGE_2$: intracellular PGE2; mRNA: messenger RNA; PGE_2: prostaglandin E2; PGT: prostaglandin uptake transport; RLU: relative luminescence units; RT-PCR: reverse transcription polymerase chain reaction; SD: standard deviation; siRNA: small interfering RNA [Color figure can be viewed at wileyonlinelibrary.com]

exhibit increased proliferation when compared with normal cells. Second, the most threatening feature of malignancy in cancer is the potential for invasion and metastases: cancer cell detachment is the initial event in metastases formation of carcinomas and tumor cells often show a decrease in cell–cell and/or cell–matrix adhesion. Besides cell detachment, the migratory activity of cancer cells also contributes to metastasis. Taking into account these considerations and our aim of studying in PC3 cells the role of COX in PGE_2 effects, we first asked whether inhibition of COX prevented protumorigenic effects of PGE_2 such as cell proliferation, detachment, migration, invasion, and HIF-1α upregulation. To this end, PC3 cells were preincubated with diclofenac (to inhibit both COX-1 and COX-2 isoenzymes) or with celecoxib (to inhibit COX-2 activity; in the case of HIF-1α the effect of a second COX-2 inhibitor, nimesulide, was also assessed), before being exposed to PGE_2. As shown in Figure 1, both NSAIDs prevented the effects of PGE_2 on cell proliferation (without affecting cell survival as assessed by annexin V/propidium iodide staining, results are not shown), adhesion to collagen I, migration, invasion, and HIF-1α upregulation (see Supporting Information Figure S.1 for quantification of the western blot analysis), which indicated that these PGE_2 effects are dependent on COX activity. It is worth mentioning that all NSAIDs used

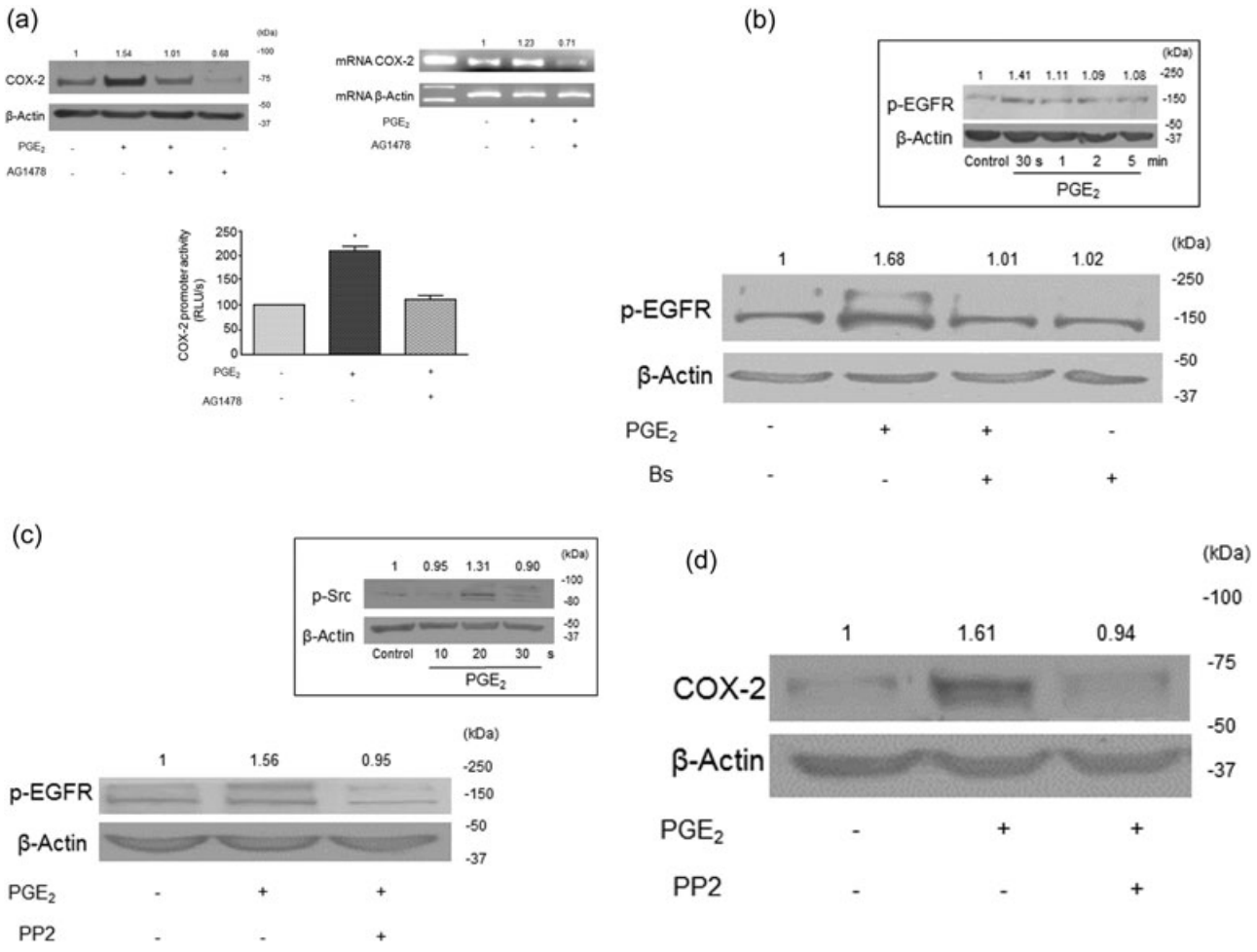

FIGURE 3 c-Src-dependent activation of EGFR mediates the upregulation of COX-2 by PGE_2. (a) The inhibitor of EGFR activation AG1478 prevents the upregulation of COX-2 by PGE_2. Upper panel: Treatments: 1 μM AG1478/1 hr and then PGE_2 for 2 hr (left) or 20 min (right). Lower panel: Transfected cells were treated with AG1478 and then with PGE_2 for 30 min. Luciferase activity was expressed as in Figure 2b. Statistical analysis: $^*p < 0.01$ versus (b) Treatment with PGE_2 results in Bs-sensitive increase in EGFR tyrosine phosphorylation. Serum starved cells were treated with 10 μM Bs/1 hr and then with PGE_2/30 s. Inset: PGE_2 increases EGFR tyrosine phosphorylation. (c) PGE_2-induced tyrosin phosphorylation of EGFR is dependent on c-Src. Serum starved cells were treated with the inhibitor of c-Src phosphorylation PP2 (10 μM/1 hr) and then with PGE_2 for 30 s. Inset: PGE_2 increases c-Src phosphorylation. (d) The inhibitor of c-Src phosphorylation PP2 prevents the upregulation of COX-2 by PGE_2. Serum starved cells were treated with the inhibitor of c-Src phosphorylation PP2 (10 μM/1 hr) and then with PGE_2 for 2 hr. General information: All experiments were repeated three times. PC3 cells were treated with 1 μM PGE_2. Western blot analysis: Normalized density ratio of COX-1, COX-2, p-EGFR, and p-Src over β-actin is indicated for each band. Autoradiographs are representative examples of at least three independent experiments. RT-PCR: Equal mRNA loading was confirmed by assessing the expression of GADPH mRNA. Normalized density ratio of COX-2 over GAPDH is indicated for each band. Bs: bromosulfophtalein; COX-2: cyclooxygenase-2; EGFR: epidermal growth factor receptor; mRNA: messenger RNA; PP2: protein phosphatase 2; PGE_2: prostaglandin E2; RT-PCR: reverse transcription polymerase chain reaction

inhibited the basal expression of HIF-1α (results are not shown, except for diclofenac in Figure 1 3, right). This fact is in good agreement with previous studies (Liu et al., 2002) and indicates that COX-2 activity plays a critical role in the regulation of HIF-1α expression. On the other hand, the fact that the inhibitory action of diclofenac was not stronger than that of celecoxib suggests that COX-2 is the COX isoenzyme which specifically mediates the studied effects of PGE_2 on PC3 cells.

3.2 | PGE_2 upregulates the expression of its own synthesizing enzyme COX-2 in a PGT-dependent manner

PGE_2 induces COX-2 in human colon cancer cells, breast cancer cells and PC cells, although the latter has only been analyzed at the messenger RNA (mRNA) level but not at the protein level of COX-2 regulation (Tjandrawinata et al., 1997; Yoshida et al., 2013). Because PGE_2-induced COX-2 upregulation might contribute to the protumoral actions of PGE_2 in PC3 cells, thereby explaining the inhibitory effect of celecoxib on these actions, we studied the effect of PGE_2 in the expression of COX isoenzymes. Our results indicated that COX-2 expression, but not COX-1 expression, increased over time (Figure 2a, upper panel and Supporting Information Figure S.2a, upper panel). In fact, COX-2 behaved as an early-response protein because its expression increased 30 min after treatment with PGE_2 (results are not shown). Parallely to the increase in COX-2 expression, there was a diclofenac-sensitive rise in the levels of $iPGE_2$ (Figure 2a, middle panel). Further mechanistic studies (Figure 2a, lower panel and Supporting Information Figure S.2a, lower panel) showed that PGE_2-induced increase in COX-2 protein expression was prevented by the inhibitor of transcription Act. D and that treatment of PGE_2 determined an increase in COX-2 mRNA expression. These results indicate that induction of COX-2 mRNA contributed to the increase in COX-2 protein expression.

