UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

Maestría y Doctorado en Ciencias Bioquímicas

PAPEL DE LA INTERACCIÓN ENTRE LOS RECEPTORES INTRACELULARES A PROGESTERONA Y LA PROTEÍNA cSRC, EN LA MIGRACIÓN E INVASIÓN DE CÉLULAS DE GLIOBLASTOMA HUMANO

TESIS

QUE PARA OPTAR POR EL GRADO DE:

Doctora en Ciencias

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Ciudad Universitaria, Cd. Mx., febrero 2022

CGEP/PMDCB/1855/2021
Asunto: jurado de examen

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Presente

Los miembros del Subcomité Académico en reunión ordinaria del 18 de octubre del presente año, conocieron su solicitud de asignación de **JURADO DE EXAMEN** para optar por el grado de **Doctorado en Ciencias**, con la réplica de la tesis **"Papel de la interacción entre los receptores intracelulares a progesterona y la proteína cSrc, en la migración e invasión de células de glioblastoma humano"**, dirigida por el Dr. **Camacho Arroyo Ignacio.**

De su análisis se acordó nombrar el siguiente jurado integrado por los doctores:

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Agradecimientos

Hoy cumplo una meta que comenzó a gestarse cuando empecé mis estudios universitarios. Como casi siempre ocurre, este camino empezó soñando y luego trabajando mucho para hacerlo realidad. Puedo decir con orgullo que me siento satisfecha de lo logrado. Sin embargo, he tenido la dicha de contar con un grupo de personas que más que satisfactoria, hicieron de esta experiencia una etapa entrañable. No creo que este espacio sea suficiente para agradecerles, pero voy a intentarlo…

A mi familia, especialmente a mi madre por ser siempre la luz más grande y cálida de mi camino, para ella no solo es mi agradecimiento también la dedicatoria de esta tesis. A mis tíos Annia y Raúl por su apoyo incondicional en cada momento de esta etapa, aconsejando sabiamente en los difíciles y celebrando con mucha alegría las victorias.

A mis amigos por ser esa dosis de frescura y risas tan necesaria para alivianar la vida, especialmente a Angela, Carmen, Karla y Fernanda, compañeras imprescindibles de viajes, juegos y del placer de compartir una rica comida.

A mi asesor Ignacio Camacho Arroyo, gracias por la confianza desde el primer encuentro, por dejarme ser y llevar a cabo mis ideas y, sobre todo, gracias por la crítica sabia y constructiva.

A mis colegas, porque tuve la suerte de trabajar con compañeros muy talentosos, que me motivaron en todo momento a ser una mejor profesional.

A los miembros de mi comité tutor Leticia Rocha Zavaleta y Martha Robles Flores y a los miembros de mi jurado Ernesto Soto Reyes Solís, Marco Antonio Velasco Velázquez, Elizabeth Langley McCarron, Alfonso León del Río y Edgar Ricardo Vázquez Martínez, por la excelente asesoría y revisión de este trabajo.

Por último, pero no menos importante, a la Facultad de Química de la UNAM por brindarme la infraestructura para desarrollar este trabajo, a CONACyT, DGAPA (PAPIIT

IN217120) y al Programa de Apoyo a los Estudios del Posgrado (PAEP) por su apoyo económico para cursar los estudios de Doctorado.

A las mujeres de mi vida,

mi madre Odalys y mi abuela América, por ayudarme a volar muy alto.

Science is not the truth. Science is finding the truth.
When science changes its opinion, it didn’t lie to you.
It learned more.

Índice

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Resumen

Los glioblastomas (GB) son los tumores cerebrales más frecuentes y agresivos en adultos. Una vez que se han diagnosticado, incluso con el uso de distintas terapias, la sobrevida media de los pacientes no supera los 15 meses. Entre los factores responsables de la alta tasa de recurrencia y de su mal pronóstico están la resistencia a la terapia y la gran capacidad de migración e invasión de las células que componen al GB. En los últimos diez años se ha demostrado que la progesterona (P4) a concentraciones fisiológicas (10-50 nM), induce la proliferación, migración e invasión de las células de GB humano a través de su receptor intracelular (RP). Aunque este receptor es principalmente conocido por su función como factor de transcripción, también puede inducir efectos no genómicos. Las acciones no genómicas del RP en líneas celulares de cáncer de mama en parte son consecuencia de su interacción con la cinasa cSRC, la cual juega un papel fundamental en la progresión de los GB. Sin embargo, en el contexto de estos tumores, no se han investigado los mecanismos no genómicos del RP a través de cSRC. En este trabajo se determinó la interacción entre el RP y cSRC y sus efectos en células de GB humanos. Los resultados mostraron que la P4 y el R5020 (agonista específico del RP) activaron a la proteína cSRC, ya que ambas progestinas aumentaron la fosforilación de cSRC en las líneas celulares U251 y U87 derivadas de GB humanos. Cuando se utilizó un siRNA contra la expresión del RP, se abolió la activación de cSRC por la P4. Ensayos de co-inmunoprecipitación y de inmunofluorescencia mostraron que cSRC y el RP interactúan y colocalizan tanto en el núcleo como en la región perinuclear de las células. El tratamiento con P4 también promovió el aumento y la disminución de la fosforilación de la cinasa de adhesión focal (FAK) (Y397, Y576/Y577) y Paxilina (Pax) (Y118) respectivamente, componentes de señalización y anclaje en las estructuras de adhesión que participan en la migración e invasión de células tumorales. El efecto de la P4 sobre la fosforilación de FAK (Y576/Y577) se abolió con el uso de un siRNA contra la expresión de cSRC. La P4 incrementó la expresión de la metaloproteinasa de matriz-9 (MMP-9) a través de cSRC. Los ensayos funcionales de migración e invasión demostraron que el silenciamiento de cSRC disminuyó el efecto inductor de la P4 sobre estos procesos. A partir de los resultados obtenidos se concluye que la P4 induce la

activación de la proteína cSRC a través del RP y esta activación participa en la regulación de la migración e invasión de células de GB humanos.

Abstract

Glioblastomas (GBs) are the most frequent and aggressive brain tumors in adults. Once diagnosed, even with the use of different therapies, the average survival of patients does not exceed 15 months. Resistance to therapy and the high capacity for migration and invasion of the cells that make up GB are the factors responsible for the high recurrence rate and poor prognosis. In the last ten years, it has been demonstrated that progesterone (P4) at physiological concentrations (10-50 nM), induces proliferation, migration, and invasion of human GB cells through its intracellular receptor (PR). Although this receptor is mainly known for its function as a ligand-regulated transcription factor, it can also induce non-genomic effects. The non-genomic actions of the PR in breast cancer cell lines are in part a consequence of its interaction with the cSRC kinase, which plays a key role in the progression of GBs. However, in the context of these tumors, non-genomic mechanisms of PR through cSRC have not been investigated. In this work, we determined the interaction between PR and cSRC and its effects in human GB cells. The results showed that P4 and R5020 (PR-specific agonist) activated the cSRC protein, as both progestins increased cSRC phosphorylation in human GB-derived U251 and U87 cell lines. When a siRNA against the PR gene was used, the activation of cSRC by P4 was abolished. Co-immunoprecipitation and immunofluorescence assays showed that cSRC and PR interact and colocalize in the nucleus and the perinuclear region of cells. P4 treatment also promoted increased and decreased phosphorylation of focal adhesion kinase (FAK) (Y397, Y576/Y577) and Paxillin (Pax) (Y118) respectively, signaling and anchoring components in adhesion structures involved in tumor cell migration and invasion. The effect of P4 on FAK (Y576/Y577) phosphorylation was abolished by using a siRNA against cSRC. P4 induced increased expression of matrix metalloproteinase-9 (MMP-9) through cSRC. Functional migration and invasion assays showed that cSRC silencing decreased the positive effect of P4 on these processes. We concluded that P4 induces cSRC protein activation through the PR and this activation participates in the regulation of migration and invasion of human GB cells.

I. Introducción

El Glioblastoma (GB) es el tumor cerebral primario (no derivado de metástasis), con la mayor incidencia y agresividad en adultos. La sobrevida promedio para estos pacientes es de 15 meses después del diagnóstico, solo el 25% sobrevive por dos años y alrededor del 5% alcanza los cinco años, pero con un gran deterioro en las funciones ejecutivas y cognitivas (1). Hasta el momento no existe una terapia que funcione para extender la sobrevida promedio por más de 15 meses. Se ha reportado que las hormonas sexuales como la progesterona (P4) inducen el crecimiento de los glioblastomas (GB) a través del receptor a progesterona (RP) (2) el cual presenta dos isoformas (RP-A y RP-B) con una regulación, expresión y función diferentes (3–5). En los GB, el RP-B es la isoforma predominante (6). Se ha demostrado que la P4 incrementa la capacidad migratoria e invasiva en las líneas celulares D54 y U251 (derivadas de GB humanos), efectos que fueron revertidos por el RU486, antagonista del RP, o por el silenciamiento en la expresión de este receptor (7). Asimismo, el bloqueo del RP en células U87 (derivadas de GB humanos) implantadas en la corteza cerebral de la rata, disminuyó la infiltración y el incremento del área del tumor provocado por la P4 (6).

No todos los efectos de la P4 se deben a la conocida actividad transcripcional del RP. La activación de la cascada cSRC/p21ras/MAP cinasa, en células de cáncer de mama, fue uno de los primeros efectos no transcripcionales regulados por la P4 que se reportaron (9). Sin embargo, no se conocía qué mecanismo permitía al RP mediar esta activación. En 2001, Boonyaratanakornkit y colaboradores demostraron la presencia de un motivo de poliprolina en el dominio amino terminal del receptor, que es necesario para mediar la interacción con el dominio SH3 de varias moléculas citoplásmicas (10). Entre las moléculas que estos autores identificaron como activadas por los RP se encontró a cSRC, proteína que pertenece a la familia de las cinasas SRC (SFKs). Existen nueve miembros en esta familia, de los cuales cinco (FYN, cSRC, YES, LYN y LCK) son expresados en gliomas humanos (11). Las proteínas de la familia SFKs, tienen un papel fundamental en la regulación del desarrollo y progresión de los GB. La importancia sobre estos procesos se evaluó en un modelo de ratones transgénicos que expresan constitutivamente una variante mutada de cSRC (v-SRC), en los cuales se observó el desarrollo de tumores gliales con características moleculares y morfológicas muy

similares a las de un GB humano (12). En 2015, Lewis-Tuffin y colaboradores observaron que el tratamiento con dasatanib, un potente inhibidor de SFKs, redujo significativamente la migración en cinco líneas celulares derivadas de gliomas humanos en comparación con las que solo recibieron vehículo (13).

Se considera que en los GB existe un grupo de células con fenotipo troncal canceroso, responsable de la recurrencia y la resistencia a las terapias. Uno de los marcadores que distingue a estas células es la glicoproteína de membrana CD133. En células CD133+ de GB, se observó que la inactivación de FYN y cSRC, disminuyó la capacidad migratoria de estas células (14).

De manera independiente, se ha estudiado el papel del RP y cSRC en la progresión de los GB, sin embargo, se desconoce si existe una interacción entre estas proteínas y de ser así, sus efectos en la regulación de procesos como la migración e invasión en los GB.

II. Antecedentes

2.1. Tumores primarios del Sistema Nervioso Central

Los tumores cerebrales primarios son un grupo de neoplasias heterogéneas que se derivan de células del Sistema Nervioso Central (SNC). Los síntomas de estos pacientes pueden ser generales o específicos y dependen de la localización, del tamaño y de la velocidad de crecimiento de estas neoplasias. Los únicos factores de riesgo que se han asociado al desarrollo de estos tumores son la exposición a altas dosis de radiaciones ionizantes y algunos síndromes genéticos (15,16). La elección de tratamiento es personalizada, pues depende del tipo de tumor, localización, grado de malignidad, de la condición física y edad del paciente. Por lo general, incluye resección quirúrgica, radio y quimioterapia. No obstante, solo el 33.4% de estos pacientes tiene una tasa global de supervivencia a cinco años (17). Los gliomas representan el 75% de los tumores cerebrales primarios (aquellos que se originan en el cerebro y no como consecuencia de una metástasis) malignos en adultos. El origen de estos tumores no se conoce con exactitud, pero algunas evidencias apuntan a que la acumulación sucesiva de mutaciones en células troncales neurales multipotentes, células progenitoras y células gliales está involucrado (18).

2.1.1. Características generales de los glioblastomas

El GB, es el más frecuente y agresivo de los gliomas primarios en adultos. Los GB representan el 56.6% de todos los gliomas y el 14.7% de los tumores cerebrales primarios **(Figura 1)**. La edad media de presentación es de 64 años, pero pueden ocurrir en cualquier momento de la vida, incluyendo la infancia. La prevalencia de los GB es mayor en hombres que en mujeres en una proporción 1.6:1. Se ha reportado, que los GB se localizan principalmente en los lóbulos cerebrales, siguiendo la siguiente distribución: lóbulo frontal 40%, temporal 29%, parietal 14% y occipital 3% y el 14% restante en estructuras como el tallo cerebral y el cerebelo. Hace más de una década se pensaba que los GB se originaban solamente de células gliales, sin embargo, hoy sabemos que estos tumores son extremadamente heterogéneos y están compuestos por múltiples tipos de células incluyendo aquellas con propiedades troncales (18).

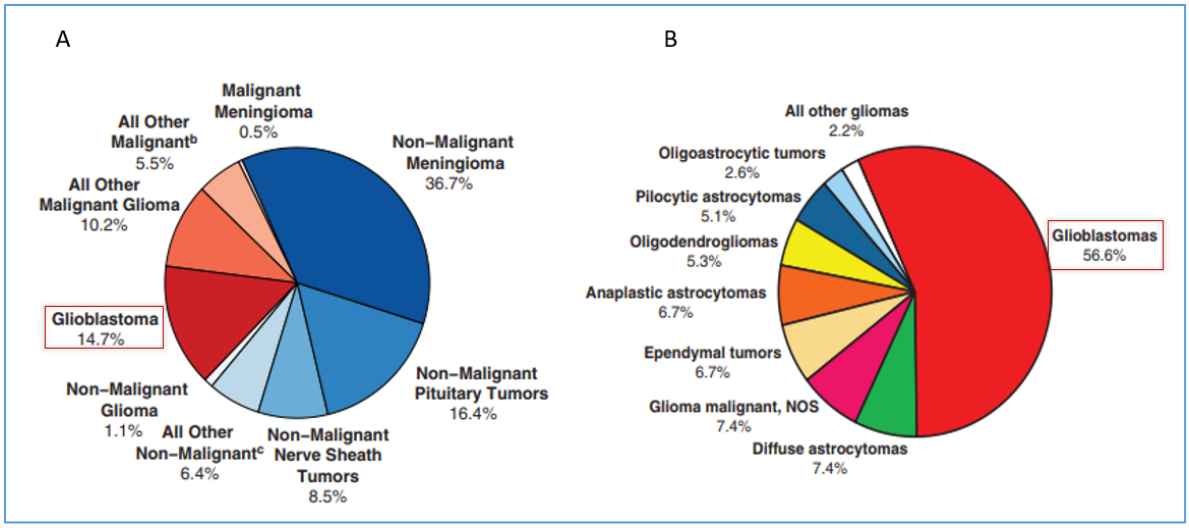

Figura 1. Proporción de los GB entre los tumores cerebrales primarios (14.7%) (**A**) y los gliomas (56.6%) (**B**). Tomado y modificado de Ostrom y colaboradores (18).

Existen varias clasificaciones para los GB, una de ellas es la que los agrupa en primarios o *de novo*, para aquellos que se originan a partir de células precursoras y secundarios para lo que se originan a partir de un glioma astrocítico (astrocitoma) de menor grado. Los GB primarios y secundarios también se diferencian en las alteraciones moleculares que presentan (19). Por ejemplo, la sobreexpresión del receptor del factor de crecimiento epidérmico (EGFR), mutaciones en el homólogo de fosfatasa y tensina (PTEN) y la pérdida del cromosoma 10q son alteraciones genéticas frecuentes de los GB primarios, mientras que los secundarios se caracterizan por presentar mutaciones en la enzima isocitrato deshidrogenasa 1 (IDH1), en p53 y por la pérdida del cromosoma 19q (20). En una clasificación molecular y clínica más específica, los GB se han agrupado en diferentes subtipos moleculares. De acuerdo con la clasificación de Philips y colaboradores, existen tres subtipos, que se agrupan en proneural, proliferativo y mesenquimal (21). Mientras que Verhaak y colaboradores establecieron cuatro subtipos, que incluyen al proneural y mesenquimal, pero separan al subtipo proliferativo en neural y clásico (22) **(Figura 2).** Una clasificación más reciente establecida por Teo y colaboradores en 2019 se basa en el análisis de seis diferentes bases de datos validadas en ratones con xenoinjertos ortotópicos de pacientes. Estos autores establecieron tres subtipos moleculares: clásico, mesenquimal y el que definieron como proneural/neural,

ya que mostró características moleculares de ambos subtipos. En el modelo de xenoinjertos ortotópicos, este último subtipo demostró una mejor respuesta a la temozolomida (fármaco oral alquilante) en comparación con la radioterapia (23). Los patrones de progresión de la enfermedad, resultados de supervivencia y alteraciones moleculares son claramente diferentes entre los subtipos, siendo el mesenquimal el más agresivo y de peor pronóstico (24).

Phillips et al. 2006		Proneural	Proliferative	Mesenchymal
	Signature	NCAM, GABBR1, SNAP91	PCNA, TOP2A, EGFR	VEGF, VEGFR1, VEGFR2, PECAM1
	Chromosome Gain/loss	None	Gain on Chr. 7, loss on Chr. 10	Gain on Chr. 7, loss on Chr. 10
	Biological process	Neurogenesis	Proliferation	Angiogenesis

Verhaak et al. 2010		Proneural	Neural	Classical	Mesenchymal
	Signature	PDGFRA, OLIG2, DDL3, SOX2, NKX2-2	MBP/MAL, NEFL, SLC12A5, SYT1, GABRA1	EGFR, AKT2, SMO,GAS1, GLI2, NOTCH3, JAG1, LFNG	YKL40, MET, CD44, MERTYK, TRADD, RELB, TNFRSF1A
	Mutated genes	TP53, PI3K, IDH1, PDGFRA	None	PTEN, CHKN2, PDGFRA	NF-κB, NF1

Figura 2. Clasificación molecular de los GB. Distinción entre los subtipos moleculares según las clasificaciones de Phillips y colaboradores (Proneural, Proliferativo y Mesenquimal) (21) y Verhaak y colaboradores (Proneural, Neural, Clásico y Mesenquimal) (22). En cada subtipo de la clasificación de Phillips y colaboradores se enumeran los genes que componen su firma molecular (Signature), si existe ganancia o pérdida de cromosomas (Chromosome Gain/loss) y los procesos biológicos asociados a estas características moleculares (Bilogical process). En la clasificación de Verhaak y colaboradores se describe la firma molecular y se enumeran las principales mutaciones (Mutated genes). Tomado y modificado de Zhang y colaboradores (25).

Existen solo dos factores de riesgo claramente establecidos para el desarrollo de los GB. Se ha visto que los pacientes que reciben radioterapia como tratamiento para otro tipo de tumor o condición, tienen un mayor riesgo de desarrollar un GB. También algunas enfermedades genéticas como la neurofibromatosis 1 y 2, la esclerosis tuberosa, el síndrome de Li-Fraumeni y el síndrome de Turcot se han asociado con un mayor riesgo de desarrollar GB (15,16). La presentación clínica de estos tumores puede ser muy variable de acuerdo con el tamaño y la localización. Con frecuencia, los pacientes presentan síntomas asociados con el incremento de la presión intracraneal tales como dolores de cabeza y pérdida progresiva de determinadas habilidades, entre ellas la visión o el habla (20). Las convulsiones se presentan en el 20-50% de los pacientes con GB y pueden ser el primer síntoma en el 45% de los pacientes (26).

La elección del tratamiento para un GB de nuevo diagnóstico requiere de un equipo multidisciplinario de profesionales de la salud en varias de sus ramas y especialistas en diagnóstico molecular. La terapia estándar por elección se compone de la máxima resección quirúrgica posible, seguida de radioterapia concomitante con temozolomida y posteriormente quimioterapia adyuvante nuevamente con temozolomida (27). No obstante, los pacientes tienen un pronóstico extremadamente desfavorable con una supervivencia media de aproximadamente 14 meses y una tasa de supervivencia a cinco años de solo el 5.1% (28) **(Figura 3).** Existen varios factores que influyen en el pronóstico, uno de ellos es el estado de metilación del promotor del gen que codifica la enzima O6-metilguanina-ADN-metiltransferasa (MGMT). El gen MGMT se localiza en la banda cromosómica 10q26 y su producto de expresión es una enzima que elimina los grupos metilo en la posición O^6 de la guanina, evitando así la activación de la apoptosis. Si el promotor del gen que codifica para la MGMT presenta un estado de metilación alto, su expresión estará disminuida y el paciente tendrá un mejor pronóstico, ya que la terapia con temozolomida será más efectiva. Lo contrario ocurre en caso de un estado de metilación bajo (29). Otro importante factor que promueve un pronóstico desfavorable de los pacientes es la gran capacidad de migración e invasión de las células de GB hacia el parénquima cerebral, lo que a su vez contribuye a la imposibilidad de remover en su totalidad al tumor y a una tasa de recurrencia excepcionalmente alta (19).

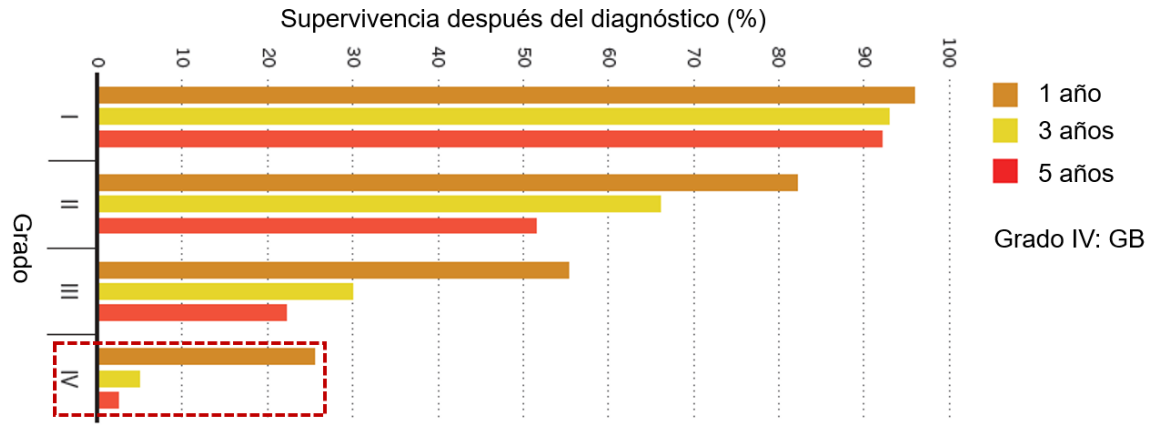

Figura 3. Supervivencia de pacientes con astrocitomas después del diagnóstico. A medida que aumenta el grado del tumor la supervivencia disminuye, llegando a ser de aproximadamente el 5% en la supervivencia a 5 años para los astrocitomas de mayor grado (IV). Tomado y modificado de Gould y colaboradores (28).

2.1.2. Mecanismos de migración e invasión en los glioblastomas

La naturaleza invasiva de los GB promueve la destrucción local del parénquima cerebral circundante y es una de las principales causas de la recurrencia en los pacientes. Incluso con los métodos más avanzados de imagenología es muy difícil detectar a las células que han migrado fuera del área del tumor primario. Aunque las metástasis extracraneales son muy poco frecuentes en estos tumores, las células de GB pueden migrar, incluso, hacia el hemisferio contralateral, haciendo que la resección quirúrgica completa sea imposible. Después de la resección quirúrgica de un glioma maligno, invariablemente se manifestará un tumor recurrente (30) **(Figura 4).** Fundamentalmente, los GB invaden el tejido cerebral sano mediante dos vías: a través del espacio perivascular, es decir alrededor de los vasos sanguíneos o mediante las fibras nerviosas mielinizadas (31). Para favorecer la migración, los GB producen componentes de la matriz extracelular (MEC), como la colágena o la fibronectina, permitiendo una mayor activación de las integrinas (32).

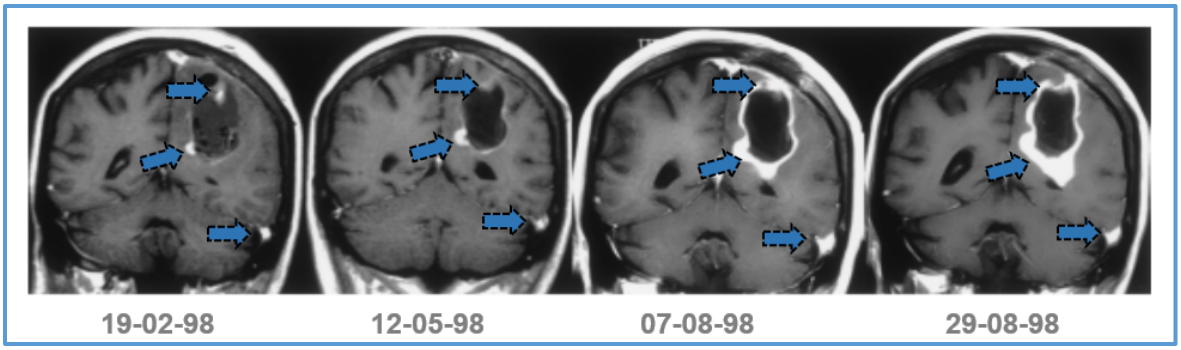

Figura 4. Progresión de un GB después de una resección quirúrgica incompleta. Imágenes captadas por resonancia magnética de un GB localizado en el lóbulo parietal izquierdo. En la figura se observa el incremento del área del tumor (flechas azules) en un periodo de aproximadamente 6 meses (19-02-98---29-08-98) después de la cirugía. Tomado y modificado de Giese y colaboradores (30).

Las interacciones con la MEC juegan un papel fundamental en la capacidad migratoria e invasiva de las células tumorales. Los GB se caracterizan por tener un centro necrótico rodeado de un área donde las células se encuentran en un ambiente altamente hipóxico y de falta de nutrientes. Dichas condiciones, promueven que las células invadan hacia zonas con ambientes más favorables (33). El fenómeno de migración-invasión de una célula involucra cinco etapas fundamentales: extensión de protrusiones en el frente celular, interacción con la MEC, contracción del citoplasma, desprendimiento del sitio original y degradación de la MEC para efectuar la migración-invasión a través de esta (34,35). La interacción con la MEC se lleva a cabo principalmente a través de las integrinas, asociadas a los complejos de adhesión focal, que reconocen diferentes componentes de la MEC, como laminina, colágena y fibronectina (36). La unión del receptor CD44 con el ácido hialurónico (AH), principal componente de la MEC en el cerebro, también contribuye a esta interacción (37). Abandonar el sitio de origen requiere una serie de cambios moleculares que conducen a un proceso muy similar a la transición epitelio-mesénquima (EMT), como la disminución en la expresión de moléculas de adhesión neuronal y de la E-cadherina y el aumento en la expresión de la N-cadherina y del receptor CD44 (38). En la degradación de la MEC participan diversos tipos de proteinasas, incluyendo las denominadas metaloproteinasas de matriz. En el caso de los GB, las metaloproteinasas de matriz-2 y -9 (MMP-2 y -9) juegan un papel fundamental

(39). El movimiento de la célula, así como su coordinación son imprescindibles para que la célula invada hacia otras áreas fuera del sitio de origen. Para que esto ocurra se requiere del reordenamiento del citoesqueleto y de la formación de lamelipodia y filopodia, procesos en los que son imprescindibles las proteínas de la familia de Rho GTPasas (40).

2.1.2.1. Complejos de adhesión focal y glioblastomas

La adhesión a la MEC es un mecanismo fundamental en el mantenimiento de la morfología, migración, proliferación, diferenciación y supervivencia celular. En las células tumorales, es imprescindible para conferir la capacidad invasiva, que en el caso de los GB es responsable de la alta tasa de recurrencia y escasa sobrevida de los pacientes. Las integrinas constituyen uno de los receptores más importantes en la mediación de la adhesión celular a los componentes de la MEC. Las integrinas se expresan en la superficie celular como heterodímeros de subunidades α y β, asociados de manera no covalente. Ambas subunidades son proteínas transmembranales de tipo I que contienen un largo dominio extracelular encargado de reconocer a los ligandos de la MEC (motivos tripéptidos Arg-Gly-Asp de las proteínas fibronectina, laminina, vitronectina y colágena) y una porción citoplasmática que recluta múltiples proteínas intracelulares (36). Las integrinas αvβ3, αvβ5 y α6β1 se encuentran sobreexpresadas en los GB y tienen una gran relevancia en los procesos de invasión de estos tumores (41).

La adhesión de las células a la MEC induce el agrupamiento de las integrinas en la superficie celular. Por otra parte, la región citoplasmática actúa como plataforma para el reclutamiento de proteínas adaptadoras y de andamiaje, así como de proteínas de señalización hacia la cara interna de la membrana plasmática, sitio donde se forman estructuras denominadas complejos de adhesión focal **(Figura 5).** Las adhesiones focales constituyen puntos de anclaje en el frente migratorio que establecen la polaridad y la fuerza de tracción necesarias para el desplazamiento de las células durante la migración. Las proteínas adaptadoras de las adhesiones focales como la talina, Paxilina (Pax), tenascina, p130Cas y α-actinina proporcionan uniones sólidas al citoesqueleto de actina y, por tanto, conectan firmemente a la célula con la MEC. Por su parte, las cinasas

y fosfatasas que también son reclutadas a las adhesiones focales transmiten la señalización desde las integrinas al interior celular, para regular con alta precisión diferentes procesos celulares. Una de las moléculas de señalización más importantes de estos complejos es la cinasa de adhesión focal (FAK), de tipo no receptor, que ejerce su actividad catalítica sobre los residuos de tirosina de una variedad de proteínas (42).

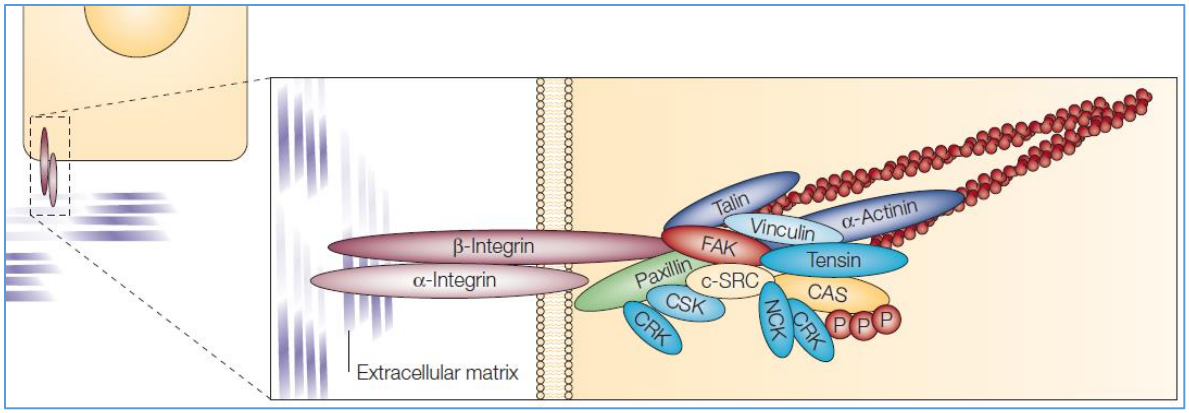

Figura 5. Organización del complejo de adhesión focal. En las adhesiones focales, los heterodímeros de las subunidades α- y β-integrina se unen a la matriz extracelular a través de sus dominios extracelulares. Sus dominios citoplásmicos se unen a un complejo formado por una serie de proteínas, como Pax, talina, vinculina, tensina y α-actinina, que conectan las integrinas con el citoesqueleto de actina. Varias moléculas de señalización también se asocian a este complejo, como FAK y cSRC, que una vez activadas inducen el recambio de la adhesión focal, para promover la motilidad celular. La fosforilación de Pax permite la correcta formación de la estructura de adhesión focal y regula la señalización hacia otras proteínas. Tomado de Yeatman y colaboradores (43).

La formación de las adhesiones focales conduce a la activación de FAK mediante la autofosforilación del residuo Y397, que a su vez crea un sitio para el reclutamiento de proteínas con dominios SH2 como cSRC, FYN, p85 o PLCy (44,45). cSRC fosforila a otros residuos de tirosina en FAK (Y576/577) y a otras proteínas de las adhesiones, creando así sitios adicionales para la unión de distintas proteínas con dominios SH2 que contribuyen a la amplificación de la señal (46). Una vez activada, FAK promueve además la activación de vías de señalización como Ras/Erk y PI3K/Akt (47). En biopsias de GB se han encontrado mayores niveles de FAK con respecto al tejido cerebral normal, y de

igual manera se ha confirmado una mayor expresión de esta cinasa en las células que componen el frente invasivo de gliomas de alto grado (48). Como se había mencionado, el funcionamiento de los complejos de adhesión focal también depende de un grupo de proteínas adaptadoras. Una vez fosforilada, la proteína adaptadora Pax estabiliza la unión de FAK y cSRC a las zonas de adhesión focal. En un mecanismo de retroalimentación positivo, cSRC y FAK fosforilan a la proteína adaptadora Pax en los residuos de tirosina 118 y 31, lo que facilita el reclutamiento de otras proteínas adaptadoras como p130Cas (49). Sun y colaboradores encontraron que la sobreexpresión de la proteína adaptadora Pax se correlaciona con la progresión del tumor y predice tiempos cortos de sobrevida en pacientes con GB (50). Las adhesiones focales son estructuras dinámicas y el recambio de la formación del sitio de adhesión en el frente celular y la ruptura en la parte posterior son esenciales para el movimiento celular. En este proceso intervienen proteínas de la familia Rho GTPasas, siendo las más importantes y mejor caracterizadas: RhoA, responsables de la coordinación de la contractibilidad entre el cuerpo celular y la parte posterior de la célula; RAC-1 que regula la formación de protrusiones en el frente migratorio y Cdc42 que modula la polaridad de la célula (51). Se ha encontrado que la actividad de Cdc42 y RAC-1 está aumentada en los gliomas con respecto al tejido normal (40). La fosforilación de la α-actinina por FAK promueve su entrecruzamiento con los filamentos de actina. Las estructuras resultantes son fundamentales para la generación de la fuerza de tracción que posibilita el desplazamiento de la célula (52).

Se ha demostrado que la P4 promueve la migración e invasión de líneas celulares derivadas de GB humanos (7) y de tumores implantados ortotópicamente en la corteza cerebral de la rata (8), sin embargo, los mecanismos moleculares que intervienen en estos efectos no se conocen con exactitud.

2.2. Características generales de la P4

La P4 o pregn-4-en-3,20-diona es una de las formas de progestágenos más activas, sintetizadas por el organismo. Esta hormona se deriva del colesterol y su estructura está compuesta de 21 átomos de carbono. Fue descubierta en los primeros años del siglo XX por su función en el mantenimiento del embarazo y purificada por primera vez en 1935

(53). La síntesis de esta hormona esteroide se lleva a cabo en las gónadas, placenta, corteza adrenal y en el SNC. A partir del colesterol la acción de la enzima de escisión de la cadena lateral del colesterol (P450scc) da lugar a la pregnenolona y posteriormente la enzima 3β-hidroxiesteroide deshidrogenasa (3β-HSD) transforma la pregnenolona en P4 (54). Además de su papel en la regulación de las funciones reproductivas, en el SNC, la P4 interviene en la diferenciación sexual, la conducta reproductiva, el estado de ánimo, el sueño, el aprendizaje, la memoria, la neuroprotección y el crecimiento tumoral (8,55–58). Debido a su estructura lipofílica, esta hormona atraviesa fácilmente la barrera hematoencefálica e interactúa con sus receptores en diversas células del SNC regulando procesos como la formación de nuevas espinas dendríticas, la reorganización del citoesqueleto, la mielinización y la neurogénesis (59). Por lo tanto, la expresión de los receptores a P4 en las diferentes áreas del cerebro es fundamental para que esta hormona ejerza sus funciones en el SNC.

2.2.1. La P4 y sus receptores en los glioblastomas

En las dos últimas décadas se ha acumulado evidencia sobre el papel de la P4 en la progresión de los GB. La primera evidencia que sugirió la participación de la P4 en la progresión de los GB fue la detección del RP en estos tumores. Más importante que la presencia del receptor, es el hecho de que su contenido se incrementa a medida que aumenta la malignidad de los astrocitomas (4,60,61). Se ha demostrado que, a través del RP, la P4 induce la proliferación, migración e invasión de células de GB humano en modelos *in vitro* e *in vivo* (5,6,61). Además del RP, la P4 ejerce sus efectos a través de los receptores membranales a P4 (mPRs), miembros de la familia de los receptores adipoQ acoplados a proteína G. Estudios recientes han detectado la expresión del mPRα, mPRβ, mPRδ, y mPRε en células de GB y el incremento de la proliferación e invasión por la activación específica del mPRα (63–65).