We have previously found that an $iPGE_2$-activated mechanism, requiring the previous transport of PGE_2 to the inside the cell, mediates the pro-tumorigenic effects of PGE_2 on PC3 cells (Madrigal-Martínez et al., 2015). Because COX-2 also mediates these effects (Figure 1), we reasoned that $iPGE_2$ might also be responsible for PGE_2-induced COX-2 upregulation. To test this hypothesis, we assessed the effect of inhibiting PGT (i.e., the PGE_2 uptake transporter) on the upregulation of COX-2 by PGE_2. Figure 2b (upper panel) shows that $iPGE_2$ increased shortly after treatment with PGE_2 in a PGT-inhibitor sensitive manner and that, as expected, knockdown or pharmacological inhibition of PGT resulted in prevention of PGE_2-induced increase in both COX-2 expression (Figure 2b, middle panel and Supporting Information Figure S.2b) and activity of a COX-2 promoter construct transfected in PC3 cells (Figure 2b, lower panel). In summary, the results shown in Figure 2 indicate that PGE_2 upregulates the expression of its own synthesizing enzyme COX-2 in a PGT-dependent manner.

3.3 | c-Src-dependent activation of EGFR mediates the upregulation of COX-2 by PGE_2

In PC3 cells, PGE_2 augments the phosphorylation of EGFR (Jain et al., 2008) and we have previously found that the transactivation of EGFR by EP2 receptor and further EGFR-dependent increase in HIF-1α expression, are critical events leading to the protumorigenic effects of PGE_2 on PC3 cells (Fernández-Martínez & Lucio-Cazaña, 2015a). In consequence, it was likely that EGFR mediated the upregulation of COX-2 by PGE_2. We explored this possibility by assessing the prevention of PGE_2-induced increase in COX-2 expression by the inhibitor of EGFR activation, AG1478. The results shown in Figure 3a (and Supporting Information Figure S.3a) in which AG1478 prevented the increase in COX-2 protein and mRNA and in the activity of a COX-2 promoter construct transfected in PC3 cells, confirmed our hypothesis. Furthermore, treatment with PGE_2 resulted in increased phosphorylation of EGFR, which was prevented by the inhibitor of PGT transporter bromosulfophtalein (Figure 3b and Supporting Information Figure S.3b). The latter results confirmed that activation of EGFR mediated the upregulation of COX-2 by PGE_2 and highlighted the role in the mechanism of action of PGE_2 of its PGT-dependent uptake by PC3 cells.

Our previous studies have shown that c-Src mediates the transactivation of EGFR in human renal proximal tubular HK-2 cells treated with PGE_2 (Fernandez-Martínez & Lucio-Cazaña, 2015b). To assess the relevance of c-Src in the mechanism through which PGE_2 increases the expression of COX-2 in PC3, we studied the effect of c-Src inhibitor PP2 on PGE_2-induced EGFR phosphorylation and COX-2 upregulation. Our results indicated (Figure 3c–d and Supporting Information Figure S.3c–d) that PP2 prevented both effects of PGE_2 and that c-Src phosphorylation increased upon treatment with PGE_2. These results indicate that c-Src mediates the phosphorylation of EGFR that leads to the upregulation of COX-2 by PGE_2.

3.4 | Erk1/2/p38/MSK-1 and PI3k/Akt pathways mediate the EGFR-dependent-increase in COX-2 expression upon treatment with PGE_2

We have previously found in human renal proximal tubular HK-2 cells that PI3k/Akt and Erk1/2/p38/MSK-1 pathways mediate the EGFR-dependent increase in HIF-1α upon treatment with PGE_2 (Fernandez-Martínez & Lucio-Cazaña, 2015b, unpublished results). Because PGE_2 also induces an EGFR-, COX-2-dependent increase in HIF-1α expression in PC3 cells (Fernández-Martínez & Lucio-Cazaña, 2015a; Figure 1). We studied the role of PI3k/Akt and Erk1/2/p38/MSK-1 pathways as possible mediators of the EGFR-dependent increase in COX-2 induced by PGE_2. To this end, we first studied whether pretreatment with LY294002 and wortmannin (inhibitors of PI3k), PD98059 (inhibitor of the mitogen-activated protein kinase kinase (MAPKK) which is upstream of Erk1/2), SB203580 (p38 MAPK inhibitor), or H89 (MSK-1 inhibitor) or siRNA MSK-1 (given that H89 inhibits PKA too, we also inhibited

MSK-1 through knocking it down) prevented the PGE_2-induced increase in COX-2 expression, which was confirmed by the results shown in Figure 4a and Supporting Information Figure S.4a. These inhibitors also prevented the increase in the activity of a COX-2 promoter construct in PC3 cells treated with PGE_2 (Figure 4a). Furthermore, treatment with PGE_2 resulted in phosphorylation of Akt, Erk1/2, p38, and MSK-1 (Figure 4b and Supporting Information Figure S.4b) (which is activated downstream of the ERK or p38 MAPK pathways in vivo (Vermeulen, Vanden Berghe, Beck, De Bosscher, & Haegeman, 2009), this effect being prevented by EGFR inhibitor AG1478 (Figure 4c and Supporting Information Figure S.4c) (of note, PGE_2-induced MSK-1 phosphorylation was prevented by PD98059 and SB203580, but not by LY294002 (Figure 4d and Supporting Information Figure S.4d), which indicates that the pathways PI3k/Akt and Erk1/2/p38/MSK-1 are independent).

Taken together, the results shown in Figure 4 indicate that PI3k/Akt and Erk1/2/p38/MSK-1 pathways mediate the EGFR-dependent-increase in COX-2 expression upon treatment with PGE_2.

3.5 | PGE_2 increases transcriptionally the expression of HIF-1α through an intracrine mechanism involving the same signaling pathways that mediate COX-2 upregulation

In PC3 cells, we have demonstrated that the protumoral effects of PGE_2 are mediated by the BG-sensitive increase in HIF-1α expression and activity (Madrigal-Martínez et al., 2015). However, we did not studied the mechanisms and signaling pathways leading to HIF-1α upregulation. To address this issue, we first studied the mechanisms through which PGE_2 increases HIF-1α expression. Previous studies indicate that upregulation of HIF-1α by PGE_2 in HK-2 cells is not due to an Act. D insensitive increase in HIF-1α protein stability—which is the canonical pathway of hypoxia-induced HIF-1α accumulation (Ivan et al., 2001; Jaakkola et al., 2001; Lando, Peet, Whelan, Gorman, & Whitelaw, 2002)—but to an Act. D-sensitive, protein stability-independent mechanism (Fernández-Martínez, Arenas Jiménez, & Lucio Cazaña, 2012). To test if this was also the case in PC3 cells, we first analyzed the effect of PGE_2 on HIF-1α expression. As shown in Figure 5a left and Supporting Information Figure S.5a left, PGE_2

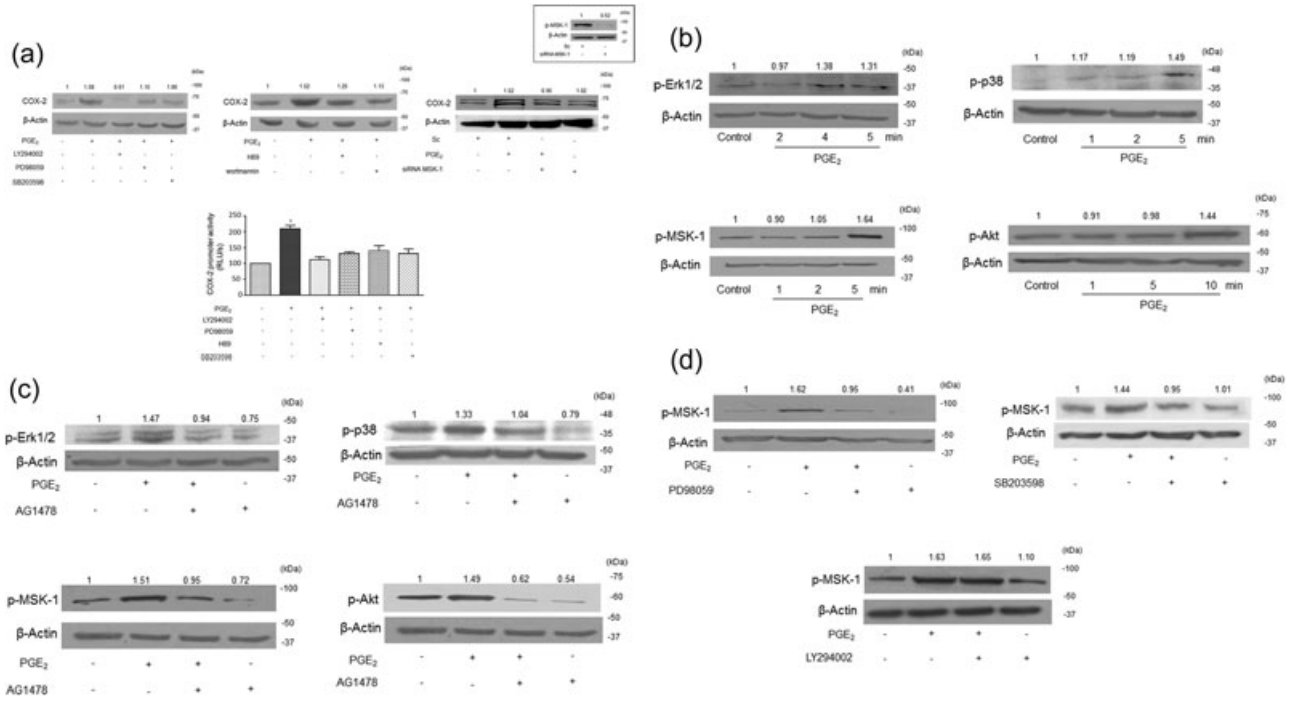

FIGURE 4 Erk1/2/p38/MSK-1 and PI3k/Akt pathways mediate the EGFR-dependent-increase in COX-2 expression in PC3 cells upon treatment with PGE_2. (a) Inhibition of Erk1/2/p38/MSK-1 and PI3k/Akt pathways prevents the upregulation of COX-2 by PGE_2 PC3 cells. Upper panel PC3 cells were preincubated with pharmacological inhibitors of both pathways (MSK-1 was also knocked down as shown in the right panel) before being treated with PGE_2 for 2 hr. Lower panel: Cells which were transfected with a human COX-2 gene promoter were pretreated with the kinase inhibitors and treated with PGE_2 for 30 min. Luciferase activity was expressed as in Figure 2b. Statistical analysis: $^*p < 0.01$ versus other groups. (b) PGE_2 increases Erk1/2, p38, MSK-1, and Akt phosphorylation. (c) PGE_2 increases the phosphorylation of Erk1/2, p38, MSK-1, and Akt in an EGFR-dependent manner. Cells were treated for 1 hr with AG1478 and then with PGE_2 for 5 min (p-Erk1/2, p-p38, and p-MSK-1) or 10 min (p-Akt). (d) PGE_2-induced increase in MSK1 phosphorylation is sensitive to inhibition of Erk1/2 and p38 but not to inhibition of PI3K. Cells were treated with the kinase inhibitors before being treated with PGE_2 for 5 min. General information: Treatments: PC3 cells were treated for 1 hr with 20 μM PD98059, 10 μM SB203580, 1 μM H89, 10 μM LY294002, 1 μM wortmannin, or 1 μM AG1478 and then cells were treated with 1 μM PGE_2. Western blot autoradiographs are representative examples of at least three independent experiments. Normalized density ratio of COX-2, p-Erk1/2, p-p38, p-MSK-1 and p-Akt and over β-actin is indicated for each band. COX-2: cyclooxygenase-2; EGFR: epidermal growth factor receptor; Erk1/2: extracellular signal–regulated kinases 1/2; MSK-1: mitogen- and stress-activated protein kinase-1; PGE2: prostaglandin E2; PI3k: phosphoinositide 3-kinase/Akt

determined an increase in the expression of HIF-1α mRNA in PC3 cells. Furthermore, preincubation of PC3 cells with the inhibitor of transcription Act. D prevent the upregulation of HIF-1α protein in PGE_2-treated cells (Figure 5a, right and Supporting Information Figure S.5a, right). These results indicate the intervention of transcriptional mechanisms, but they do not rule out the hypothetical contribution of stabilization of HIF-1α protein. This issue was addressed by analyzing the effect of PGE_2 on in the turnover of HIF-1α. To this end, we studied the time-course of HIF-1α expression (western blot analysis) in PC3 cells that were first incubated for 6 hr under control conditions or with PGE_2 and then treated for 0–30 min with translation inhibitor CHX. As shown in Figure 5a right, inset and Supporting Information Figure S.5a right, inset, HIF-1α protein half-life under hypoxia was not modified by PGE_2. Altogether, these results demonstrate that transcriptional mechanisms are responsible for the increase in HIF-1α expression induced by PGE_2.

Finally, we sought to confirm the relevance of PGE_2-induced increase in COX-2 expression in the upregulation of HIF-1α by PGE_2. To this end, we studied the effect on PGE_2-induced increase in HIF-1α expression of the inhibitors of COX-2 upregulation. Figure 5b and Supporting Information Figure S.5b. shows that all the maneuvers that prevent the upregulation of COX-2 by PGE_2 (i.e., genetic knockdown of PGT and MSK-1, and pharmacological inhibition of c-Src, EGFR, Erk1/2, p38, MSK-1, and PI3K), also prevented the upregulation of HIF-1α by PGE_2. These results highlight the relevance of PGE_2-induced increase in COX-2 expression in the upregulation of HIF-1α by PGE_2 (Figure 6).

4 | DISCUSSION

In this study, we have shown that COX-2 activity is an absolute requirement for the protumorigenic intracrine effects of PGE_2 in PC3 cells. Furthermore, our data show a novel positive feedback loop through which $iPGE_2$ upregulates COX-2 and that might underlie the intracrine effects of PGE_2 in PC3 cells. Thus, the current data suggest that PGE_2 may contribute to the maintenance of PC cell growth by exerting an enhancing effect on its own synthesis through the continuous upregulation of COX-2 by newly synthesized PGE_2.

PGE_2 has been previously found to induce COX-2 in mouse skin (Ansari, Sung, He, & Fischer, 2007) and in several human cancer cell types including PC (both androgen-dependent and androgen-independent), breast and colon (Tjandrawinata et al., 1997; Yoshida et al., 2013). However, this is the first time, to the best of our knowledge, that it is shown that the pro-tumorigenic effects of PGE_2 are strictly dependent on COX-2 activity. Obviously, further studies will be needed to determine whether COX-2 is equally relevant for the protumorigenic effects of PGE_2 in cancer cell types other than PC3 cells. The same is true for the role of $iPGE_2$ in the upregulation of COX-2, because our study has the relevance of this intracrine

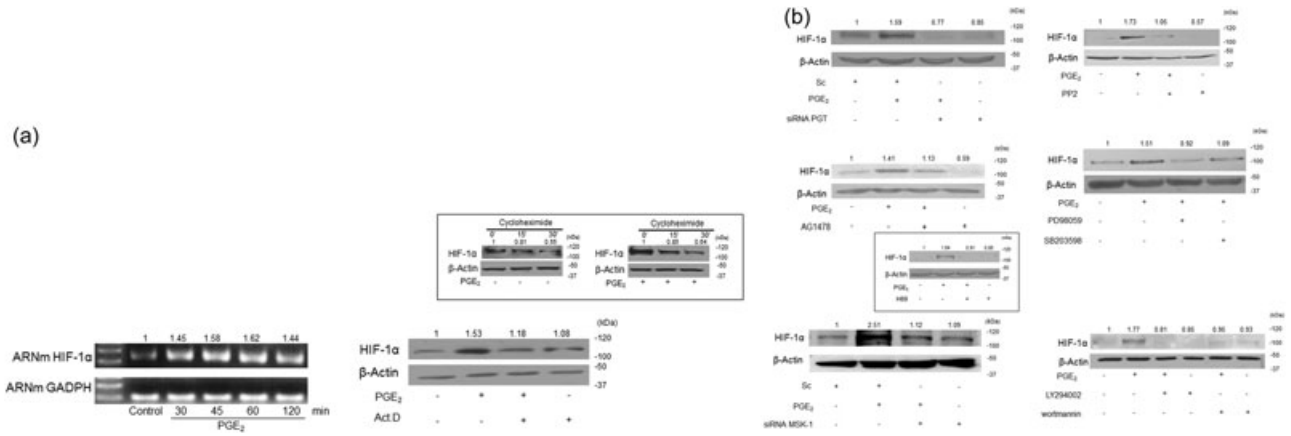

FIGURE 5 PGE_2 increases transcriptionally the expression of HIF-1α in PC3 cells through an intracrine mechanism involving the same signaling pathways that mediate COX-2 upregulation. (a) PGE_2 increases transcriptionally the expression of HIF-1α. Left: PGE_2 increases the expression of HIF-1α mRNA Right: Act. D prevents PGE_2-induced HIF-1α upregulation. PC3 cells were treated with Act. D and then with PGE_2 for 2 hr. Inset: PGE_2 does not increase the stability of HIF-1α Cells were incubated for 6 hr with or without PGE_2. Thereafter, the protein translation inhibitor CHX (50 μg/ml) was added for up to 30 min. (b) PGE_2 increases the expression of HIF-1α in PC3 cells through a Src, EGFR, Erk1/2, p38, MSK-1, and PI3K-dependent intracrine mechanism. Upper panel Left: siRNA PGT prevents PGE_2-induced HIF-1α upregulation. Right: The inhibitor of c-Src phosphorylation PP2 prevents the upregulation of HIF-1α by PGE_2 Middle panel Left: The inhibitor of EGFR activation AG1478 prevents the upregulation of HIF-1α by PGE_2. Right: PGE_2-induced increase in HIF-1α expression is sensitive to inhibitors of Erk1/2 and p38. Lower panel Left: siRNA MSK-1 prevents PGE_2-induced HIF-1α upregulation. Inset: The inhibitor of MSK1 H89 prevents the upregulation of HIF-1α by PGE_2. Right: Inhibitors of PI3K activation prevent the upregulation of HIF-1α by PGE_2. General information: Pretreatments: 1 μg/ml Act. D, 10 μM PP2, 1 μM AG1478, 20 μM PD98059, 10 μM SB203580, 1 μM H89, 10 μM LY294002, or 1 μM wortmannin. All pretreatments lasted 1 hr and then cells were treated with 1 μM PGE_2 for 6 hr (unless otherwise indicated). Western blot autoradiographs are representative examples of at least three independent experiments. Normalized density ratio of HIF-1α over β-actin is indicated for each band. Act. D: actinomycin D; CHX: cycloheximide; COX-2: cyclooxygenase-2; EGFR: epidermal growth factor receptor; Erk1/2: Extracellular signal -regulated kinases 1/2; HIF-1α: hypoxia-inducible factor-1α; mRNA: messenger RNA; MSK-1: mitogen- and stress-activated protein kinase-1; PGE_2: prostaglandin E2; PP2: protein phosphatase 2; PGT: prostaglandin transporter; siRNA: small interfering RNA