2.2.2. Mecanismos de acción del receptor intracelular a progesterona (RP)

El RP pertenece a la familia de los receptores a hormonas esteroides, los cuales actúan como factores de transcripción regulados por ligando. En los humanos, la transcripción del gen del RP (11q-22-23) se da bajo el control de dos promotores diferentes que

generan dos isoformas: RP-A y RP-B. Estructuralmente se diferencian por la extensión del extremo amino terminal de la isoforma B que tiene 164 aminoácidos más que la isoforma RP-A. La cadena polipeptídica de ambas isoformas está conformada por la región del Dominio N-Terminal (NTD). En el caso del RP-B esta región cuenta con dos dominios funcionales de activación (AF-1 y AF-3), mientras que en el caso del RP-A esta región está conformada por un dominio de activación y otro de inhibición (AF-1 e IF). El resto de la estructura está formada por la región de unión al ADN (DBD), una región bisagra (H) y la región C-terminal, que contiene el dominio de unión a ligando (LBD) y el dominio de activación AF-2 **(Figura 6)** (66). El análisis de las estructuras tridimensionales atómicas de los dominios DBD y LBD demuestra un alto nivel de conservación con aquellos de la familia de los receptores nucleares, sin embargo, ocurre todo lo contrario con la región NTD. En el extremo N-terminal, AF-1 es responsable de la activación independiente de ligando del RP, ya que interactúa con diferentes proteínas correguladoras y es blanco de modificaciones postraduccionales que contribuyen a la regulación de la actividad del receptor. Dependiendo del gen blanco, este dominio puede funcionar de manera independiente o interactuar con el AF-2. El dominio DBD contiene dos motivos en forma de dedos de zinc que están formados por dos hélices α y dos módulos de unión a zinc. Esta conformación permite el reconocimiento y la discriminación de los elementos de respuesta a P4 (ERP), que son secuencias específicas localizadas en la región promotora de los genes blanco de la P4. Por su parte, el dominio LBD está conformado por 12 hélices *α* y una hoja *β* plegada. Cuando la P4 se pone en contacto con la hendidura del dominio LBD, la estructura de 12 hélices α funciona como una tapa que cierra dicha hendidura. El dominio AF-2 presenta una hendidura hidrofóbica que le permite interactuar con los motivos LXXLL (cinco aminoácidos donde L es leucina y X cualquier aminoácido), presentes en algunos coactivadores como SRC-1. Los antagonistas del RP funcionan desplazando esta hendidura hidrofóbica y por tanto interrumpiendo la actividad del AF-2. La región H, ubicada entre los dominios de LBD y DBD, presenta una señal de localización nuclear (NLS) que es necesaria para la translocación del RP, desde el citoplasma hacia el núcleo y, además, juega un papel importante en la dimerización e interacción con proteínas correguladoras del receptor. La presencia del dominio AF-3 e IF en el extremo N-terminal del RP-B y RP-A

respectivamente, contribuyen a la función particular de cada una de estas isoformas. El dominio AF-3 media la interacción entre el extremo N-terminal y el C-terminal, y está demostrado que mutaciones en este sitio inhiben la actividad transcripcional del RP-B. El dominio IF interviene en la función represora del RP-A sobre la actividad transcripcional del RP-B (3). Las diferencias funcionales de ambas isoformas fueron determinadas por primera vez en estudios con ratones knockout. En los animales deficientes de RP-B se interrumpió el desarrollo de las glándulas mamarias, mientras que aquellos deficientes de RP-A no fueron capaces de ovular y por tanto resultaron infértiles (67,68).

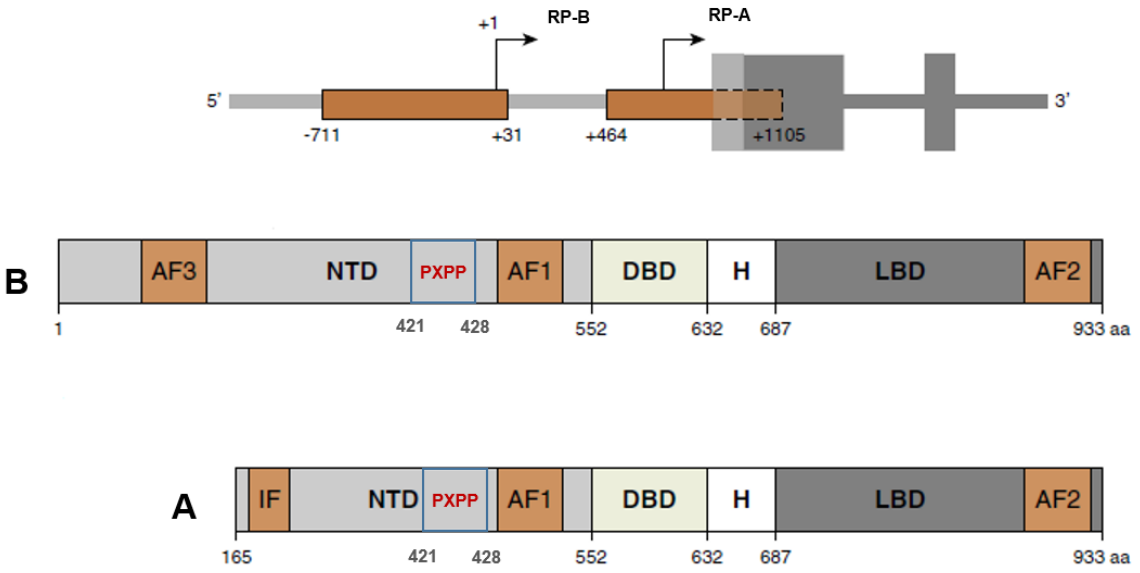

Figura 6. Gen del RP Humano y sus isoformas RP-B (**B**) y RP-A (**A**). El RP en humanos se transcribe a partir de un mismo gen (11q-22-23) bajo la acción de dos promotores diferentes lo que da lugar a la isoforma RP-A y RP-B, siendo esta última la más larga por 164 aminoácidos. Ambas isoformas tienen una estructura muy similar, comparten la región de unión al ADN (DBD), una región bisagra (H) y la región C-terminal, que contiene el dominio de unión a ligando (LBD) y la función de activación AF-2. Las isoformas son diferentes en el extremo N-terminal. En el caso del RP-B cuenta con dos dominios funcionales de activación (AF-1 y AF-3), y en el caso del RP-A un dominio funcional de activación y otro de inhibición (AF-1 e IF). Ambas isoformas tienen un motivo de

poliprolina (PXPP) entre los aminoácidos 421 y 428. Tomado y modificado de Camacho-Arroyo y colaboradores (3)**.**

El mecanismo de acción clásico o genómico de la P4 comienza una vez que esta hormona se une al RP. En ausencia de la P4 el RP se mantiene en el citoplasma en una conformación inactiva, a través de la interacción con chaperonas como la proteína de choque térmico Hsp90 y otras co-chaperonas. Una vez que la P4 interactúa con el dominio LBD, induce un cambio conformacional en el receptor que favorece su fosforilación, disociación del complejo de chaperonas, dimerización y translocación al núcleo. Una vez en el núcleo, los receptores se unen a los ERPs ubicados en las regiones reguladoras de los genes blanco. La transcripción se inicia con el reclutamiento de co-activadores y complejos remodeladores de la cromatina. Cuando los receptores se disocian del ADN, son marcados para degradación a través de la vía del proteosoma 26S (3).

Además del mecanismo de acción clásico o genómico, el RP puede activar señales extra nucleares a través de un mecanismo no clásico o no genómico. En el citoplasma o incluso anclado a la membrana plasmática, este receptor interactúa con otras moléculas citoplásmicas lo que conduce a la activación de diversas cascadas de señalización. Mediante un motivo de poliprolina (PXPP) ubicado entre los aminoácidos 421-428 del extremo amino terminal **(Figura 6)**, este receptor interactúa con los dominios SH3 de varias moléculas como la cinasa cSRC (69). En células de cáncer de mama esta interacción ha sido ampliamente estudiada y se conocen varios de los efectos que desencadena. En 1998, Migliaccio y colaboradores encontraron que en la línea celular de cáncer de mama T47D la P4 activó la vía de señalización cSRC/p21/Erk mediante la acción del RP y el receptor a estrógenos (RE) (9). Este hallazgo contribuyó a explicar las bases moleculares del efecto de la P4 sobre el crecimiento de los tumores de mama.

2.2.3. Efectos de la P4 mediados por el RP en los GB

La P4 es la hormona sexual más estudiada en el contexto de los GB. Desde 1997, algunos estudios han relacionado el contenido del RP con la malignidad de los

astrocitomas. Por ejemplo, Khalid y colaboradores encontraron que el RP se expresó más en GB (astrocitoma grado IV) que en astrocitomas grados I y II provenientes de biopsias de 86 pacientes (60). Además, en nuestro laboratorio, González-Agüero y colaboradores hallaron que el nivel de la proteína del RP se observó en el 83% de las biopsias de astrocitomas de grado III, mientras que el 100% de las biopsias de GB expresaron al RP. Estos autores también encontraron que el RP-B predominó sobre el RP-A en las biopsias analizadas (4). Recientemente, Arcos-Montoya y colaboradores reportaron que el contenido del RP, determinado por inmunofluorescencia, fue mayor en muestras derivadas de pacientes con GB, con respecto a las muestras de pacientes con astrocitomas de menor grado o tejido normal (61). Para comprender el papel del RP en la progresión de los GB, algunos investigadores han realizado ensayos funcionales mediante la estimulación o inhibición del receptor. González-Agüero y colaboradores trataron líneas celulares derivadas de GB humanos con P4 a una concentración de 10 nM y observaron un aumento significativo en la tasa de proliferación comparada con el vehículo. Cuando se añadió al tratamiento RU486, un antagonista del RP, se bloqueó el efecto de la P4, lo que sugiere que esta hormona induce la proliferación de células de GB a través del RP (62). La acción de la P4 sobre la proliferación de células de GB también se ha demostrado en modelos *in vivo*. En este caso, el tratamiento con P4 aumentó el área de los tumores que se desarrollaron en la corteza motora de ratas macho después de la implantación de células de astrocitoma humano grado III, con respecto al vehículo. Este efecto se bloqueó cuando se administraron en conjunto RU486 y P4. Resultados similares se obtuvieron al implantar la línea celular derivada de GB humano U87 en la corteza cerebral de ratas, y al utilizar oligonucleótidos anti sentido (ODN) contra la expresión del RP (6,56). Los efectos de la P4 sobre las células de GB no se limitan a modificar eventos asociados a la proliferación. En 2016, en nuestro laboratorio, Piña-Medina y colaboradores encontraron que la P4 incrementó la capacidad migratoria e invasiva de la línea celular derivada de GB, U251, con respecto al vehículo. Este efecto fue parcialmente bloqueado cuando se adicionaron RU486 u oligonucleótidos anti sentidos contra la expresión del RP. Además de los ensayos funcionales, estos autores determinaron el efecto de la P4 sobre la activación de la cofilina, proteína fundamental en la regulación del ensamblaje y desensamblaje del citoesqueleto. Los resultados

demostraron que la P4 modificó la tasa p-cofilina/cofilina, efecto que fue modificado con la adición de RU486 (7).

En células de cáncer de mama, la P4 promueve mecanismos no genómicos a través de la interacción entre el RP y cSRC. Este evento conduce a la activación de cSRC y otras proteínas más abajo en la cascada de señalización que a su vez incrementan la capacidad de proliferación, migración e invasión (9,10,70).

2.3. Las proteínas cinasas de la familia SRC

Con la culminación del Proyecto Genoma Humano (HGP) se identificaron 518 genes únicos que codifican cinasas (71), de los cuales 90 se traducen para dar lugar a proteínas tirosina cinasas (PTKs) (72). Estas cinasas se dividen en dos grupos, las proteínas tirosina cinasas de tipo receptor (RPTKs) y las proteínas tirosina cinasas de tipo no receptor (NRPTKs) (73) (**Figura 7A**). Mientras que las RPTKs son proteínas integrales de membrana, las NRPTKs son intracelulares, aunque pueden tener anclajes lipídicos y encontrarse asociadas a membranas. Las NRPTKs se localizan en la cara interna de la membrana plasmática, el citoplasma, las membranas de vesículas endocíticas y el núcleo (74). Las NRPTKs están divididas en las subfamilias SRC, JAK, ABL, TEC, ACK, CSK, FAK, FES, FRK y SYK (75) (**Figura 7A**). La subfamilia SRC incluye a su vez a las cinasas cSRC, FYN, YES, BLK, YRK, FGR, HCK, LCK y LYN, de las cuales, FYN, cSRC, YES, LYN y LCK son expresadas en gliomas humanos (11). La más relacionada con la progresión de diferentes tipos de cáncer es cSRC (43). Los dominios estructurales de la subfamilia SRC son desde el N-terminal: el dominio SH4, SH3, SH2 y SH1. SH1 es el dominio catalítico; SH2 y SH3 son adhesivos moleculares importantes para la interacción proteína-proteína; mientras que el dominio SH4 del extremo N-terminal, es importante para el anclaje a la membrana. Entre los dominios SH4 y SH3 se encuentra una región cuya secuencia varía considerablemente entre los diferentes miembros de la subfamilia SRC. El dominio SH4, situado en el extremo N-terminal contiene un sitio de miristoilación crítico para el anclaje a la membrana (75) (**Figura 7B**).

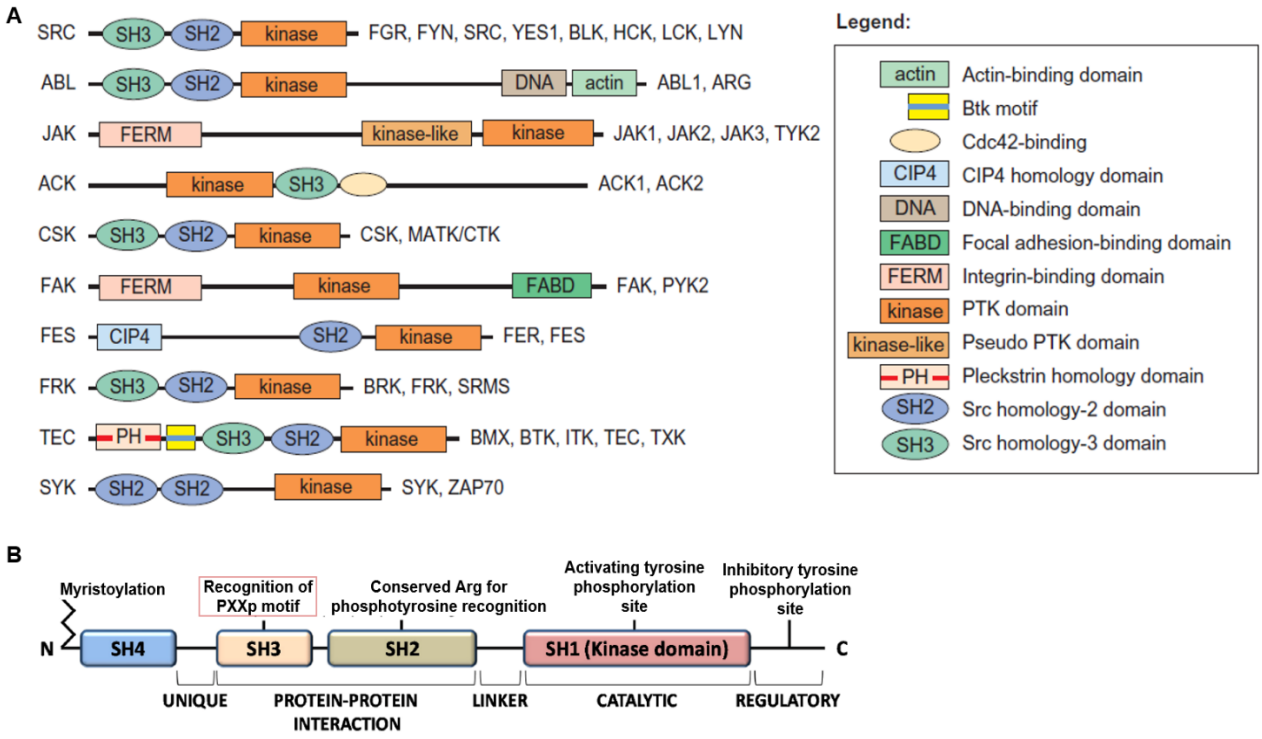

Figura 7: Grupo de las proteínas tirosina cinasas de tipo no receptor (NRPTKs). (**A**) Estructura de las cinasas que componen el grupo de las NRPTKs. (**B**) Estructura y función de los dominios que componen a las cinasas de la familia SRC. (**A**) Tomado y modificado de Brady, Scott T. y Lau, Lit Fui (74). (**B**) Tomado y modificado de Bagnato G y colaboradores (76).

2.3.1. Estructura, función y mecanismos de regulación de cSRC

En 1911, Peyton Rous describió por primera vez un virus que causaba el crecimiento de tumores transmisibles entre pollos (77). Esta idea resultó muy controversial porque hasta aquel momento no existían evidencias sobre la transmisión del cáncer a través de agentes infecciosos. En la década de 1950 se demostró definitivamente que las células infectadas por el virus del sarcoma de Rous (VRS) se transformaban en células tumorales. La confirmación definitiva de que el gen v-SRC del VRS causaba los tumores en los pollos, se obtuvo cuando mutaciones en este gen que conferían sensibilidad a ciertas temperaturas impidieron que el VRS transformara las células a temperaturas no permisivas (78,79). Las secuencias celulares (cSRC) homólogas al gen v-SRC del VRS están presentes en el ADN genómico de todos los vertebrados examinados y se han

conservado evolutivamente (80). A diferencia de v-SRC, el gen humano *SRC* codifica para una proteína (cSRC) cuya activación se puede modular a través del dominio de regulación negativa ubicado en su extremo C-terminal y no posee capacidad transformante inherente (81). Tanto la forma aviar de la proteína cSRC, como la humana, están compuestas por un extremo C-terminal que contiene un residuo de tirosina (Tyr527, aves; Tyr530, humanos) fundamental para la regulación negativa de la proteína, un dominio amino-terminal único y cuatro dominios de homología cSRC (SH). El dominio cinasa SH1 contiene el sitio de autofosforilación (Tyr416, aves; Tyr419, humano), el dominio SH2 interactúa con la Tyr530 del extremo C-terminal, el dominio SH3 favorece el contacto intramolecular con el dominio cinasa en la forma inactiva de la proteína, y el dominio SH4 contiene el sitio de miristoilación que es importante para la localización en la membrana. Para la completa activación de cSRC, el sitio de autofosforilación es imprescindible, mientras que el extremo C-terminal y los dominios SH2 y SH3 juegan un papel fundamental en la regulación negativa. En su estado inactivo, cSRC mantiene una conformación estructural cerrada gracias a la interacción entre sus dominios. En su forma fosforilada, la Tyr530 interactúa con el dominio SH2 propiciando a su vez la interacción entre los dominios SH1 y SH3, de manera que el sitio de autofosforilación permanece bloqueado y por lo tanto cSRC se encuentra en un estado inactivo (**Figura 8**) (43,82,83).

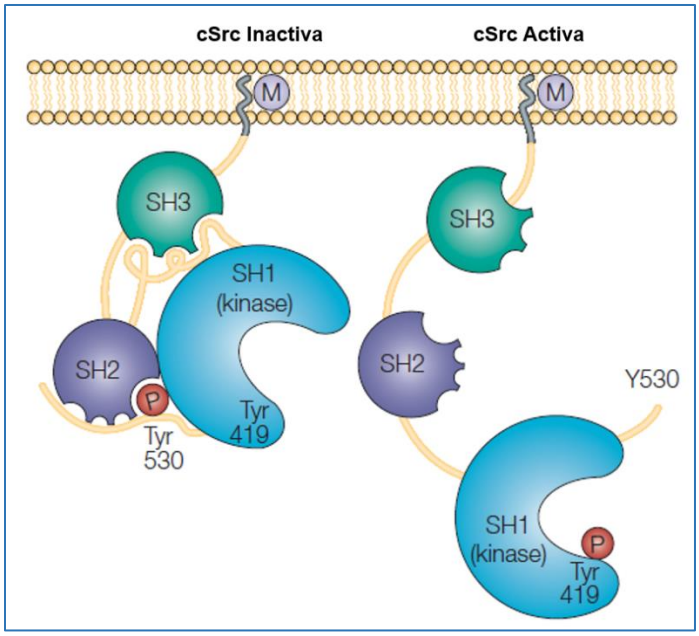

Figura 8: Estructura de cSRC en su conformación inactiva y activa. En su estado inactivo, cSRC mantiene una conformación cerrada gracias a las interacciones entre el dominio catalítico (cinasa SH1) con el dominio SH3 y del dominio SH2 con el residuo Tyr530 fosforilado del extremo C-terminal. La acción de determinadas fosfatasas sobre la fosforilación del residuo Tyr530 o el desplazamiento del dominio SH3 debido a la interacción con otras proteínas, induce un cambio conformacional que expone al residuo Tyr419, facilitando su autofosforilación y por lo tanto la activación de la proteína. Tomado y modificado de Yeatman y colaboradores (43).

La inactivación de cSRC a través de la fosforilación del residuo Tyr530 se lleva a cabo por la tirosina cinasa de cSRC (CSK) y su homólogo CHK (84). CSK está estructuralmente relacionada con cSRC, pero carece del dominio regulador negativo que se encuentra en el extremo C-terminal de cSRC. Masaki y colaboradores demostraron que la reducción de la actividad de CSK se correlaciona inversamente con el aumento de la actividad de cSRC en el carcinoma colorrectal (85). Por el contrario, diversas fosfatasas que funcionan como activadores de cSRC se encargan de eliminar el fosfato en el residuo Tyr530 (86). En estudios *in vitro* e *in vivo* se ha observado que la proteína tirosina fosfatasa-α (PTPα) desfosforila el residuo de tirosina terminal (Tyr 530). La eliminación de este fosfato afecta la interacción inhibidora con el dominio SH2, lo cual a

su vez libera el residuo Tyr419 y permite su autofosforilación (87). Además de la fosforilación-desfosforilación del residuo Tyr530 que implica una regulación intramolecular, la activación de cSRC está mediada por interacciones intermoleculares. Los dominios SH2 de diversos receptores tirosina-cinasa como EGFR (88) y el receptor del factor de crecimiento derivado de plaquetas (PDGFR) (89), así como los motivos de poliprolina de algunas proteínas citoplásmicas como FAK o receptores nucleares como el RP, el RE y el receptor de andrógenos (RA) (9,10,43,90), son los responsables de esta regulación. Tanto el dominio SH2 como el motivo de poliprolina de estas proteínas pueden desplazar las interacciones intramoleculares SH2-Tyr530 y SH3-dominio auto catalítico, liberando a cSRC de su conformación cerrada y exponiendo el dominio auto catalítico.

2.3.2. cSRC en el cáncer

Los efectos de la activación de cSRC en el cáncer varían de acuerdo con el grado de malignidad de los tumores. Las investigaciones sugieren que en células precancerosas cSRC contribuye al proceso de tumorigénesis a través del incremento de la proliferación celular. cSRC está sobreexpresada y/o activada en una amplia gama de tumores que también sobreexpresan varios receptores de tirosina cinasas, lo que indica la posibilidad de interacciones sinérgicas para promover la tumorigénesis. Tice DA y colaboradores encontraron que la transfección conjunta de EGFR y cSRC en fibroblastos, dio lugar a un aumento de la proliferación (88). Se ha demostrado que cSRC interactúa con las vías reguladoras del ciclo celular y las cascadas de transducción de señales relacionadas con la proliferación. Por ejemplo, varios autores han reportado la interacción de cSRC con las vías de señalización de Ras para promover la transformación de fibroblastos y linfocitos (91,92). Sin embargo, en el cáncer avanzado, la actividad de cSRC no se correlaciona con una mayor tasa de proliferación si no con eventos asociados a la migración e invasión de las células, procesos que finalmente conducen a la metástasis hacia órganos distantes (93,94). La sobreexpresión de la proteína cSRC y el aumento de su actividad específica son fenómenos muy frecuentes en numerosos tipos de cáncer. En particular, los cánceres del tracto gastrointestinal, como el cáncer colorrectal, muestran un aumento progresivo de la actividad de cSRC a medida que avanza el

estadio del tumor (95,96). Además del cáncer colorrectal, se ha demostrado un aumento de la actividad de cSRC en otras neoplasias gastrointestinales, como las hepatocelulares, pancreáticas, gástricas y esofágicas, así como en los cánceres de mama, ovario y pulmón (43). En las células tumorales, cSRC ejerce sus efectos a través de una serie de mecanismos mediados por interacciones con varios sustratos y proteínas de unión. La motilidad y la invasión de las células tumorales dependen, entre otros factores, de la pérdida de la adhesión célula-célula mediada por la E-cadherina. En este contexto cSRC promueve la ubiquitinación de la E-cadherina, lo que conduce a su endocitosis y por consiguiente a la disrupción del contacto célula-célula. El recambio de las adhesiones focales también es necesario para conferir la capacidad de motilidad e invasión a las células tumorales, en este caso, se sabe que cSRC lo promueve a través de varios mecanismos. Uno de ellos es la activación de FAK, que a su vez fosforila sustratos como Pax, CAS y p190 RHOGAP para provocar cambios en el citoesqueleto que conducen al desensamblaje de las adhesiones focales. De manera independiente a FAK, cSRC regula la organización del citoesqueleto y de las adhesiones focales a través de su interacción con la p120 catenina y la cortactina. La fosforilación de la proteína R-Ras asociada a Ras (RRAS) por cSRC inhibe la función de las integrinas, lo que también contribuye al recambio de las adhesiones focales. Además, la actividad de cSRC provoca cambios en la expresión de varios genes que participan en la progresión tumoral. La activación de FAK estimula la vía de señalización de la cinasa amino terminal c-JUN (JNK), lo que conduce a un aumento en la expresión de las MMP-2 y -9. Todos estos eventos moleculares regulados por cSRC orquestan el escenario ideal para la migración e invasión a través de la MEC y membrana basal, la extravasación hacia vasos sanguíneos o linfáticos y finalmente la metástasis hacia otros órganos (43).

2.3.2.1. Papel de cSRC en la progresión de los GB

La expresión y actividad de las cinasas de la subfamilia SRC: cSRC, FYN, LYN y YES, es mayor en células de GB que en astrocitomas de menor grado o tejido normal (11,97). De acuerdo con los datos obtenidos a través del Proyecto del Atlas del Genoma del Cáncer, el aumento de la actividad de las cinasas de la subfamilia SRC en los GB, no se debe a la amplificación o mutación de los genes (98,99). Uno de los primeros estudios

sobre el papel de cSRC en los GB se llevó a cabo en ratones transgénicos que expresaban constitutivamente la variante mutada de cSRC que no posee el dominio regulador negativo del extremo C-terminal (v-SRC). En estos animales se observó el desarrollo de tumores gliales con características moleculares y morfológicas muy similares a las de un GB humano (12). La participación de cSRC en la migración e invasión de células de GB se ha estudiado en modelos *in vitro* e *in vivo*. El papel de cSRC en la regulación de la motilidad de las células de GB fue reportado por Angers-Loustau y colaboradores en 2004. En este trabajo se implantaron esferoides de la línea celular de GB humano (U251) en matrices tridimensionales de colágena tipo I y se observó que los inhibidores farmacológicos específicos de la familia cSRC, PP2 y SU6656, redujeron significativamente la invasión de las células. Además se observó que PP2 interfirió con el reordenamiento de los filamentos de actina en la formación del lamelipodio (100). En 2015, Lewis-Tuffin y colaboradores encontraron que el silenciamiento de cSRC, FYN, YES y LYN disminuyó la proliferación y migración en las líneas celulares LN229, U87, U251, TP483 y SF767 derivadas de GB humanos (13). En células de GB con expresión positiva para el marcador de troncalidad CD133, la inhibición de la expresión y actividad de cSRC y FYN disminuyó la capacidad migratoria e invasiva de estas células (14). Kobayashi y colaboradores demostraron que las proteínas Oct3 y 4, reguladoras de la auto renovación de células troncales, inducen la sobreexpresión de cSRC y FAK en células de GB, en consecuencia las células con sobreexpresión de Oct3 y 4 mostraron una mayor capacidad de migración e invasión (101). En 2017, se demostró que en células troncales de GB ($SOX2^{+}$/$Nestina^{+}$) derivadas de pacientes, la adición de un péptido penetrador, cuya secuencia correspondía a los aminoácidos 266–283 de la secuencia de la conexina 43, redujo de manera significativa la motilidad y capacidad invasiva de estas células a través de la inhibición de cSRC y FAK. La región que corresponde a los residuos 266-283, dentro del dominio C-terminal de la CX43, recluta a cSRC junto con sus inhibidores, CSK y PTEN. Como consecuencia, se produce una disminución de la fosforilación de FAK en los residuos Y576 y Y577 por los bajos niveles de cSRC activa (102).

2.3.2.2. Interacción cSRC-RP

La primera evidencia sobre el mecanismo no genómico del RP fue reportada en 1998 por Migliaccio y colaboradores. Estos autores encontraron que el tratamiento con P4 indujo rápidamente la activación de cSRC y ERK2 en células T47D derivadas de un carcinoma de mama ductal humano. Además, se determinó que la activación de cSRC fue dependiente de la interacción con el RP y el RE. Más específicamente, se propuso que el RP no interactúa directamente con cSRC sino con el extremo N-terminal del RE, formando un complejo RP-RE-cSRC que es el responsable de desencadenar la activación de la cinasa (9). Tres años más tarde Boonyaratanakornkit y colaboradores describieron el mecanismo por el cual el RP induce la activación de vías de señalización asociadas a cSRC. Al contrario de la hipótesis de Migliaccio y colaboradores, estos autores sugirieron que el RP se une directamente con el dominio SH3 de cSRC, a través de un motivo de poliprolina ubicado en su extremo N-terminal. Con el objetivo de determinar si esta secuencia era imprescindible para la interacción entre el motivo de poliprolina del RP y el dominio SH3, tres prolinas (P422A, P423A y P426A) se convirtieron en alaninas mediante mutagénesis dirigida al sitio. El receptor mutante (PR-BmPro) se expresó en el sistema de baculovirus y se comprobó su unión a los dominios SH3 de un grupo de cinasas (cSRC, HCK, FYN, p85, GRB) mediante un ensayo de arrastre con glutatión S transferasa (GST). Posteriormente, Boonyaratanakornkit aclaró estas diferencias al demostrar que RE y RP tienen la capacidad de interactuar directamente con cSRC. El RE lo hace con el dominio SH2 mientras que el RP lo hace exclusivamente con el dominio SH3 y ambas interacciones pueden ocurrir de manera independiente o en un complejo ternario. Estos autores además encontraron que la activación de cSRC a través del RP incrementó la expresión de genes sin ERP, como la ciclina D1 (103). En 2010, Fu y colaboradores demostraron que en células de cáncer de mama tratadas con P4, la interacción RP-cSRC aumentó la capacidad migratoria e invasiva a través de la activación de FAK, la vía PI3K/Akt y el complejo de GTPasas pequeñas RhoA/Rho (70). En células endoteliales umbilicales humanas el RP ejerce un efecto muy similar al descrito previamente en las células de cáncer de mama. El tratamiento con P4 también provocó un aumento en la activación de FAK y la capacidad migratoria de estas células, efectos que son disminuidos por la adición de inhibidores del

RP y de cSRC (104). Como hemos descrito previamente, se conoce que el RP juega un papel fundamental en la activación de la migración e invasión en las células de GB. Sin embargo, los mecanismos moleculares que orquestan estos procesos no se han investigado en su totalidad y no existen evidencias de la interacción RP-cSRC y sus efectos en estos tumores.

III. Planteamiento del problema

Además de su función como factores de transcripción, el RP puede actuar como transductor de señales a través de la interacción con dominios SH3 de moléculas citoplásmicas, como cSRC. Se ha demostrado que, de manera independiente, el RP y cSRC participan en la regulación de los procesos de migración e invasión de los GB. Sin embargo, se desconoce si ambas proteínas interactúan y los efectos que dicha acción desencadena en estos tumores. El estudio de la interacción entre el RP y la proteína cSRC podría aportar valiosa información sobre los mecanismos involucrados en la migración e invasión en las células de GB humano.

IV. Hipótesis

La activación de cSRC a través de su interacción con el RP estará involucrada en el aumento de la migración e invasión de células de GB humano tratadas con P4.

V. Objetivos

5.1 Objetivo general

Caracterizar el papel de la activación de cSRC a través de su interacción con el RP, en la capacidad de migración e invasión en líneas celulares derivadas de GB humanos.

5.1.1 Objetivos particulares

1. Conocer el efecto de la P4 en la activación de la proteína cSRC en las líneas celulares derivadas de GB humanos U251 y U87.
2. Evaluar la participación del RP en la activación de la proteína cSRC en las células U251 y U87.
3. Determinar la interacción entre el RP y cSRC en células de GB humanos.
4. Evaluar el papel de cSRC en la activación de FAK y Pax en células U251 y U87 tratadas con P4.
5. Evaluar la participación de cSRC en la regulación de la expresión de las MMP-2 y -9 en las células U251 y U87 tratadas con P4.
6. Determinar el efecto de cSRC sobre la capacidad de migración e invasión en células de GB tratadas con P4.

VI. Metodología

6.1. Cultivo de células

Se utilizaron células U251 y U87, derivadas de GB humanos y procedentes de la Colección de Cultivos Americana (ATCC). Se sembraron 2 x 10^5 células en placas de 35 mm x 12 mm y se mantuvieron, hasta alcanzar una confluencia del 80%, en medio DMEM (In vitro, S.A., D.F., MEX) suplementado con SFB al 10%, piruvato 1 mM, glutamina 2 mM, y aminoácidos no esenciales 0.1 mM (GIBCO, NY, USA) y con penicilina/estreptomicina al 1% a 37 °C con 5% de CO_2.

6.2. Tratamientos

24 h antes de los tratamientos se sustituyó el medio de cultivo anteriormente mencionado por medio DMEM (In vitro, S.A., D.F., MEX) sin rojo de fenol y libre de hormonas, suplementado con SFB al 10% también libre de hormonas. Las células se trataron con P4 (10, 50 y 250 nM), con R5020 (10 nM), PP2 (1µM) o con vehículo (DMSO al 0.01%). Los tiempos de tratamiento fueron de 10 min para evaluar la activación de cSRC, de 20 min para FAK y Pax respectivamente y de 3 y 6 h para la expresión de las MMP-2 y -9. Para la detección de la activación de cSRC, FAK y Pax o la expresión de la MMP-9 en las células U251 cuya expresión del RP o cSRC fue disminuida por silenciamiento, se procedió de la misma forma, una vez terminados los tiempos de transfección que se describirán más adelante.

6.3. Extracción de proteínas

Después de tratadas, las células fueron homogenizadas en buffer RIPA con un coctel de inhibidores de proteasas (Sigma Aldrich, St Louis, MO USA, # P8340) y un grupo de inhibidores de fosfatasas (NaF, Na_4P2O_7, Na_3VO_4 y Na_3VO_4). Las proteínas fueron obtenidas por centrifugación a 12500 rpm durante 5 min y cuantificadas utilizando el espectrofotómetro NanoDrop-2000 (Thermo Scientific, MA, USA).

6.4. Western blot

Para la separación de las proteínas se depositaron aproximadamente 30 µg en un gel de poliacrilamida a una concentración del 8.5% para cSRC, Pax y MMP-2 y al 7.5% para FAK y MMP-9, en condiciones desnaturalizantes. Las proteínas fueron transferidas a una membrana de nitrocelulosa en condiciones semi secas en un transblot (BIO-RAD) durante 30 min a 25 V para las proteínas de 60 kDa (cSRC), 68 kDa (Pax) y 83 y 73 kD de la forma escindida y total de la MMP-2 y durante 1 h a 25 V para las proteínas de 125 kDa (FAK) y 93 y 83 kDa de la forma escindida y total de la MMP-9. El bloqueo se realizó con albúmina sérica bovina al 5% a 37°C durante 2 h. Las membranas fueron incubadas con los anticuerpos primarios contra las forma fosforilada y total de las proteínas cSRC, Pax y FAK (phospho SRC Tyr-416 Cell Signaling, MA, USA, Ref 2101; SRC Cell Signaling, MA, USA, Ref 2108; phospho paxillin Tyr-118 Cell Signaling, MA, USA, Ref 2541; paxillin Cell Signaling, MA, USA, Ref 2542; phospho FAK Tyr-397 Cell Signaling, MA, USA, Ref 3283; FAK Cell Signaling, MA, USA, Ref 3285) y de las proteínas MMP-2 y MMP-9 (MMP-2 Cell Signaling, MA, USA, Ref. 4042; MMP-9 Cell Signaling, MA, USA. USA, Ref. 3852). Todos los anticuerpos se utilizaron en una dilución 1/1000. Como control de carga se detectó a la proteína α-tubulina a una dilución 1:1000 (Sigma Aldrich, St Louis, MO USA, Ref T3195). Todos los anticuerpos fueron incubados durante 48 h excepto el anticuerpo contra α-tubulina que fue incubado por 24 h. Posteriormente, las membranas fueron incubadas con el anticuerpo secundario contra conejo (1:10000) (Santa Cruz Biotechnology, TX, USA) durante 45 min en agitación y a temperatura ambiente. Por recomendación de Cell Signaling, los anticuerpos primarios y secundarios fueron removidos de las membranas con una solución con Tris-HCl pH 6.8 a 0.06 M, SDS al 2 % y β-mercaptoetanol al 0.7 % durante 30 min a 50 °C en agitación. La señal quimioluminiscente fue detectada por exposición de las membranas al sustrato SuperSignal West Fento (Thermo Scientific # 34096) con placas Kodak Biomax Light Film (Sigma-Aldrich, MO, USA).