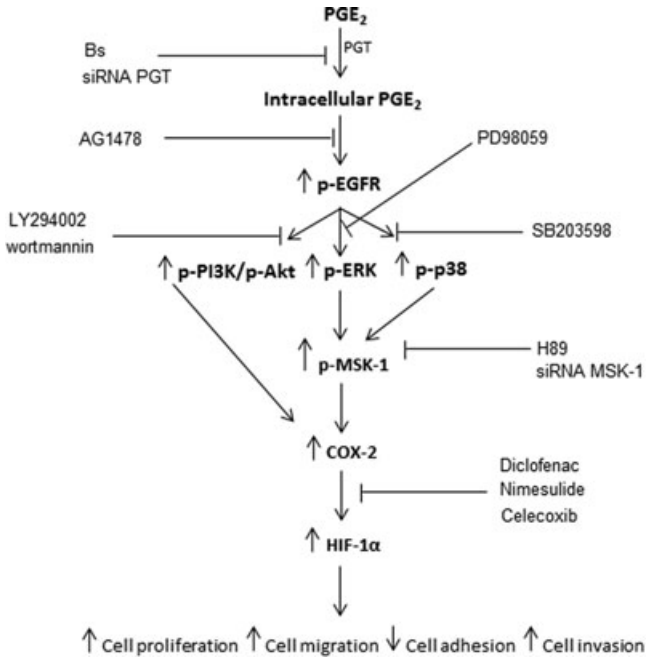

FIGURE 6 Proposed pathway by which PGE_2 upregulates COX-2. COX-2: cyclooxygenase-2; EGFR: epidermal growth factor receptor; ERK: Extracellular signal -regulated kinases 1/2; HIF-1α: hypoxia-inducible factor-1α; MSK-1: Mitogen- and stress-activated protein kinase-1; PGE2: prostaglandin E2; PI3k: phosphoinositide 3-kinase/Akt; PGT: prostaglandin transporter; siRNA: small interfering RNA

mechanism of PGE_2 action demonstrated only in PC3 cells. Regarding the implication of EP receptors in our current results, both transactivation of EGFR and EGFR-dependent increase in HIF-1α expression in PGE_2-treated PC3 cells are mediated by EP2 receptors (Fernández-Martínez & Lucio-Cazaña, 2015a).

We have previously shown that the stimulatory effect of PGE_2 on tumor phenotypes in PC3 cells (i.e., enhanced cell proliferation, migration, invasion, angiogenesis, and loss of cell adhesion to collagen I) is critically dependent on the increase in HIF-1α expression (Fernández-Martínez & Lucio-Cazaña, 2015a). Here, we show that, in turn, COX-2 activity is required by PGE_2 for upregulating HIF-1α. Hence, we reasoned that the mechanism through which PGE_2 increases COX-2 expression in PC3 cells must be the essentially the same that mediates the increase in HIF-1α expression. Indeed, our data confirmed that, as for PGE_2-induced HIF-1α upregulation, PGE_2-induced COX-2 upregulation involved the $iPGE_2$-dependent activation of EGFR through a Src-, Ca^{2+}-dependent mechanism. Further studies demonstrated that the increasing effect of EGFR on COX-2 expression was mediated by kinases PI3K and MSK-1 (the latter previously activated by MAP kinases p38 and Erk1/2). Interestingly, most of the components of the pathway leading to COX-2 upregulation, such are c-Src, EGFR, PI3k-Akt, and Erk1/2, have been suggested to play relevant roles in PC, as indicated in several review articles (Georgi et al., 2014; Guérin, Fischel, Ferrero, Bozec, & Milano, 2010; Toren & Zoubeidi, 2014; Vlaeminck-Guillem, Gillet, & Rimokh, 2014). Consequently, the positive feedback loop of COX-2 upregulation triggered by $iPGE_2$ might also contribute to maintain a constant stimulation on these kinases involved in the progression of PC. Additional studies are needed to explore this hypothesis.

COX-2 is activated at both the transcriptional and posttranscriptional levels, by growth factors, cytokines and mitogens (Gallego, Diaz-Prado, Jimenez-Fonseca, Garcia-Campelo, & Cassinello-Espinosa, 2007). COX-2-derived PGs stimulate cell proliferation, invasion, and angiogenesis and therefore COX-2 has been associated with malignant tumor growth and metastasis. Accordingly, COX-2 has been found at increased levels in PC as well as in other malignant neoplasms such are lung cancer, breast cancer, and colon cancer (Harris, Beebe-Donk, & Alshafie, 2007). COX-2 has been found to be upregulated throughout the entire prostate tumorigenic process (i.e., from hyperplasia to metastatic PC; Elkahwaji, 2012), and it could contribute to the progression of PC because COX-2 expression is lower in T1-T2 stages than in T3–T4 stages (Shao et al., 2012). Yet, the evidence from clinical studies on the protective effects of NSAIDs that preferentially inhibit COX-2 activity is far from being conclusive: whereas no special effect was found in several studies (Antonarakis et al., 2009; James et al., 2012; Smith et al., 2006; Sooriakumaran et al., 2009; Vinogradova, Coupland, & Hippisley-Cox, 2011), in a couple of studies they reduced the risk of PC (Doat et al., 2017; Harris et al., 2007). More recently, a preprostatectomy study on the biological effects of celecoxib or placebo found that celecoxib had no effect on apoptosis or androgen receptor levels in benign prostate or cancerous tissues (Flamiatos et al., 2017). Interestingly, in this study celecoxib did not reduce prostate prostaglandins (Flamiatos et al., 2017), which might explain why celecoxib, and perhaps other anti-COX-2 agents, do not always demonstrate a beneficial effect in the prevention or treatment of PC. It should also be taken into account the possibility that overall survival in studies reaching negative results might be enhanced if they had a longer duration. Whatever the reasons behind the absence of conclusive evidence on the protective effects on PC of NSAID that preferentially inhibit COX-2 activity, alternative PGE_2-based pharmacological approaches might be more effective considering that PGE_2 seems to be the main pro-carcinogenic prostanoid (Wang & Dubois, 2010). PGE_2-based pharmacological approaches might also have less safety concerns than COX-2 based ones -particularly unacceptable cardiovascular side effects-because they preserve production and effects of other prostanoids (for instance, antithrombotic prostacyclin) as they act downstream of COX-2 (Wang & Dubois, 2010). Because we have found that the pro-tumorigenic effects of PGE_2 in PC3 cells are critically dependent on its transport by PGT to the inside of the cell (Madrigal-Martínez et al., 2015), it is relevant to evaluate whether inhibitors of PGT have better specificity for the prevention or treatment of PC and result in minimal adverse effects.

ACKNOWLEDGMENTS

This study was supported by grant SAF2011-26838 from the Spanish Ministerio de Ciencia e Innovación.

CONFLICTS OF INTEREST

There are no conflicts of interest to disclose.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Madrigal-Martínez A, Constâncio V, Lucio-Cazaña FJ, Fernández-Martínez AB. PROSTAGLANDIN E_2 stimulates cancer-related phenotypes in prostate cancer PC3 cells through cyclooxygenase-2. *J Cell Physiol*. 2018;1–12. https://doi.org/10.1002/jcp.27515

Conclusiones

5. Conclusiones

1. En células PC3, una línea celular representativa del CP humano resistente a castración, el tratamiento con PGE_2 potencia fenotipos tumorales malignos como aumento de la proliferación celular, pérdida de adhesión al colágeno, aumento de la capacidad de migración y de invasión, incremento de la expresión de HIF-1α que, a su vez, incrementa la producción de VEGF y la angiogénesis *in vitro*. La producción endógena de PGE_2 también contribuye probablemente a la agresividad intrínseca de las células PC3, ya que la inhibición de la actividad de COXs atenúa el valor basal de los fenotipos tumorales analizados.

2. Para ejercer los efectos sobre los fenotipos tumorales malignos, PGE_2 debe ingresar al interior celular mediante el transportador de prostaglandinas PGT y activar receptores EPs intracelulares (presumiblemente EP2-EP4). Ello da lugar a la transactivación de EGFR (vía Src) por receptores EPs y posterior aumento transcripcional de COX-2, de forma dependiente de quinasas, lo que promueve un incremento sostenido de la producción de PGE_2. Este hecho sugiere que, al menos durante las primeras horas posteriores a la exposición a PGE_2, puede existir una retroalimentación positiva entre PGE_2 intracelular y COX-2, que contribuye a la potenciación de los fenotipos tumorales gracias al mantenimiento de un elevado contenido celular en PGE_2.

3. En células RWPE-1, una línea celular epitelial no maligna de próstata humana, el tratamiento con PGE_2 provoca efectos similares a los descritos en células PC3 mediante mecanismos análogos. De esta manera, PGE_2 debe ingresar primero al interior celular vía PGT y activar receptores EPs intracelulares, lo que provoca la transactivación de EGFR. Finalmente, se produce un incremento de la proliferación celular, de la producción de VEGF, de la angiogénesis, de la capacidad de migración y una pérdida de adhesión al colágeno que dependen del aumento de la expresión de HIF-1α inducido por EGFR.

   Teniendo en cuenta las conclusiones anteriores sobre el papel de PGE_2 intracelular en las líneas celulares epiteliales prostáticas estudiadas, nuestros resultados sugieren que la activación de receptores EPs intracelulares por PGE_2 podría tener un papel fundamental en la progresión de la hiperplasia benigna de próstata y del cáncer de próstata resistente a castración. En consecuencia, el bloqueo de la entrada de PGE_2 al interior celular, mediante la inhibición de PGT, debería ser evaluado como una nueva estrategia terapéutica para reducir la progresión de las citadas alteraciones proliferativas prostáticas.

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Anexo

7. Anexo.

7.1. Materiales

7.1.1. Reactivos

Medios, materiales y "otros" utilizados en cultivos celulares:

- Medio RPMI 1640 de Sigma (St. Louis, MO, EEUU), medio basal para células endoteliales de American Type Culture Collection (ATCC) (Manassas, VA, EEUU), medio de crecimiento de queratinocitos y medio Opti-MEN™ de Gibco Life Technologies (Carlsbad, CA, EEUU).
- Frascos de cultivo, placas, pipetas, etc. de plástico de BD Biosciences (Palo Alto, CA, EEUU), transwell (Corning Costar, Cambridge, UK) y NUNC™ Brand Products (Roskilde, Dinamarca).
- Suero bovino fetal de Sigma (St. Louis, MO, EEUU), antibiótico y antimicótico (penicilina-estreptomicina-anfotericina B), tampón fosfato salino (PBS) (pH 7,4) tripsina/EDTA (0,25%) de Gibco Life Technologies (Carlsbad, CA, EEUU).

Reactivos empleados en cultivos celulares:

- PGE_2, colágeno tipo I y bromuro de 3-(4,5-dimetiltiazol-2-il)-2,5-difenil-tetrazol (MTT) de Sigma (St. Louis, MO, EEUU), 16,16-PGE_2 de Cayman Chemical (Ann Arbor, MI, EEUU), Matriz de Matrigel™* y 5-bromo-2-desoxiuridina (BrdU) de BD Biosciences (Palo Alto, CA, EEUU), Lipofectamina™ 2000 de Invitrogen (Carlsbad, CA, EEUU), giemsa de Merck (Darmstadt, Germany).
- Plásmidos que contienen los genes reporteros de HRE y Renilla reformis unidos a luciferasa p9HIF1-Luc y pRL-CMV, respectivamente. Ambos plásmidos fueron cedidos por el grupo del Dr. Manuel Ortiz Landazuri (Servicio de Inmunología, Hospital Princesa, Madrid, España), plásmido que contiene el gen reportero del promotor COX-2 humano unido a

* La Matriz de Matrigel™ procede de la matriz extracelular de la membrana basal del tumor de ratón EHS (Engelbreth-Holm-Swarm). Contiene diversas proteínas de matriz extracelular como laminina, colágeno tipo IV, entactina/nidógeno, heparán sulfato y proteoglicanos. Además, contiene el activador tisular del plasminógeno y diversos factores de crecimiento.

luciferasa phPES2-Luc, fue cedido por el Dr. Hiroyasu Inoue (National Cardiovascular Center Research Institute, Osaka Japón).

- SiRNA de MSK-1 (sc-35977) y siRNA de PGT (sc-78211) de Santa Cruz Biotechnology (Temecula, CA, EUUU), scramble siRNA (AM4637) de Applied Biosystem (Foster City, CA, EEUU).
- Antagonistas EPs: AH6809 y GW627368X de Cayman Chemical (Ann Arbor, MI, EEUU).
- Inhibidores COX: diclofenaco (COX-1/COX-2) de Cayman Chemical (Ann Arbor, MI, EEUU), nimesulida (COX-2) de Sigma (St. Louis, MO, EEUU) y celecoxib (COX-2) de Cayman Chemical (Ann Arbor, MI, EEUU).
- Otros Inhibidores farmacológicos: actinomicina D, AG1478, verde de bromocresol, bromosulftaleína, cicloheximida, YC1, LY294002, H89, PD98059, SB203580 y wortmanina de Sigma (St. Louis, MO, EEUU), PP2 de Calbiochem Millipore (Darmstadt, Alemania).

Reactivos utilizados en la elaboración de tampones y disoluciones.

- Ácido acético, glicerol, HCl y metanol de Panreac (Barcelona, España).
- Albúmina sérica bovina (BSA), azul de bromofenol, dimetilsulfóxido (DMSO), glicina, borato sódico, β-mercaptoetanol, Tris-HCl (Trizma® base), Tritón X-100 y Tween-20 de Sigma (St. Louis, MO, EEUU).
- EDTA y SDS de Merck (Darmstadt, Alemania).

Reactivos empleados en los ensayos de RT-PCR:

- TRi-Reagent y dietilpirocarbonato (DEPC) de Sigma (St. Louis, MO EEUU).
- Cloroformo, isopropanol y etanol absoluto de Panreac (Barcelona, España).
- Hexámeros aleatorios, oligo dT y enzima retrotranscriptasa de Takara (Shiga, Japón).
- $MgCl_2$, dNTPS y Taq Polimerasa de Biotools (Barcelona, España).
- Agarosa D1 de Conda (Madrid, España).
- Greensafe Premium de Nzytec (Lisboa, Portugal).
- Nucleic Acid Stain de Biotium (Hayward, CA, EEUU).

- Marcadores de peso molecular de NIPON Genetic Europe GmbH (Düren, Alemania).

Anticuerpos y reactivos utilizados en la extracción, detección y cuantificación de proteínas:

Anticuerpos específicos:

Anticuerpo 1ª	Casa comercial
COX-2	Abcam (Cambridge, UK)
COX-1	Santa Cruz Biotechnology (Temecula, CA, EEUU)
PGE_2	Abcam (Cambridge, UK)
EP2	Cayman Chemical (Ann Arbor, MI, EEUU)
EP4	Cayman Chemical (Ann Arbor, MI, EEUU)
HIF-1α	BD Biosciences (Palo Alto, CA, EEUU)
PGT	Cayman Chemical (Ann Arbor, MI, EEUU)
p-EGFR (Tyr_{1173})	Santa Cruz Biotechnology (Temecula, CA, EEUU)
p-MSK-1 (Tyr_{1173})	Santa Cruz Biotechnology (Temecula, CA, EEUU)
p-Erk1/2 (Thr_{202}/ Tyr_{204})	Cell Signaling (Danvers, MA, EEUU)
p-AKT (Ser_{473})	Cell Signaling (Danvers, MA, EEUU)
p-p38 (Thr_{180}/Tyr_{182})	Cell Signaling (Danvers, MA, EEUU)
p-c-Src (Tyr_{416})	Calbiochem Millipore (Darmstadt, Alemania)
β-Actina	Sigma (St. Louis, MO EEUU)
BrdU	BD Biosciences (Palo Alto, CA, EEUU)

Anticuerpo 2ª	Casa comercial
Anti IgG de conejo	Calbiochem Millipore (Darmstadt, Alemania)
Anti IgG de ratón	Sigma (St. Louis, MO EEUU)
Alexa Fluor® 488 anti-conejo	Invitrogen (Eugene, Oregon EEUU)
Alexa Fluor® 488 anti-ratón	Invitrogen (Carlsbad, CA, EEUU)
Alexa Fluor® 633 anti-ratón	Invitrogen (Carlsbad, CA, EEUU)

Reactivos utilizados:

- Acrilamida/bis-acrilamida al 40% de National Diagnostic (Atlanta, GE, EEUU).
- Temed de Sigma (St. Louis, MO EEUU).

- Tampón Lisis Pasivo 5x de Promega (Madison, WI, EEUU).
- Combinado de inhibidores de proteasas de Roche Diagnostics Gmbh (Mannheim, Alemania).
- Reactivo Bradford para el ensayo de cuantificación de proteínas (Dye-Reagent) de BioRad (Richmond, CA, EE.UU).
- Persulfato amónico, marcadores de peso molecular y membranas de nitrocelulosa de BioRad (Richmond, CA, EEUU).
- Reactivo de detención para Western Blotting Amersham ECL (Barcelona, España).
- Películas fotográficas CURIX RP2 PLUS AGFA (Mortsei, Bélgica).
- Kit de ELISA para $VEGF_{165}$ humano DuoSet R&D System (Minneapolis, MN, EEUU).

7.1.2. Modelos experimentales

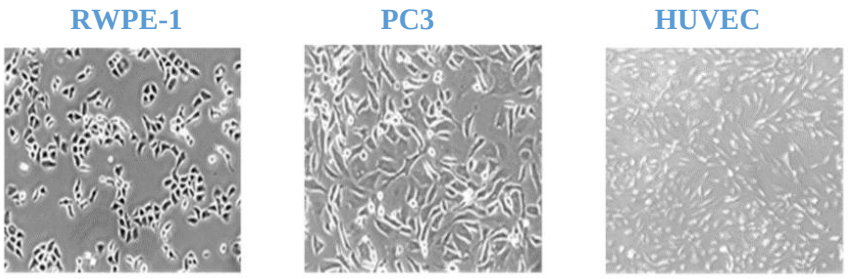
Líneas celulares

La línea celular **RWPE-1** es representativa de una línea epitelial de próstata humana no maligna, y procede de células de la zona periférica de una próstata adulta histológicamente normal. Son células que contienen un plásmido que porta una copia simple del genoma del virus del papiloma humano 18 (HPV-18). Se mantuvieron en medio de crecimiento de queratinocitos, suplementado con 50 µg/ml de extracto de pituitaria bovina, con 5 ng/ml de EGF recombinante humano y con 1% de antibiótico y antimicótico.

La línea celular de cáncer de próstata humana **PC3** es representativa del CP humano resistente a castración, procede de un adenocarcinoma prostático de grado IV con capacidad de metástasis en hueso. Se mantuvieron en medio RPMI-1640 con 10% de suero bovino fetal y con 1% de antibiótico y antimicótico.