6.5. Silenciamiento de las proteínas cSRC y RP

Para los experimentos de silenciamiento las condiciones de cultivo variaron con respecto a las que se describieron anteriormente. En esta ocasión se sembraron 1 x 10^5 células, en medio DMEM (In vitro, S.A., D.F., MEX) suplementado con SFB al 10%, piruvato 1 mM, glutamina 2 mM, y aminoácidos no esenciales 0.1 mM (GIBCO, NY, USA) y con penicilina/estreptomicina al 1% a 37 °C con 5% de CO_2. Después de 24 h, el medio de crecimiento fue sustituido por el medio de transfección: DMEM (In vitro, S.A., D.F., MEX) sin antibiótico, sin SFB, sin rojo de fenol y libre de hormonas con los respectivos tratamientos: lipofectamina (0.5%), 100 nM de siRNA contra la expresión del RP (Ambion, PGR Silencer Select, Pre-designed cat#4392420) o cSRC (Ambion, SRC Silencer Select, Pre-designed cat # 4392420) según el caso, así como 100 nM de siRNA control (Silencer Select Negative Control #1). El medio de transfección fue retirado a las 12 h y sustituido por medio DMEM (In vitro, S.A., D.F., MEX) sin antibiótico, sin rojo fenol y suplementado con SFB al 10% libre de hormonas. Después de 48 h de transfección las células fueron colectadas para la extracción de ARN, en el caso del RP, o proteína en el caso de cSRC. Para la extracción del ARN se utilizó el reactivo de trizol (Ambiom by Life Technologies, ref: 10296028) y la expresión del RP, después del silenciamiento, se detectó a nivel de ARNm por RT-PCR. Una vez extraído el ARN, se llevó a cabo su cuantificación y la determinación de su calidad a través de las relaciones 260/280, 260/230 y por su visualización en un gel de integridad. Tanto la concentración como la calidad del ARN fueron aceptables para la metodología de RT-PCR, que se llevó a cabo con los paquetes: Platinum Pfx DNA Polymerase Cat# 11708-039 y LightCycler FastStart DNA Master SYBR Green I Cat# 12 239 264 001. Los oligonucleótidos que se utilizaron para la PCR fueron los siguientes: RP: FW: 5´-ACATGGTAGCTGTGGGAAGG-3´, RV: 5´-GCTAAGCCAGCAAGAAATGG-3´, 18S: FW 5´ -AGT GAA ACT GCG AAT GGC- 3´, RV: 5´-CTG ACC GGG TTG GTT TTG-3´. En el caso de cSRC, la expresión posterior al silenciamiento se detectó a nivel de proteína y se procedió como fue descrito en las secciones de extracción de proteínas y western blot.

6.6. Inmunofluorescencia

Para la detección de las proteínas cSRC y RP, se sembraron 15 000 células por pozo sobre cubreobjetos, en medio DMEM (In vitro, S.A., D.F., MEX) suplementado con SFB al 10%, piruvato 1 mM, glutamina 2 mM, y aminoácidos no esenciales 0.1 mM (GIBCO, NY, USA) y con penicilina/estreptomicina al 1% a 37 °C con 5% de CO2. A las 24 h de sembradas el medio se reemplazó por DMEM (In vitro, S.A., D.F., MEX) sin rojo de fenol y libre de hormonas, suplementado con SFB al 10% también libre de hormonas por otras 24 h, posteriormente, las células se fijaron en paraformaldehído por 20 min, diluido hasta el 2% en el mismo medio que contenía a las células. Una vez transcurrido este tiempo, las células se lavaron tres veces con tampón fosfato salino (PBS) por 5 min y se permeabilizaron y bloquearon al mismo tiempo en una solución de PBS con Tritón al 0.2% y albúmina sérica bovina al 1%. Posteriormente las células se incubaron con los anticuerpos primarios contra cSRC (Cell Signaling, MA, USA, Ref 2108) 1/500; y RP (Santa Cruz Biotechnology Dallas, Texas, USA Ref B-30 sc-811) 1/200 por 48 h en una solución de PBS con glicina al 1%, Triton al 0.2% y albúmina sérica bovina al 1%. Al retirar el anticuerpo primario, las células se lavaron tres veces con PBS por 5 min. A continuación, se incubaron con el anticuerpo secundario contra conejo (1/1000) alexa fluor 568 (Invitrogen, Oregon, USA Ref A11011) para el anticuerpo primario contra cSRC y con el anticuerpo secundario contra ratón (1/1000) alexa fluor 488 (Invitrogen, Oregon, USA Ref 1890503) para el anticuerpo primario contra el RP, durante 45 min, en total oscuridad. Después de la incubación con el anticuerpo secundario las células se lavaron tres veces con PBS por 5 min y se incubó con Hoechst 1/12000 (Thermo Scientific, Rochester, NY, USA, Ref 33342) por 7 min en total oscuridad. Finalmente, las células se lavaron tres veces con PBS por 5 min y se montaron en el medio Aqua-Poly/Mount (Polysciences, Valley Road, Warrington, PA, Ref 18606). Las muestras se visualizaron en un microscopio Olympus Bx43F para detectar la fluorescencia alexa 568, alexa fluor 488 y Hoechst. La colocalización se estableció fusionando las imágenes mediante el software ImageJ (National Institute of Health).

6.7. Co-inmunoprecipitación

Las células se lisaron en una solución tampón que contenía Tris-HCl 50 mM (pH 7.4), NaCl 150 mM, EDTA 1 mM, Triton X-100 al 1%, SDS al 0.1% y un cóctel de inhibidores de proteasas (Sigma Aldrich, St Louis, MO USA, # P8340) a 4 °C durante toda la noche. Posteriormente, los lisados celulares se centrifugaron a 12500 rpm durante 15 min. Del sobrenadante se tomó el volumen correspondiente a 1 mg de proteína y se incubó con 2 µg de anticuerpo anti-RP (Santa Cruz Biotechnology Dallas, Texas, EE. UU. Ref. B-30 sc-811) y 50 µL de proteína acoplada a sefarosa más agarosa A/G (sc-2003; Santa Cruz Biotechnology) bajo agitación permanente a 4 °C durante toda la noche. Al día siguiente, las muestras se centrifugaron y las perlas de A/G se lavaron tres veces con una solución tampón que contenía (Tris HCl 20 mM; NaCl 150 mM; EDTA 1 mM, Triton X-100 0,1 y un cóctel de inhibidores de proteasas (Sigma Aldrich, St Louis, MO EE. UU., # P8340) pH 7.5). Finalmente, las muestras se desnaturalizaron hirviendo en tampón de carga (Tris 120 mM, pH 6.8; SDS al 4%; glicerol al 0.2%; β-mercaptoetanol al 5% y azul de bromofenol 10 mg/mL) y se separaron en SDS-PAGE. La detección de cSRC y el RP se realizó como se describió previamente en la sección de Western blot. Para detectar el RP se eliminaron previamente los anticuerpos primarios y secundarios.

6.8. Ensayos de migración e invasión

Para evaluar la capacidad de migración de las células, una vez terminado el tiempo de transfección (de acuerdo con el protocolo descrito anteriormente), se realizó la herida en cada uno de los pozos con una punta de micropipeta desechable de 200 µL, se retiró el medio y las células despegadas en este proceso se eliminaron con dos lavados de PBS. Se añadieron 2 mL de DMEM sin rojo fenol y libre de hormonas y posteriormente Ara-C (inhibidor de la proliferación), a una concentración de 10 µM. Las células se incubaron por 1 h a 37 °C y pasado este tiempo, sin retirar el medio, se aplicaron los tratamientos correspondientes con P4 (50 nM) o su vehículo (DMSO al 0.01%) en las células transfectadas con el siRNA control o contra la expresión del cSRC. Finalmente, se tomaron fotos a las 0, 6 y 12 h, utilizando como referencia la cuadrícula previamente dibujada en las cajas. Se calculó la capacidad de cierre de la herida por la fórmula $100 - \frac{tx\ x\ 100}{t0}$. En el caso de la invasión las células se cultivaron como se describe en la sección

"Cultivo de células". Los insertos Transwell (membrana de 8.0 µm; Corning, USA) se colocaron en una placa de 6 pocillos y se cubrieron con 2 mg/mL de matrigel (Sigma Aldrich, EE. UU.) que se diluyeron previamente en DMEM sin rojo fenol y libre de SFB. Se colocó 1 mL de esta dilución en el inserto e inmediatamente se incubó la placa a 37 °C durante 2 h en una atmósfera de 95% de aire y 5% de CO_2. A continuación, se añadieron 20000 células suspendidas en 1.5 mL de medio libre de suero con 10 µM de Ara-C (Sigma Aldrich, EE. UU.) en el inserto. Los pocillos inferiores se llenaron con 2 mL de DMEM con 10% de SFB como quimioatrayente. Los tratamientos con P4 o PP2 (inhibidor de la actividad de cSRC) se realizaron en los insertos y las células se incubaron a 37 °C, 5% de CO2 y 95% de aire durante 24 h. Las células invasoras de la superficie inferior de la membrana se fijaron con paraformaldehído al 4% durante 20 min y se tiñeron con cristal violeta al 0.1% durante 20 min. A continuación, los insertos se lavaron tres veces durante 10 min con PBS para eliminar el exceso de colorante. Por último, los insertos se observaron con un microscopio invertido (CKX41, Olympus, JPN) y se tomaron las correspondientes imágenes con un aumento 100x en una cámara Infinity1-2C. El número de células que invadieron el matrigel se contó en 5 campos seleccionados al azar mediante el uso del software ImageJ1 (NIH, EE. UU.).

6.9. Análisis bioinformático de la expresión de los genes PXN (Pax) y PTPN12 (PTP-PEST) en GB

Los recuentos de RNA-Seq de 196 gliomas de grado II, 223 de grado III y 139 de grado IV (GB) se obtuvieron de los proyectos GB y Gliomas de bajo grado del repositorio de la base de datos: The Cancer Genome Atlas (TCGA) (https://portal.gdc.cancer.gov/). Los datos se descargaron y procesaron mediante el uso del paquete TCGAbiolinks versión 2.12.6 para R.17. Además, se obtuvieron perfiles de expresión de 249 muestras de corteza cerebral sana de la base de datos GTEx (https://gtexportal.org/home/). Los datos se normalizaron por DESeq2 versión 1.22.2 y se representaron gráficamente. La correlación de la expresión génica en los GB se analizó en el paquete TCGAbiolinks para R.

7. Análisis *in sílico*

Para el análisis *in sílico* se utilizaron tres bases de datos diseñadas para la predicción de sitios de fosforilación: NetPhos 3.1, KinasePhos, y GPS 5.0. NetPhos 3.1 que predicen sitios de fosforilación utilizando conjuntos de redes neuronales. Se realizan predicciones tanto genéricas como específicas de las cinasas (105). KinasePhos identifica sitios de fosforilación específicos de las proteínas cinasas a partir de las secuencias y los patrones de acoplamiento (106), mientras que GPS 5.0 utiliza métodos de determinación del peso de la posición y de optimización de la matriz de puntuación (107). Todas estas bases de datos predicen la fosforilación de residuos de serina, treonina o tirosina en proteínas eucariotas.

7.1. Análisis estadístico

Los datos se analizaron con el programa Graph Pad Prism 5 (GraphPad Software, Inc., USA). Se realizó un ANOVA de una vía seguida de una prueba de comparaciones múltiples de Bonferroni (**Figuras 9A; 10C** y **D; 12C, D** y **E; 14B; 16A** y **B; 17B** y **D**) y posteriormente la prueba t student para establecer las diferencias estadísticas entre 2 grupos comparables (**Figuras 9B, 10A y B, 11B, 12A y B, 15A y B**). Los valores de $p < 0.05$ se consideraron estadísticamente significativos.

VII. Resultados

7.1. La P4 induce la activación de cSRC en células de GB humanos

Para determinar el papel de la P4 en la activación de cSRC en células de GB, se administró P4 a tres concentraciones diferentes (10, 50 y 250 nM) a la línea celular U251 durante 10 min y la fosforilación de cSRC (Y416) se determinó mediante western blot. La concentración de 50 nM fue la más efectiva en inducir un incremento en la tasa p-cSRC (Y416)/cSRC (**Figura 9A**). Teniendo en cuenta este resultado, en las células U87 solo se analizó la concentración de 50 nM, que a su vez también indujo un incremento en la tasa p-cSRC (Y416)/cSRC (**Figura 9B**).

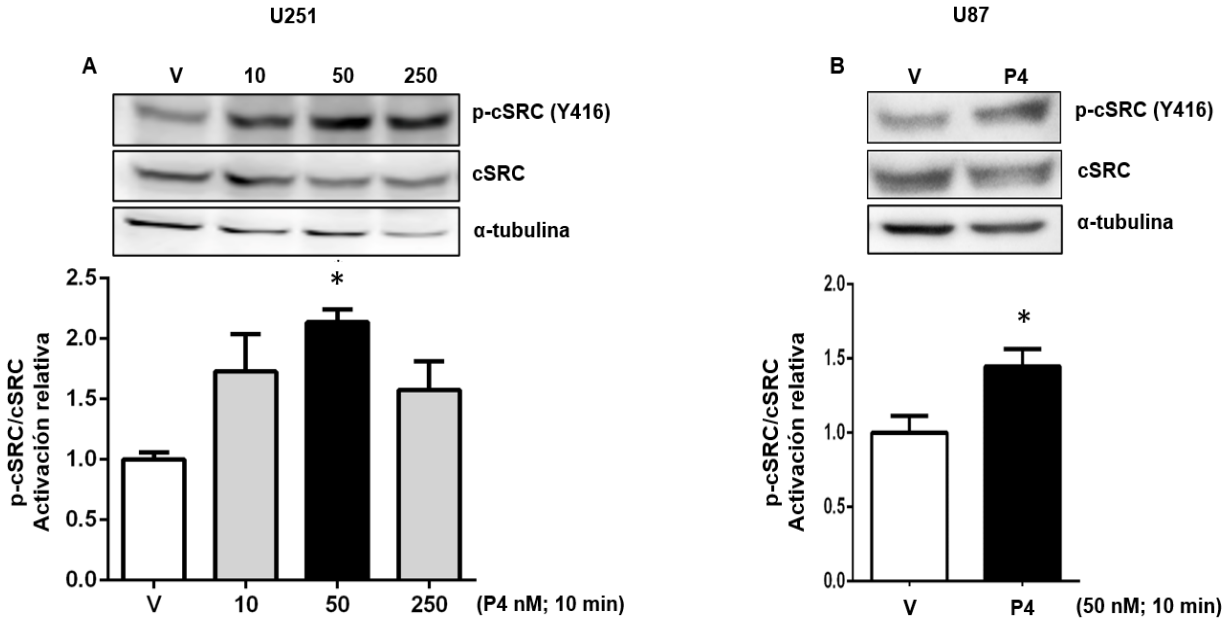

Figura 9. Activación de cSRC por la P4 en células de GB humano. Las células U251 (**A**) y U87 (**B**) se trataron con P4 (10, 50 y 250 nM) o vehículo (V, DMSO 0.01%) durante 10 min. Los paneles superiores muestran las imágenes representativas del western blot para p-cSRC, cSRC y α-tubulina. El panel inferior muestra el análisis densitométrico, donde los datos se normalizaron con respecto al vehículo. Los resultados están expresados como ± S.E.M. n = 4; *p <0.05 vs. V.

7.2. La activación de cSRC por la P4 está mediada por el RP en células de GB humanos

Además del RP la P4 tiene afinidad por otros receptores, como se describió en la sección 2.2.1. de los antecedentes (65,108). El R5020 es una progestina sintética que funciona como un agonista del RP. Teniendo en cuenta la alta afinidad del R5020 por el RP (Kd ≈ 2 nM) (109) sobre otros receptores (110,111), en este trabajo se incluyó el tratamiento de las células con 10 nM de R5020. Como en el caso de la P4, el R5020 aumentó la tasa p-cSRC (Y416)/cSRC en las células U251 y U87 (**Figuras 10A** y **B**). Posteriormente, para confirmar que la activación de cSRC por la P4 está mediada por el RP, las células U251 se transfectaron con un siRNA comercial contra la expresión del RP o con un siRNA control (secuencia aleatoria) y se trataron con P4 (50 nM) durante 10 min. La eficiencia de la transfección fue superior al 50% (**Figura 10C**). La P4 indujo la activación de cSRC en las células transfectadas con el siRNA control como se describió en el experimento que corresponde a la Figura 9. Por el contrario, en las células transfectadas con el siRNA contra la expresión del RP se bloqueó el aumento de la tasa p-cSRC (Y416)/cSRC inducido por la P4 (**Figura 10D**). Este resultado demuestra la participación del RP en la activación de cSRC por la P4 en células de GB humano. Considerando el corto tiempo (10 min) en el que se observa la activación de cSRC, este resultado sugiere que al igual que en las células de cáncer de mama, en este caso, el RP induce la activación de cSRC a través del mecanismo no genómico que se describió en la sección 2.3.1.

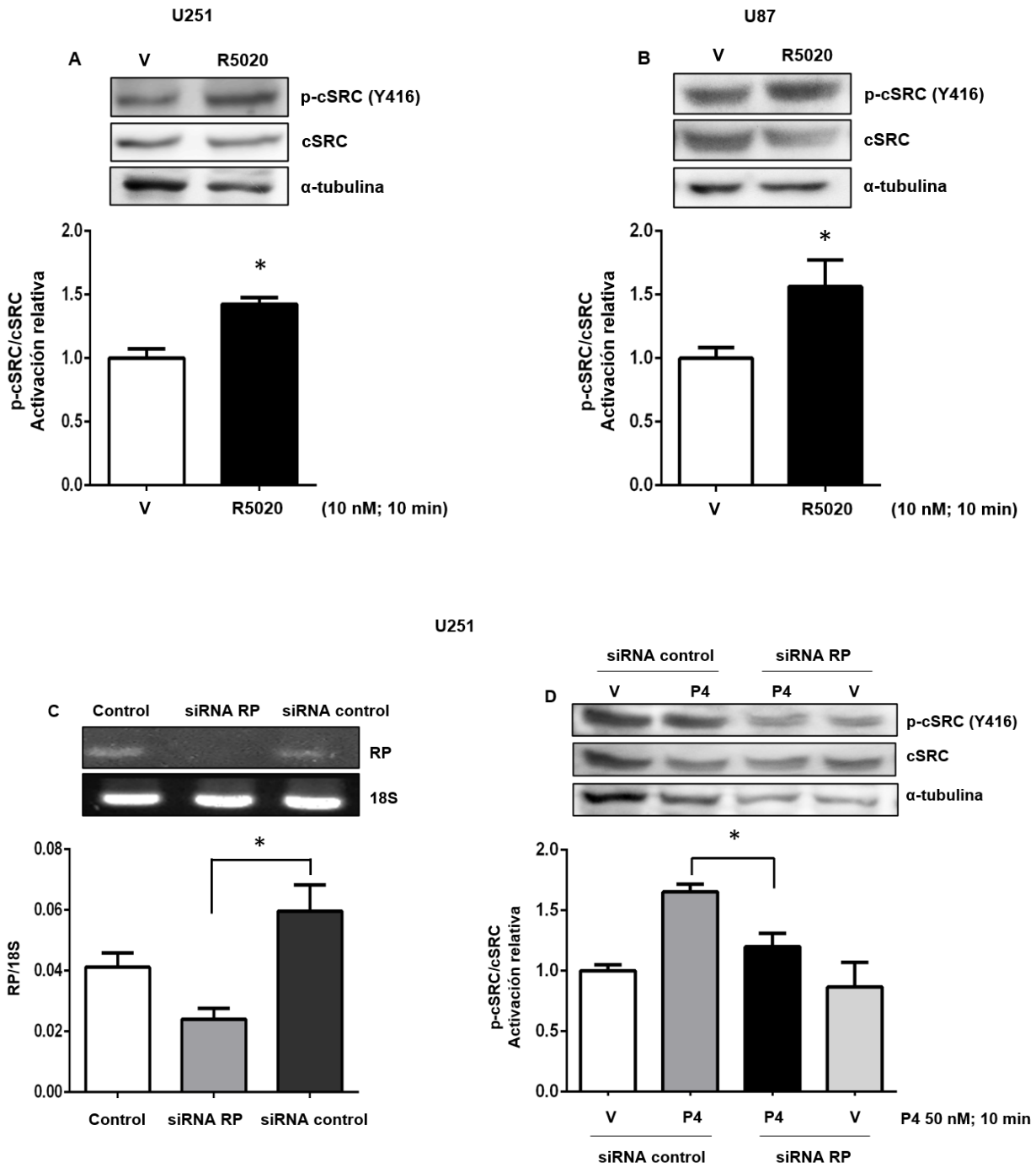

Figura 10. Participación del RP en la activación de cSRC por la P4 en células de GB. Las células U251 (**A**) y U87 (**B**) se trataron con R5020 (10 nM) o vehículo (V, DMSO 0.01%) durante 10 min. Las células U251 (**C**) se transfectaron con un siRNA contra la expresión del RP o un siRNA control (secuencia aleatoria) (100 nM) o solo se trataron con lipofectamina (Control). (**D**) Las células transfectadas se trataron con P4 (50 nM) o

vehículo (V, DMSO 0,01%) durante 10 min. Los paneles superiores muestran (**A**, **B** y **D**) las imágenes representativas del western blot para p-cSRC, cSRC y α-tubulina, (**C**) imagen representativa de la RT-PCR para las bandas correspondientes al RP y al 18S. Los paneles inferiores muestran el análisis densitométrico de (**A**, **B** y **D**) Western blot y (**C**) RT-PCR. Los resultados se expresan como la media ± S.E.M. (**A**, **B** y **D**) n = 4 * p <0.05 vs. v, (C) n = 3 *p <0.05 vs. siRNA control.

7.3. El RP y cSRC interactúan en células de GB humano

El mecanismo no genómico del RP tiene lugar a través de la interacción entre el motivo de poliprolina ubicado en el extremo N-terminal de este receptor y el dominio SH3 de la cinasa cSRC (10). Para evaluar la interacción de estas proteínas en las células U251 derivadas de un GB humano, primero se realizó un ensayo de inmunofluorescencia como se describe en la sección de metodología. De acuerdo con la imagen de inmunofluorescencia (**Figura 11A**), ambas proteínas co-localizan principalmente en el núcleo y en el espacio perinuclear. Aunque este ensayo no demuestra la interacción física, sí sugiere la posibilidad de que esta ocurra ya que relaciona a dos proteínas en un mismo lugar e instante de tiempo. Con el objetivo de corroborar este resultado, se llevó a cabo un ensayo de co-inmunoprecipitación donde además de evidenciarse la interacción entre ambas proteínas se observa que la P4 potencia el efecto a los 5 min de tratamiento (**Figura 11B**).

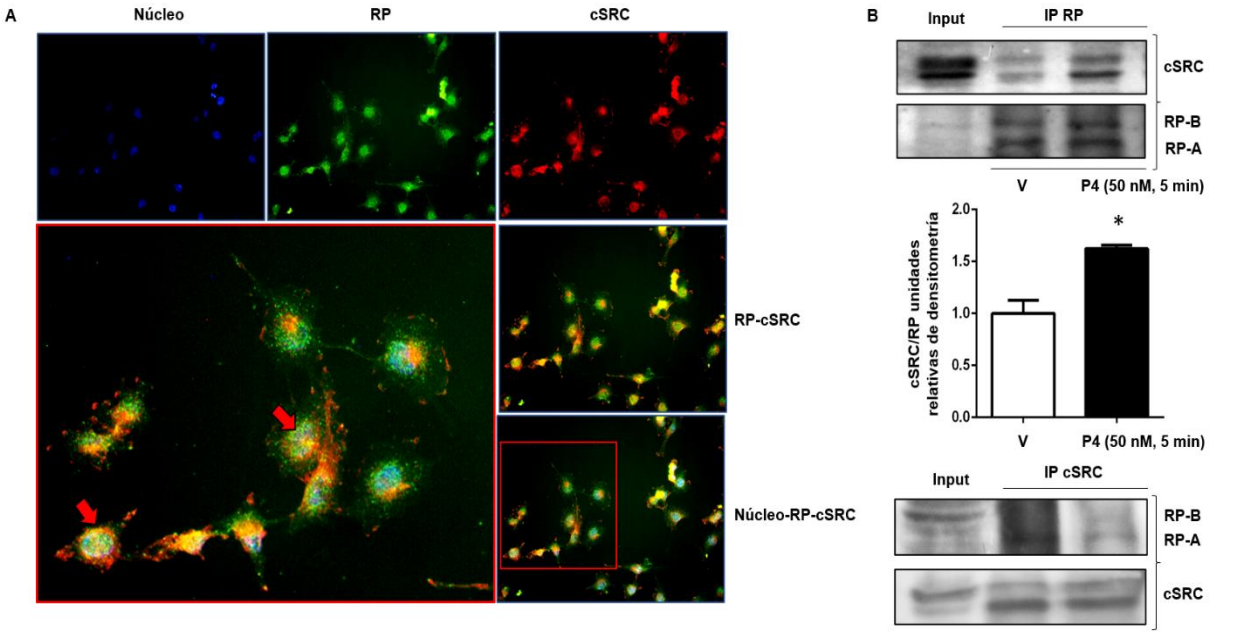

Figura 11. Interacción entre el RP y cSRC en las células U251. Los ensayos de inmunofluorescencia se llevaron a cabo en condiciones basales. (**A**) Señal del núcleo (azul), células inmunopositivas para el RP (verde), cSRC (roja), unión de ambas señales (RP-cSRC) y unión con la señal del núcleo (Núcleo-RP-cSRC). En la imagen amplificada de la unión total, las flechas indican las áreas de co-localización entre el RP y cSRC en el núcleo y el espacio perinuclear principalmente. (**B**) Las células U251 se trataron con P4 (50 nM) o vehículo (V, DMSO 0,01%) durante 5 min, se inmunoprecipitaron con un Ac contra el RP y se reveló por western blot la señal de ambas proteínas. También se muestra la inmunoprecipitación del RP utilizando un Ac contra cSRC (IP cSRC). Los resultados se expresan como la media ± S.E.M. n = 3 *p <0.05 vs V.

7.4. cSRC tiene sitios blancos de fosforilación en el RP

La fosforilación de los receptores nucleares, incluido el RP, tiene una gran relevancia en los efectos regulados por estas proteínas. La fosforilación del RP sobre los residuos de serina se ha estudiado ampliamente (112); sin embargo, en relación con los residuos de tirosina no hay información sobre las proteínas que están involucradas y los efectos que puede tener esta modificación sobre la actividad del receptor. Teniendo en cuenta la interacción entre cSRC y el RP, se decidió evaluar el papel potencial de cSRC sobre la

fosforilación del RP mediante el uso de tres bases de datos diferentes (NetPhos 3.1, KinasePhos y GPS 5.0). En todas ellas se encontró el mismo residuo putativo de tirosina en el aminoácido 87 del RP (**Tabla 1**). Aunque este resultado debe ser confirmado en ensayos experimentales, la información obtenida por las bases de datos abre la posibilidad para futuras investigaciones de las funciones y la regulación del RP por cSRC.

Tabla 1. Análisis *in silico* de los potenciales sitios de fosforilación de cSRC sobre el RP.

	ID	Posición	AA	Cinasa	Score	E-value	Péptido
Netphos 3.1	Receptor de progesterona *Homo sapiens*	87	Y	cSRC	0.516		VEGAYSRAE
				EGFR	0.444		
KinasePhos	AAA60081.1	87	Y	cSRC		13	VEGAYSRAE
GPS 5.0		87	Y	TK/cSRC/SRCA/YES1	27.217		LSDVEGAYSRAEATR

7.5. cSRC regula la activación de FAK y Pax en células de GB humanos tratadas con P4

FAK y Pax son componentes fundamentales de las adhesiones focales, ya que facilitan y regulan la formación de estos sistemas. Para evaluar si la P4 induce la activación de FAK y Pax, las células U251 y U87 fueron tratadas con P4 a 50 nM durante 20 min. La activación de estas proteínas se determinó mediante western blot con el uso de anticuerpos específicos para los residuos de tirosina 397 (Y397) y 576/577 (Y576/577) de FAK y el residuo de tirosina 118 (Y118) de Pax. La P4 (50 nM) aumentó la tasa p-FAK/FAK (Y397 e Y576/577) en las células U251 y la tasa p-FAK (Y576/577)/FAK en las células U87, mientras que, en el caso de la proteína Pax, la P4 disminuyó la tasa p-Pax (Y118)/Pax en las células U251 a los 20 min (**Figura 12A** y **B**). cSRC es una de las cinasas más involucradas en la fosforilación de los componentes de las adhesiones focales, principalmente de FAK (29). Para determinar el papel de cSRC en la fosforilación de FAK y Pax, las células U251 se transfectaron con un siRNA comercial contra la expresión de cSRC o con un siRNA control (secuencia aleatoria) y se trataron con P4 (50 nM) durante 20 min. La eficiencia del siRNA para disminuir la expresión de cSRC fue del 50% (**Figura 12C**). El siRNA contra la expresión de cSRC bloqueó el aumento de la

tasa p-FAK (Y576/577)/FAK inducido por la P4 (**Figura 12D**), pero no la disminución de la relación p-Pax/Pax (**Figura 12E**). Este resultado sugiere que la P4 participa en la regulación de las adhesiones focales a través de cSRC en las células de GB.

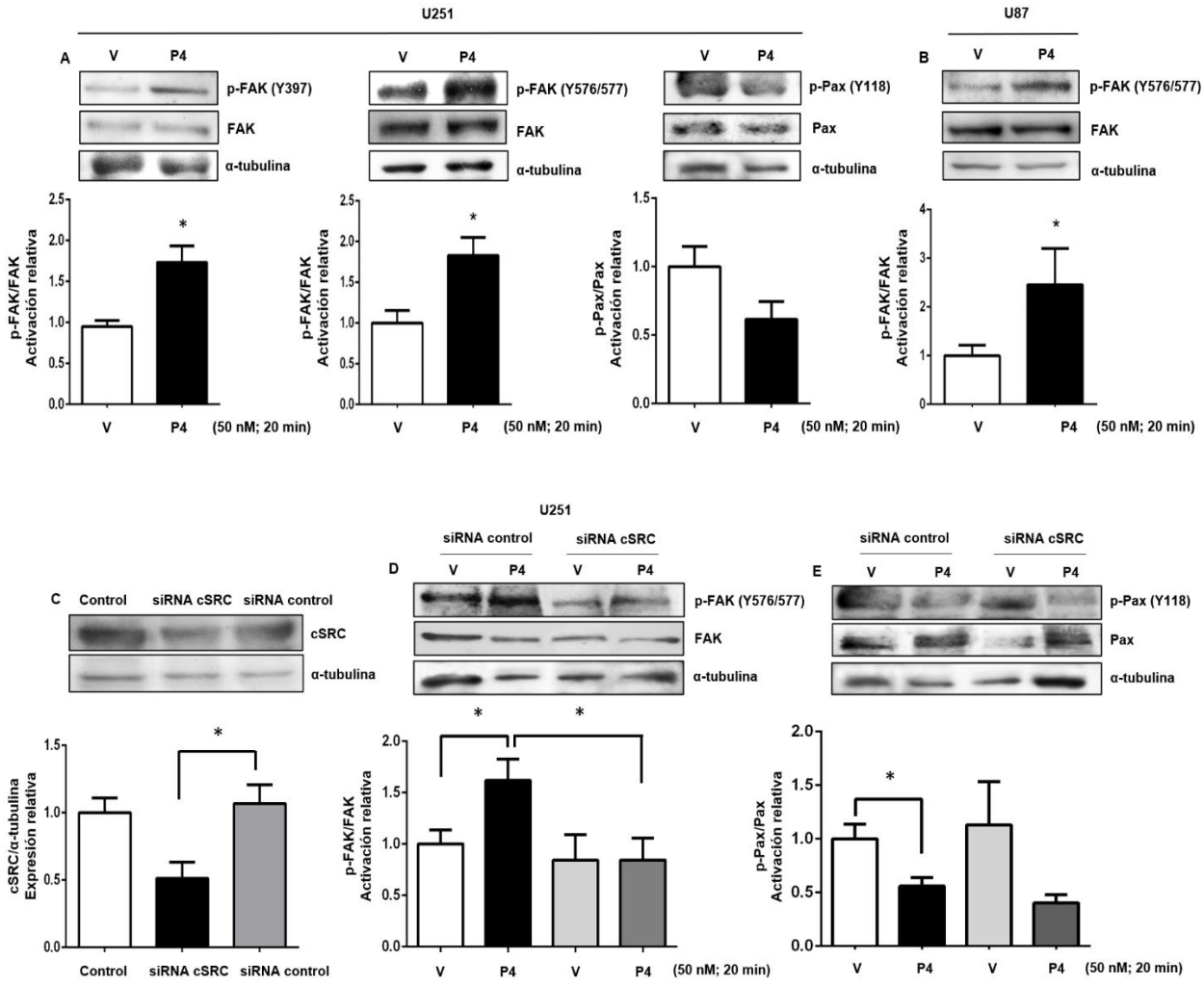

Figura 12. Participación de cSRC en la activación de FAK y Pax por la P4 en células de GB humanos. Las células U251 (**A**) y U87 (**B**) se trataron con P4 (50 nM) o vehículo (V, DMSO 0,01%) durante 20 min. Las células U251 (**C**) se transfectaron con un siRNA contra la expresión de cSRC o un siRNA control (secuencia aleatoria) (100 nM) o solo se trataron con lipofectamina (Control). (**D** y **E**) las células transfectadas con el siRNA contra cSRC o el siRNA control se trataron con P4 (50 nM) o vehículo (V, DMSO 0.01%) durante 20 min. Los paneles superiores muestran las imágenes representativas del western blots para cSRC, p-FAK, FAK, p-Pax, Pax y α-tubulina. Los paneles inferiores muestran el

análisis densitométrico. Los datos se normalizaron respecto al vehículo o al control. Los resultados se expresan como la media ± S.E.M. n = 4 *p <0.05.

Una de las proteínas estrechamente relacionadas con la desfosforilación de Pax es la tirosina fosfatasa PTP-PEST. Shen y colaboradores encontraron que PTP-PEST co-inmunoprecipita con FAK y Pax en células de embriones de pollo (113). Estos autores también demostraron que la sobreexpresión de PTP-PEST está asociada a la disminución de la fosforilación de Pax (114). En los GB, PTP-PEST regula los eventos de invasión mediante la ubiquitinación dependiente de la fosforilación de proteínas focales esenciales como Cas, FAK, Pax y cSRC (115). Por medio de un análisis bioinformático, en este trabajo se comparó la expresión de los genes que codifican para las proteínas Pax (PNX) y PTP-PEST (PTPN12) entre tejido normal y astrocitomas de diferentes grados, así como entre los cuatro subtipos moleculares de GB descritos por Verhaak y colaboradores. Se estableció la correlación entre la expresión de ambos genes en los GB. PNX y PTPN12 tienen una mayor expresión en astrocitomas de grado II, III y IV (GB) con respecto al tejido normal y dentro de los GB, tienen una mayor expresión en el subtipo mesenquimal que a su vez se ha asociado con el peor pronóstico (**Figura 13A**), lo cual sugiere que ambos genes participan en la progresión de los GB. Además, el análisis mostró un valor de 0.65 de correlación en la expresión de ambos genes en los GB (**Figura 13B**) lo que sugiere que ambas proteínas participan en una misma ruta de señalización.

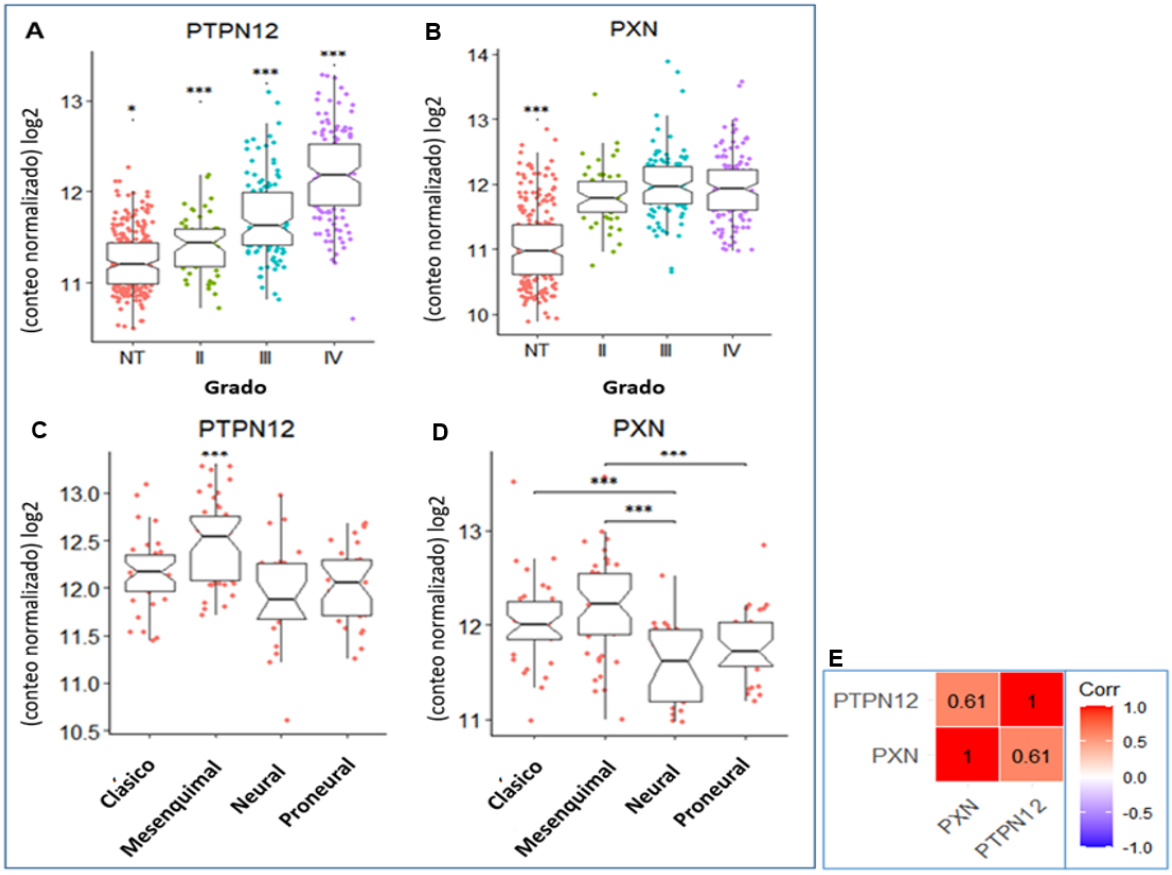

Figura 13. Expresión y correlación de PXN (Pax) y PTPN12 (PTP-PEST) a partir de las bases de datos de los proyectos GB y Gliomas de bajo grado del Atlas del Genoma del Cáncer (TCGA). (**A** y **B**) Comparación de la expresión de PXN y PTPN12 entre tejido normal (NT) y astrocitomas de grado II, III y IV. (**C** y **D**) Comparación entre los subtipos moleculares (clásico, mesenquimal, neural y proneural) definidos por Verhaak y colaboradores. (**E**) Correlación entre la expresión de PXN Y PTPN12 en GB.