Las células endoteliales de la vena umbilical humana **(HUVEC)**, son una línea humana hipodiploide de origen endotelial donde el 72% de las células presentan 45 cromosomas y el porcentaje de células poliploides es del 15,8%. Estas células tienen una vida aproximada de 50-60 duplicaciones celulares. Se mantuvieron en medio basal de las células endoteliales con 1% de antibiótico y antimicótico, además de ser suplementado con 5 ng/ml de VEGF, 5 ng/ml de EGF, 5 ng/ml del factor de crecimiento de fibroblastos, 15 ng/ml del factor de crecimiento insulínico tipo 1, 10 mM de L-glutamina, 0,75 unidades/ml de sulfato de heparina, 1 µg/ml de hidrocortisona y 2% de suero bovino fetal.

En la siguiente imagen, se puede observar las diferencias morfológicas de las tres líneas celulares. Las fotografías se tomaron en un microscopio invertido acoplado a una cámara Nikon Diaphot 300 (10x).



Las tres líneas celulares proceden de ATCC (Manassas, VA EEUU). Las células se cultivaron a 37ºC, en ambiente húmedo y con un 5% de CO_2, el medio se renovó cada 3 días. Las células previamente lavadas con PBS, se levantaron del frasco de cultivos con 0,5% de tripsina/EDTA, una vez despegadas las células del frasco, se neutralizó el efecto de la tripsina añadiendo medio completo. Entonces, las células se centrifugaron a 500 x g, durante 5 min y se resuspendieron en medio completo. A continuación, se procedió a la siembra de las células tanto en frascos de cultivo para mantener la línea celular, como en placas de cultivo de diferentes tamaños para realizar diferentes experimentos. En determinados experimentos, las células se deprivaron antes de ser tratadas. Dicha deprivación consistía en cultivarlas durante 24 h, con medio RPMI-1640 libre de suero (células PC3), o con medio de crecimiento de queratinocitos libre de EGF/extracto de pituitaria bovina (RWPE-1).

7.2. Métodos

7.2.1. Ensayo de MTT

El ensayo de MTT se caracteriza por la conversión de sal tetrazolio de MTT (de color amarillo y soluble en agua) en sal de formazán (de color azul oscuro e insoluble en agua), gracias a la acción de la enzima mitocondrial succinato deshidrogenasa, localizada en la membrana interna de la mitocondria de las células vivas. Este método permite valorar de una manera aproximada cambios en el número de células vivas tras la exposición a un tratamiento. Para ello, las células se sembraron en una placa de 96 pocillos (10^4 células/pocillo) y se deprivaron de suero. A continuación, las células se trataron como se indica en la sección de resultados, pasado el tiempo indicado se añadió 0,1 mg/ml

de MTT y se incubaron durante 2 h, a 37ºC, en oscuridad. Entonces, se retiró el medio y se añadió 100 µl DMSO por pocillo para disolver el precipitado de formazán. La intensidad del color azul se correlaciona con la actividad metabólica de las células, y dicha actividad metabólica se puede relacionar con el número de células si se asume que el tratamiento no altera la actividad de la mitocondria. El producto de la reacción se cuantificó midiendo la absorbancia a 570-630 nm en un fotómetro de microplacas (ELX 800 Bio Tek Intruments, INC).

7.2.2. Ensayo de proliferación con BrdU

Se trata de un ensayo complementario al método anterior, en el que la síntesis de DNA se analiza mediante la captación de BrdU (un derivado de la uridina que se puede incorporar al DNA en lugar de la timidina). Para ello, se sembraron las células (6 x 10^4 células/pocillo) sobre un cristal estéril de 12 mm^2. Entonces, las células se deprivaron de suero durante 24 h antes de ser tratadas como se indica en la sección de resultados y a continuación, las células se incubaron con 10 µM de BrdU durante las últimas 16 h del tratamiento. Después, se fijaron con 2% de paraformaldehído durante 15 min y el DNA se desnaturalizó parcialmente con 2 M de HCl durante 30 min. Este paso es fundamental, porque el anticuerpo monoclonal anti-BrdU se une a las moléculas de BrdU incorporadas únicamente cuando el DNA es de cadena sencilla. Para neutralizar el efecto del HCl, las células se incubaron con 0,1 M de borato sódico durante 2 min, a temperatura ambiente. A continuación, se permeabilizó la membrana de las células con 0,2% de Tritón X-100 y 3% de BSA en PBS durante 5 min, e inmediatamente después se lavaron 3 veces con PBS durante 10 min. Entonces, se incubó primero con el anticuerpo monoclonal anti-BrdU (1:50) durante 2 h a temperatura ambiente y después, con el anticuerpo secundario Alexa Fluor® 488 anti-ratón (1:2000), durante 1 h, a 37ºC, en oscuridad. Posteriormente, las células se lavaron 3 veces con PBS y se procedió al montaje de los cristales sobre los portas con el reactivo Prolong Gold antifade que contenía DAPI (4 ',6-diamino-2-fenilindol), un marcador fluorescente que se une a regiones de adenina y timina del DNA. Por último, se visualizaron las muestras en el microscopio confocal (LEICA TCS-SP) (Heidelberg, Alemania), y se realizó un contaje manual en distintos campos elegidos de manera aleatoria, del número de total de células mediante el marcaje con DAPI y del número de células BrdU positivas (células que proliferan al incorporar BrdU en el DNA, dan una fluorescencia verde del BrdU). El análisis cuantitativo se realizó comparando las células BrdU positivas con el número total de células.

7.2.3. Ensayo de adhesión celular

El ensayo de adhesión celular al colágeno permite realizar una estimación de la capacidad que tienen las células tumorales para desprenderse del tejido (que es consecuencia de su menor adhesión a la matriz extracelular) y así poder diseminarse a otras partes del organismo. En la práctica, las células cultivadas se tripsinizaron, y se resuspendieron (2,5 x 10^5 células/ml) en medio RPMI-1640 libre de suero con 0,1% BSA, en el caso de las células PC3, y en medio de crecimiento de queratinocitos libre de EGF/extracto de pituitaria bovina con 0,1% BSA, en el caso de las células RWPE-1. A continuación, se realizaron los tratamientos como se indica en la sección de resultados. Las células se trataron en suspensión y se mantuvieron a 37ºC, con agitaciones cada 5 min hasta final del tratamiento. Fue entonces cuando se añadieron las células sobre las placas de 96 pocillos, cubiertas previamente con 100 µl de colágeno tipo I (8µg/cm^2, en ácido acético 10 mM), que simula la matriz extracelular. A continuación, se analizó la evolución de la adhesión de las células tumorales al colágeno I en diferentes tiempos a 37ºC. Las células adheridas en cada intervalo de tiempo se cuantificaron mediante ensayo de MTT (sección 5.2.1), previa aspiración del medio que contenía las células no adheridas y posterior sustitución por medio fresco y el reactivo.

7.2.4. Ensayo de cierre de herida

El ensayo de cierre de herida se realizó para estimar la capacidad de migración de las células tumorales (a mayor migración celular, mayor capacidad de diseminación). Las células se sembraron en placas de 24 pocillos (3 x 10^5 células/pocillo) y, pasadas 24 h de la siembra, se realizó una brecha longitudinal de ~ 1 mm de anchura (herida) en la monocapa de células mediante una punta amarilla de una pipeta estéril en el centro del pocillo. A continuación, las células se sometieron a los correspondientes tratamientos (ver sección de resultados). Finalmente, se realizaron fotografías a distintos tiempos en un microscopio invertido acoplado a una cámara Nikon Diaphot 300 (10x) para documentar el cierre progresivo de la “herida” mediante la cuantificación de su anchura. Se asignó un valor de 100 al promedio de las medidas del ancho de la herida de la monocapa de las muestras a tiempo 0 h. Este valor se tomó como referencia para calcular el grado de cierre de la herida a cada tiempo.

7.2.5. Ensayo de migración e invasión en *transwell*

Para ambos ensayos se usaron pocillos tipo *transwell* con membranas de policarbonato con un tamaño de poro de 8 µm. La siembra de células tumorales sobre estas membranas permite cuantificar cuántas células tienen la capacidad de atravesarla, lo que constituye una estimación de su capacidad migratoria. Si los *transwell* se recubren previamente con 50 µl de Matriz de Matrigel™ diluido en PBS (1:10), entonces el paso de las células a través de la Matriz de Matrigel™ puede utilizarse como un índice de su capacidad de invasión, ya que este producto emula las características de una matriz extracelular.

Para realizar ambos ensayos, las células se prepararon a una concentración final de 5 x 10^5/ml en medio RPMI-1640 libre de suero, en el caso de las células PC3, o en medio de crecimiento de queratinocitos libre de EGF/extracto de pituitaria bovina en el caso de las células RWPE-1. A continuación, se trataron en suspensión como se indica en la sección de resultados y se mantuvieron a 37ºC, con agitaciones cada 5 min hasta final del tratamiento. Entonces, se añadió a la parte de superior del *transwell* 0,2 ml de la suspensión celular y 0,6 ml de medio completo a la placa donde reposaba la cámara *transwell*. Las células se incubaron durante 24 h o 48 h para permitir la migración o la invasión a la parte inferior de la membrana del *transwell*. Las células que migraron o invadieron se fijaron con metanol durante 5 min y se tiñeron con Giemsa durante 5 min. Las células situadas en la parte superior de la membrana se retiraron con la ayuda de un hisopo y, finalmente, se realizaron fotografías de diferentes campos con un microscopio invertido acoplado a una cámara Nikon Diaphot 300 (20x o 10x), y se procedió al recuento de las células.

7.2.6. Determinación de la secreción de VEGF

Esta técnica nos permite medir mediante un ensayo de tipo ELISA la cantidad de VEGF secretado por las células.

Para realizar este ensayo, las células se sembraron en placas de 24 pocillos (8 x 10^4 RWPE-1/pocillo) o (5 x 10^4 PC3/pocillo) y se deprivaron de suero durante 24 h antes de recibir los correspondientes tratamientos (ver sección de resultados). Después, el medio que presumiblemente contenía VEGF se guardó a -80°C hasta su uso. La cantidad de VEGF producido se analizó siguiendo las instrucciones indicadas por el fabricante. En primer lugar, se incubó el anticuerpo primario anti-VEGF en una placa de 96 pocillos durante toda la noche a 4°C. Entonces, se realizaron 3 lavados con PBS y 0,05% de Tween-20 y se bloqueó con una solución que contenía 0,05% de Tween-20 y 5% de BSA en PBS durante 1 h, a temperatura ambiente. Por otro lado, se preparó una curva estándar con

diluciones seriadas a partir de una concentración de VEGF de 4000 pg/ml. Esta curva estándar y las muestras de estudio, se añadieron a la placa de 96 pocillos y se incubaron durante 2 h. Posteriormente, se realizaron 3 lavados y después se incubó con el anticuerpo secundario conjugado con biotina durante 2 h. Pasado ese tiempo las muestras se volvieron a lavar y se incubaron durante 20 min con el complejo estreptavidina/peroxidasa. A continuación, las muestras se lavaron y se añadió finalmente la solución sustrato que contiene peróxido de hidrógeno, como sustrato específico de la peroxidasa, y el colorante ABTS (2,2' azinobis (3-etilbenzotiazolin 6-ácido sulfónico) en forma de sal diamónica) para visualizar la reacción. La cuantificación de la reacción se realizó en un fotómetro de microplacas a una longitud de onda de 405 nm (ELX 800 Bio Tek Intruments, INC).

7.2.7. Ensayo de angiogénesis “in vitro”

Las células endoteliales HUVEC tienen capacidad para formar una red de capilares en el interior de una matriz en presencia de factores inductores adecuados. Este ensayo permite realizar una estimación de la capacidad de las células tumorales para inducir procesos de neoangiogénesis. Para ello, las células se sembraron en placas de 24 pocillos (8×10^4 RWPE-1/pocillo) o (5×10^4 PC3/pocillo). Pasadas 24 h de su deprivación de suero, las células se trataron como se indica en la sección de resultados. Al finalizar los tratamientos, se recogió el medio secretado por las células RWPE-1 o PC3 y se utilizó posteriormente para el ensayo de angiogénesis que se detallará a continuación. En primer lugar, las células HUVEC se tripsinizaron para levantarlas del frasco de cultivo, se centrifugaron a 500 x g, durante 5 min, y se lavaron 2 veces en PBS para eliminar los posibles factores de crecimiento del medio de cultivo. Paralelamente, los pocillos de placas de 96 pocillos se recubrieron con 40 µl de matriz de Matrigel™, y para su polimerización, se incubaron a 37ºC durante 2 h. Pasado el tiempo, se sembraron las células HUVEC (25×10^3 células/pocillo) y se añadió 100 µl del medio secretado por las células RWPE-1 o PC3. Las células HUVEC se incubaron a 37ºC, durante 3 h, tiempo suficiente para permitir la formación de “tubos” en la matriz. Entonces, se tomaron varias microfotografías de las estructuras formadas en diferentes campos, mediante un microscopio invertido acoplado a una cámara Nikon Diaphot 300 (10x), y se cuantificó el número de “tubos” usando el programa ImageJ (NIH, Bethesda).

7.2.8. Aislamiento de RNA y RT-PCR

Las células se sembraron en placas de 6 pocillos (5 x 10^5 RWPE-1/pocillo) o (3 x 10^5 PC3/pocillo) y se trataron como se indica en la sección de resultados. El RNA total fue aislado con el reactivo TRi-Reagent siguiendo los diferentes pasos indicados por el fabricante. Lo primero fue incubar las células con 0,5 ml de reactivo TRi-Reagent durante 5 min para disociar los complejos nucleoproteícos. A continuación, las muestras se recogieron y se centrifugaron a 12.000 x g, durante 10 min, a 4ºC. Una vez realizada la centrifugación, al sobrenadante de cada muestra se le añadió 0,1 ml de cloroformo (para permitir la disociación de ácidos nucleicos y proteínas), se agitó y se incubó durante 10 min, a 4ºC. Entonces se centrifugó nuevamente a 12.000 x g, durante 10 min, separándose la mezcla en tres fases (orgánica, interfase y acuosa). De las tres fases, se extrajo la fase acuosa donde está la fracción de RNA. Posteriormente, se procedió a la precipitación del RNA mediante la adición de isopropanol (1/1 respecto al volumen de la fase acuosa). Entonces, las muestras se centrifugaron a 12.000 x g durante 10 min a 4ºC, el precipitado se lavó a 4ºC dos veces con etanol al 70% y se dejó secar. A continuación, las muestras se resuspendieron en un volumen de agua tratada con 0,1% de dietilpirocarbonato (DEPC), que evita la actividad de las ribonucleasas. La concentración y calidad del RNA aislado, se determinó en un espectrofotómetro Nanodrop®, midiendo la absorción de luz a dos longitudes de onda, 260nm y 280 nm. Mediante la relación A260/A280 se pudo estimar la pureza del ácido nucleico, ya que las proteínas absorben luz a 280 nm; los valores pueden variar desde 1,8 para DNA puro hasta 2 para un contenido exclusivo de RNA. Una vez, obtuvimos la concentración de RNA aislado, se convirtió un microgramo del total de RNA en DNA complementario mediante la reacción de la transcripción inversa: 50 pmol de hexámeros aleatorios y 0,5 µl de la enzima retrotranscriptasa (200 U/µl), suplementado con 25 pmol de oligo dT. Las condiciones fueron 15 minutos a 42ºC, para permitir la elongación del DNA complementario, y 3 minutos a 95ºC para inactivar la enzima retrotranscriptasa. A continuación, el DNA complementario se amplificó mediante la reacción de PCR, utilizando *primers* específicos. La amplificación del DNA se llevó a cabo a partir de 2 µl de producto final obtenido en la reacción de transcripción y 18 µl de un "mix" que preparamos previamente en hielo. Este mix contenía 1,6 µl de $MgCl_2$ (50 mM), 0.4 µl de dNTPs (10 mM), primers (0,5 µM), 0,5 µl enzima Taq Polimerasa de Biotools (5 U/µl), 2 µl del tampón de PCR 10X comercial suministrado con la enzima, agua DEPC (hasta 18 µl). Las condiciones para realizar la reacción fueron: desnaturalización inicial de la doble hebra de DNA a 94°C, durante 5 min, seguido de la desnaturalización completa de la doble hebra de DNA a 95°C durante 1 min, anillamiento de los *primers* a sus secuencias complementarias a 60-70°C durante 1 min. Por último, la elongación de la cadena a 72°C, durante 1 min. Cada ciclo se repitió entre 25-40 veces en función

de la expresión del gen de estudio. La señal fue normalizada con los niveles de expresión de β-Actina o GAPDH. Los productos de PCR se separaron por electroforesis en geles de agarosa al 2%, y se visualizaron gracias a la tinción con Greensafe Premium de Nzytec y a la exposición a luz UV.

Secuencias de los *primers* del gen a amplificar y el número de ciclos empleados:

mRNA	Sentido 5'-3'	Antisentido 5'-3'	Nº ciclos
HIF-1α	GAA AGC GCA AGT CCT CAA AG	TGG GTA GGA GAT GGA GAT GC	37
VEGF-A	ATC TTC AAG CCA TCC TGT GTG C	TCA CCG CCT CGG CTT GTC ACA T	40
COX-2	GAT ACT CAG GCA GAG ATG ATC TAC CC	AGA CCA GGC ACC AGA CCA AAG A	37
β-Actina	AGA AGG ATT CCT ATG TGG GCG	CAT GTC CCA GTT GGT GAC	25
GAPDH	CAA GGG CAT CCT GGG CTA C	TTG AAG TCA GAG ACC TG	26

7.2.9. Purificación de plásmidos

El aislamiento y purificación de cada uno de los plásmidos (HRE, promotor de COX-2 o Renilla) se realizó siguiendo los diferentes pasos sugeridos por el fabricante del kit "Endofree Plasmid Maxi kit" de Macherey-Nagel (Düren, Alemania). Para ello, las bacterias con el plásmido de interés se incubaron en agitación constante toda la noche a 37°C, en 250 ml de medio LB (Caldo de Lisogenia) con un antibiótico concreto. La elección del antibiótico se basó en la resistencia que tiene cada plásmido al antibiótico, con el fin de eliminar todas las bacterias que no tuviesen el plásmido incorporado. Entonces, se centrifugó a 6000 x g, durante 15 min, a 4°C, el pellet se resuspendió con una solución que incluía el kit, e inmediatamente después, se incubó con una solución lisis durante 5 min, a temperatura ambiente. Transcurrido ese tiempo, se añadió una solución de neutralización para eliminar el efecto de la "solución lisis". A continuación, se procedió a separar los plásmidos del resto del lisado de bacterias, utilizando columnas con filtro. Para realizar el proceso de filtrado, primero se preparó la columna añadiendo una solución de equilibrado. Después, se añadió a la columna el lisado de bacterias, y posteriormente se lavó el filtro con un solución de lavado para dejar únicamente en el

filtro los plásmidos. Por último, se utilizó una solución de elución para recoger el plásmido del filtro. Una vez obtenido el plásmido, se procedió a su precipitación mediante la adición de isopropanol y posterior centrifugación a 12.000 x g durante 10 min a 4ºC. El plásmido precipitado se lavó a 4ºC dos veces con etanol al 70% y se dejó secar. Las concentraciones finales se obtuvieron midiendo las muestras en un espectrofotómetro Nanodrop®.