7.6. Papel de cSRC en la migración de células de GB humano tratadas con P4

Como se esperaba de acuerdo con los resultados presentados hasta este punto, el silenciamiento de cSRC disminuyó la capacidad migratoria de las células U251 tratadas con P4, lo que se ve reflejado en el porcentaje de cierre de la herida (**Figura 14**). A las 6 y 12 h de tratamiento con la P4, las células que fueron transfectadas con el siRNA contra la expresión de cSRC cubrieron una menor área de la herida con respecto a las que se transfectaron con el siRNA control. Este resultado y los anteriores sugieren que cSRC

participa en los eventos que conducen a la migración de células de GB a través de la regulación de la fosforilación de las proteínas Pax y FAK en las células de GB estimuladas con P4.

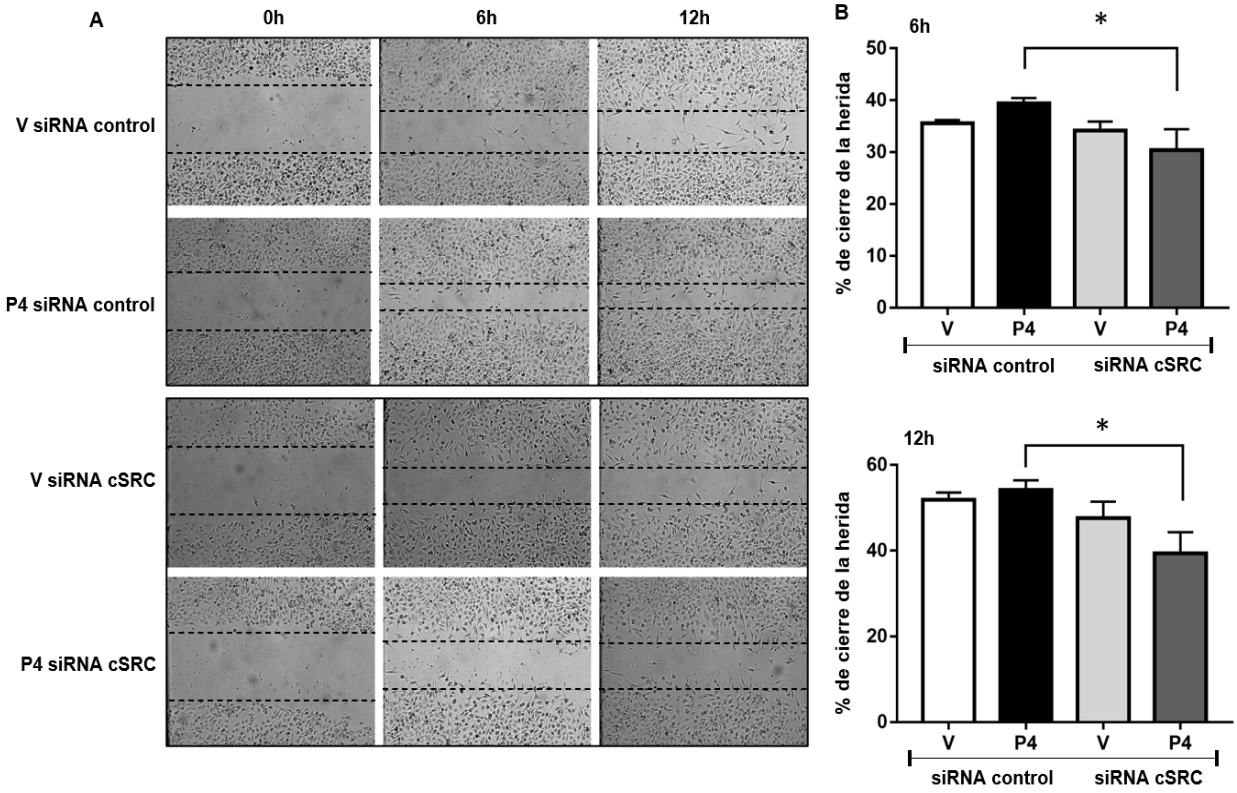

Figura 14. cSRC participa en la migración celular inducida por la P4. Las células U251 transfectadas con un siRNA contra la expresión de cSRC y un siRNA control (secuencia aleatoria) (100 nM) se trataron con P4 (50 nM) o vehículo (V, DMSO 0.01%). Se tomaron fotografías del área de la herida a las 0, 6 y 12 h y se capturaron con un aumento de 100X. (**A**) Imagen representativa del cierre de la herida. (**B**) cuantificación del cierre de la herida (%). Los resultados se expresan como la media ± S.E.M. n = 4; *p<0.05.

7.7. Efecto de la P4 en la expresión de las MMP-2 y -9 en células de GB humanos

Las MMP-2 y -9 desempeñan un importante papel en la capacidad de las células de GB para invadir el parénquima cerebral que rodea al tumor. La expresión de ambas metaloproteinasas es mayor en GB que en el tejido cerebral normal. Además, una elevada expresión de ambas metaloproteinasas es un indicador de mal pronóstico para

la recurrencia de los GB (116–118). La P4 indujo un aumento de la expresión de la MMP-9 a las 3 y 6 h de tratamiento en las células U251 y a las 6 h de tratamiento en las células U87 (**Figura 15B**), sin embargo, en el caso de la MMP-2 no se observaron diferencias significativas en el cambio de la expresión.

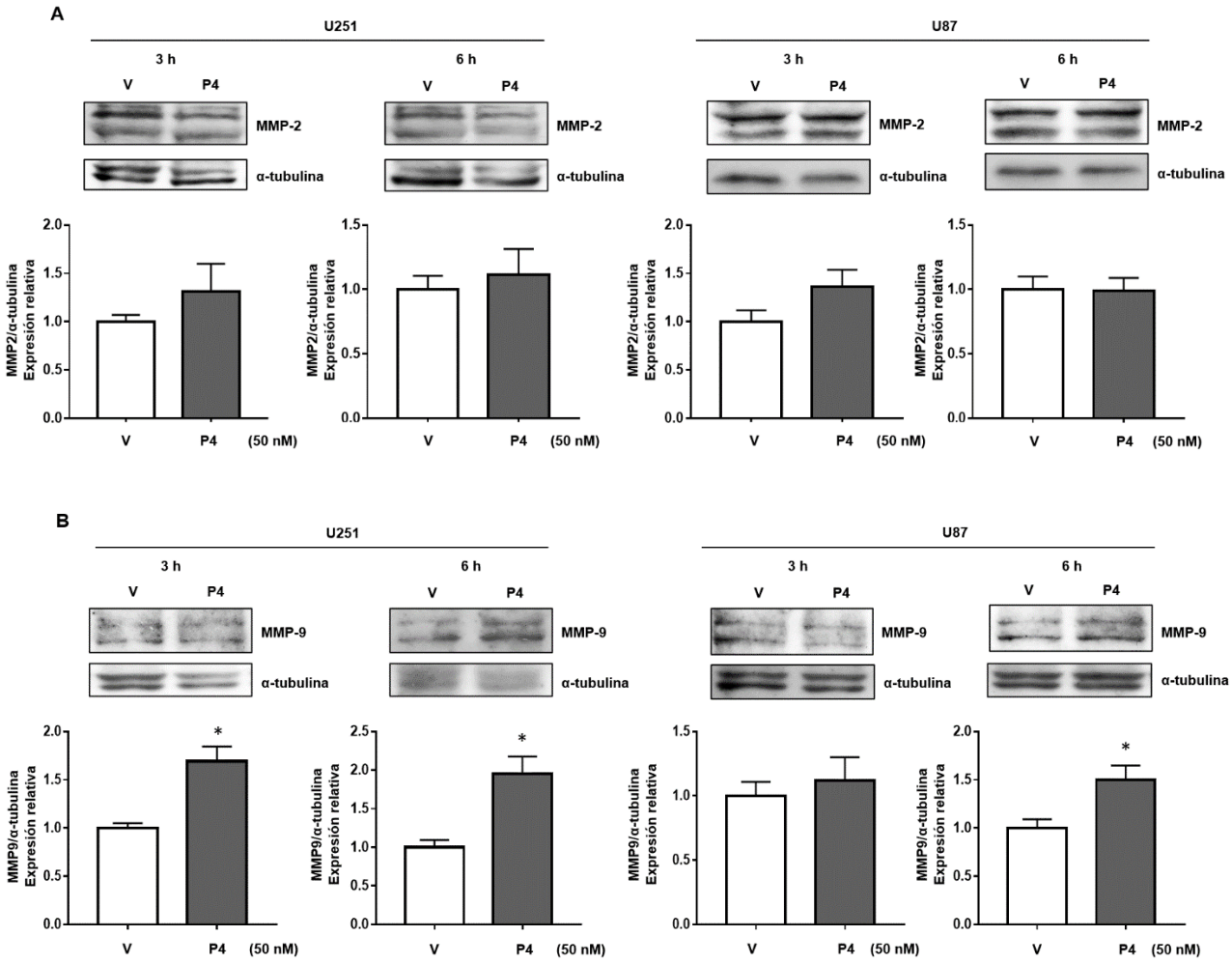

Figura 15. La P4 aumenta la expresión de la MMP-9 en células de GB humanos. Las células U251 y U87 se trataron con P4 (50 nM) o vehículo (V, DMSO 0.01%) durante 3 y 6 h. Los paneles superiores muestran las imágenes representativas del western blot para MMP-2 (**A**), MMP-9 (**B**) y α-tubulina. Los paneles inferiores muestran el análisis densitométrico. Los datos se normalizaron con respecto al vehículo. Los resultados se expresan como la media ± S.E.M. n = 3; *p <0.05 vs. V.

7.8. cSRC participa en la regulación de la expresión de la MMP-9 en células de GB humanos tratadas con P4

Considerando la capacidad de la P4 para aumentar el contenido de la proteína MMP-9 en las células derivadas de GB humanos y antecedentes que relacionan a cSRC con el incremento de la actividad y expresión de la MMP-9 en células derivadas cáncer de mama (119), se decidió evaluar el papel de la cinasa cSRC sobre la expresión de la MMP-9 en el contexto de este trabajo. Para cumplir este objetivo ambas líneas celulares (U251 y U87) se transfectaron con un siRNA contra la expresión de cSRC o con un siRNA control (secuencia aleatoria) y se trataron con P4 (50 nM) durante 6 h. En las células transfectadas con el siRNA contra cSRC el efecto de la P4 fue significativamente menor que en aquellas que solo recibieron el siRNA control (**Figura 16A y B**). Este resultado sugiere que, al menos parcialmente, cSRC está involucrada en el efecto de la P4 sobre la expresión de la MMP-9.

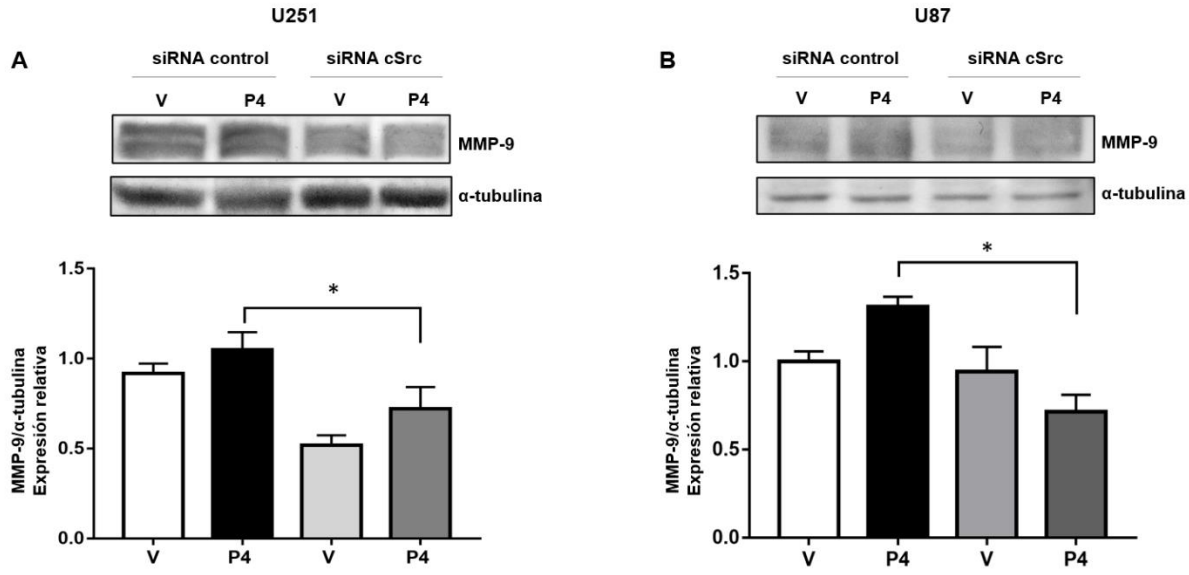

Figura 16. Participación de cSRC en el incremento de la expresión de la MMP-9 por la P4 en células de GB humanos. Las células U251 (**A**) y U87 (**B**) se transfectaron con un siRNA contra la expresión de cSRC o un siRNA control (secuencia aleatoria) (100 nM) y se trataron con P4 (50 nM) o vehículo (V, DMSO 0.01%) durante 6 h. Los paneles superiores muestran las imágenes representativas del western blot para la MMP-9 y la α-tubulina. Los paneles inferiores muestran el análisis densitométrico. Los datos se

normalizaron respecto al vehículo. Los resultados se expresan como la media ± S.E.M. n = 4 *p <0.05.

7.9. Papel de cSRC en la capacidad invasiva de células de GB humanos tratadas con P4

Considerando el efecto de la P4 en el aumento de la expresión de la MMP-9 (**Figura 15B**) y la participación de cSRC en este evento (**Figura 16A y B**), en este trabajo también se evaluó el papel de cSRC en la capacidad de invasión de células de GB humano a través de un ensayo en cámara de Boyden con matrigel (**Figura 17**). La P4 incrementó el número de células que invaden a través del matrigel (**Figura 17**) y este efecto se revirtió cuando las células U87 fueron tratadas además con el inhibidor farmacológico de cSRC, PP2 (**Figura 17C y D**). Aunque en el caso de las células U251 el efecto del PP2 sobre la acción de la P4 no fue estadísticamente significativo, si se observa una tendencia bastante marcada en la disminución de la capacidad invasiva de las células tratadas con P4+PP2 con respecto a las que solo recibieron P4 (**Figura 17A** y **B**).

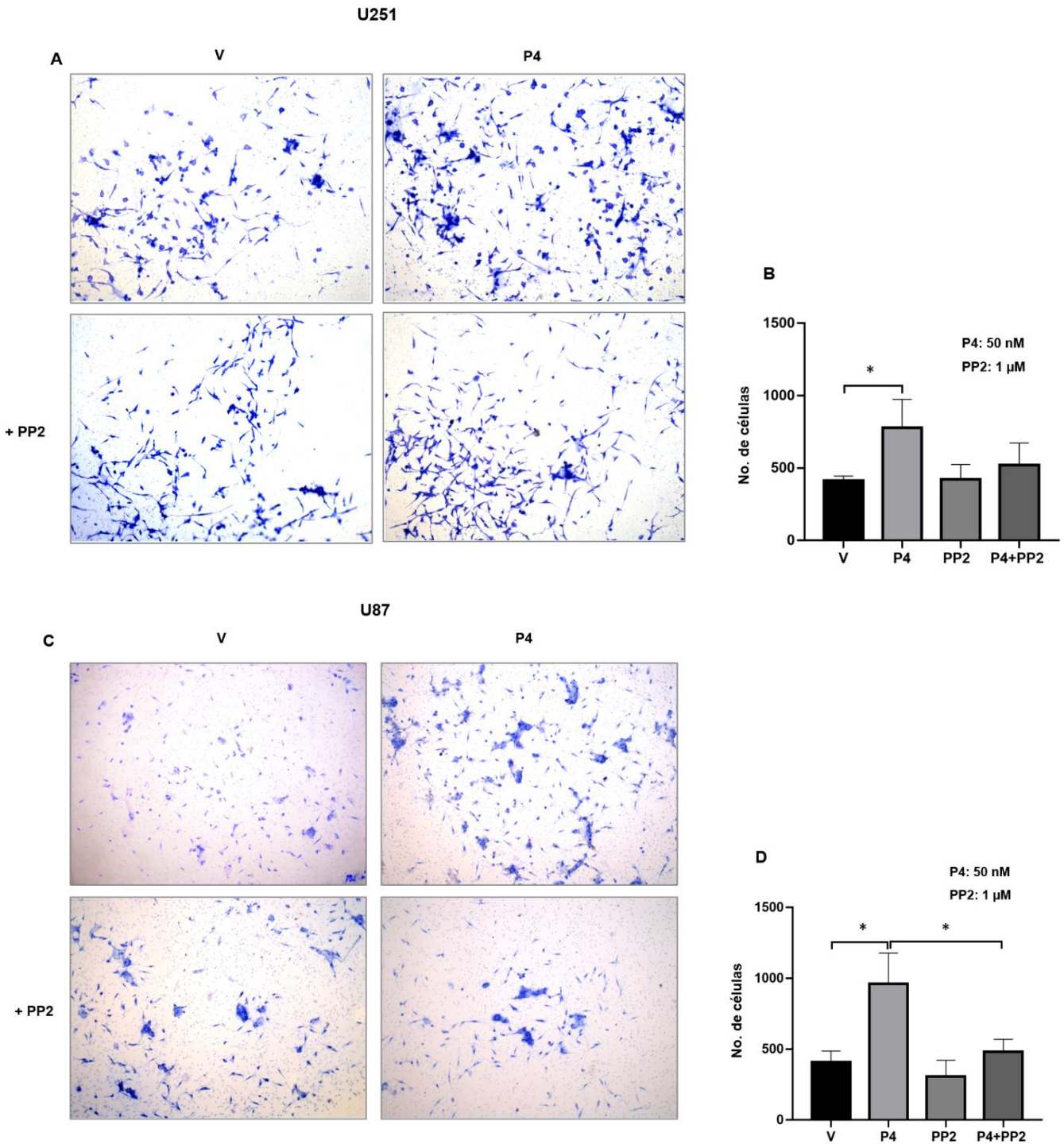

Figura 17. cSRC participa en la invasión celular inducida por la P4 en células de GB humanos. Las células U251 y U87 se trataron con vehículo (V, DMSO 0.01%), P4 (50 nM), PP2 (1µM), o la combinación de P4 (50 nM) + PP2 (1 µM) durante 24 h. Las células que invadieron a través del matrigel se fijaron con paraformaldehído, se tiñeron y se

tomaron fotografías con un aumento de 100X. (**A** y **C**) Imágenes representativas de las células de U251 y U87 que atravesaron el matrigel. (**B** y **D**) Cuantificación del número de células que invadieron a través del matrigel. Los resultados se expresan como la media ± S.E.M. n = 4; *p<0.05.

VIII. Discusión

El GB es el tumor cerebral más agresivo y frecuente en adultos. Los pacientes con GB tienen una supervivencia global de 14 meses (120). Uno de los factores que influye en el mal pronóstico de estos pacientes es la alta capacidad de las células del GB para migrar e invadir el parénquima cerebral que rodea al tumor, lo que a su vez hace extremadamente difícil una resección quirúrgica completa (121). Existen diversas vías de señalización implicadas en los procesos de migración e invasión de los GB; algunas son activadas por la proteína cSRC que pertenece a la familia de las cinasas SRC (SFK) (13). De todos los miembros de la familia (FYN, YES, BLK, YRK, FGR, HCK, LCK Y LYN) cSRC es la que con mayor frecuencia se relaciona con procesos involucrados en la progresión del cáncer (95). Esta cinasa participa en la migración e invasión de múltiples tumores malignos a través de la regulación de la contracción acto-miosina, la polimerización de la actina (122) y la proteólisis de la MEC (123). En los GB, cSRC desempeñan un papel esencial en los acontecimientos relacionados con la motilidad y la alteración de la MEC (13,100,102).

Se ha demostrado que el RP activado por la P4 promueve la migración e invasión de las células de GB (5,6). Sin embargo, no hay información sobre la posible interacción entre el RP y cSRC en el contexto de estos tumores cerebrales. En este trabajo, investigamos en primer lugar la capacidad de la P4 para activar a cSRC a través del RP y cómo esta activación regula la fosforilación/desfosforilación o expresión de proteínas involucradas en la migración e invasión de las células de GB humano.

Las células U251 y U87 fueron tratadas con P4 durante 10 min, y la activación de cSRC se evaluó mediante western blot. El aumento de la tasa p-cSRC (Y416)/cSRC en las células U251 fue significativo a una concentración de 50 nM. El residuo de tirosina Y416 está localizado en el dominio SH1, y su autofosforilación es necesaria para la completa activación de cSRC (43). Cuando las células U87 fueron tratadas con P4, también se observó un aumento significativo en la tasa p-cSRC (Y416)/cSRC. Por lo tanto, la P4 induce la activación de cSRC en las líneas celulares derivadas de GB humanos.

El gen *SRC* fue uno de los primeros protooncogenes descubiertos y hasta el momento de los más estudiados (124); no obstante, su papel en la progresión del cáncer no se conoce con exactitud. En un principio, esta cinasa fue relacionada principalmente con los

eventos de proliferación (125). Sin embargo, las investigaciones más recientes han reportado, que en neoplasias de alto grado de malignidad, cSRC no participa en eventos de proliferación sino que regula procesos como la adhesión, la invasión y la motilidad (43,126). En el cáncer colorrectal, el aumento de la actividad de cSRC, en lugar de su sobreexpresión, se asocia con la metástasis (96,123). Algunos autores consideran que cSRC induce la proliferación celular en las primeras etapas del desarrollo del cáncer, pero regula los procesos de migración e invasión en el caso de tumores completamente diferenciados (94). Teniendo en cuenta la evidencia que existe sobre el papel de cSRC en la regulación de los procesos de migración e invasión de las células de GB, la activación de cSRC por la P4 en el contexto de este trabajo, podría estar involucrada en la inducción de los eventos previamente mencionados.

La cinasa de tipo no receptor FAK, fue uno de los primeros sustratos de cSRC identificados. La actividad de FAK está estrechamente relacionada con la regulación del recambio de las estructuras celulares conocidas como adhesiones focales (127). En los complejos de adhesión focal, cSRC induce la fosforilación de FAK y facilita el ensamblaje y la liberación de las proteínas que componen estas estructuras, evento que es esencial para la migración celular. El complejo FAK-cSRC también fosforila a Pax, que recluta otros componentes a los sitios de adhesión focal (43). Hecker y Gladson encontraron que la expresión de FAK en muestras de biopsias de astrocitoma anaplásico y GB fue más elevada en comparación con la de muestras de biopsia cerebral no neoplásica (128). En este trabajo, evaluamos la capacidad de la P4 para activar a FAK y Pax. La P4 promovió el aumento de la tasa p-FAK (Y397)/FAK y p-FAK (Y576/577)/FAK que corresponde al sitio de autofosforilación y a los residuos de tirosina directamente fosforilados por cSRC, respectivamente. Para determinar si la P4 induce la fosforilación de FAK a través de cSRC, se transfectaron células U251 con un siRNA comercial contra la expresión de cSRC o con un siRNA control (secuencia aleatoria que no reconoce un blanco particular) y se trataron con P4 en las mismas condiciones de los experimentos anteriores. En este caso, la P4 no logró inducir la activación de FAK en las células transfectadas con el siRNA contra cSRC. Además, en el ensayo de migración realizado por la metodología del cierre de la herida, a las 6 y 12 h de tratamiento con la P4, las células que fueron transfectadas con el siRNA contra de cSRC cubrieron una menor área

de la herida con respecto a las que se transfectaron con el siRNA control. Este resultado demuestra la participación de cSRC en la activación de FAK y la capacidad de migración de células de GB tratadas con P4.

Pax es una proteína multifuncional que desempeña un papel de andamiaje en las adhesiones focales. La sobreexpresión de esta proteína se ha asociado a astrocitomas de alto grado y a una disminución de la supervivencia (50). Tras la activación de las integrinas, Pax se fosforila principalmente en los residuos de tirosina, Y31 e Y118, pero este estado de fosforilación es dinámico y pasa por eventos de fosforilación-desfosforilación. Zaidel-Bar y colaboradores encontraron que, en su estado fosforilado, Pax se asocia a los complejos y a las adhesiones focales, mientras que en su estado no fosforilado se encuentra en las adhesiones fibrilares. Estos autores propusieron un modelo en el que Pax se fosforila inicialmente y se recluta hacia las adhesiones o complejos focales. La velocidad de este reclutamiento está regulada por la presencia de ambos estados. Finalmente, bajo la fuerza mecánica ejercida por la contracción acto-miosina, Pax se desfosforila a un ritmo elevado y la fosforilación se restablece a una baja velocidad (129). Este resultado sugiere que la P4 podría inducir el reclutamiento de Pax hacia las adhesiones fibrilares y además contribuir con el estado dinámico de fosforilación/desfosforilación, que es imprescindible para el desplazamiento de las células sobre la MEC. Cuando las células U251 se transfectaron con el siRNA contra la expresión de cSRC, la reducción de la tasa p-Pax (Y118)/Pax fue más evidente, lo que está en consonancia con el papel de cSRC en la fosforilación de Pax. Una de las proteínas estrechamente relacionada con la desfosforilación de Pax es la tirosina fosfatasa PTP-PEST. Shen y colaboradores informaron que PTP-PEST co-immunoprecipita con FAK y Pax en células de embrión de pollo (113). Estos autores también demostraron que la expresión de PTP-PEST disminuye la fosforilación de Pax (114). En células de GB la PTP-PEST regula los eventos de invasión mediante la ubiquitinación, dependiente de fosforilación, de proteínas focales esenciales como Cas, FAK, Pax y cSRC (115). El análisis bioinformático, que se realizó como parte de este proyecto, reveló que la expresión del ARNm de PXN (Pax) y PTPN12 (PTP-PEST) es mayor en los astrocitomas (grados II, III y IV o GB) en comparación con tejido de cerebro normal y además mostraron la mayor expresión en el subtipo mesenquimal (subtipo

molecular de GB más agresivo y de peor pronóstico) (24) entre los cuatros subtipos moleculares definidos por Verhaak y colaboradores (22). Este resultado en conjunto con los antecedentes previamente expuestos, sugieren que el efecto de la P4 sobre Pax en el contexto de este trabajo, puede estar mediado por PTP-PEST. El análisis de la correlación de la expresión génica reveló un valor de 0.61 (correlación positiva significativa). Este resultado refuerza la posibilidad de que ambas proteínas (Pax y PTP-PEST) participan juntas en la regulación de procesos involucrados en la migración de los GB y Pax sea un sustrato para la actividad fosfatasa de PTP-PEST.

En 2013, Matías-Sánchez y colaboradores encontraron que el RP, estimulado por la P4 o la progestina sintética acetato de medroxiprogesterona, tiene un papel esencial en la polimerización de los filamentos de actina, la ramificación y la formación de complejos de adhesión focal en las neuronas corticales. El mecanismo molecular propuesto por estos autores implica la activación de FAK, y de otras proteínas relacionadas con la migración, como las de la familia WAVE y la moesina. La fosforilación de esta última fue promovida por el RP a través de Ras y Rho-cinasa 2. Por lo tanto, no debemos subestimar el papel de estas últimas proteínas en los efectos de la P4 (130).

Los efectos inducidos por la P4 sobre la activación de cSRC también podrían estar mediados por los receptores membranales a P4 (mPRs), que pertenecen a la familia de los receptores adipoQ acoplados a proteína G. Se han identificado cinco subtipos de mPRs (mPRα, mPRβ, mPRδ, mPRε, y mPRγ) y de estos, la expresión de los cuatro primeros se ha verificado en células de GB humanos (63,64). Es importante destacar que la activación del mPRα por el agonista ORG 02-0, induce proliferación, migración e invasión a través de la activación de cSRC y Akt en células de GB humanos (65). Considerando esta posibilidad, se usó un agonista del RP (R5020) para tratar a las células U251 y U87, y en ambos casos, se observó un aumento de la tasa p-cSRC (Y416)/cSRC. Teniendo en cuenta la alta afinidad del R5020 por el RP y que esta progestina no se transforma en los metabolitos activos de la P4 (53), tiene sentido pensar que, en las condiciones de esta investigación, la activación de cSRC por la P4 ocurrió a través del RP y no por otro receptor o metabolito. Para determinar si la activación de cSRC por la P4 fue inducida por acción del RP, las células U251 se transfectaron con un siRNA contra la expresión del RP o con un siRNA control y se trataron con 50 nM de P4

durante 10 min. En esta ocasión, la P4 no indujo la activación de cSRC en las células transfectadas con el siRNA contra el RP. Por lo tanto, el efecto de la P4 sobre la activación de cSRC está mediado por el RP en estas células de GB humanos. Aunque el RP es ampliamente conocido por su papel como factor de transcripción (131,132), en las dos últimas décadas la atención se ha centrado en las acciones que puede ejercer fuera del núcleo (133). Algunas de las acciones no genómicas del RP se deben a un motivo de poliprolina (aminoácidos 421-428) que puede interactuar con el dominio SH3 de varias proteínas, incluida cSRC. Una vez que se produce esta interacción, se eliminan las uniones intramoleculares que mantienen a cSRC en una configuración cerrada, y el sitio de autofosforilación queda expuesto (10). Este mecanismo se ha estudiado ampliamente en el cáncer de mama. Se ha demostrado que en líneas celulares derivadas de cáncer de mama, la P4 promueve la interacción entre el RP y cSRC lo que a su vez conduce a un incremento de la proliferación (103), la migración y la invasión (70). Para determinar si existe una interacción entre el RP y cSRC, en células derivadas de GB humanos, se llevaron a cabo los ensayos de colocalización por inmunofluorescencia y co-inmunoprecipitación. En el primero se observó la colocalización de las proteínas cSRC y RP en la región nuclear y perinuclear de las células U251 y en el caso de la co-inmunoprecipitación, SRC precipitó con ambas isoformas del RP (isoformas A y B) y este efecto se potenció a los 5 min de tratamiento con P4. Este resultado sugiere que la activación de cSRC por la P4 se debe al cambio conformacional que experimenta cSRC al interactuar con el motivo de poliprolina del RP. Sin embargo, este resultado debe interpretarse con reserva, ya que algunos autores han reportado que, en líneas celulares de cáncer de mama, la activación de cSRC a través del RP depende del RE, cuyo subtipo REα desempeña un papel fundamental en la progresión del cáncer de mama ya que a través de la activación de la vía cSRC/Erk induce el incremento de la proliferación celular. Tanto los estrógenos como las progestinas pueden ejercer este efecto; sin embargo, de acuerdo con los estudios realizados por Migliaccio y colaboradores y Ballaré y colaboradores, en el caso de la P4 la activación de cSRC requiere de un complejo que incluya a REα, RP y cSRC (9,134). Boonyaratanakornkit y colaboradores, por el contrario, apoyan la idea de la autosuficiencia del RP para inducir la activación de cSRC. Estos autores encontraron que en células de cáncer de mama negativas para la

expresión del RE, la P4 promovió la activación de cSRC a través del RP (10). En los GB, el estradiol induce el crecimiento celular, la migración, la invasión y la transición epitelio mesénquima (EMT) a través de la activación del REα (135,136). Por lo tanto, no podemos descartar la idea del papel del REα en la interacción RP-cSRC.

La fosforilación del RP es una modificación postraduccional ampliamente estudiada. Entre otras funciones, está directamente relacionada con regular la actividad transcripcional de este receptor y su degradación por el proteosoma. La fosforilación de los residuos de serina y treonina del RP ha sido frecuentemente reportada (137,138); sin embargo, no hay informes sobre las cinasas que participan en la fosforilación de los residuos de tirosina ni de la repercusión que este evento puede tener para la función del receptor. Por lo tanto, se realizó un análisis *in silico* en tres bases de datos para buscar sitios de fosforilación putativos de cSRC sobre el RP. Las bases de datos NetPhos 3.1, KinasePhos y GPS 5.0. reportaron al residuo de tirosina en la posición 87 con la probabilidad más alta de ser fosforilado por cSRC. Aun cuando son necesarios una serie de experimentos *in vitro* e *in vivo* para demostrar esta predicción, podemos especular sobre el posible papel de esta modificación postraduccional tomando como referencia estudios que se han publicado en este contexto, pero con el RE. La fosforilación del RE por las proteínas SFKs se ha estudiado en líneas celulares de cáncer de mama y su inhibición altera la estabilidad, actividad transcripcional y unión al ligando de este receptor. Entonces, la interacción del RP y cSRC en las células de GB podría ser bilateral e implicar las acciones genómicas y no genómicas de la P4 (139).

La colonización de áreas del cerebro alejadas del sitio de inicio del tumor, además de la habilidad de desplazarse sobre la MEC, requiere de la degradación de esta última. Las endopeptidasas dependientes de zinc de la familia de las metaloproteinasas de matriz son un grupo de proteínas imprescindibles para esta función. Específicamente las MMP-2 y -9 han resultado de gran interés en el contexto de los GB. El incremento en la expresión de estas dos endopeptidasas correlaciona con un mal pronóstico para la recurrencia de los GB (118). En este trabajo, se observó que el tratamiento con 50 nM de P4 durante 6 h aumenta la expresión de la MMP-9 en las células U251 y U87, sin embargo, en la expresión de la MMP-2 no hubo cambios estadísticamente significativos. Cuando ambas líneas celulares se transfectaron con un siRNA contra la expresión de

cSRC, el tratamiento con P4 tuvo un efecto significativamente menor, sobre la expresión de la MMP-9 que en aquellas que se transfectaron con el siRNA control. En la línea celular de cáncer de mama MCF-7, la inhibición de la actividad de la cinasas cSRC y FAK bloqueó la secreción de la MMP-9 (119). Gautam y colaboradores reportaron que en la línea celular de cáncer de mama triple negativa, MDA-MB-231, la inhibición de la actividad de cSRC disminuye la expresión de MMP-9 y la invasión celular (140). De manera muy interesante, en células de fibroblastos, una forma mutada de cSRC (v-SRC) y FAK, participan en la disminución de la actividad y expresión de la MMP-9 pero no de la MMP-2 (127). Teniendo en cuenta estos antecedentes y lo que se ha descrito en la sección de resultados del presente trabajo, es posible que en el contexto de los GB la activación de cSRC por la P4 esté principalmente relacionada con la regulación de la MMP-9 pero no de la MMP-2. Por lo tanto, la disminución en la capacidad invasiva de las células de glioblastoma tratadas con P4 y PP2, inhibidor de cSRC, con respecto a las que solo recibieron P4, podría estar relacionada con el efecto de la P4 sobre la activación de cSRC y el papel de esta cinasa en el incremento de la expresión de la MMP-9.

IX. Conclusiones

1. La P4 induce la activación de la proteína cSRC en células derivadas de GB humanos.
2. El RP participa en la activación de la proteína cSRC en las células de GB.
3. La activación de cSRC mediada por el RP ocurre a través de la interacción entre ambas proteínas.
4. La activación de cSRC por la P4 regula la fosforilación de las proteínas FAK y Pax en células de GB humano.
5. cSRC participa en el incremento en la expresión de la MMP-9 en células de GB tratadas con P4.
6. La migración e invasión promovidas por la P4 en células de GB humano está mediada, en parte, por la activación de cSRC.
7. **La activación de cSRC a través de su interacción con el RP está involucrada en la inducción de los procesos de migración e invasión de células de GB tratadas con P4.**

X. Perspectivas

1. Considerando el potencial papel (análisis *in silico* Tabla 1) de cSRC sobre la fosforilación del RP en el residuo Y87, resultaría de gran interés comprobar este efecto en experimentos *in vitro*.
2. Teniendo en cuenta la interacción RP-cSRC y que los efectos genómicos de este receptor se modifican por su fosforilación, evaluar el efecto de cSRC sobre la actividad transcripcional del RP, aportaría valiosa información sobre los efectos de ambas proteínas en la progresión de los GB.
3. Se ha comprobado que el RE participa en la progresión de los GB, y en el contexto del cáncer de mama, además estabiliza la unión RP-cSRC. Por esta razón sería de gran interés evaluar el papel del RE en la interacción entre RP y cSRC en los GB.
4. Comprobar el papel de la fosfatasa PTP-PEST en la desfosforilación de la Pax y sobre la capacidad de migración de células de GB, teniendo en cuenta que en los análisis de expresión de mRNA (**Figura 13A**) ambas proteínas están sobreexpresadas en los GB con respecto al tejido normal y en el subtipo molecular mesenquimal, que es el más agresivo y de peor pronóstico en los GB.
5. Determinar el efecto de la P4 a través del RP en la activación de cSRC en células troncales derivadas de glioma, ya que esta población celular juega un papel fundamental en el mal pronóstico y recurrencia de los GB.