7.2.10. Ensayo de genes reporteros con luciferasa

Las células se sembraron en placas de 6 pocillos (3 x 10^5 PC3/pocillo) o (5 x 10^5 RWPE-1/pocillo). Pasadas 24 h, las células se co-transfectaron con el plásmido de interés (1 µg/pocillo del plásmido que contenía el HRE o con 0,5 µg/pocillo del plásmido que contenía el promotor COX-2) y con 0,5 µg/pocillo del plásmido que contenía el control de transfección (Renilla). Para transfectar se utilizó como vehículo la Lipofectamina™ 2000 y como medio Opti-MEN™ †. Entonces se preparó en un eppendorf "A" 450 µl/pocillo de Opti-MEN™ y 50 µl/pocillo de Lipofectamina™ 2000, y en otro eppendorf "B" 450 µl de Opti-MEN™ con los plásmidos de HRE y Renilla o el promotor de COX-2 y Renilla. Se incubó durante 5 min a temperatura ambiente y se añadió la mezcla del eppendorf "A" al eppendorf "B", después se dejó incubar la mezcla 20 min, a temperatura ambiente. Mientras tanto, se lavaron las células 3 veces con medio libre de antibiótico y antimicótico, en el último lavado se dejó a las células con 800 µl de medio libre de antibiótico y antimicótico. Transcurrido los 20 min se añadió el contenido del eppendorf "B" a las células y se incubaron las células a 37ºC, durante 24 h. Entonces, las células transfectadas se trataron como se indica en la sección de resultados. A continuación, se lisaron las células con un tampón lisis de Promega, se mantuvieron 30 min en hielo y se centrifugaron a 4000 x g, durante 10 min, a 4ºC. Después, se recogió el sobrenadante y se mantuvieron las muestras en hielo hasta su análisis. Para medir la actividad luciferasa del HRE, del promotor de COX-2 y de la Renilla se utilizó el Kit Dual-luciferase Reporter Assay System de Promega (Madison, WI, EEUU) y el luminómetro Berthhold detection Systems FB12 Luminometer. Entonces, se añadió 100 µl del reactivo LARII y 20 µl de la muestra en un tubo. A continuación, se procedió a medir en el luminómetro la luminiscencia de la actividad luciferasa de luciérnaga del HRE o del promotor de COX-2 (en función del plásmido transfectado en las células). Después, a la mezcla LARII-muestra se le añadió 50 µl del reactivo Stop and Glo, e inmediatamente, se midió la luminiscencia de la actividad luciferasa de la Renilla. Una vez se realizaron las dos

† Es un medio ideal para utilizarlo en las transfecciones con lípidos catiónicos como la Lipofectamina™ 2000.

medidas, la actividad luciferasa de luciérnaga del HRE o del promotor de COX-2 se normalizó con los valores obtenidos de la actividad luciferasa de la Renilla. De este modo se obtuvieron los valores RLU (unidades de luciferasa relativas) de cada muestra.

7.2.11. Silenciamiento de genes

Se trata de un ensayo que permite eliminar el mRNA que codifica por una proteína dada, mediante el uso de pequeñas moléculas de RNA bicatenario de 21 a 27 nucleótidos, denominadas RNA de interferencia o siRNA. De tal modo que la cadena complementaria del siRNA se unirá a la secuencia del mRNA formando una doble hebra, la cuál será destruida por la propia célula. En consecuencia, la expresión del gen que codifica para la proteína de interés disminuirá o desaparecerá por completo. Para realizar el silenciamiento de nuestros genes de interés se sembraron las células PC3 en placas de 6 pocillos (3 x 10^5 células/pocillo). Cuando las células alcanzaron un 70-80% de confluencia se realizó la transfección de los siRNA de elección. Para ello, se utilizó el medio Opti-MEN™ y Lipofectamina™ 2000 como vehículo para transportar al interior celular los siRNA de PGT, MSK-1 y scramble‡. Entonces se preparó en un eppendorf "A" 450 µl/pocillo de Opti-MEN™ y 50 µl/pocillo de Lipofectamina™ 2000, y en otro eppendorf "B" 450 µl/pocillo de Opti-MEN™ con los siRNA de PGT (6 µl/pocillo) y siRNA de scramble (3 µl/pocillo) o siRNA de MSK-1 (6 µl/pocillo) y siRNA de scramble (3 µl/pocillo). Se incubó durante 5 min a temperatura ambiente y se añadió la mezcla del eppendorf "A" al eppendorf "B", después se dejó incubar la mezcla 20 min, a temperatura ambiente. Durante el tiempo de incubación, se lavaron las células 3 veces con medio libre de antibiótico y antimicótico, en el último lavado se dejó a las células con 800 µl de medio libre de antibiótico y antimicótico. Transcurrido los 20 min se añadió el contenido del eppendorf "B" a las células y se incubaron las células a 37ºC, durante 24 h. Para determinar la eficacia de la técnica de silenciamiento se analizó la expresión de las diferentes proteínas mediante ensayo de inmunofijación de proteínas explicaremos en detalle a continuación.

7.2.12. Inmunofijación de proteínas

Las células se sembraron en placas de 6 pocillos (3 x 10^5 células PC3/pocillo) o (4 x 10^5 células RWPE-1/pocillo) y se trataron como se indica en la sección de resultados. A continuación, se

‡ Se trata de una mezcla inactiva de RNAs de cadena corta que sirve como control negativo del procedimiento de silenciamiento.

lisaron con un tampón lisis de Promega, con un combinado de inhibidores de proteasas (PMSF 0,5 mM, y 2 µg/ml de aprotinina, leupeptina y pepstatina), se mantuvieron 30 min en hielo y se centrifugaron a 4000 x g, durante 10 min, a 4ºC. Después, se recogió el sobrenadante y se desnaturalizaron las proteínas contenidas en el sobrenadante en dos pasos, primero añadiendo una solución (que contenía 50 % de glicerol, 15% de SDS, 3,5 M de β mercaptoetanol, 0,125% de azul de bromofenol) y segundo incubando las muestras durante 5 min, a 95ºC. Las proteínas se separaron por electroforesis en gel de acrilamida/bisacrilamida al 8, 10 o 15% en condiciones desnaturalizantes, a temperatura ambiente y se transfirieron a una membrana de nitrocelulosa, en condiciones semisecas con un tampón de transferencia (48 mM Tris, 39m M Glicina, 0,04%, SDS, y 20% Metanol), durante 45 min, a 11 V. La membrana se bloqueó con leche desnatada al 5% y Tween-20 al 0,05% en PBS durante 1 h, a temperatura ambiente. Posteriormente, la membrana se incubó con el anticuerpo primario correspondiente a 4ºC, durante toda la noche. Transcurrido ese tiempo la membrana se lavó 3 veces con PBS, y se incubó con el anticuerpo secundario correspondiente acoplado a peroxidasa durante 1 h, a temperatura ambiente. La membrana se volvió a lavar con PBS y se incubó durante 1 minuto con el reactivo de detección de Western Blotting Amersham ECL para realizar el revelado. El análisis densitométrico de las bandas se realizó con el programa Quantity One (BioRad).

Anticuerpos específicos utilizados en la inmunofijación:

Anticuerpo 1ª	Peso molecular de la proteína	Dilución	Anticuerpo 2ª
COX-2	72 kDa	1:2.000	Conejo
COX-1	72 kDa	1:1.000	Ratón
p-EGFR	170 kDa	1:500	Conejo
HIF-1α	110 kDa	1:1.000	Ratón
PGT	71 kDa	1:1.000	Conejo
p-MSK-1	90 kDa	1:500	Conejo
p-AKT	60 kDa	1:1.000	Conejo
p-c-Src	60 kDa	1:2.000	Conejo
pErk1/2	44-42 kDa	1:1.000	Conejo
p-p38	38 kDa	1:1.000	Conejo
β-Actina	50 kDa	1:40.000	Ratón

7.2.13. Inmunocitoquímica

Las células se sembraron en placas de 24 pocillos a razón de 10^4 células/pocillo sobre un cristal estéril de 12 mm^2 y entonces se trataron como se indica en la sección de resultados. A continuación, las células se fijaron con 2% de paraformaldehído durante 10 min y la membrana se permeabilizó con 0,1% de Tritón X-100 en PBS durante 10 min, a temperatura ambiente. Las células se bloquearon con 4% de BSA en PBS, a temperatura ambiente, durante 1 h. Después, se incubaron con los anticuerpos primarios correspondientes, a 4ºC durante toda la noche y se incubaron con Alexa Fluor® 633 anti-ratón o Alexa Fluor® 488 anti-conejo en oscuridad durante 1 h. Finalmente, se volvió a lavar y se procedió al montaje de los cristales con el reactivo Prolong Gold antifade con DAPI sobre portas. Las muestras se visualizaron y analizaron en el microscopio confocal (LEICA TCS-SP) (Heidelberg, Alemania).

Anticuerpos específicos utilizados en la inmunocitoquímica:

Anticuerpo 1ª	Dilución	Anticuerpo 2ª
PGE_2	1:50	Conejo
EP2	1:500	Conejo
EP4	1:500	Conejo
HIF-1α	1:100	Ratón