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XII. Anexos

12.1 Artículos en revistas indizadas

12.1.1 Artículos derivados del proyecto de Doctorado

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 26 March 2021
doi: 10.3389/fendo.2021.640298

Intracellular Progesterone Receptor and cSrc Protein Working Together to Regulate the Activity of Proteins Involved in Migration and Invasion of Human Glioblastoma Cells

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Edited by:
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Specialty section:
This article was submitted to Cancer Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *11 December 2020*
Accepted: *08 March 2021*
Published: *26 March 2021*

Citation:
Bello-Alvarez C, Moral-Morales AD, González-Arenas A and Camacho-Arroyo I (2021) Intracellular Progesterone Receptor and cSrc Protein Working Together to Regulate the Activity of Proteins Involved in Migration and Invasion of Human Glioblastoma Cells. Front. Endocrinol. 12:640298. doi: 10.3389/fendo.2021.640298

Glioblastomas are the most common and aggressive primary brain tumors in adults, and patients with glioblastoma have a median survival of 15 months. Some alternative therapies, such as Src family kinase inhibitors, have failed presumably because other signaling pathways compensate for their effects. In the last ten years, it has been proven that sex hormones such as progesterone (P4) can induce growth, migration, and invasion of glioblastoma cells through its intracellular progesterone receptor (PR), which is mostly known for its role as a transcription factor, but it can also induce non-genomic actions. These non-classic actions are, in part, a consequence of its interaction with cSrc, which plays a significant role in the progression of glioblastomas. We studied the relation between PR and cSrc, and its effects in human glioblastoma cells. Our results showed that P4 and R5020 (specific PR agonist) activated cSrc protein since both progestins increased the p-cSrc (Y416)/cSrc ratio in U251 and U87 human glioblastoma derived cell lines. When siRNA against the PR gene was used, the activation of cSrc by P4 was abolished. The co-immunoprecipitation assay showed that cSrc and PR interact in U251 cells. P4 treatment also promoted the increase in the p-Fak (Y397) (Y576/577)/Fak and the decrease in p-Paxillin (Y118)/Paxillin ratio, which are significant components of the focal adhesion complex and essential for migration and invasion processes. A siRNA against cSrc gene blocked the increase in the p-Fak (Y576/Y577)/Fak ratio and the migration induced by P4, but not the decrease in p-Paxillin (Y118)/Paxillin ratio. We analyzed the potential role of cSrc over PR phosphorylation in three databases, and one putative tyrosine residue in the amino acid 87 of PR was found. Our results showed that P4 induces the activation of cSrc protein through its PR. The latter and cSrc could interact in a bidirectional mode for regulating the activity of proteins involved in migration and invasion of glioblastomas.

Keywords: glioblastoma, progesterone receptor, cSrc, non-genomic actions, focal adhesion kinase, paxillin

INTRODUCTION

Astrocytomas are the most common primary brain tumors in the central nervous system (CNS). The WHO classifies these tumors according to the degree of malignancy in a range from I to IV. Grade IV represents the most malignant astrocytoma, also known as glioblastoma (1). Patients with glioblastoma have an overall survival of 15 months, even when receiving the standard therapy consisting of the maximum bearable surgical removal followed by radiotherapy and chemotherapy with temozolomide (2). The current standard treatment for glioblastoma has remained unchanged for more than ten years (3). Some alternatives, such as the use of angiogenesis blockers or Src family kinase inhibitors, have been tested in clinical assays, but none of them with successful outcomes (4, 5) The poor prognosis of patients with glioblastoma is a consequence of the high rate of recurrence of these tumors promoted by the inherently radio-resistance and chemo-resistance and the high rate of migration and tumor invasion cells (6). Glioblastoma cells can spread to the surrounding brain parenchyma, which makes extremely difficult the complete resection of the tumor, and finally provokes the recurrence of glioblastoma (7).

Migration and invasion of tumor cells to the normal brain are complex processes that involve multiple steps and molecular signaling. In this context, the focal adhesion complex has a significant role. Some of their structural and regulator components, including non-receptor cytoplasmic tyrosine kinase cSrc, Focal adhesion kinase (Fak), Paxillin (Pax), Tyrosine-protein phosphatase non-receptor type 12 (PTP-PEST), and integrins have been associated with the spread of glioblastoma cells (8–11). The tyrosine kinase Fak acts as a regulator and scaffold protein since it can recruit cSrc and Pax to the specific sites in the focal adhesion complex. In turn, cSrc can phosphorylate other proteins, including Fak and Pax to form an active complex able to mediate the cell-extracellular matrix (ECM) adhesion, protrusion of cytoplasm to form the leading edge, cell contraction, recruitment of proteases, and detachment of the trailing edge (12). The role of PTP-PEST in glioblastomas has been associated with the stability of focal adhesion substrates (Fak, Pax, among others) by the regulation of their phosphorylation-dependent ubiquitination (11).

Lewis-Tuffin and colleagues demonstrated that some Src Family Kinase members, such as cSrc, Fyn, Yes, and Lyn, have an essential role in the motility of glioblastoma cells since the knockdown of these kinases reduces the rate of migration in three different cell lines (8). Some stem cell markers, such as Oct-3/4 have been related to increased migration and invasion of glioma stem cells through cSrc and Fak upregulation (13).

As a result of the higher prevalence of glioblastoma in men than women (14), sex hormones and their receptors have gained particular attention. Several studies have demonstrated a central role of progesterone (P4) in the promotion of proliferation (15, 16), migration, and invasion (17) of glioblastoma cells. One of the proteins with a great affinity for P4 is the progesterone receptor (PR), which belongs to the nuclear receptor family, and acts as a ligand-inducible transcription factor (18). When oligonucleotide antisense against PR or RU486, an antagonist of PR, was administered, the effect of P4 over migration and invasion on human glioblastoma cells was significantly diminished (17). These results suggest that PR has a significant role in promoting the progression of glioblastomas. In some breast cancer cell lines, it has been proven that P4 activates cSrc through PR, and in turn, increases migration and invasion rate (19). However, the role of PR in cSrc activation and their participation in the migration and invasion of glioblastoma cells is unknown. In this work, we studied the interplay between PR and cSrc, and its effects on the activity of proteins involved in migration and invasion of glioblastoma cells. To study the potential relationship between these proteins, glioblastoma-derived cell lines were treated with P4 or R5020 (PR agonist), and the phosphorylated/non-phosphorylated ratio of cSrc was measured by western blot. P4 and R5020 increased cSrc phosphorylation. To confirm the participation of PR in the cSrc phosphorylation, cells were transfected with a commercial siRNA against PR. Cells transfected with the PR siRNA were unable to increase cSrc phosphorylation. To investigate the physical interaction between PR and cSrc, we performed a co-immunoprecipitation assay, and interaction between PR and cSrc was observed. *In silico* analysis showed that cSrc could participate in the phosphorylation of PR in the amino acid 87. The role of cSrc activation by P4 in the switch Fak-phosphofak and Pax-phosphopax ratios and the migratory capacity of glioblastoma cells was determined by western blot and wound-healing assay in cells transfected with a commercial siRNA against cSrc. Fak phosphorylation and migration decreased in cells transfected with siRNA against cSrc compared to cells treated with control siRNA. Findings of this work suggest for the first time that cSrc and PR interact in glioblastoma cells. P4 through PR induces cSrc activation, which in turn participates in regulating the activity of proteins involved in the migration and invasion of glioblastomas.

MATERIALS AND METHODS

Cell Culture and Treatments

U251 and U87 (ATCC, USA) human glioblastoma derived cell lines were plated in 10 cm dishes and sustained in DMEM medium (*In vitro*, S.A., D.F., MEX), supplemented with 10% fetal bovine serum (FBS), 1 mM pyruvate, 2 mM glutamine, 0.1 mM non-essential amino acids (GIBCO, NY, USA) at 37°C, 5% CO2. The culture medium mentioned above was replaced by DMEM medium (*In vitro*, S.A., CDMX., MEX) without phenol red and free of hormones, supplemented with charcoal-stripped serum FBS (sFBS) (Hyclone, Utah), 24 h before the treatments. Cells were treated with P4 (10, 50 and 250 nM), 10 nM of R5020 (progestin with high affinity for PR (Kd ≈ 2 nM)) (20) or vehicle (DMSO 0.001%). Cell treatments lasted 10 and 20 min to assess cSrc, Fak, and Pax phosphorylation.

Abbreviations: CNS, central nervous system; Fak, Focal adhesion kinase; Pax, paxillin; ECM, extracellular matrix; PTP-PEST, (tyrosine-protein phosphatase non-receptor type 12); TCGA, The Cancer Genome Atlas; P4, progesterone; PR, progesterone receptor; SFK, Src Family Kinase; ER, estrogen receptor, mPRs, membrane progesterone receptors; PAQR, Progestin and AdipoQ Receptor.

Protein Extraction and Western Blotting

Activation of cSrc, Fak, and Pax was determined by measuring protein phosphorylation. Cells were treated with P4 (10, 50, and 250 nM), R5020 (10 nM), or vehicle (DMSO 0.001%), and western blot was used to determine the content of p-cSrc, p-Fak, and p-Pax. After treatments, cells were homogenized in RIPA buffer with a cocktail of protease inhibitors (Sigma Aldrich, St Louis, MO USA, # P8340) and a group of phosphatase inhibitors (NaF, $Na_4P_2O_7$, and Na_3VO_4). Proteins were obtained by centrifugation at 12,500 rpm for 5 min and quantified using the NanoDrop-2000 spectrophotometer (Thermo Scientific, MA, USA). For protein separation, 30 µg were loaded on a polyacrylamide gel at a concentration of 8.5% for cSrc and Pax, and 7.5% for Fak, under denaturing conditions. Proteins were transferred to a nitrocellulose membrane under semi-dry conditions in a transfer (BIO-RAD) for 30 min at 25 V in the case of the 60 kDa (cSrc) and 68 kDa (Pax) proteins and 1 h at 25 V in the case of the 125 kDa protein (Fak). Blocking was performed with 5% bovine serum albumin at 37°C for 2 h. Membranes were incubated with the primary antibodies against the phosphorylated and total forms of the cSrc, Pax, and Fak proteins (phospho Src Tyr-416 Cell Signaling, MA, USA, Ref. 2101; Src Cell Signaling, MA, USA. USA, Ref. 2108; phospho Pax Tyr-118 Cell Signaling, MA, USA, Ref. 2541; Pax Cell Signaling, MA, USA, Ref. 2542; phospho Fak Tyr-397 Cell Signaling, MA, USA, Ref. 3283; Fak Cell Signaling, MA, USA, Ref. 3285). Antibodies against the total and phosphorylated forms were used in a 1/500 dilution. As a loading control, the alpha-tubulin protein was detected at a 1/1,000 dilution (Santa Cruz Biotechnology, St. Louis, TX, USA, Ref. sc-398103). All the antibodies were incubated for 48 h except that against alpha-tubulin, which was incubated for 24 h. Subsequently, the membranes were incubated with the secondary antibody against rabbit (Thermo Scientific, USA, Ref. 1858415) or mouse (Santa Cruz Biotechnology, TX, USA, Ref. sc-516102) (1/10,000) with shaking and at room temperature for 45 min. The primary and secondary antibodies were removed from the membranes with a solution containing Tris–HCl pH 6.8 at 0.06 M, SDS at 2%, and β-mercaptoethanol at 0.7% for 30 min at 50°C at stirring. The chemiluminescent signal was detected by exposing the membranes to the SuperSignal West Fento substrate (Thermo Scientific # 34096) with Kodak Biomax Light Film plates (Sigma-Aldrich, MO, USA).

siRNA Transfection

Commercial siRNA against PR was used to test if P4 induced the cSrc activation through its PR. Briefly, 2.5×10^5 U251 cells were plated in 6-well dishes in DMEM medium supplemented with 10% FBS, and 24 h later, the medium was replaced with DMEM phenol red-free medium without FBS and antibiotics. Cells were transfected with a PR siRNA (100 nM) or with control siRNA that does not induce specific mRNA degradation using Lipofectamine RNAiMAX (Thermo Scientific, USA). The medium was refreshed 12 h after the addition of a PR siRNA or control siRNA, and 48 h after siRNAs addition, the cells were harvested for total RNA extraction to determine the efficiency of the transfection. The same protocol was used with Commercial siRNA against cSrc to test the interplay between P4, cSrc, and Fak and Pax activation. In this case, the efficiency of transfection was determined by western blot, and 48 h after transfection, the cells were harvested for protein extraction as previously described.

RNA Extraction and RT-PCR

RNA extraction was performed using TRIzol reagent (Invitrogen, USA) and following the manufacturer's instructions. One µg of total RNA was used to synthesize the first-strand cDNA in a reaction carried out by M-MLV reverse transcriptase (Thermo Scientific, USA) following the manufacturer's protocol. The efficiency of transfection was determined by RT-PCR from 2 µl of synthesized cDNA. PCR conditions were: 5 min incubation at 94°C followed by 28 cycles of 15 s at 94°C, 30 s at 60°C, and 30 s at 68°C, and a final incubation for 60 s at 68°C. The 18S ribosomal RNA gene was used as an internal expression control. The primers used were PR forward 5′-CCCGCCCTATCTCAACTACC-3′ and reverse 5′-GTTGTGCTGCCCTTCCATTTG-3'). 18S forward 5′-AGTGAAACTGCAATGGCTC-3′ and reverse 5′-TGACCGGGTTGGTTTTGAT-3'.

Migration Assay

The wound-healing assay was performed to determine the cell migration of U251 cells. 2.5×10^5 cells were plated in 6-well slides with DMEM high glucose supplemented until reaching 70% confluence. Then, cells were transfected as was described in the previous section. About 48 h after transfection and in 90% of confluence, a scratch was made using a 200 µl pipette tip. Floating cells were removed with PBS and DMEM medium (*In vitro*, S.A., CDMX., MEX) without phenol red and free of hormones, supplemented with 10% SFB also free of hormones were added again. Cytosine β-D-arabinofuranoside hydrochloride (10 µM, Ara-C, C1768, Sigma-Aldrich, St. Louis, MO, USA) was used to inhibit cell proliferation 1 h before adding the treatments. Cells were treated with P4 (50 nM), or vehicle (DMSO 0.001%). Four random fields were chosen per treatment to determine cell migration after 0, 6, and 12 h of treatment. Photographs were taken with an Infinity 1-2C camera (Lumenera, CA) connected to the inverted microscope Olympus CKX41 (Olympus, JPN).

Co-Immunoprecipitation

Cell cultures were lysed in a buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and a cocktail of protease inhibitors (Sigma Aldrich, St. Louis, MO USA, # P8340) at 4°C overnight. Cell lysates were centrifuged at 12,500 rpm for 15 min. One mg of total protein present in the supernatant was incubated with 2 µg of antibody anti-PR (Santa Cruz Biotechnology Dallas, Texas, USA Ref B-30 sc-811) and 50 µL of sepharose-coupled protein A/G plus-agarose (sc-2003; Santa Cruz Biotechnology) under permanent agitation at 4°C overnight. The next day, samples were centrifuged, and the pellets washed three times with buffer (20 mM Tris HCl; 150 mM NaCl; 1mM EDTA, 0.1 Triton X-100,

and a cocktail of protease inhibitors (Sigma Aldrich, St. Louis, MO USA, # P8340 pH 7.5). Finally, the samples were denatured by boiling in loading buffer (120 mM Tris, pH 6.8; 4% SDS; 0.2% glycerol; 5% β-mercaptoethanol; and 10 mg/ml bromophenol blue) and separated in SDS-PAGE. Western blot for cSrc was done as previously described in the Protein extraction and Western blotting section.

TCGA Data Analysis

RNA-Seq counts from 196 grade II, 223 grade III, and 139 grade IV gliomas were obtained from Glioblastoma and Low-Grade Glioma projects of The Cancer Genome Atlas (TCGA) repository (https://portal.gdc.cancer.gov/). The data were downloaded and processed using TCGAbiolinks package version 2.12.6 for R.17 Additionally, expression profiles of 249 healthy brain cortex samples were obtained from the GTEx database (https://gtexportal.org/home/). Data were normalized by DESeq2 version 1.22.2 and plotted. Gene expression correlation in glioblastoma, from the TCGAbiolinks package for R.

Statistical Analysis

Data were and analyzed using Graph Pad Prism 5 program (GraphPad Software, Inc., USA). A one-way ANOVA with Bonferroni *post hoc* test (**Figures 1A, E, F**, **2C–E**, **3B**) or t-student test were used to establish the statistical differences between comparable groups. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Activation of cSrc by P4 Is Mediated by PR in Glioblastoma Cells

The role of PR and cSrc in breast cancer has been broadly studied. The stimulation of breast cancer cells with P4 activated cSrc through PR, and induced various signaling pathways that conducted to cancer progression (21–23). To test the potential role of P4 in cSrc activation in glioblastoma cells, at the beginning of the study, a time-dependence assay (0–60 min) using P4 (10 nM) was performed in U251 cells (**Supplementary Figure 1**), however, a significant effect on p-cSrc/cSrc ratio was not observed, and we decided to test higher P4 concentrations, at 10 (**Figures 1A, B**) and 15 min (**Supplementary Figure 2**). U251, and U87 cells were treated with three different concentrations of P4 (10, 50 and, 250 nM) for 10 min, and the phosphorylation of cSrc (Y416) was determined by western blot. P4 induced cSrc activation at 50 nM in U251 and U87 cells (**Figures 1A, B**). P4 has affinity for other receptors besides PR (24, 25); Nevertheless, because of the high affinity of R5020 for PR (Kd ≈ 2 nM) (20) over other receptors (AR 1% binding affinity) (26–28), cells were also treated with 10 nM of R5020. As in the case of P4, R5020 increased the p-cSrc (Y416)/cSrc ratio in U251 and U87 cells (**Figures 1C, D**). To finally demonstrate that cSrc activation by P4 was mediated by its intracellular PR, U251 cells were transfected with a commercial siRNA against PR or control siRNA (scramble sequence) and treated with P4 for 10 min at 50 nM. The efficiency of transfection was higher than 50% (**Figure 1E**). P4 induced the activation of cSrc in cells with control siRNA as in the previous experiments. In contrast, the siRNA against PR blocked the increase in p-cSrc (Y416)/cSrc ratio induced by P4 (**Figure 1F**). This result demonstrates the participation of PR in the cSrc activation by P4 (**Figure 4**). Considering the short time (10 min) for the activation of cSrc, this result suggests that PR exerts this effect through nongenomic actions.

cSrc and PR Interact in U251 Cells

Non-genomic actions of PR are associated with the polyproline domain which can interact with SH3 domains of a variety of proteins including cSrc. In breast cancer cell lines, a physical interaction between cSrc and PR has been demonstrated (22). Co-immunoprecipitation assay was performed to evaluate the interaction between PR and cSrc. U251 cells were immunoprecipitated with antibodies against PR, and a western blot was carried out. In both vehicle and P4 treated cells, a band corresponding to cSrc was detected, indicating that PR (isoforms A and B) and cSrc directly or indirectly interact in glioblastoma cells. The treatment with P4 increased the interaction between PR and cSrc (**Figure 1G**). This result suggests that activation of cSrc is possible because of the physical interaction between cSrc and PR in glioblastoma cells (**Figure 4**).

P4 Induces a Switch in Fak-Phosphofak and Pax-Phosphopax Ratios Through cSrc in Glioblastoma Cells

Fak and Pax are two of the most critical components of the focal adhesion complex, fundamental to regulating cell migration and invasion. To test if P4 was able to induce Fak and Pax activation, U251 and U87 cells were treated with P4 at 50 nM for 20 min, and their phosphorylation was determined by western blot. P4 (50 nM) increased the p-Fak/Fak ratio (Y397 and Y576/577) in U251 cells and the p-Fak/Fak ratio (Y576/577) in U87 cells, while in the case of Pax, P4 decreased the p-Pax/Pax ratio (Y118) in U251 cells at 20 min (**Figures 2A, B**). cSrc is one of the major kinases implicated in the phosphorylation of focal adhesion complex components, especially Fak (29). To test the role of cSrc in the phosphorylation of Fak and Pax, U251 cells were transfected with a commercial siRNA against cSrc or control siRNA (scramble sequence) and treated with P4 (50 nM) for 20 min. The efficiency of transfection was 50% (**Figure 2C**). The siRNA against cSrc blocked the increase in the p-Fak/Fak (Y576/577) ratio induced by P4 (**Figure 2D**), but not the decrease in p-Pax/Pax ratio (**Figure 2E**). This result indicates that P4 is involved in regulating focal adhesion complex through PR and cSrc in glioblastoma cells (**Figure 4**).

Silencing of cSrc Reduces the Migration Induced by P4

Previous results suggest that P4 and cSrc have a fundamental role in the migration of glioblastoma cells, which in turn, participate in the recurrence of this tumor (17, 30). To determine whether

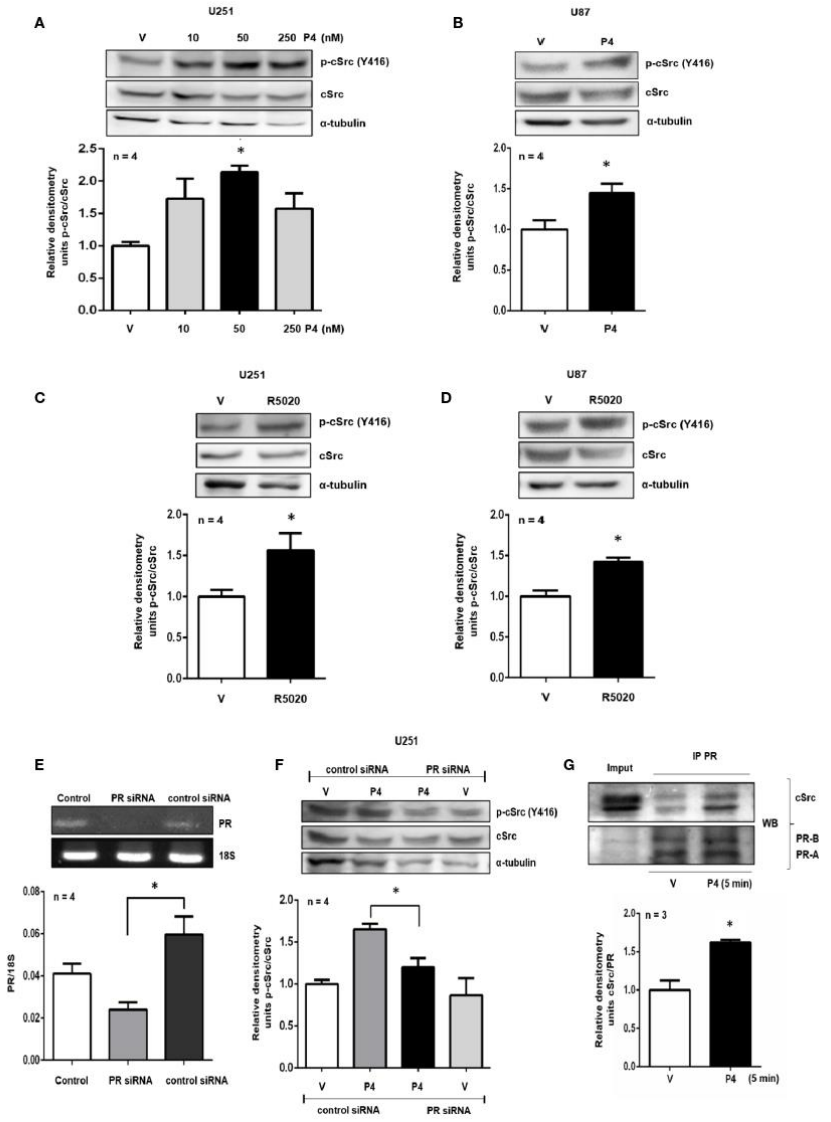

FIGURE 1 | P4 induces the activation of cSrc through PR. **(A, B)** U251 and U87 cells were treated with P4 (10, 50 and 250 nM) and P4 (50 nM) respectively or vehicle (V, DMSO 0.01%) for 10 min. **(C, D)** U251 and U87 cells were treated with R5020 (10 nM) or vehicle (V, DMSO 0.01%) for 10 min. **(E)** U251 cells were transfected with PR siRNA and a control siRNA (an aleatory RNA sequence) (100 nM) or were only treated with lipofectamine (Control). **(F)** Transfected cells with PR siRNA or control siRNA were treated with P4 (50 nM) or vehicle (V, DMSO 0.01%) for 10 min. Upper panels show the representative western blots for p-cSrc, cSrc, and α-tubulin or representative RT-PCR bands for PR and 18S mRNA. Lower panels show the densitometric analysis. **(G)** U251 cells were treated with P4 (50 nM) or vehicle (V, DMSO 0.01%) for 5 min and co-immunoprecipitated with PR. Data were normalized respect to the vehicle or control. Results are expressed as the mean ± S.E.M. **(A–F)** n = 4 **(G)** n = 3; *p < 0.05.

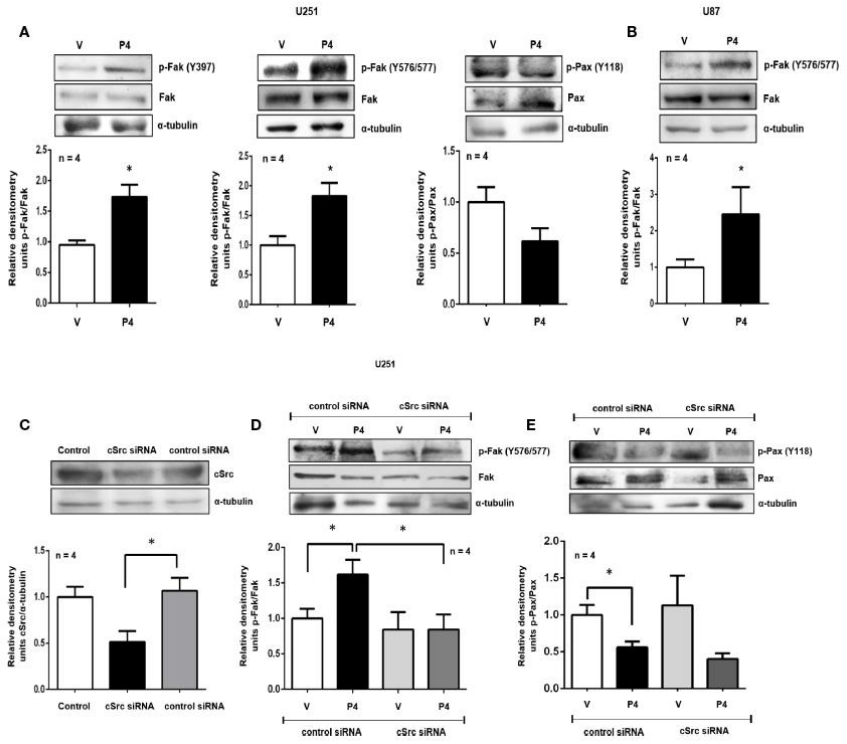

FIGURE 2 | P4 induces the activation of Fak and Pax through cSrc in glioblastoma cells. **(A, B)** U251 and U87 cells were treated with P4 (50 nM) or vehicle (V, DMSO 0.01%) for 20 min. **(C)** U251 cells were transfected with cSrc siRNA and a control siRNA (an aleatory RNA sequence) (100 nM) or were only treated with lipofectamine (Control). **(D, E)** Transfected cells with cSrc siRNA or control siRNA were treated with P4 (50 nM) or vehicle (V, DMSO 0.01%) for 20 min. Upper panels show the representative western blots for, cSrc, p-Fak, Fak, p-Pax, Pax, and α-tubulin. Lower panels show the densitometric analysis. Data were normalized respect to the vehicle or control. Results are expressed as the mean ± S.E.M. n = 4; *p < 0.05.

silencing of cSrc modify the migration induced by P4 in U251 cells, a scratch-wound assay was performed. In cells transfected with control siRNA and treated with P4, a slight increase in migration was observed as compared to vehicle at 6 and 12 h after treatment. This increase was inhibited in cells transfected with cSrc siRNA (**Figures 3A, B**). This result demonstrates that the migration of glioblastoma cells induced by P4 is related to the activation of cSrc and reinforces the previous molecular findings.

cSrc Has Several Putative Phosphorylation Sites Over PR

Phosphorylation of nuclear receptors, including PR, has great relevance in functions executed by these proteins. PR phosphorylation has been broadly studied in serine residues; however, there is scarce information about tyrosine residues. The potential role of active cSrc over PR phosphorylation was determined using three different databases (NetPhos 3.1, KinasePhos, and GPS 5.0). We found the same putative tyrosine residue in the amino acid 87 of PR in all of them. In the GPS 5.0 database, this residue presented the highest score, which means that it also has the highest potential for phosphorylation (**Table 1**). Even when this result must be confirmed in experimental assays, the information obtained by the databases opens the possibility of future investigation of functions and regulation of PR by cSrc phosphorylation (**Figure 4**).

PXN (Pax) and PTPN12 (PTP-PEST) Expression Depends on Tumor Grade and Glioblastoma Subtype

PXN and PTPN12 expression data from 196 grade II, 223 grade III, and 139 grade IV (glioblastoma) astrocytomas were obtained from TCGA and compared to 249 healthy human brain cortex samples from the GTEx database. The expression of these genes was also compared among the four subtypes of glioblastomas defined by Verhaak and colleagues (31). The PXN and PTPN12 mRNA expression increased in glioblastomas compared to normal brain,

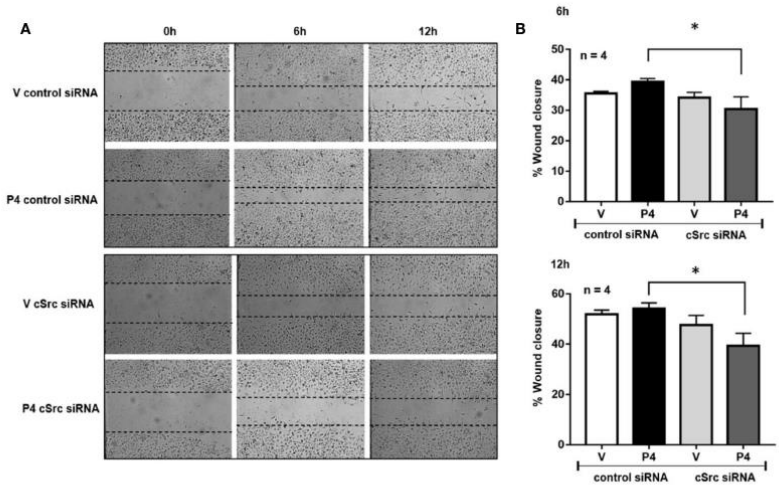

FIGURE 3 | cSrc participates in cell migration induced by P4. U251 cells transfected with cSrc siRNA and a control siRNA (an aleatory RNA sequence) (100 nM) were treated with P4 (50 nM) or vehicle (V, DMSO 0.01%) in cells. Photographs from the scratch area were taken at 0, 6 and 12 h and were captured with 400× magnification. **(A)** Representative image of the scratch area. **(B)** Graph of the wound closure (%), determined in the scratch area. Results are expressed as the mean ± S.E.M. n = 4; *p < 0.05.

and in the case of PTPN12, the expression was higher in glioblastomas compared to astrocytomas grades II and III (**Supplementary Figure 3A**). The analysis of expression among the four subtypes of glioblastomas showed that PXN and PTPN12 have the highest levels of expression in the mesenchymal subtype (the most aggressive glioblastoma subtype) (32) (**Supplementary Figure 3A**). The analysis of gene expression correlation between PXN and PTPN12 revealed a value of 0.61 (significant positive correlation) (**Supplementary Figure 3B**).

DISCUSSION

Glioblastoma is the most malignant brain tumor. Patients with glioblastoma have an overall survival of 14 months (1). One of the main influencing factors in the poor prognosis of these patients is the high capacity of glioblastoma cells to migrate and invade the brain parenchyma surrounding the tumor, which in turn makes extremely difficult a complete surgical resection (33). Several molecular signals are implicated in the processes of migration and invasion in glioblastoma; some are activated by cSrc kinase protein that belongs to the Src Family Kinase (SFK) (8, 34). Of all of the other family members (FYN, YES, BLK, YRK, FGR, HCK, LCK, and LYN) cSrc is the most often associated with cancer progression (35). This kinase has been associated with migration and invasion of multiple malignancies through the regulation of actomyosin contraction, actin polymerization (36), and ECM proteolysis (37). In glioblastomas, SFKs play an essential role in events related to motility and disruption of ECM.

It has been demonstrated that PR activated by P4 promotes the migration and invasion of glioblastoma cells (17). However, there is no information about the possible interplay between PR and cSrc in glioblastoma cells. In this work, we first investigated the capacity of P4 to activate cSrc through its PR and how this activation regulates the phosphorylation/dephosphorylation of kinases related to migration and invasion of glioblastoma cells. U251 and U87 cells were treated with P4 for 10 min, and the activation of cSrc was evaluated by western blot. The most effective concentration of P4 was 50 nM. The increase in the p-cSrc (Y416)/cSrc ratio in U251 cells was evident. Y416 is the amino acid residue localized in the domain SH1, which contains the autophosphorylation site required for the full cSrc activation (38). When U87 cells were treated with P4, a significant increase in the p-cSrc (Y416)/cSrc ratio was observed. Therefore, P4 induces the activation of cSrc in human glioblastoma derived cell lines.

In colorectal cancer, the increasing activity of cSrc rather than its overexpression is associated with metastasis (39, 40). cSrc is one of the first and most studied proto-oncogenes (41); however, its role in cancer progression is not entirely understood. The central role attributed to this kinase was increasing cellular proliferation (42). However, most recent investigations have found that cSrc regulates processes such as adhesion, invasion, and motility (38, 43). For example, the overexpression of cSrc in colon cancer does not induce the proliferation rate increase, but it facilitates the spread of cells (37). Some colleagues consider that cSrc induces cellular proliferation at the first stage of cancer development but regulates migration and invasion processes at the later stages (29). It has been demonstrated an essential role of

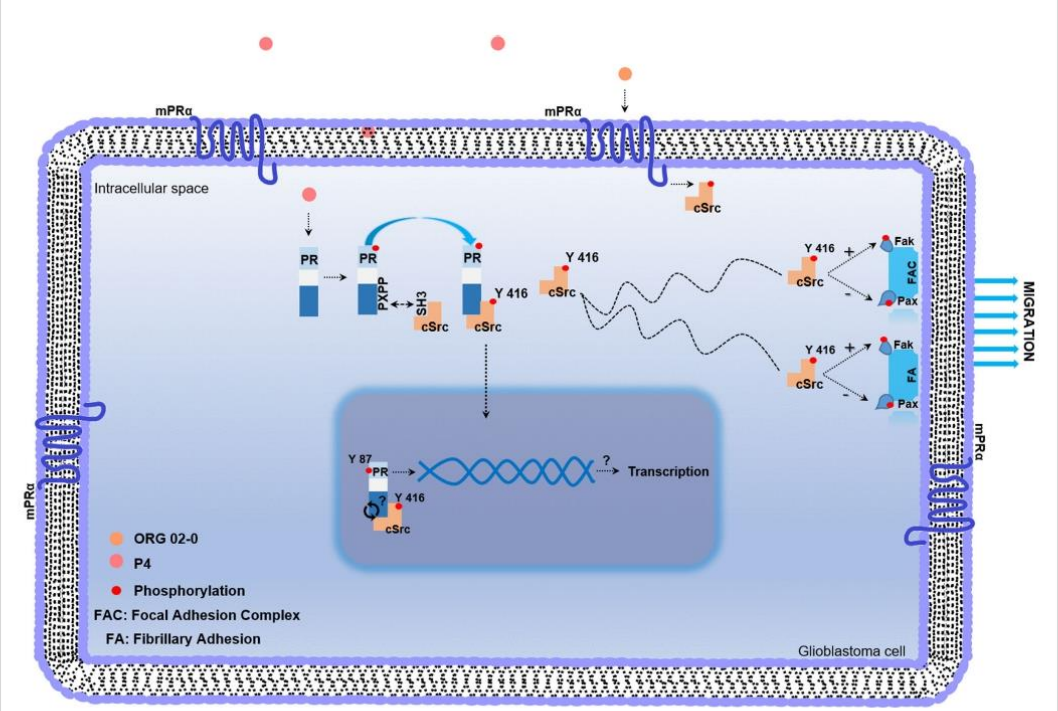

FIGURE 4 | Working model of non-genomic PR mechanism of action in glioblastoma cell. Once P4 enters the cell, it induces phosphorylation of PR in serine and threonine residues. After activation, PR recruits cSrc through the interaction between the polyproline (PXPP) and the SH3 domains of PR and cSrc, respectively. This interaction induces a conformational change in cSrc, which exposes its catalytic domain, and promotes the autophosphorylation of cSrc. Once activated, cSrc participates in regulating the phosphorylation of focal adhesion and fibrillary adhesion components such as Fak and Pax, which finally lead to the migration process. mPRs are expressed in glioblastoma cells and the agonist of mPRα (ORG 02-0) induces the activation of cSrc. The putative role of cSrc in the tyrosine 87 phosphorylation of PR could be involved in the transcriptional activity of PR.

TABLE 1 | *In silico* analysis of putative phosphorylation sites of cSrc over PR.

	ID	Position	AA	Kinase	Score	E-value	Peptide
Netphos 3.1	Progesterone Receptor Homo sapiens AAA60081.1	87	Y	SRC	0.516		VEGAYSRAE
				EGFR	0.444		
KinasePhos		87	Y	SRC		13	VEGAYSRAE
GPS 5.0		87	Y	TK/SRC/SRCA/YES1	27.217		LSDVEGAYSRAEATR

Three different databases to predict phosphorylation sites were used to analyze the potential role of cSrc in PR phosphorylation. One putative tyrosine residue in the amino acid 87of PR was found in three databases: NetPhos 3.1, KinasePhos, and GPS 5.0.

SFKs in the motility of glioblastoma cells (30, 44). The activation of cSrc by P4 may be involved in the regulation of events associated with the migration and invasion of glioblastoma cells.

One of the first identified substrates of cSrc was Fak, a non-receptor tyrosine kinase closely related to regulating a variety of cellular processes, including cell migration (45). At the focal adhesion complexes, cSrc induces Fak's phosphorylation and facilitates the turnover of these junctions, an essential step to cell migration. The complex Fak-cSrc can also phosphorylate Pax, which recruits other components to focal adhesion sites (38). It has been reported elevated levels of Fak expression in anaplastic astrocytoma and glioblastoma tumor biopsy samples compared to normal brain (46). In this work, we evaluated the capacity of P4 to activate Fak and Pax. P4 promoted the increase in the p-Fak(Y397)/Fak and p-Fak(Y576/577)/Fak ratio that corresponds to the autophosphorylation site and to another site directly phosphorylated by cSrc, respectively. Thus, P4 induces Fak's phosphorylation, including in the tyrosine residues directly related to cSrc and with the turnover of focal adhesions (38). To determine if P4 induces the phosphorylation of Fak through cSrc, we

transfected U251 cells with a commercial siRNA against cSrc or with control siRNA and treated them with P4 in the same conditions of the previous experiments. In this case, P4 failed to induce Fak activation in cells transfected with siRNA against cSrc.

Pax is a multifunctional protein that plays a scaffolding role at focal adhesions. Overexpression of this protein has been associated with high-grade astrocytomas as well with a poor survival (10). Upon integrin activation, Pax is mainly phosphorylated at two different tyrosine residues, namely Y31 and Y118, but this phosphorylation state is not permanent. Zaidel-Bar and colleagues found that tyrosine-phosphorylated Pax is associated with focal complex and focal adhesions, while non-phosphorylated Pax is associated with fibrillary adhesions. These colleagues proposed a hypothetical model in which Pax is initially phosphorylated and recruited to integrin adhesions. The rate of this recruitment is regulated by the presence of both phosphopax and Pax. Finally, phosphopax is dephosphorylated at a high rate under mechanical force, and the phosphorylation is reestablished at a low rate (47). In this work, we observed that P4 decreased the p-Pax(Y118)/Pax ratio in U251 cells 20 min after the treatment. This result suggests that P4 should induce Pax recruitment towards the integrins at fibrillary adhesions and contributes to the presence of both phosphorylated and unphosphorylated state, which is necessary to migration processes. When U251 cells were transfected with the siRNA against cSrc, the reduction in p-Pax(Y118)/Pax was more evident, which is in line with the role of cSrc in Pax phosphorylation. One of the proteins closely related to the dephosphorylation of Pax is the tyrosine phosphatase PTP-PEST. Shen and colleagues found that PTP-PEST coimmunoprecipitates with Fak and Pax in chicken embryo cells (48). These colleagues also demonstrated that the expression of PTP-PEST decreases the phosphotyrosine on Pax (49). In glioblastoma, PTP-PEST regulates the invasion events by phosphorylation-dependent ubiquitination of essential focal proteins such as Cas, Fak, Pax, and Src (11). Bioinformatic analysis revealed that PXN and PTPN12 mRNA expression was higher in astrocytomas (Grades II, III, and IV) compared to normal brain and showed the highest expression in the mesenchymal subtype (the most aggressive glioblastoma subtype, associated with bad prognostic) (32). The analysis of gene expression correlation revealed a value of 0.61 (significant positive correlation). These results suggest that these proteins together are implicated in the progression of glioblastomas. In the same line, the scratch-wound assay analysis showed that silencing of cSrc in U251 cells abolished the increase in cell migration induced by P4. Interestingly in 2013, Matias-Sanchez and colleagues found that PR, stimulated by P4 and the synthetic progestin medroxyprogesterone acetate, have an essential role in the actin polymerization, branching, and focal adhesion complex formation in cortical neurons. The molecular mechanism proposed by these colleagues involucrate the activation of Fak, and other proteins related to migration, such as WAVE and moesin. Phosphorylation of the latter was promoted by PR through the Ras homolog gene family, member A and Rho-associated kinase-2. Therefore, we should not underestimate the role of these last proteins in P4 effects (50).

The observed effects induced by P4 could also be mediated by membrane progesterone receptors (mPRs), G protein-coupled receptors that belong to the Progestin and AdipoQ Receptor Family (PAQR). Five subtypes of mPRs (mPRα, mPRβ, mPRδ, mPRϵ, and mPRγ) have been identified, and they are expressed in human glioblastoma cells (51, 52). Importantly, the activation of mPRα by ORG 02-0, a specific mPRα agonist, induces proliferation, migration, and invasion through the activation of cSrc and Akt in human derived glioblastoma cells (24).

P4 can exert its effects through various receptors in glioblastoma cells (24, 25). Therefore, an agonist of PR (R5020) was used to treat the U251 and U87 cells, and in both cases, an increase in p-cSrc (Y416)/cSrc ratio was observed. Considering the high affinity of R5020 for the PR and that this progestin is unable to be transformed into the active metabolites of P4 (53), it makes sense to think that results previously described are a consequence of the action of P4 through its PR and not by another receptor or metabolite. To determine if cSrc activation by P4 was induced through PR, a more specific assay was conducted. U251 cells were transfected with a commercial siRNA against PR or with control siRNA and treated with P4 in the same conditions of the previous experiments. As is shown in **Figure 1F**, P4 failed to induce cSrc activation in cells transfected with siRNA against PR. Thereby, the effect of P4 over cSrc activation is mediated by PR in these human glioblastoma cells.

Even though PR is widely known for its role as a transcription factor (53, 54), in the last two decades the attention has been focused on the actions that it can exert out of the nucleus (55). Non-genomics actions of PR are due to a polyproline domain (amino acids 396–456) that can interact with the SH3 domain of several proteins, including cSrc. Once this interaction occurs, the intramolecular interactions that hold cSrc in a closed configuration are disrupted, and the autophosphorylation site is exposed (22). This mechanism has been broadly studied in breast cancer. It has been demonstrated that in breast cancer cells, P4 can promote the interaction between PR and cSrc and, in turn, inducing proliferation (56), migration, and invasion (19). To test the interaction between PR and cSrc we performed a co-immunoprecipitation assay. This assay shows that PR (isoforms A and B) and cSrc interact in glioblastoma cells and that P4 enhances this interaction. This result suggests that activation of cSrc by P4 is due to a conformational change in cSrc that enables the autocatalytic domain to be exposed. However, this result must be interpreted with care since some colleagues have found that in breast cancer cell lines, the activation of cSrc through the PR is dependent on the estrogen receptor (ER). ERα plays an essential role in breast cancer cells by activating the Src/Erk pathway and increasing cell proliferation. Estrogens or progestins can induce this effect; however, according to studies conducted by Migliaccio and colleagues and Ballaré and colleagues, it is necessary to form a complex including ERα, PR, and Src (21, 23). Boonyaratanakornkit and colleagues, on the contrary, support the idea of PR self-sufficiency to induce cSrc activation without ER. They found that in breast cancer cells no expressing ER, P4 induced the activation of cSrc through PR (22). In glioblastomas, estradiol increased cell growth, migration, invasion, and the epithelial–mesenchymal transition (EMT) through activation of ERα (57, 58); therefore, we cannot dismiss the idea of the role of ERα in the PR-cSrc interaction.

Phosphorylation of PR is a post-translational modification broadly studied. Among other functions, it is directly related to regulating the transcriptional activity of this receptor and the degradation by the proteasome (59, 60). Serine and threonine phosphorylation of PR has been widely investigated (60–62); however, there are no reports about PR tyrosine phosphorylation. Therefore, we performed an *in silico* analysis in three databases to search putative phosphorylation sites of cSrc over PR. NetPhos 3.1, KinasePhos, and GPS 5.0. predicted the putative tyrosine residue in the amino acid 87 with the highest score. Even when *in vitro* and *in vivo* experiments are mandatory to demonstrate this prediction, we can speculate about this post-translational modification's possible role. The phosphorylation of the estrogen receptor by SFKs proteins has been studied in breast cancer cell lines. Its inhibition reduces its stability and transcriptional activity and alters the ligand binding (63).

Then, the interaction of PR and cSrc in glioblastoma cells could be bilateral and involve the genomic and non-genomic actions of P4. In conclusion, this work is the first report in demonstrating the interaction between cSrc and PR in human glioblastoma cells. This interaction induces cSrc activation, which in turn participates in the regulation of the activity of proteins involved in the migration and invasion of glioblastomas. The results presented here open new perspectives for the treatment of glioblastomas.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://portal.gdc.cancer.gov/

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by CB-A and AD-M. The first draft of the manuscript was written by CB-A and AD-M and reviewed by IC-A. AG-A participated in the analysis of results, and all authors commented the versions of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was financially supported by DGAPA-PAPIIT (IN217120), UNAM, Mexico and by a scholarship to CB-A from Consejo Nacional de Ciencia y Tecnología (277679), Mexico.

ACKNOWLEDGMENTS

The authors thank Carmen J. Zamora-Sánchez (Facultad de Química, UNAM) and Ángela Patricia Moreno-Londoño (Facultad de Medicina, UNAM), for their technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.640298/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Bello-Alvarez and Camacho-Arroyo *Biology of Sex Differences* (2021) 12:28
https://doi.org/10.1186/s13293-021-00372-5

Biology of Sex Differences

REVIEW Open Access

Impact of sex in the prevalence and progression of glioblastomas: the role of gonadal steroid hormones

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Abstract

Background: As in other types of cancers, sex is an essential factor in the origin and progression of glioblastomas. Research in the field of endocrinology and cancer suggests that gonadal steroid hormones play an important role in the progression and prevalence of glioblastomas. In the present review, we aim to discuss the actions and mechanism triggered by gonadal steroid hormones in glioblastomas.

Main body: Glioblastoma is the most common malignant primary brain tumor. According to the epidemiological data, glioblastomas are more frequent in men than in women in a 1.6/1 proportion both in children and adults. This evidence, and the knowledge about sex influence over the prevalence of countless diseases, suggest that male gonadal steroid hormones, such as testosterone, promote glioblastomas growth. In contrast, a protective role of female gonadal steroid hormones (estradiol and progesterone) against glioblastomas has been questioned. Several pieces of evidence demonstrate a variety of effects induced by female and male gonadal steroid hormones in glioblastomas. Several studies indicate that pregnancy, a physiological state with the highest progesterone and estradiol levels, accelerates the progression of low-grade astrocytomas to glioblastomas and increases the symptoms associated with these tumors. In vitro studies have demonstrated that progesterone has a dual role in glioblastoma cells: physiological concentrations promote cell proliferation, migration, and invasion while very high doses (out physiological range) reduce cell proliferation and increases cell death.

Conclusion: Gonadal steroid hormones can stimulate the progression of glioblastomas through the increase in proliferation, migration, and invasion. However, the effects mentioned above depend on the concentrations of these hormones and the receptor involved in hormone actions. Estradiol and progesterone can exert promoter or protective effects while the role of testosterone has been always associated to glioblastomas progression.

Keywords: Glioblastoma, Estradiol, Progesterone, Testosterone, Sex differences, Progression, Prevalence

Background

Astrocytomas are the most common malignant brain tumors. They are classified according to their malignancy in four grades from I to IV, astrocytoma grade IV, or glioblastoma presents the worst prognostic [1]. Glioblastoma has an average prevalence male-to-female ratio of 1.6/1 (see Fig. 1), and this datum is independent of race, age, economic status, or geographical location [2]. For a long time, the prevalence of glioblastomas in men with respect to women has suggested that sex and specifically, gonadal steroid hormones should participate in glioblastomas growth. Nowadays, a large number of pieces of evidence about the role of these hormones in glioblastomas origin and progression have emerged [3].

Progesterone (P4) and estradiol (E2) are the main female gonadal steroid hormones, while testosterone (T) is

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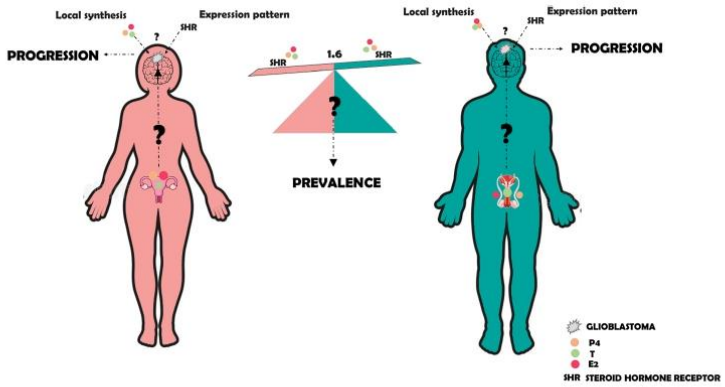

Fig. 1 Influence of E2, P4, and T in the prevalence and progression of glioblastoma. Women and men synthesize E2, P4, and T from cholesterol, in the reproductive system, adrenal gland, and brain. Gonadal steroid hormones can also reach the brain by crossing the blood-brain barrier. The evidence accumulated to date suggests that E2, P4, and T from both sources have an important role in glioblastoma progression. However, the significance of E2, P4, and T in the 1.6 males:females prevalence of glioblastoma has not been completely determined. Results obtained nowadays suggest that the role of these hormones in glioblastomas depends on the concentration of hormones and the availability of the isoforms or subtypes of their receptors

the typical male gonadal steroid hormone (see Fig. 1). Besides their classical actions in reproduction, their role in immunological response [4], neuroprotection [5], brain plasticity [6], and cancer are broadly known [7]. The central nervous system (CNS) can synthesize gonadal steroid hormones from cholesterol. Steroids that are synthesized in the CNS are also known as neurosteroids [7]. Recently, the study of neurosteroid functions in the CNS has been extended to brain tumors, mainly glioblastomas. The effects of gonadal steroid hormones over glioblastomas depend on the specific hormone concentration (Table 1) [9], the administration time [10], and the receptor involved in hormone actions [21, 22].

For these reasons, the precise role of this kind of hormone in glioblastomas is controversial. Besides, the intrinsic molecular, cellular, and tissue differences of each sex also impact the prevalence and progression of glioblastomas.

Even when receiving the standard therapy, consisting of surgical resection followed by radiotherapy and chemotherapy with temozolomide (TMZ), patients with glioblastoma relapse in a period not much longer than a year. This therapy has remained without changes for more than a decade [23]. Compile and analyze the evidence about the actions of gonadal steroid hormones in glioblastomas is an essential step in the understanding of the whole picture of this complex disease. According to accumulated knowledge until the present, gonadal steroid hormones exert a variety of important actions in glioblastoma progression through the promotion of proliferation, migration, and invasion. Considering these facts, in this review, we discussed the influence of these steroids in the prevalence and progression of glioblastomas in the context of the molecular differences between sexes.

Influences of sex differences in cancers of non-reproductive tissues

Gonadal steroid hormones have a high impact on the incidence and evolution of several cancers in males and females. Sex hormone levels are different between them, and therefore their role in pathophysiological processes. However, sex hormones do not constitute the unique factor influencing the differential prevalence and course of cancer in males and females. Recently, the intrinsic genetic conditions have gained great relevance. Most important is the fact that besides sex chromosomes, the autosomal genes also contribute to this heterogeneity. The regulation of the autosomal genome is sexually dimorphic [24]. Reinius and cols found hundreds of genes with sex-biased expression patterns in the brain cortex of male and female humans and macaques [25].

Interestingly, the brain cortex is the main localization site of glioblastomas. The differences in gene expression patterns between sexes have a significant influence on predisposition to develop certain diseases. Several cancer types are more frequent and have a worse prognosis in men than in women [26]. According to Global Cancers Statistics, colon, rectum, stomach, liver, esophagus, bladder, skin, pancreas, Kaposi sarcoma, lip, and oral cavity cancers have a higher incidence in men than in women. In colorectal cancers, it has been reported that estrogens

Table 1 Effects of low and high concentrations of P4 on glioblastoma cells in in vitro and in vivo models

P4	
Low doses	**High doses**
• *In vitro effects*	• *In vitro effects*
- Induces proliferation through PR actions (10 nM) (ref [8])	- Decreases cell viability (20–80 μM) (ref [9])
- Increases the expression of EGFR and cyclin D1 through its PR and the recruitment of steroid receptor coactivator-1 (SRC-1) (10 nM) (ref [10])	- Enhances toxicity of TMZ (5 and 80 μM) (ref [11])
- Induces the expression of progesterone-induced blocking factor (PIBF) (10 nM) (ref [12])	- Changes in detoxification mechanisms, stress, immune response, and glucose metabolism (100 and 300 μM) (ref [13])
- Stimulates the unphosphorylated state of cofilin (10 nM) (ref [14])	- Reduction of glycolytic metabolism by decreasing the Glut1 expression (8 and 100 μM) (ref [15])
- Induces migration and invasion through PR actions (10 nM) (ref [14])	
- Increases the number of glioma stem cells from U251 cells (10 nM) (ref [16])	
- Allopregnanolone and 5alpha-dihydroprogesterone (P4 metabolites) increase the number of glioblastoma cells (10 nM) (refs [17, 18])	
- 5alpha-dihydroprogesterone increases the migration of glioblastoma cells (10 nM) (ref [18])	
• *In vivo effects*	• *In vivo effects*
- Increases the area and infiltration of tumor through PR actions (1 mg) (refs [8, 19, 20])	- Reduction of tumor volume (8 and 100 μM) (ref [9]) (100 and 200 μM) (ref [15])
	- Induction of cell senescence by attenuating the signaling pathway PI3K/Akt/mTOR (8 and 100 μM) (ref [9])

play a protective role against colorectal cancer development in female mice [27]. In contrast, testosterone has the opposite effect in male rats [28]. Nevertheless, the differences in the prevalence of colorectal cancer between both sexes are present both in children and adults. The last idea suggests that other factors besides gonadal steroid hormones influence the prevalence and prognosis of cancer between females and males. In kidney cancer, one potential explanation for the higher prevalence in men is the X chromosome-encoded mutations since these are more frequently found in tumors derived from males. Specifically, the mutation in the KDM5C gene has a significant impact on tumorigenesis [29]. Besides, other authors have reported that gonadal steroid hormones also exert effects on kidney cancer cells. E2 inhibited the growth of renal cell carcinoma in an estrogen receptor (ER)-dependent pathway [30], and on the contrary, dihydrotestosterone (DHT) promoted renal carcinoma growth [31] via androgen receptors. Gastric cancer is more frequent in men than in women in a 2:1 proportion. As in the previous examples in this type of cancer, E2 plays a protective role since it induced apoptosis at different concentrations by activating caspase 3 and inhibiting Bcl-2 and Bcl-xL [32]. Interestingly, in gastric cancer, the protective role of E2 depends on the activated ER subtype [33]. One of the few examples regarding a higher prevalence in women than in men (2.9 times) is thyroid cancer. It has been suggested that E2 induces ERα expression over ERβ, promoting proliferation and growth [34]. Some of the tissues previously mentioned can synthesize gonadal steroid hormones, which have an essential role in maintaining cellular homeostasis. For example, in the intestine, estrogens are necessary to preserve the epithelial barriers and reduce the permeability. However, other organs such as kidney express ERs but it cannot synthesize gonadal steroid hormones from cholesterol [35].

Yuan and cols analyzed the molecular differences between male and female patients in 13 cancer types, including glioblastomas. They found two sex-effect groups with different profiles according to the prevalence and mortality. The weak sex-effect group contained few sex-biased genes associated with prevalence and mortality ratios more similar between females and males. In contrast, the strong sex-effect group contained a greater number of molecular signatures influenced by sex and more separated ratios of prevalence and mortality between females and males. In this study, glioblastomas were located in weak sex-effect groups containing few sex-biased genes [26]. As the previous types of cancers discussed, brain tumors including glioblastomas are susceptible to gonadal steroid hormones action. It is also important considering the ability of CNS to synthesize gonadal steroid hormones from cholesterol and that these hormones can regulate the expression of several genes. In the brain, gonadal steroid hormones display a variety of actions that conduce to brain plasticity [6], for example in the amygdala and hypothalamus of fetal rats,

E2 promotes neurogenesis through the increase in proliferation and survival of new neurons [36]. Neuroprotective effects of E2 have been demonstrated in primary cultures of hippocampal neurons since this hormone prevented neuronal death induced by glucose deprivation [37]. In orchidectomized rodent males, T or DHT increases spine dendritic density [38]. In primary cultures of rat cortical neurons, P4 increases the phosphorylation of a group of proteins involucrate in cytoskeleton remodeling, such as focal adhesion kinase and Wiskott-Aldrich syndrome protein family member 1 [39]. It has been observed that P4 regulates sexual behavior increasing the lordosis through the PR-B isoform in females rats [40]. In the case of glioblastomas, it has been reported that P4 and E2 increased the expression of proteins closely associated with angiogenesis and cell proliferation, such as vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), and Cyclin D1 in human glioblastoma derived cell lines. In the case of E2, this effect was induced through ERα [10, 22].

Prevalence of brain tumors in female and male patients

In 15 countries on six continents, it has been reported an evident prevalence of brain tumors in males in comparison with females ranged from > 1 to 3.5 for astrocytomas (including glioblastomas), medulloblastomas, ependymomas, and oligodendrogliomas [41]. Neuroblastomas, the most common extracranial solid tumors in children, originated from neural crest cells, have a modest difference in the prevalence between males and females (boys:girls ratio = 1.2) [42]. The prevalence of vestibular schwannomas, benign and slow-growing brain tumors, depends on age. Between 35 and 54 years, the incidence is higher in females, whereas between 65 and 84 years, the incidence is higher in males [43]. Chordomas, tumors of the sarcoma family that occur midline along the spinal axis, affect men more commonly than women in an approximately 2:1 ratio [44]. In medulloblastomas and glioblastomas, the male predominance is evident both in the pediatric and adult populations [45]. This suggests that other factors besides gonadal steroid hormones are involved in the prevalence of brain tumors.

The dysregulation of the MAPK pathway is a frequent mechanism involved in the over-proliferation of many cancer types such as astrocytomas. In some areas of the male mice brain, it has been observed a higher activation of the MAPK pathway, independent of gonadal steroid hormone status [46]. On the contrary, in vitro studies have shown a greater activation of the MAPK pathway in female astrocytes. However, when female astrocytes were treated with E2, the inhibition effect over the MAPK pathway was more potent than in male astrocytes. Furthermore, in female astrocytes, the effect of E2 triggers a higher apoptosis rate [47]. This latter effect in females could be associated with ER's different expression patterns between males and females [48]. This evidence is insufficient to explain the differences between females and males in the prevalence and progression of brain tumors. However, considering E2 and its receptors are involved in the regulation of MAPKs signaling, this hormone could play an essential role in the prevalence and progression of astrocytomas through its interaction with this pathway.

Glioblastoma

Glioblastoma or astrocytoma grade IV is the most common malignant primary brain tumor, representing 56% of all gliomas. Patients with glioblastoma have an extremely poor prognosis with overall survival of 15 months, and to date, there is no effective therapy for the treatment of this malignancy. The standard gold therapy for glioblastoma has been unmodified for more than ten years [49]. Epidemiological data refers that primary glioblastomas are more common in men (1.6 males:females ratio) while secondary glioblastomas (evolve from low-grade astrocytoma) appear more frequently in women (0.65 males:females ratio) [50]. The higher incidence of primary glioblastomas in men suggests a potential inductor role of male gonadal steroid hormones in the occurrence of these tumors. In secondary glioblastomas, these data suggest that female gonadal steroid hormones are related to the progression (from low-grade astrocytomas) rather than the prevalence. Other factors besides gonadal steroid hormones, such as intrinsic genetic and molecular differences, have been linked to glioblastoma prevalence in the male population.

Intrinsic genetic and molecular differences in the prevalence and progression of glioblastomas

The contribution of sex chromosomes to sex differences has long been recognized. The differences in the dosage of X-linked genes have been associated with the sex-specific genetic architecture of some diseases [24], such as dyskeratosis congenita [51], and severe combined immunodeficiency syndromes [52]. Dunfords and cols provided pieces of evidence to support the EXITS theory. Some tumor suppressor genes (TSGs) in X-chromosome escape from X inactivation; these genes are known as EXITS genes for "escape from X-inactivation tumor suppressors." These authors suggest that mutations in TSGs that escape X-inactivation have a significant influence on a male predominance of cancer, or in other words, that biallelic expression of EXITS genes confers a protection status against cancer in women. In the specific case of glioblastomas, these authors found proof of biallelic expression in females of KDM6A (encodes the lysine-specific demethylase 6A), KDM5C (encodes the lysine demethylase 5C), DDX3X (encodes a DEAD-box helicase 3 X-linked), and ATRX

(encodes the ATRX chromatin remodeler). A higher expression of these genes was detected in females with respect to males [53]. These results are just a proof of clear intrinsic genetic differences between men and women with glioblastoma. However, the functional role of these genes in glioblastomas prevalence has not been demonstrated. The transition to the persistent state of glioblastoma stem cell (GSC) is dependent on the upregulation and widespread redistribution of histone demethylases KDM6A/B [54]. Sun and cols went a little further and found that male astrocytes from mesenchymal glioblastoma carried out a greater inactivation of tumor suppressor RB than female astrocytes [55]. Also, these authors questioned the basis of difference in the RB regulation between males and females, and they found that female astrocytes respond with greater activation of the cyclin–cyclin-dependent kinase (CDK) complexes inhibitors p16 and p21 against serum deprivation or the potent antineoplastic etoposide than male astrocytes. These results were obtained under conditions that promoted the growth arrest dependent on Rb; thereby, the difference in the status of p16 and p21 is a critical element in the higher prevalence of glioblastomas in males [56].

Gonadal steroid hormones in the prevalence and progression of glioblastomas: E2

Based on the average male-to-female ratio of 1.6/1 for glioblastomas, female gonadal steroid hormones have been considered as possible factor protection against them [57]. However, this hypothesis excludes secondary glioblastomas, which are more frequent in females than in males [50]. Nuclear steroid receptors play an essential role in the actions of gonadal steroid hormones. E2 can induce various effects through its classical receptors, ERα, and ERβ (see Fig. 2). In the glioblastoma context, results obtained about this hormone's role are heterogeneous and depend on the ER subtypes expression. Batistatou and cols in 2004 found that the expression of ERβ proportionally decreases according to the grade of malignancy of astrocytomas. Two years later, the same authors found that the low expression of ERβ was correlated with the worst survival of patients with astrocytomas [58, 59]. Sareddy and cols found similar results in terms of the decrease of ERβ expression with glial tumors' progression [60]. However, another group (Li and cols) found that ERβ5 is the isoform predominant in human gliomas, and its expression increase with the malignancy grade [61].

Recently, Hernández-Vega and cols found a slight but significant increase in the mRNA levels of ERα and ERβ in 155 glioblastomas compared to 167 low-grade gliomas. It was also determined that the mRNA expression of ERα and ERβ was higher in the mesenchymal glioblastoma subtype compared to the other three subtypes defined by Verhaak and cols [62]. Besides, using TCGA analysis, a higher expression of both ER subtypes was associated with a poor clinical outcome [63]. On the contrary, Jimenez and cols found that the lowest expression of ERα mRNA was associated with a bad prognosis [64].

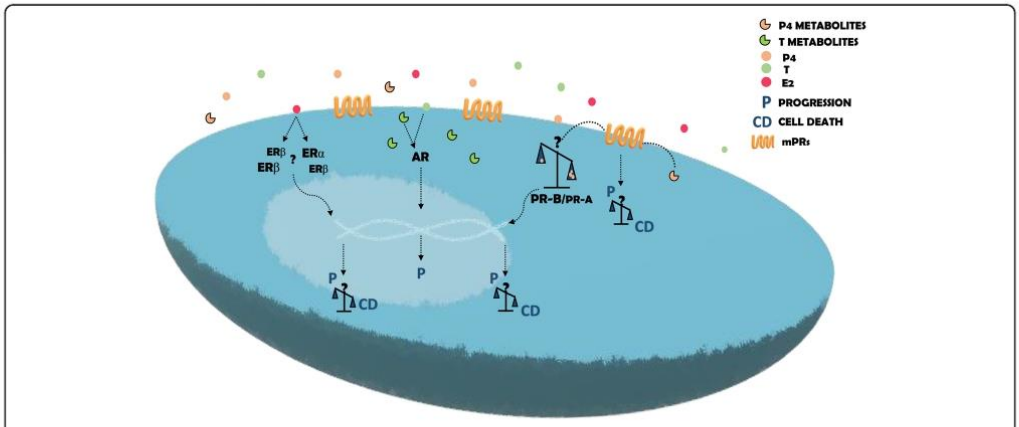

Fig. 2 Molecular mechanisms of E2, P4, and T action in glioblastoma cells. Once in glioblastoma cells, E2, P4, and T can activate intracellular o membrane receptors, and according to the cellular context, different effects will be triggered. Effects induced by E2 are mainly mediated by the ERα/ERβ ratio. In a context with an ERα prevalence, E2 is associated with glioblastoma progression through enhancing proliferation, EMT, migration, and invasion, while ERβ prevalence is associated with cell death. In the case of P4 and its metabolites, a dual behavior is associated with the concentration of the former. Low levels of P4 induce an increase in proliferation, migration, and invasion while high levels induce processes related to cell death. P4 exerts its action through the PR or mPRs. The effects of T and its metabolites have been always associated with events that promote the progression of glioblastomas

Hönikl and cols, through the analysis of 60 tissue samples, determined that the high expression of ERα and aromatase was correlated with a significantly higher survival probability of GBM patients, regardless of gender [65]. González-Arenas and cols demonstrated that estrogens can induce the growth of human astrocytomas through the ERα and by recruitment of coactivators SRC-1 and SRC-3 [22]. In contrast, the ERβ agonist DPN inhibited the cell proliferation of glioblastoma-derived cell lines T98G, U87, LN229, and U138 and stimulated cell death in a xenograft model in mice [60]. Despite the contradiction related to the value of ERα and ERβ as a prognostic factor and their expression in gliomas of different malignant grade, these results suggest that E2 has a protective role against gliomas through its ERβ; however, this effect may depend on the amount of each ERβ isoform (see Fig. 2). In breast cancer cells, the same dual behavior of E2 through ERα and ERβ has been found [66].

It would be interesting to establish a relationship between levels of ER subtypes in males and females and their influence over the regulation of p16 and p21 effects in each sex.

An interesting data obtained by Wei Yang and cols suggested that male glioblastoma patients could have a better response to inhibitors of the cell cycle. In contrast, female patients would do better with integrin signaling inhibitors [67]. According to Sun and cols, this hypothesis is based on results who found that female astrocytes have a stronger antiproliferative response than male astrocytes by the p16 and p21 activation [55]. Considering that integrins are essential to change the cell interactions with the ECM, one of the steps to epithelial-mesenchymal transition, based on Yang's results and the last pieces of evidence exposed, it could be assumed that E2 plays a role in the epithelial-mesenchymal transition of glioblastomas. Importantly, Hernández-Vega and cols have recently demonstrated that E2 induced the epithelial-mesenchymal transition in human glioblastoma cells through the activation of ERα. These authors found that E2 and PPT, an agonist of ERα, modified glioblastoma cell morphology, increased cell migration, and the expression of EMT markers such as vimentin N-cadherin. At the same time, the treatment with MPP, an antagonist of ERα, blocked the E2 and PPT effects. The agonist of ERβ, DNP, did not affect any processes [63].

After these pieces of evidence, the role of E2 in the prevalence or progression of glioblastoma is still controversial since it seems that E2 actions in glioblastomas depend on cell context (see Figs. 1 and 2).

Gonadal steroid hormones in prevalence and progression in glioblastoma: P4

The most studied hormone in the context of glioblastomas is P4. Several pieces of evidence have implicated this hormone and its reduced metabolites in the progression of glioblastomas. Since 1997, some studies have related the progesterone receptor (PR) content with astrocytomas malignancy. For example, Khalid and cols found that PR was more expressed in glioblastomas (astrocytoma grade IV) than in astrocytomas grade I and II from biopsies of 86 patients [68]. Also, González-Agüero and cols found that PR content at the protein level was observed in 83% of astrocytomas grade III biopsies while 100% of glioblastoma biopsies were positive for PR signal. These authors also found that the predominant isoform in the biopsies analyzed was PR-B over PR-A (see Fig. 2) [69].

Interestingly, there exist some pieces of evidence about the behavior of astrocytomas during pregnancy. This period is characterized by the highest increase in P4 levels, up to 200-fold. In 2016, Hanada and cols reported the quickest ever progression of diffuse astrocytoma during pregnancy. A 21-year-old woman was diagnosed with a low-grade astrocytoma during her first pregnancy. In the second pregnancy, at the 4th month, the biopsy revealed the presence of diffuse astrocytoma, and 2 months later, the tumor was surgically removed. The histological diagnostic confirmed a glioblastoma [70]. In 2018, Peeters and cols carried out a multi-institutional retrospective study with 50 pregnant women diagnosed with a glioma. Of 24 women who had been diagnosed with a glioma before pregnancy, 87% of the cases showed an increase in the growth rate of the tumor, and 38% of the cases showed a clinical deterioration with seizures that were only resolved after delivery. In the case of 28 women diagnosed with glioma during pregnancy, the tumors were discovered during the second (29%) and the third (54%) trimesters. In 21.4% of the cases, the clinical deterioration improved after delivery. These authors concluded that pregnancy can unfavorably impact over glioma progression [71], and parity attenuates this effect. These facts are in line with the results of in vitro assays detailed below.

To understand the role of PR in glioblastomas progression, some authors have carried out functional assays by stimulating or inhibiting PR. González-Agüero and cols treated cell lines derived from human glioblastomas with P4 at 10 nM (Table 1). They observed a significant increase in the proliferation rate of these cells compared to the vehicle. When these authors used RU486, an antagonist of PR, the latter's effect was blocked, which suggests that P4 induces the proliferation of glioblastoma cells through its PR [8]. These findings suggest a potential role of P4 via its intracellular receptor in the progression and malignancy of astrocytomas. However, these pieces of evidence do not correspond to the supposed protective role of female hormones in glioblastomas. Previously, this review mentioned the

importance of the specific concentration of hormones. Regarding this fact, it is essential to cite a group of authors, Atif and cols, who found a dual behavior in the effects induced by P4 depending on the concentration. In the proliferation assays conducted by these authors, cell lines derived from glioblastomas showed an increase in viability at physiological concentrations, as the Camacho-Arroyo group has reported. In contrast, at very high concentrations (Table 1), P4 decreased cell viability [9]. Besides the antiproliferative effects induced by P4 at high concentrations, it has been found that P4 enhances the cytotoxic effects of temozolomide in glioblastoma cells and reduces its toxicity in normal cells [11]. Antiproliferative effects of high concentrations of P4 have been related to the reduction of glycolytic metabolism and the induction of cell senescence by decreasing the Glut1 expression and attenuating the signaling pathway PI3K/Akt/mTOR [15]. A recent proteomic analysis, conducted by Altinoz and cols suggests that suppressive actions of high doses of P4 on glioblastoma are induced by changes in detoxification mechanisms, stress, immune response, and glucose metabolism [13]. High doses of medroxyprogesterone acetate, a synthetic variant of P4, have also shown antiproliferative effects on glioblastoma-derived cell lines such as C6 and U87 [72].

The action of P4 over the proliferation of glioblastoma cells has also been demonstrated in in vivo models. In this case, P4 increased the area of tumor derived from human astrocytoma grade III cells that were implanted in the motor cortex of male rats. This effect was blocked when RU486 was administrated together with P4 [19]. Similar results were obtained when U87 human glioblastoma-derived cell line was implanted in the cerebral cortex of rats, and phosphorothioated antisense oligodeoxynucleotides (ODNs) against PR expression was used [20].

P4 also promoted the migration and invasion of U251 and U87 human glioblastoma-derived cell lines, and when RU486 or oligonucleotide antisense against PR were used together with P4, the effect of P4 was blocked. P4 also stimulated the unphosphorylated state of cofilin, which is fundamental in the actin cytoskeleton remodeling [14]. In 2014 Germán-Castelán and cols implanted U373 cells in Wistar adult male rats' motor cortex and observed that in rats treated with P4, the tumor grew and infiltrated to deeper structures of the brain in a higher proportion compared to vehicle [19].

According to its capacity to stimulate proliferation in glioblastoma cells, P4 also promotes the overexpression of some genes closely related to molecular proliferation pathways. In human glioblastoma derive cells, P4 increased the expression of EGFR and cyclin D1 through its PR and the recruitment of steroid receptor coactivator-1 (SRC-1) [10].

Another evidence about the role of P4 in the proliferation of glioblastoma cells is related to the capacity of these hormones to induce the expression of progesterone-induced blocking factor (PIBF) [12]. This protein has been associated with the proliferative and immunologic effects of P4 in some malignancies [73]. PIBF has been associated with the suppression of anti-tumor immunity in a mode like those used by the embryo during pregnancy [74]. Kyurkchiev and cols found that PIBF was intracellularly expressed by primary culture cells derived from six samples of glioblastoma patients. Considering the immunosuppressant role of PIBF, these authors suggested that glioblastoma cells escape from the immune system by expressing this factor [75]. In cells derived from human glioblastoma, P4 induces the increase of the expression of PIBF, which in turn leads to the increase of the number of cells and in the JAK1 and STAT6 phosphorylation at 20 min [76].

Therefore, in glioblastoma cells, P4 can modulate the immune and growth response through increased PIBF expression.

Not only P4 but also its metabolites such as allopregnanolone and 5alpha-dihydroprogesterone (DHP) can induce an increase in the proliferation of glioblastoma cells. Zamora-Sánchez CJ and cols found that allopregnanolone and 5alpha-dihydroprogesterone, both reduced metabolites from P4, increased the number of U87 and U251 cells, similarly to P4 [17, 18]. Besides, DHP increased the migration of glioblastoma cells. These effects reinforce the role of P4 over the growth of glioma cells.

Standard care treatment for glioblastomas provides only an overall survival of 15 months. The poor benefices of the standard gold therapy (maximal safe surgical resection followed by combined chemotherapy with temozolomide and radiation) for glioblastomas are attributed to cancer stem cells, which provide the tumor the capacity of invasion, resistance to the therapy, and recurrence. In this context, Piña-Medina and cols demonstrated that PR expression is higher in glioma stem cells (GSCs) than in glioblastoma cell lines and that P4 was able to increase the number GSCs from U251 cells [16]. Chek and cols found that a 43-year-old male with glioblastoma multiforme exclusively treated with RU486 showed a significant improvement of quality of life since the patient improved speech and movement of his hands after 2 weeks of starting the treatment with RU486 [77].

PR is not the only receptor involved in P4 effects in glioblastoma cells. P4 can also interact with membrane progesterone receptors (mPRs) (see Fig. 2) and the progesterone receptor membrane component-1 and 2 (PGRMC1-2). In immortalized granulosa cells, the depletion of PGRMC1 and PGRMC2 attenuated some actions exerted by P4 [78]. P4 decreased PGRMC1 mRNA content in LN-229 spheroids, while in U87 spheroids, P4

increased the protein content of the PGRMC1 [79]. mPRs are G protein-coupled receptors members of the progestin and adipoQ receptor (PAQR) Family and five subtypes have been described (mPRα, mPRβ, mPRγ, mPRδ, and mPRε) [80]. In the context of glioblastoma, expression of mPRα, mPRβ, mPRγ [81], mPRδ, and mPRε has been detected in U87 and U251 cells. The analysis of expression data from TCGA revealed that mPRβ, mPRδ, and mPRε were downregulated in GBM compared to normal tissues. mPRδ expression was negatively correlated to the tumor grade, while mPRε expression was independent of the tumor grade. The analysis of the clinical outcome of both mPRs showed that the low expression of mPRδ was correlated to poor prognosis. At the same time, the contrary behavior was observed in the case of mPRε [82]. In U87 and U251 cells, Org OD 02-0, a specific mPRα agonist, increased cell proliferation, migration, and invasion. The addition of siRNA against mPRα blocked the agonist effects [83].

Until this point, it seems very clear the potential role of P4 (at low concentration) (Table 1) and its metabolites to promote the progression of glioblastoma cells by interacting with PR or mPRs (see Fig. 2).

Sex steroid effects on GBMs are derived from hormones synthetized in the brain or in the endocrine glands?

Neurons, glial cells [84, 85], and glioblastoma-derived cell lines such as U87 and C6 [86] can synthesize neurosteroids from cholesterol. Besides, gonadal steroid hormones can also reach the brain. The effect of these hormones does not depend on their origin in the organism, but on the concentration and the receptor subtype they interact with. For this reason, it is difficult to relate the effects of E2, P4, and T on glioblastomas with the organ they were synthesized. In the hippocampus, for example, the concentration of estradiol (E2) is sixfold higher than in plasma [87]. In this brain region, E2 plays an essential role in brain plasticity through the induction of proliferation and the increase in the frequency of multiple synapse boutons in CA1 neurons [88]. If we consider the concentration of E2 in this area compared to that in plasma, the most logical thought is to attribute the effects described above to E2 synthesized in the hippocampus. In contrast, in the subventricular zone, the levels of circulating testosterone (T) are fundamental to induce proliferation, since the subventricular zone of castrated rats exhibited less 5-Bromo-20-deoxyuridine (BrdU) incorporation than control or castrated animals treated with T [89]. Then, at least in events related to brain plasticity, the specific brain region is an important factor to consider. In the case of glioblastomas, there is no information about differences in gonadal steroid hormones concentration among plasma, tumor, and tumor microenvironment; however, in one study conducted by Bao and cols, serum levels of T were higher in patients with glioblastoma than in non-cancer patients [90]. A pilot study with 36 biopsies of astrocytoma patients revealed that E2 concentration was higher in astrocytomas grade IV (glioblastomas) than in grade II or III astrocytomas. Besides, the highest expression of aromatase was associated with the worst survival prognostic in glioblastoma patients [64]. In another study by Plunkett and cols, in which nude rats received intracerebral implants of U87MG cells, ovariectomized female rats died first than sham-ovariectomized animals [91], which suggest the relevance of gonadal steroid hormones in glioblastoma progression. Taking into consideration these pieces of evidence, we consider that gonadal steroid hormones and neurosteroids have an essential role in the effects discussed in this review.

Risk of glioblastoma associated to exogenous female gonadal steroid hormones

Up to this point, we have discussed the role of endogenous E2 and P4 in the prevalence and progression of glioblastomas. However, several studies have reported the significance of exogenous female gonadal steroid hormones, such as the administered as contraceptives or hormone replacement therapies, and the risk of developing a glioblastoma. In 2006, Wigertz and cols studied hormonal contraceptives and hormone replacement therapy on the glioma risk. These authors included 115 glioma cases and 323 controls; however, their results did not show any correlation between hormone usage and glioma risk [92]. Over the years, this result has been contradicted by other authors. In 2011, Cowppli-Bony and cols collected the available information from 20 articles with data related to the influence of exogenous female gonadal steroid hormones on the risk of developing a glioma. The analysis showed that replacement therapy and oral contraceptives were associated with a reduced glioma risk [93]. In another analysis, which incorporated 11 studies with 4860 cases and 14,740 controls, a lower risk of glioma was observed in women who were ever users of oral contraceptive and hormone replacement therapy than those who had never used them [94]. Anic and cols carried out a case-control study of 507 glioma cases, 247 meningioma cases, and 695 community-based controls. These authors found that glioma cases were less likely to be presented in women who used long-term oral contraceptives [95]. However, another study conducted by Andersen and cols showed that the use of hormonal contraceptives for an extended period increases glioma risk. This study was performed using Denmark's national administrative and health registries with 317 glioma cases and 2126 controls [96]. Most recently, in 2018, a meta-analysis of case-control and cohort studies showed that a decreased risk

of gliomas is associated with the use of hormonal contraceptives and hormone replacement therapy. Despite the contradictions, the evidence suggests that the use of exogenous female gonadal steroid hormones decreases the probability of developing a glioma [97], which supports the idea of female gonadal steroid hormones' protection against glioma development. However, it is essential to note that the most used contraceptives combine estrogens and progestins, then the effects of this therapy are caused by this combination, and not by only estrogens or only progestins. González-Arenas and cols found that tibolone, a selective tissue estrogenic activity regulator, commonly used in the treatment of menopausal symptoms, increased the proliferation of U251 and U87 derived glioblastoma cells lines, and this effect was blocked by ERs or PRs antagonists, ICI 182, 780, and RU 486 [98].

Gonadal steroid hormones in prevalence and progression in glioblastoma: T

Regarding the role of male gonadal steroid hormones, the first study about the relationship between T levels and glioblastoma was conducted in 2017 by Bao and cols. These authors found that T's levels in the serum of glioma patients were higher than in the control group and the benign brain tumor group. However, the levels of T in the serum did not change among other astrocytomas WHO grades. In this study, the authors also discovered that the androgen receptor (AR) promoted the proliferation of cell lines derived from human glioblastoma through suppressing p53 [90]. There are also no data on the difference in AR levels between men and women in glioblastomas, but in animal models [48] and humans [99, 100], the content of AR is higher in a variety of male brain areas compared with the female brain. More than two decades ago, Brentani and cols detected AR's expression in 42% of 12 glioblastoma samples [101]. Paoletti and cols found the expression of AR in 21.6% of 57 samples from 25 glioblastomas, 18 anaplastic astrocytomas, and 14 other types of astrocytomas [102]. Over the years, other authors have found similar results regarding AR in glioblastoma cells [103–105]. In 2014, Liu and cols compared the expression of AR between high-grade astrocytic tissue and low-grade astrocytic tissue, and the levels of AR were significantly lower in high grade astrocytic compared to low-grade astrocytic tissue, and the expression of this receptor was negatively correlated to the differentiation of astrocytic tumors [106]. Yu and cols found a higher AR expression in 22 samples from male GBM patients than the expression in the normal periphery brain. These authors also detected the AR in eight human GBM cell lines: A172, LN-18, LN-229, M059, T-98G, U87-MG, U118-MG, and U138-MG. In this study, the authors discovered that DHT antagonized the cell growth induced by TGFβ and increased the apoptosis rate [107]. However, in 2018, another group found that AR antagonists induced cell death in T98G, U87MG, and A172 cell lines [108]. A more extensive study carried out by Rodriguez-Lozano and cols found that T induced the proliferation, migration, and invasion of U251 and U87 cells. These effects were blocked when flutamide, an antagonist of AR, was used [109]. T is principally metabolized to DHT by the enzyme 5α-reductase (5αR). In cell lines derived from human glioblastomas, this metabolite increased the proliferation, migration, and invasion, whereas the treatment with finasteride and dutasteride, both inhibitors of 5αR, blocked the effects induced by T, which means that T promotes the previous effects in glioblastoma cells through its metabolite DHT. In this study, the TCGA Data Analysis of mRNA expression of AR, 5αR1, and 5αR2 from 196 grade II, 223 grade III, and 139 grades IV were compared with 249 healthy brain cortex samples. This analysis showed that AR and 5αR2 expression was higher in all astrocytomas than the healthy brain; however, no statistically significant differences were observed between astrocytoma grades [110]. Werner and cols determined that AR expression at the transcript and protein levels in LN18, T98G glioblastoma cell lines, patient-derived xenografts (PDX), and human tumors overlapped with the expression of this receptor in primary prostate cancer. These authors also found that anti-androgens' treatment slowed the growth and increased sensitivity to radiation of LN18, T98G, and U87 cell lines and patient-derived xenografts (in vitro and in vivo) [111]. These results suggest that T is involved in the growth of these tumors, regardless of the tumors' grade, in the progression toward a more proliferative, migratory, and invasive state (see Fig. 2). However, to establish an association between T and the prevalence of glioblastomas in men, AR and T levels in a larger group of biopsies from male and female glioblastoma patients must be measured.

Conclusions

After compiling and analyzing the main actions of gonadal steroid hormones in glioblastoma cells, the more evident conclusion is that all of them can stimulate the progression of glioblastomas through the increase in proliferation, migration, and invasion. However, there are essential factors to consider since the effects mentioned above depend on the concentrations of hormones and the receptor involved in their actions. The actions exerted by female gonadal steroid hormones, E2 and P4, are the most controversial and complex. Depending on the type of receptor, ERα or ERβ, or hormone concentration, the actions of E2 and P4, respectively, have been associated with either a promoting or a protective role.

Studies about T's effects are less contradictory, and its role in glioblastoma progression has been broadly demonstrated by several authors in distinct models (see Fig. 2).

Regarding the role of gonadal steroid hormones in the prevalence of glioblastoma, the pieces of evidence found until the present is not sufficient to establish a conclusion. However, considering that they participate in the regulation of expression of several genes through their receptors, signaling pathways that have been associated with the prevalence of glioblastoma in both sexes could also be regulated by these steroids. The exogenous female gonadal steroid hormones consumed as hormonal contraceptives and hormone replacement therapy have been associated with a lower risk of developing glioblastoma, but more studies are required.

Perspectives and significance

In general, this review highlights the urgency of finding more conclusive results about the role of gonadal steroid hormones in the progression and prevalence of glioblastomas, paying special attention to the differences between men and women. To answer this question, it is necessary to generate work models that allow the establishment of the differences between the concentration of hormones and the expression of their receptors in serum, tumor, and the tumor microenvironment in both sexes (see Figs. 1 and 2). These studies could be carried out in animal models, organotypic brain slice cultures, and patient biopsies. Complete knowledge in this area would establish an important precedent to determine the role of gonadal steroid hormones in the prevalence of glioblastomas and to establish more personalized and efficient therapies. Once the role of gonadal steroid hormones and their receptor in glioblastoma progression and prevalence have been completely demonstrated, new options for glioblastoma therapy could be incorporated, for example, agonists or antagonists of gonadal steroid hormones receptors, enzyme inhibitors, the gonadal steroid hormones themselves in concentrations effective to suppress tumor growth, and their genes and protein targets.

Abbreviations

P4: Progesterone; E2: Estradiol; T: Testosterone; CNS: Central nervous system; TMZ: Temozolomide; EGFR: Epidermal growth factor receptor; ER: Estrogen receptor; PR: Progesterone receptor; AR: Androgen receptor; CDK: Cyclin-dependent kinase; TSGs: Tumor suppressor genes; GSC: Glioblastoma stem cell; DHP: Dihydroprogesterone; PAQR: Progestin and adipoQ receptor; mPRs: Membrane progesterone receptors; PGRMC 1-2: Progesterone receptor membrane component-1 and 2; DHT: 5α-dihydrotestosterone; 5αR: 5α-reductase; PDX: Patient-derived xenografts

Acknowledgements

Not applicable.

Authors' contributions

The first draft of the manuscript was written by Claudia Bello-Alvarez and reviewed by Ignacio Camacho-Arroyo. Both authors commented the versions of the manuscript. All authors read and approved the final manuscript.

Funding

This work was financially supported by DGAPA -PAPIIT (IN217120, UNAM, México and by a scholarship to Claudia Bello-Alvarez from Consejo Nacional de Ciencia y Tecnología (277679), México.

Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Received: 22 December 2020 Accepted: 4 March 2021
Published online: 22 March 2021

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Publisher's Note

12.1.2 Otras publicaciones

frontiers in Endocrinology

ORIGINAL RESEARCH
published: xx January 2019
doi: 10.3389/fendo.2019.00016

Testosterone Promotes Glioblastoma Cell Proliferation, Migration, and Invasion Through Androgen Receptor Activation

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Cancer Endocrinology, a section of the journal Frontiers in Endocrinology

Received: 10 October 2018
Accepted: 10 January 2019
Published: xx January 2019

Citation:
Rodríguez-Lozano DC, Piña-Medina AG, Hansberg-Pastor V, Bello-Alvarez C and Camacho-Arroyo I (2019) Testosterone Promotes Glioblastoma Cell Proliferation, Migration, and Invasion Through Androgen Receptor Activation. Front. Endocrinol. 10:16. doi: 10.3389/fendo.2019.00016

Glioblastomas (GBM) are the most frequent and aggressive human brain tumors due to their high capacity to migrate and invade normal brain tissue. Epidemiological data report that GBM occur in a greater proportion in men than in women (3:2), suggesting the participation of sex hormones in the development of these tumors. It has been reported an increase in testosterone (T) levels in patients with GBM. In addition, androgen receptor (AR) is overexpressed in human GBM, and genetic silencing of AR, and its pharmacological inhibition, induce GBM cell death *in vivo* and *in vitro*. However, the role of T in proliferation, migration and invasion in human GBM cell lines has not been evaluated. We observed that T increased the number of U87, U251, and D54 cells derived from human GBM due to an increase in cell proliferation. This induction was blocked with flutamide, an antagonist of AR. T also induced migration and invasion of GBM cells that flutamide partially blocked. These data suggest that T through AR contributes to the progression of GBM by promoting proliferation, migration, and invasion.

Keywords: Glioblastomas, testosterone, androgen receptor, cell proliferation, cell migration, cell invasion

INTRODUCTION

Glioblastomas (GBM) or grade IV astrocytomas are the most aggressive and frequent tumors in the Central Nervous System (CNS). They arise from uncontrolled proliferation of astrocytes, precursor glial cells, and cancer stem cells, and they are generally located in the brain cortex, basal ganglia and thalamus (1). GBM present their highest incidence in humans between 45 and 70 years-old, and the average survival time after diagnosis is around 12–16 months (2). GBM treatment mainly consists of surgical resection, as well as radio and/or chemotherapy. However, due to its infiltration capacity, it is practically impossible to completely extract the tumor, and it relapses (3, 4).

The CNS is an important target for sexual steroids such as androgens (5). Testosterone (T) is the main circulating androgen in men, and in addition to sexual functions, this hormone and its main metabolite, dihydrotestosterone (DHT), regulate diverse functions in the brain such as neuronal differentiation and brain masculinization (6), emotional states, impulsive and aggressive behavior, learning and memory (7, 8). Rapid modulation of dendritic spines induced by hippocampal T and DHT are essential in synaptic plasticity (9). Besides, androgens are involved in the regulation of

pathological processes such as tumor growth (10, 11). They can exert their multiple effects through the interaction with its intracellular receptor (AR), a transcription factor that once activated, binds to specific DNA sequences called androgen response elements located in gene promoter regions, thus regulating their expression (12, 13).

It has been found that AR expression was higher in biopsies of GBM patients as compared with that in normal brain tissue (14). Bao et al. (15) observed that AR expression increases according to astrocytomas grade, thus, GBM presented the highest expression. Overexpression of AR has also been observed in several cell lines derived from human GBM (14). In addition, it has been reported that genetic silencing of AR and its pharmacological inhibition induce GBM Cell death *in vivo* and *in vitro*, decreasing GBM growth (15, 16).

Besides, it has been reported an increase in testosterone (T) levels in patients with gliomas as compared with patients with a benign tumor or brain injury (15). These data suggest that T-activated AR signaling should play a role in the physiopathology of GBM. This proposal is reinforced by the fact that GBM are more frequent in men than in women in a 3:2 ratio (17). In the present study, we investigated the participation of T and AR activation in GBM cell proliferation, migration, and invasion.

MATERIALS AND METHODS

Cell Culture

U87, U251, and D54 cell lines derived from human GBM were used in this study. U87 and U251 cell lines were acquired at ATCC, and D54 cell line was generously obtained by Dr. Andrés Gutiérrez from Dr. Sontheimer's (University of Alabama, Birmingham, AL, USA). Cell lines were cultured with Dulbecco's Modified Eagle Medium with phenol red (DMEM, Biowest, FRA) and supplemented with fetal bovine serum 10% (FBS, Biowest, FRA), pyruvate (1 mM; InVitro SA, MEX), non-essential amino acids (0.1 mM; InVitro SA, MEX), and a mix of antibiotics (1 mM; InVitro SA, MEX). Cells were incubated with CO_2 at 5% and at 37°C. Cells were grown until reaching a 70–80% confluence.

Treatments

Twenty-four hours before treatments cells were grown in phenol red-free DMEM medium (In Vitro S.A., MEX) supplement with FBS (10%) without hormones (charcoal stripped, GeneTex, USA). In order to determine the T concentration that significantly modifies the number of GBM cells, they were treated with testosterone (T, 1, 10, 100 nM and 1 μM in 0.01% ethanol; Sigma, NLD), and vehicle (V, 0.01% ethanol). T (100 nM), competitive antagonist of AR: flutamide (F 5 μM; Sigma, USA), F plus T (F was added 1 h before T), and vehicle were used to evaluate proliferation. T (100 nM), F (10 μM), F plus T, and vehicle were used in migration, invasion and Western blot experiments.

Cell Counting

1×10^4 U87 cells, and 7×10^3 U251 and D54 cells were seeded in 24-well plate and grown for 24 h. Cells were treated at 0 h of each experiment as described in "Treatments" section. Cells were harvested with 1 mL PBS-EDTA (1 mM) and stained using trypan blue (0.4%) every 24 h during 120 h. In four fields per duplicate, live and dead cells were quantified with a hemocytometer (Neubauer chamber on Olympus BX41, JPN microscope) to test the effect of T and AR activation on cell growth and viability.

Cell Proliferation Assay

5-bromo-2′-deoxyuridine (BrdU) incorporation assay was used to test the proliferative effect of T. 6×10^3 U87 cells and 4×10^3 U251 and D54 cells were seeded per well in 4-well chamber slides and maintained as described in "Cell culture" section. Cells were treated with T 100 nM at 0 h of each experiment, and BrdU detection with a labeling kit (Roche, DE) was performed according to manufacturer's instructions at 120 h. Fluorescent dye Hoechst 33342 was used to stain DNA. Fluorescence signal was observed at 486 and 515–565 nm with the aid of an Olympus Bx43F fluorescence microscope (Olympus, JPN). Subsequently, F (5 μM) was used to test the role of AR in U87 and D54 cells proliferation at 72 h. The number of cells that incorporated BrdU was quantified with Image J program (NIH, USA), and the percentage of cells positive for BrdU was calculated considering the total number of cells stained with Hoechst.

Migration Assay

To determine the effect of T on migration of U87, U251, and D54 cells, the "Scratch" test was used to study collective and directional movement of cell populations. 4×10^5 cells were seeded in 6-well plates in DMEM medium and allowed to grow until reaching a confluence of 60–70%, then medium was changed by phenol red-free DMEM, supplemented with FBS (10%) without hormones (charcoal stripped). Twenty-four hours later (when monolayer was absolutely confluent), cells were washed with PBS, then 500 μL of PBS-EDTA (1 mM) were added to each well, and immediately two parallel scratches by well were made with a 200 μL pipette tip. Detached cells were removed by aspiration. One milliliter medium phenol free-red DMEM and without hormones was placed; Cytosine hydrochloride β-D-arabinofuranoside (Ara-C, inhibitor of DNA synthesis, 10 μM; Sigma, USA) was added 1 h before treatments to rule out that changes in the number of migrating cells were due to an increase in proliferation. Without removing the medium, hormonal treatments were added and two photographs per well were taken with an Infinity12C camera coupled to an inverted Olympus CKX41 microscope at 100X magnification of the "Scratch" zone at 0, 3, 6, 12, 24, and 48 h. At 24 h the medium and treatments were refreshed. The number of cells that migrated into the wound was counted using the ImageJ software.

Invasion Assay

To evaluate the effects of T on U87, U251, and D54 cells invasion, Boyden chamber assay was performed. Transwell inserts (8.0 μm membrane, Corning, USA) were placed in 6-well plate and covered with 1 mL of Matrigel (extracellular matrix gel from Engelbreth-Holm-Swarm; Sigma-Aldrich, USA) previously diluted in FBS and phenol red-free DMEM at 2 mg/mL final concentration. The inserts were incubated at 37°C

and 5% CO_2 for 2 h to allow gelation. 5×10^5 cells were seeded in the insert with 1.5 mL phenol red-free DMEM that included hormone treatments and Ara-C (10 μM). The bottom of the wells was filled with 2 mL phenol red-free DMEM with FBS (10%) as chemoattractant (18). Cells were incubated at 37°C and 5% CO_2 for 24 h. Matrigel was removed with 3 washes of PBS, and cells trapped in the porous membrane were fixed with paraformaldehyde (4%) and stained with

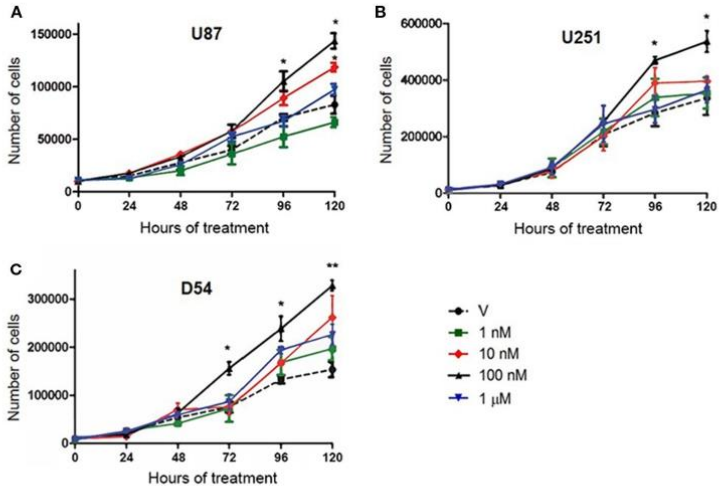

FIGURE 1 | T increases the number of cells derived from human GBM. Number of U87 **(A)**, U251 **(B)**, and D54 **(C)** cells during 120 h of treatment. Each point represents the mean ± SD, $n = 5$. *$p \leq 0.05$, **$p \leq 0.01$ T vs. V.

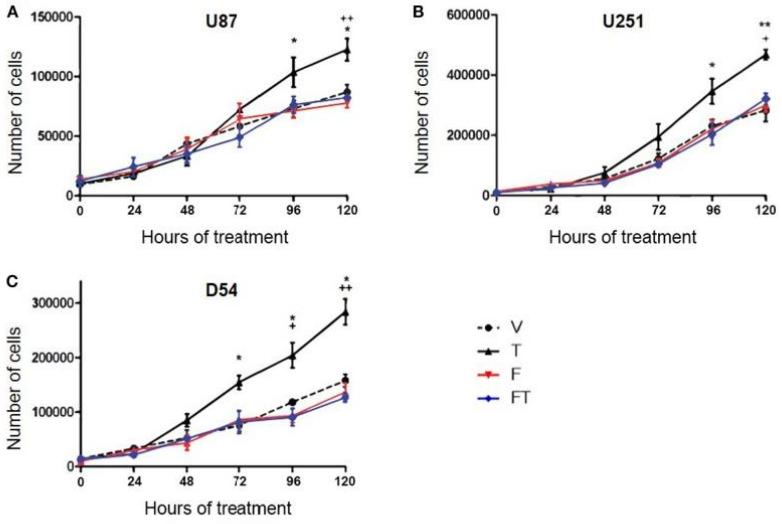

FIGURE 2 | T increases the number of GBM cells through AR. Number of U87 **(A)**, U251 **(B)**, and D54 **(C)** cells during 120 h with vehicle (V), testosterone (T 100 nM), flutamide (F 5 μM), and F plus T (FT). Each point represents the mean ± SD, $n = 5$. *$p \leq 0.05$, **$p \leq 0.01$: T vs. V; +$p \leq 0.05$, ++$p \leq 0.01$ T vs. F and FT.

crystal violet (1%). Finally, the insert was allowed to dry and observed under a microscope, five random photographs were taken per insert with an Infinity1-2C camera coupled to an inverted Olympus CKX41 microscope at a 100X magnification. The number of invading cells was quantified in the fields taken at random and the corresponding statistical tests were performed.

Western Blotting

To determine the effects of T and F on AR protein content in human GBM cells, 5×10^5 U87 cells were seeded in 6-well plates and treated for 24 h as described in "Treatments" section. Cells were lysed with RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton, 0.01% SDS, and ethylene diamine tetra acetic acid 0.5 M EDTA, 1 mL) with a mixture of protease inhibitors (p8340, Sigma-Aldrich, USA) at 4°C, and incubated for 1 h under agitation, centrifuged at 14,000 rpm at 4°C for 5 min, and supernatant was separated for storage at 4°C. Lysates were quantified with the Pierce Protein Assay reagent (Thermo Scientific, IL) in the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) at 660 nm. Thirty micrograms of total protein were mixed with Laemmli 2X buffer (100 mM Tris-base pH 6.8, 0.1% bromophenol blue, 20% glycerol, 4% SDS, 10% β-mercaptoethanol), and boiled for 5 min. Proteins were separated by electrophoresis in denaturing 7.5% polyacrylamide gels. Samples were separated at 80 volts for 2 h. Proteins were transferred to nitrocellulose membranes (Millipore, USA) in a semi-humid chamber at

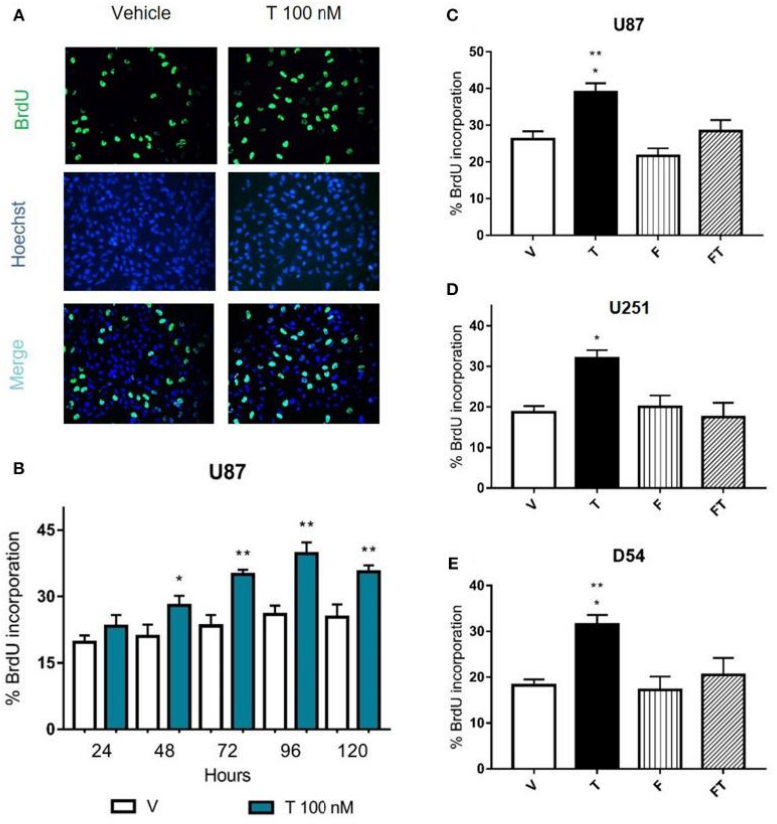

FIGURE 3 | Effects of flutamide on GBM cell proliferation. **(A,B)** Cell proliferation was measured after the treatment of testosterone (T 100 nM) during 24, 48, 72, 96, and 120 h in GBM cells by the BrdU incorporation assay. **(A)** Representative immunofluorescence images (400X magnification) of BrdU-positive U87 cells (upper panel), cell nuclei (Hoechst stain, middle panel), and merge (lower panel) are shown. **(B)** Graph represents the percentage of U87 cells incorporating BrdU. Each bar indicates the mean ± SD, $n = 4$. $^*p \leq 0.05$, $^{**}p \leq 0.01$ T vs. vehicle (V). **(C–E)** AR antagonist flutamide (F 5 μM) blocks the increase in cell proliferation induced by T. Graphs show cell proliferation of U87 **(C)**, U251 **(D)**, and D54 **(E)** cells treated 78 h with V, T, F, and F plus T (FT). Each bar indicates the mean ± SD, $n = 4$. $^*p \leq 0.05$ vs FT; $^{**}p \leq 0.01$ vs V and F **(B)**.

20 mA for 1 h. Membranes were blocked with a solution of Bovine Serum Albumin (2% BSA, InVitro SA., MEX) and 3% milk in TBS-0.1% Tween or 5% milk in TBS-0.1% at 37°C for 2 h or overnight at 4°C. Membranes were incubated with a primary anti-AR antibody at a 1:300 dilution (0.7 μg/mL, rabbit anti-AR polyclonal antibody sc-815, Santa Cruz, USA) or with monoclonal AR (D6F11; 0.08 μg/mL Cell signaling) in 5% milk blocking solution at 4°C overnight. They were then washed with 0.1% TBS-Tween 3 times for 5 min, and incubated at room temperature for 45 min with a peroxidase-conjugated anti-rabbit mouse antibody (IgG-HRP, Santa Cruz sc-2357) in a dilution of 1:7,500 (0.05 μg/mL). To remove the antibody, the membranes were washed with stripping buffer (glycine 0.1 M and SDS 0.5%, pH = 2.5) for 10 min at room temperature and blocked at 37°C for 2 h. Subsequently, they were incubated with primary anti-α-tubulin antibody in a 1:1,000 dilution (0.2 μg/mL) (mouse anti-α tubulin monoclonal antibody, Santa Cruz sc-5286) as loading control. Finally, membranes were washed with TBS-Tween 3 times for 5 min and incubated at room temperature for 45 min with a secondary antibody goat anti-mouse conjugated to peroxidase (IgG-HRP, Santa Cruz sc-2005) in a dilution of 1:5,000 (0.08 μg / mL). Chemiluminescence signals were detected exposing membranes to Kodak Biomax Light Films (Sigma-Aldrich, MO, USA) using peroxidase substrate SuperSignal West Femto Maximum Sensitivity (Thermo Scientific, MA, USA). Blot images were captured using a Canon digital camera and bands were quantified with the ImageJ software (National Institute of Health, WA, USA).

Statistical Analysis

A one-way ANOVA followed by a Tukey test were performed. GraphPad Prism 7 (GraphPad Software 7, Inc., USA) was used to calculate probability values. $p \leq 0.05$ was considered statistically significant.

RESULTS

T Increases the Number of GBM Cells

To determine whether human GBM cell number is modified by T, we evaluated U87, U251, and D54 cells growth rate through a time course experiment with T at different concentrations (1, 10, 100 nM and 1 μM). We observed a significant increase in the number of cells treated with T 100 nM in the three GBM cell lines from 72 h (D54), and 96 h (U87 and U251) of treatment. No significant difference was observed with T 1 nM and 1 μM (**Figure 1**). Viability of all cell lines remained constant with all T

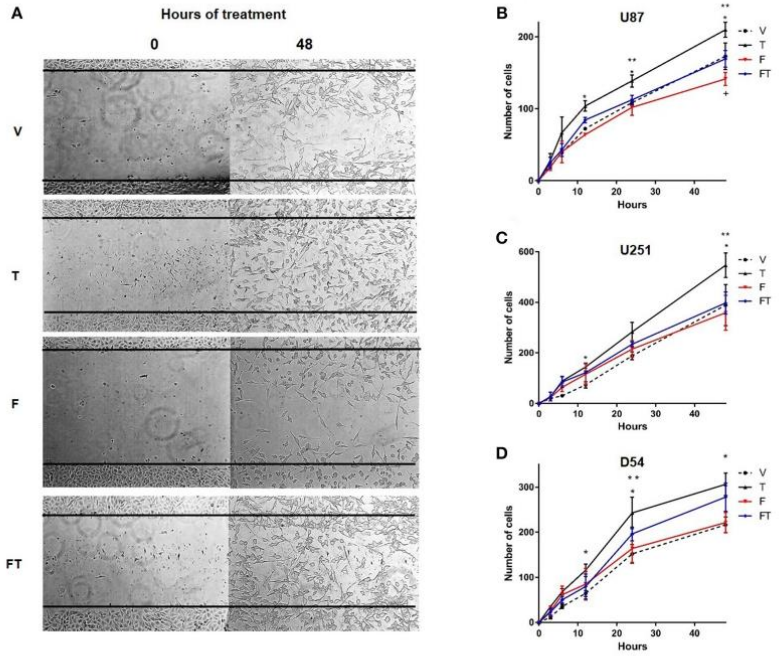

FIGURE 4 | T increases GBM cell migration. **(A)** Representative photographs from 0 to 48 h of the Scratch in D54 cells. Graphs of U87 **(B)**, U251 **(C)**, and D54 cells **(D)** show the number of cells that migrate at 0, 3, 6, 12, 24, and 48 h treated with vehicle (V), testosterone (T 100 nM), flutamide (F 10 μM), and F plus T (FT), each point represents the mean ± SD, $n = 3$. *$p \leq 0.05$ T vs V and F; **$p \leq 0.01$ T vs FT; +$p \leq 0.05$ V vs F.

concentrations throughout the 120 h of treatment with respect to control (**Supplementary Figure 1**).

T Effects on the Number of GBM Cells Are Mediated by AR

To determine if AR is involved in the increase in the number of cells induced by T, U87, U251, and D54 cell lines were treated with T (100 nM), competitive antagonist of AR: flutamide (F, 5 μM), F plus T (FT), and vehicle for 120 h. The cell count was carried out for 120 consecutive hours with trypan blue dye. As shown in **Figure 1** a significant increase in the number of U87, U251, and D54 cells treated with T (100 nM) was observed. This effect was blocked by F. The single administration of the antagonist did not significantly modify the number of cells (**Figure 2**). Viability of U87, U251, and D54 cells was not significantly modified with any of the treatments (**Supplementary Figure 2**).

Role of AR in U87, U251, and D54 Cell Proliferation

In order to know if the increase in GBM cell number induced by T is caused by changes in cell proliferation, 5-bromo-2′-deoxyuridine (BrdU) assay was performed at 24, 48, 72, 96, and 120 h in U87 cells. **Figures 3A,B** shows that T (100 nM) increased the percentage of cells that incorporated BrdU from 48 to 120 h, suggesting that the increase in number of cells is due to proliferation. To determine if T effects on proliferation are mediated by AR, U87, U251, and D54 cells were treated with antagonist F, and F plus T. Data showed that F (5 μM) blocked the proliferative effect of T, while the single administration of F did not modify cell proliferation (**Figures 3C–E**).

Role of T in Cell Migration

In order to evaluate the effects of T on GBM cell migration, Scratch assays were performed. It was observed that T (100 nM) increased the number of migrating cells with respect to vehicle from 12 to 48 h in U87 and D54 cells, and at 12 and 48 h in U251 cells. F (10 μM) completely blocked T effects in U87 and U251 cells, but only partially in D54 cells. Treatment with a single administration of F had no effect on migration of D54 and U251 cells at the times evaluated as compared with vehicle, but decreased it in U87 cells (**Figure 4** and **Supplementary Figure 3**).

Role of T in Cell Invasion

The effect of T on cell invasion was evaluated by a Boyden chambers assay. It was found that T (100 nM) increased the number of invasive U251, D54, and U87 cells as compared to vehicle at 24 h (**Figure 5** and **Supplementary Figure 4**). T increased the number of invasive U251 and D54 cells with respect to F (10 μM). F decreased U251 cell invasion induced by T,

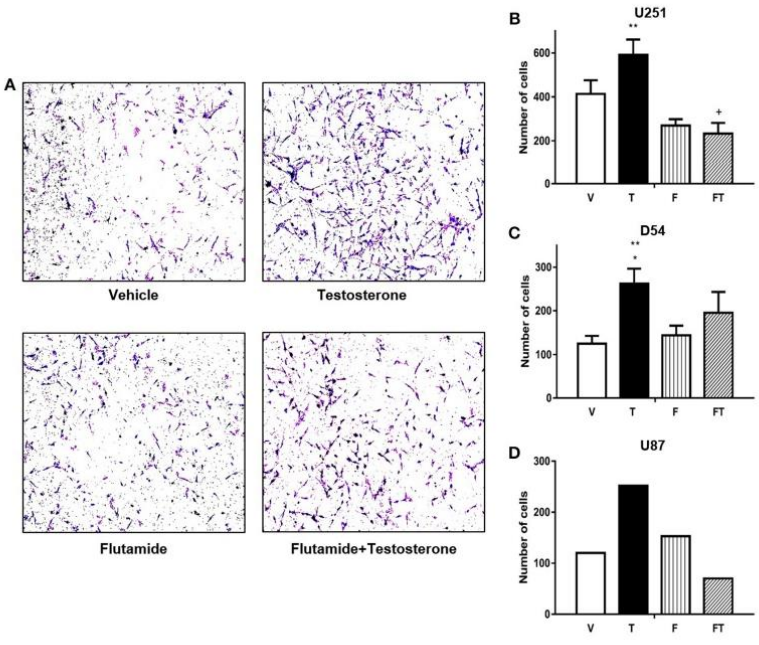

FIGURE 5 | T increases GBM cell invasion. **(A)** Representative photographs are observed in a Boyden chamber assay at 24 h with different treatments in D54 cells. Graphs of U251 **(B)**, D54 **(C)**, and U87 cells **(D)** represent number of cells that invade at 24 h with vehicle (V), testosterone (T 100 nM), flutamide (F 10 μM), and F plus T (TF). Each point represents mean ± SD, $n = 3$ (U251 and D54 cells) and $n = 1$ (U87 cells). *$p < 0.05$ vs V and F; **$p \leq$ vs 0.01 vs FT; +$p < 0.05$ FT vs V.

but it only partially blocked the invasive effect of T in D54 cells (**Figure 5**).

T Reduces AR Content in U87 Cells

We observed that AR was expressed in the three GBM cell lines used in this study (**Supplementary Figure 5**). In order to study the regulation of AR expression by T, Western blots were performed. The LnCaP and PC3 cells, both derived from prostate cancer, were used as positive and negative controls, respectively. **Figure 6** shows that AR content (determined with the use of polyclonal antibody SC-5286) is lower in U87 cells treated with T (100 nM) during 24 h than in cells treated with vehicle. Treatments with F (10 μM) and F plus T did not show significant differences as compared with vehicle and T. Interestingly, T effect was not clearly observed when AR was detected with the monoclonal antibody D6F11 (**Supplementary Figure 5**).

DISCUSSION

The participation of androgens in several physiological and pathological processes in the CNS has been widely described (5). It is also known that they are involved in prostate, colon and lung cancer growth (11, 19–21). Epidemiological data report that GBM occur in a greater proportion in men than in women (3:2), which suggests androgens participation in the development of these tumors. Different evidences suggest that sex hormones can modulate proliferation, migration and invasion of GBM (22–25). Thus, in this work we evaluated the role of T in the progression of GBM.

According to our results, T (100 nM) induces the growth of human GBM cell lines used in this study (U87, U251, and D54), without modifying the percentage of viability. In order to know that cell growth was due to an increase in proliferation, BrdU assay was performed in the three cell lines. It was found that T increased cell proliferation at 48 h and the increase in the number of cells was reflected until 96 h. This difference could be due to the fact that the replication rate of the U87 cell line is 36 h. These data agree with those reported by Merritt and Foran (26) who observed an increase in cell viability with T (1 μM) in T98G at 120 h of treatment. These data suggest that T contributes to the progression of GBM by promoting cell proliferation. Although the inductor effect of T on proliferation was observed at higher concentrations than physiological ones, it has been shown that T levels of patients with some type of astrocytoma are higher than those with some other benign tumor or brain injury (15).

Since GBM present a high infiltration capacity that involves processes of migration and invasion of surrounding tissues of the CNS (27), we evaluated for the first-time motility of GBM cells in response to T. Unlike migration, cell invasion also involves cell adhesion and extracellular matrix degradation, allowing cells to penetrate through tissue barriers such as the basement membrane or stroma (28, 29). Our results indicate that T (100 nM) increased the migration of U87, U251, and D54 cells. It is noteworthy that the increase in number of cells that migrated with T treatment was only due to a greater motility and not to an increase in cell proliferation since experiments were performed in the presence of AraC, a potent inhibitor of α, β, and δ DNA polymerases, which interferes with elongation, during replication and chain repair. Although the half-life of Ara-C is <1 h in most cell lines, more than 80% of Ara-C remains in the DNA up to 24 h (30, 31). F blocked T effects in U87 and U251 cells, but this blockade was only partial in D54 cells, revealing the participation of other mechanisms that could regulate the activity of T, such as the membrane androgens receptor, whose action by the non-classical mechanism has recently been described in prostate cancer and Sertolli cells (32). It has been described that non-genomic androgen actions regulate proliferative/migratory signaling in stromal cells (33), steroid signaling activation and intracellular localization of sex steroid receptors (34).

Similar to the effects observed in migration, T increased invasion in GBM cell lines. As mentioned, infiltration of GBM to areas of healthy brain tissue not only involves migration, but also involves the action of proteins such as metalloproteinases (MMP), therefore, it is possible that T has an effect on some MMP described in GBM (35, 36). Participation of MMPs in motility of GBM cells has been described, as well as a differentiated expression of MMPs among cell lines, which could explain the different results in U251, U87, and D54 cell invasion with T and F (37). As in the case of migration, we observed that the inductor effect of the T was completely blocked by F in U251 cells, but only partially blocked in D54 cells. We cannot rule out the participation of a non-nuclear mechanism of T or its metabolites that could mediate its effect on migration and invasion of GBM cells. Some studies have reported that aromatase (enzyme that synthesizes estradiol from T) is overexpressed in astrocytoma biopsies, besides it has been negatively correlated with the survival of patients, and positively with estradiol concentration (38). Our group previously demonstrated that estradiol increases cellular growth of GBM cells by activation of ERα (39). These

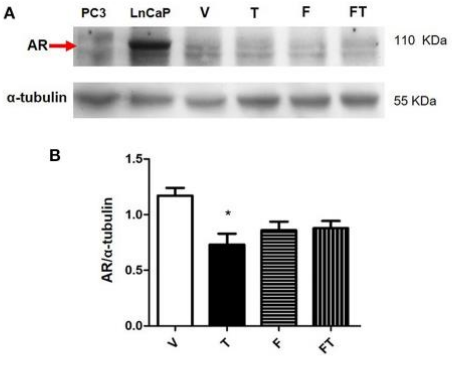

FIGURE 6 | Expression of AR in U87 cells. **(A)** Results obtained from Western blot of PC3 (negative control), LnCap (positive control), and U87 cells treated with vehicle (V), testosterone (T 100 nM), flutamide (F 10 μM), and F plus T (FT), during 24 h. **(B)** Graph represents densitometric analysis of the experiments. Each bar indicates the mean ± SD, $n = 4$. *$p < 0.05$, T vs. V.

data suggest that depending on the status of aromatase the effect should be mediated by T or estradiol. Therefore, the characterization of the expression and activity of this enzyme is fundamental.

Effects of T can be mediated by AR, a nuclear transcription factor member of the family of steroid receptors. It has been reported that AR expression is higher in patients with GBM as compared with normal brain tissue from the same patients (14), and that the expression of AR increases as the degree of malignancy of astrocytomas progresses, being grade IV (GBM) the ones that present the highest protein content (15). Overexpression of AR has also been described in 8 cell lines derived from GBM, including U87 and U251 (15). These data were replicated in our study by Western blot. We evaluated whether the effect of T can be mediated by the AR, using F, a non-steroidal antiandrogen without androgenic properties that it is suitable for using in the treatment of prostate cancer (40, 41). It was observed that F treatment blocks the effect of T on growth of the three cell lines used in this study, as well as the increase in cell proliferation induced by T at 72 h of treatment. It is important to mention that in the cell count and proliferation experiments, a lower concentration of antagonist was used because F 10 μM generated a decrease in cell viability from 72 h. Besides, recent studies have shown that genetic silencing of AR in cell lines and pharmacological inhibition of AR reduces GBM cell growth, and induces GBM cell death *in vivo* and *in vitro* (15, 16).

AR was expressed in GBM cell lines used in the present study. We analyzed the effects of T on AR protein content in U87 cell line with two different antibodies. Although we observed a reduction in AR content when we used the polyclonal antibody SC-5286, the experiments with the monoclonal antibody D6F11 did not demonstrate such reduction. Therefore, additional experiments are required to determine the effect of T in the regulation of AR in GBM cells.

In conclusion, in this work we demonstrated that T induces proliferation, migration, and invasion of human GBM cells through the interaction with AR.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

DR-L and IC-A conceived the study and wrote the paper. DR-L and CB-A performed and analyzed the experiments. AP-M and VH-P participated in the experimental design, provided technical assistance and contributed to the preparation of figures. All authors reviewed the results and approved the final version of the manuscript.

FUNDING

This work was supported by PAIP no. 5000-9170, Facultad de Química, UNAM, México.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00016/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Hindawi
BioMed Research International
Volume 2020, Article ID 2196024, 7 pages
https://doi.org/10.1155/2020/2196024

Research Article

Expression of Membrane Progesterone Receptors in Eutopic and Ectopic Endometrium of Women with Endometriosis

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Received 11 March 2020; Revised 23 May 2020; Accepted 18 June 2020; Published 14 July 2020

Academic Editor: Paul Harrison

Endometriosis is one of the most frequent gynecological diseases in reproductive age women, but its etiology is not completely understood. Endometriosis is characterized by progesterone resistance, which has been explained in part by a decrease in the expression of the intracellular progesterone receptor in the ectopic endometrium. Progesterone action is also mediated by nongenomic mechanisms via membrane progesterone receptors (mPRs) that belong to the class II members of the progesterone and adipoQ receptor (PAQR) family. The aim of the present study was to evaluate the expression at mRNA and protein levels of mPR members in the eutopic and ectopic endometrium of women with endometriosis. Total RNA and total protein were isolated from control endometrium (17 samples), eutopic endometrium (17 samples), and ectopic endometrium (9 samples). The expression of *PAQR7* (mPRα), *PAQR8* (mPRβ), and *PAQR6* (mPRδ) at mRNA and protein levels was evaluated by RT-qPCR and Western blot, whereas *PAQR5* (mPRγ) gene expression was evaluated by RT-qPCR. Statistical analysis between comparable groups was performed using one-way ANOVA followed by Tukey's multiple comparisons test with a confidence interval of 95 %. The analysis of gene expression showed that *PAQR7* and *PAQR5* expression was lower in both eutopic and ectopic endometrium as compared to the endometrium of women without endometriosis, whereas the expression of *PAQR8* and *PAQR6* was only reduced in eutopic endometrium. Furthermore, mPRα and mPRβ protein content was decreased in the ectopic endometrium of women with endometriosis. Our results demonstrate a decrease in the expression and protein content of mPRs in eutopic and ectopic endometrium of patients with endometriosis, which could contribute to the progesterone resistance observed in patients with this disease.

1. Introduction

Endometriosis is defined as the presence of endometrial glands and stroma outside the uterus, which are commonly found in the peritoneal cavity and ovaries [1–3]. Endometriosis is the leading cause of chronic and cyclic pelvic pain in reproductive age women, affecting 10-15 % of women worldwide; pain symptoms include dysmenorrhea, dyspareunia, dysuria, and dyschezia [4, 5]. Infertility is commonly associated with this disease mainly due to physical and molecular disruption in the uterus which in turn reduces implantation capacity and finally increases the risk of pregnancy loss [6]. Moreover, endometriosis negatively impacts women's quality of life by deteriorating their physical, mental, and social wellbeing [7]. The gold standard for the diagnosis of endometriosis is made by laparoscopic inspection with histologic confirmation after biopsy [8]. The aim of endometriosis treatment is to mitigate the symptoms associated with the disease and includes pharmacological therapy with nonsteroidal anti-inflammatory drugs, progestins, oral contraceptives, and gonadotropin-releasing hormone agonists, as well as surgical removal of endometrial implants and the affected tissue; however, endometriosis recurs in at least 5-15 % of the cases after most invasive surgeries [8, 9]. The etiology of this disease is far from being elucidated; however, altered estrogen signaling and progesterone resistance have been identified as the most common hallmarks of this disease [10].

Progesterone resistance in endometriosis has been attributed in part to a decrease in the expression of the B isoform of its intracellular receptor (PR-B) in the endometriotic lesions (ectopic endometrium) of women with the disease [11]. Furthermore, it has been proposed that progesterone resistance leads to an altered eutopic endometrium function in women with endometriosis, which in turn has been associated with pregnancy loss [6]. There is controversy about the alteration in the expression of PR in eutopic endometrium, suggesting that other mechanisms should be involved in progesterone resistance in this tissue [12].

Progesterone induces the decidualization of the endometrium, which is essential for embryo implantation and maintenance of pregnancy [13]. It has been demonstrated that progesterone exerts its actions by activating genomic and nongenomic mechanisms [14, 15]. Genomic action mechanisms are mediated by the PR, which acts as a ligand-dependent transcription factor that regulates the expression of progesterone-responsive genes [16–18]. Moreover, nongenomic action mechanisms are mediated in part by specific receptors localized in the plasma membrane that are not related to PR and are divided into two major groups: the membrane progesterone receptors (mPRs) that belong to the class II members of the progesterone and adipoQ receptor (PAQR) family and the progesterone receptor membrane components (PGRMCs) [19].

mPRs are G protein-coupled receptors that are encoded by five different genes: *PAQR7* (mPRα), *PAQR8* (mPRβ), *PAQR5* (mPRγ), *PAQR6* (mPRδ), and *PAQR9* (mPRε) [19, 20]. The activation of mPRs is necessary to achieve full effects of progesterone in some responsive tissues or cells to this hormone in which those effects are only partially explained by PR activation [21–23]. Importantly, we and others have demonstrated that the content and activity of these receptors are altered in many diseases, including cancer [24–27]. The expression pattern of mPRs is tissue-specific and their activation by progesterone or by the mPR specific agonist 10-ethenyl-19-norprogesterone (Org OD 02-0) regulates signaling pathways involved in mammary gland development, sexual behavior, ovulation, maintenance of pregnancy, and other processes [19, 21, 28–32]. mPRs are expressed in female reproductive and embryonic tissues, mainly in the endometrium, myometrium, ovaries, and placenta [19, 30, 33, 34]. Particularly, it has been demonstrated that *PAQR7*, *PAQR8*, *PARQ5*, and *PAQR9* are expressed in the endometrium. *PAQR7* expression is induced during the secretory phase of the menstrual cycle, whereas the expression of *PAQR5* and *PAQR9* is decreased during that phase [30]. In addition, *PAQR7* and *PAQR8* expression and the respective protein content are decreased in endometrial cancer compared to adjacent nonaffected endometrium, whereas mPRγ protein content is increased in endometrial cancer tissue [35]. To the best of our knowledge, it has not been demonstrated whether gene expression and protein content of mPRs are altered in ectopic lesions and eutopic endometrium of patients with endometriosis.

We hypothesized that the expression of mPRs is decreased in both eutopic and ectopic endometrium of patients with endometriosis compared with the endometrium of women without the disease, similar to that reported in PR. Therefore, the aim of the present study was to evaluate the mRNA expression and protein content of mPRs in eutopic and ectopic endometrium of women with endometriosis and endometrium in control subjects.

2. Materials and Methods

2.1. Participants and Tissue Collection. Seventeen patients with ovarian endometriosis (confirmed by laparoscopy and histological analysis) and seventeen women without the disease undergoing hysterectomy for benign conditions were recruited. Women included in the present study had regular menstrual cycles and did not take any hormonal treatment (including contraceptives) for at least 3 months before obtaining the sample. This study was approved by the Research and Ethical Committee from the Instituto Nacional de Perinatología in Mexico City, Mexico, reference number IRB00001944 and complied with the 1964 Declaration of Helsinki and its later amendments. All study participants signed informed consent for enrolment in the present study. Samples were collected from November 2016 to October 2019. A total of seven tissue biopsies from ovarian endometrioma, two tissue biopsies from peritoneum lesions, and seventeen biopsies from eutopic endometrium were obtained from women with a diagnosis of ovarian endometriosis at the time of resection surgery. Seventeen endometrial biopsies were obtained from women without endometriosis (controls). Endometrial samples from patients (eutopic) and controls were obtained using a Pipelle suction curette. Almost half of the samples were obtained during the proliferative phase of the menstrual

cycle. Once obtained, samples were immediately transferred and conserved in RNA later (Qiagen) at -20 °C until RNA and protein isolation.

2.2. RNA Isolation and RT-qPCR. RNA isolation was performed using the RNeasy Fibrous Tissue Kit (74704, Qiagen) following the manufacturer's instructions. RNA Integrity Number (RIN) was determined in an Agilent 2100 Bioanalyzer (Agilent Technologies). All samples included in the present study showed a RIN score > 7.0. RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). cDNA was obtained from 2 μg of total RNA using the M-MVL reverse transcriptase and oligo-$dT_{12\text{-}18}$ primers according to the manufacturer's instructions (28025013, Thermo Fisher Scientific). 20 ng of cDNA was amplified using the StepOnePlus PCR system (Thermo Fisher Scientific) and the Power SYBR Green PCR Master Mix (4367659, Thermo Fisher Scientific) following the manufacturer's protocol. Table 1 describes the oligonucleotides used in the present study. Negative controls with non-retrotranscribed RNA and without cDNA were included in all the experiments. Relative quantification of gene expression was performed by the $\Delta\Delta$Ct method (relative to the average ΔCT values of the control group), in which *18S* ribosomal RNA was used as the endogenous reference gene. All PCR reactions generated a single product of the expected size, as evidenced by melting curve analysis and agarose gel electrophoresis, respectively.

2.3. Protein Isolation and Western Blot. Tissues from biopsies were homogenized with a Polytron homogenizer using a T-PER buffer (FNN0071, Thermo Fisher Scientific) supplemented with a protease inhibitor cocktail (p8340, Sigma-Aldrich). Total proteins were obtained by centrifugation at 22000 g, at 4 °C for 5 min and quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

Protein samples (50 μg) were separated on a 12 % *v*/*v* SDS-PAGE at 80 V then were transferred to PVDF membranes (Millipore) in semidry conditions at room temperature at 25 V for 30 min. Membranes were blocked with 5 % *w*/*v* of bovine serum albumin (BSA) at 37 °C under constant agitation for 2 h. Then, they were incubated with the primary antibodies: goat polyclonal anti-mPRα and mPRβ (Santa Cruz Biotechnology 1 μg/mL; sc-50111 and sc-50109 [C-20]) and rabbit polyclonal anti mPRδ (Novus Biologicals 1 μg/mL; NPB1-59428) or mouse monoclonal anti γ-tubulin (Santa Cruz Biotechnology 1 μg/mL; sc-5286), at 4 °C for 48 h. Blots were then incubated with a rabbit anti-goat secondary antibody (Santa Cruz Biotechnology 1 : 10000; sc-2768) and goat anti-mouse secondary antibody (Santa Cruz Biotechnology 1 : 10000; sc-2005) conjugated to horseradish peroxidase at room temperature under constant agitation for 45 min.

Chemiluminescence signals were detected, exposing membranes to Kodak BioMax Light Films (Sigma-Aldrich) using the SuperSignal West Femto as peroxidase substrate (Thermo Scientific). The band density for the antigen-antibody complex was calculated as the area under a peak in a semiquantitative way using a 14.1-megapixel digital Canon camera (SD1400IS, Canon) and the ImageJ 1.45S software (National Institutes of Health).

2.4. Statistical Analysis. All data were analyzed and plotted using the GraphPad Prism 6.0e program (GraphPad Software, Inc., USA). Statistical analysis between comparable groups was performed using one-way ANOVA followed by Tukey's multiple comparisons test with a confidence interval of 95 %.

3. Results

3.1. Demographical and Clinical Data. There were no differences in the demographic data and most of the clinical data between patients with endometriosis and women without the disease (Table 2). As expected, the only difference between the study groups relies on the fact that all women with endometriosis manifested pelvic pain, which was not reported by the control women. Almost half of the women with endometriosis included in the present study had severe endometriosis and presented a previous endometriosis surgery. Other lesions were found in most of the patients involved in the present study, which included bilateral endometriomas, adhesions, peritoneal endometriosis, uterosacral ligaments, and endometriotic lesions in the appendix and pelvic wall. It was difficult to determine the precise phase of the menstrual cycle of some women recruited in the present study, since most of them did not provide that information in the medical record.

3.2. mPR Coding Genes Are Downregulated in Ectopic and Eutopic Tissue of Women with Endometriosis. Using RT-qPCR, we observed that the expression of *PAQR7*, *PAQR8*, *PAQR5*, and *PAQR6* genes was significantly downregulated in the eutopic endometrium of patients with endometriosis compared with the endometrium of women without the disease. Moreover, the expression of *PAQR7* and *PAQR5* was significantly reduced in ectopic endometrium (Figure 1). Interestingly, very similar expression levels of *PAQR7*, *PAQR8*, *PAQR5*, and *PAQR6* genes were observed between eutopic and ectopic endometrium.

3.3. mPRα and mPRβ Content Is Decreased in Ectopic Endometrium of Patients with Endometriosis. mPR protein content was quantified by Western blot. mPRα, mPRβ, and mPRδ content did not significantly change in the eutopic endometrium; however, in the ectopic endometrium of patients with endometriosis, the content of mPRα and mPRβ was significantly lower than that in the endometrium of healthy women (Figure 2).

4. Discussion

Endometriosis is a chronic and inflammatory disease in which specific etiology has not been elucidated. However, some molecular and biochemical alterations have been related to the development and progression of this pathology [36]. Progesterone resistance is one of the classical hallmarks of endometriosis, and although the mechanisms involved in this resistance have not been fully explained, it has been suggested that a decrease in the expression of PR-B in the

Table 1: Primers used in the present study.

Gene	Forward (5′-3′)	Reverse (5′-3′)	Reference
18S	CGCGGTTCTATTTTGTTGGT	AGTCGGCATCGTTTATGGTC	[27]
PAQR7	AACTGTCAAGGGAGGTGCTG	ATTGCATCCAGGCCATAATC	[27]
PAQR8	AGGACACAGCAAACAGGACA	GGCAACACAGGCAGGAATAA	[27]
PAQR5	CAGCTGTTTCACGTGTGTGTGATCCTG	GGACAGAAGTATGGCTCCAGCTATCTGAG	[35]
PAQR6	CTTTCATCTGGCTCCGTTTC	CTGGCAAACTGGATTACCT	Present study

Table 2: Characteristics of the women included in the present study.

Demographic/clinical characteristics	Patients (17)	Controls (17)
Age (mean, ±SD)	34.8 (±7.4)	31.9 (±9.6)
Term pregnancy (*n*)	10	11
Spontaneous abortion (*n*)	8	5
Pelvic pain (*n*)	17	0
Severe endometriosis (*n*)	8	Not applicable
Other lesions (*n*)	14	Not applicable
History of surgery for endometriosis before the present study (*n*)	8	Not applicable
Women recruited during proliferative phase (*n*)	8	6
Women recruited during secretory phase (*n*)	1	1
Women recruited at an unknown phase (*n*)	8	10

endometriotic lesions could be involved in this resistance [11]. PR is not the only receptor through which progesterone can exert its functions. mPRs belong to a group of cell surface receptors that activate nongenomic mechanisms of progesterone action in many progesterone-responsive cells and tissues [19]. In the present study, we have shown for the first time that gene expression and protein content of mPRs are decreased in the ectopic and eutopic endometrium of women with endometriosis compared to the endometrium of women without the disease, which in turn could explain another possibility for the molecular mechanisms involved in the lack of progesterone effects in this pathology.

The decrease in PR-B expression in the ectopic endometrium of patients with endometriosis only partially explains the progesterone resistance, since other factors such as alterations in progesterone signaling have been involved in this pathology [10, 11]. The results of the present study showed that both the expression of *PAQR7* and *PAQR5*, as well as the protein content of mPRα and mPRβ, was significantly reduced in the ectopic endometrium of patients with endometriosis compared to the endometrium of women without the disease. Further studies are required to clarify whether the differences between mRNA expression and protein content are due to the sample size used in the present study or to specific mechanisms of transcriptional or translational regulation. These findings, together with previous studies, suggest that the decrease in the content of mPRα, mPRβ, and PR-B in the ectopic endometrium of women with endometriosis contributes to the progesterone resistance observed in this disease.

In the present study, we were unable to confirm whether there was a dependence or correlation between eutopic and ectopic tissues in regard to mPR expression, since in most cases it was not possible to obtain both samples from the same patient. The dependence between samples with respect to the expression of mPRs is an interesting topic that deserves further investigation.

The decrease in the expression of PR-B in the eutopic endometrium of women with endometriosis remains controversial since some studies have not found this reduction [12]. In the present study, we have shown that the expression of *PAQR7*, *PAQR8*, *PAQR5*, and *PAQR6* genes is downregulated in the eutopic endometrium of patients with endometriosis compared to that of controls, which in turn could be associated with the progesterone resistance that leads to a reduced implantation capacity and increased risk of pregnancy loss observed in these patients [6]. However, we did not find a decrease in the protein content of mPRα, mPRβ, and mPRδ, which should be addressed in future studies with a larger sample size to compare our findings at the mRNA level. We were not able to detect mPRγ protein due to technical limitations. A decrease in the expression and protein content of other membrane progesterone receptors, PGRMC1 and PGRMC2, has also been reported in the eutopic endometrium of patients with endometriosis compared to women without the disease [37]. The consistent decrease in mRNA levels of genes encoding mPRs and PGRMCs in the eutopic endometrium of women with endometriosis suggests a probable role of plasma membrane progesterone receptors in the pathogenesis of the disease, which should be addressed in future functional studies since it has been proposed that endometriosis originated from eutopic endometrium cells [38].

It has been previously reported that genes encoding mPRs are differentially expressed during the menstrual cycle, which suggests that sex hormones regulate their expression. Particularly, *PAQR7* expression is higher in the secretory phase of the menstrual cycle than in the proliferative phase, whereas the expression of *PAQR5* and *PAQR9* is decreased in the secretory phase and *PAQR8* expression is not differentially expressed during the menstrual cycle [30]. Half of the samples were obtained during the proliferative phase of the menstrual cycle in the present study, and the expression of

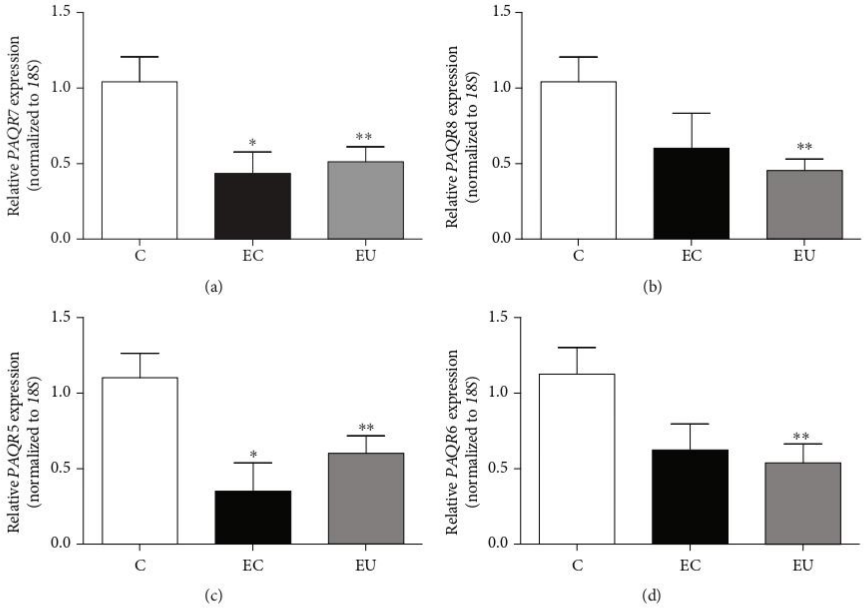

FIGURE 1: Expression levels of *PAQR* genes in ectopic and eutopic endometrium of patients with endometriosis. Total RNA was extracted from each tissue biopsy, and RT-qPCR was performed to evaluate the relative expression of (a) *PAQR7*, (b) *PAQR8*, (c) *PAQR5*, and (d) *PAQR6* genes, which was calculated by the $\Delta\Delta$Ct method. Data were normalized using *18S* transcript as a constitutive gene expression control. Results are expressed as mean ± S.E.M. Controls (C, $n = 17$), ectopic (EC, $n = 9$), and eutopic (EU, $n = 17$) endometrium of women with endometriosis. $*P < 0.05$ vs C; $**P < 0.05$ vs C.

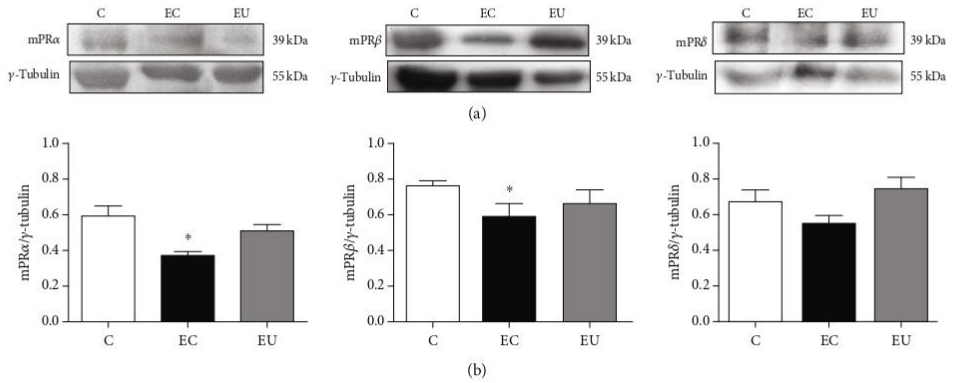

FIGURE 2: Protein content of mPRs in ectopic and eutopic endometrium of patients with endometriosis. Tissue biopsies were lysed, and proteins (50 μg) were separated by electrophoresis on 12% SDS-PAGE. Gels were transferred to PVDF membranes and then incubated with antibodies against mPRα, mPRβ, mPRδ, or γ-tubulin (used for normalization). (a) Representative images and (b) densitometric analysis of mPRα, mPRβ, or mPRδ content in controls (C), ectopic (EC), and eutopic (EU) endometrium of women with endometriosis. Results are expressed as mean ± S.E.M. of n: C = 9 (mPRα, mPRβ, and mPRδ); EU = 9 (mPRα), 6 (mPRβ), and 8 (mPRδ); and EC = 9 (mPRα and mPRβ) and 7 (mPRδ). $*P < 0.05$ vs C.

the four genes analyzed (*PAQR7*, *PAQR8*, *PAQR5*, and *PAQR6*) was decreased in the eutopic endometrium of patients with endometriosis compared to the endometrium of control women. Further studies are required to compare the expression of *PAQR* genes in patients with endometriosis during different phases of the menstrual cycle. An open

question that remains is whether the reduced expression of *PARQ* genes observed in patients with endometriosis is in part responsible for the progesterone resistance or if the latter leads to the decreased expression of those genes.

It has been recently reported that the expression of *PAQR7* and the respective protein content are decreased in endometrial cancer compared to adjacent unaffected tissue [35]. In the present study, we also found a decrease in the mRNA expression and protein content of mPRα in the ectopic endometrium of women with endometriosis, suggesting a possible connection between the alterations in endometriosis and endometrial cancer, as previously proposed [39].

5. Conclusions

The overall results of the present study demonstrate for the first time that gene expression of *PAQR7* and *PAQR5* and protein content of mPRα and mPRβ are decreased in ectopic endometrium compared to that of women without the disease and that gene expression of *PAQR7*, *PAQR8*, *PAQR5*, and *PAQR6* is decreased in eutopic endometrium. Our results reinforce the theory of progesterone resistance as part of the etiology of endometriosis. Further studies are required to elucidate the functional role of mPRs in normal, eutopic, and ectopic endometrium.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

This work was supported by the Instituto Nacional de Perinatología "Isidro Espinosa de los Reyes" (INPer; grant number 553, 212250-3000-20209-03-16).

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Sex hormones and proteins involved in brain plasticity

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Contents

Abstract

It is well known that peripheral sex steroid hormones cross the blood-brain barrier and control a broad spectrum of reproductive behaviors. However, their role in other essential brain functions was investigated since the 1980s, when the accumulation of pregnenolone and dehydroepiandrosterone in the brain of mammalian species was determined. Since then, numerous studies have demonstrated the participation of sex hormones in brain plasticity processes. Sex hormones through both genomic and non-genomic mechanisms of action are capable of inducing gene transcription or activating signaling cascades that result in the promotion of different physiological and pathological events of brain plasticity, such as remodeling or formation of dendritic spines, neurogenesis, synaptogenesis or myelination. In this chapter, we will present the effects of sex hormones and proteins involved in brain plasticity.

Vitamins and Hormones
ISSN 0083-6729
https://doi.org/10.1016/bs.vh.2020.04.002

1. Brain plasticity

1.1 What is brain plasticity?

The concept of brain plasticity (BP) is used to summarize the intrinsic abilities of the brain cells to change under physiologic or pathologic conditions in an adaptive way. Such processes occur at different timescales, from a few minutes to years. These changes involve molecular and structural characteristics of neurons and glial cells and their interactions. In this chapter, we will focus on the effects of sex steroid hormones over some plastic elements in brain synapses.

1.2 Structural and chemical changes during brain plasticity

The neural structure is continually changing. Cytoskeleton participates in regulating morphogenesis of neurons and the maintenance of mature synapses. The regulation of actin filaments (F-actin) and microtubules are essential for dendritic spines. F-actin are polymers of actin globular monomers (G-actin), their arrangement is carried out through dynamic events of polymerization and depolymerization of F-actin, which is controlled by the activity of proteins such as members of the Rho GTPases family and their effectors (Gonzalez-Billault et al., 2012; Jan & Jan, 2010), whereas microtubules are polymers of α- and β-tubulin. Besides, neuronal microtubules display a dynamic instability in neurites (Kuijpers & Hoogenraad, 2011).

The structures with more changes among life course are dendrites and axonal ends of neurons. Dendritic spines are small membranous protrusions of the neuronal surface connected to the cell by a thin neck; these can arise from the soma, dendrites, or axons, and contain the postsynaptic machinery necessary to respond to neurotransmitters (Rochefort & Konnerth, 2012).

Dendrites compute the information received from the axons of other cells. The number of dendrites, their length, diameter, and branches, limit the range and speed of information that a neuron can integrate. Moreover, dendritic spines are associated with the biochemical isolation of one synapse to another in the same dendrite (Nimchinsky, Sabatini, & Svoboda, 2002). These structures are critical because they can rapidly change their structure and even disappear due to changes in the F-actin content in a time-lapse of 2–3 min (Honkura, Matsuzaki, Noguchi, Ellis-Davies, & Kasai, 2008). Dendritic spines are of particular interest in neuroscience because they participate in synaptic plasticity and regulate mental processes such as cognition, learning, and memory (Bruel-Jungerman, Davis, & Laroche, 2007).

1.3 Brain plasticity during developmental stages

Neurogenesis and gliogenesis are the two processes that originate mature neurons and glial cells, respectively, from neural stem cells (NSC). While neurogenesis occurs early during embryonic development and early postnatal life, gliogenesis starts at late embryo developmental states and continues in postnatal life. Neurogenesis begins when neuroepithelial cells are symmetrically divided, and numerous cell layers of them are formed. The neurogenesis switch at the ventricular zone where downregulates tight junction proteins such as occludin in embryo models of chick and mice (Aaku-Saraste, Hellwig, & Huttner, 1996). Then, neuroepithelial cells give rise to radial glial cells, which eventually originates neurons, astrocytes, and oligodendrocytes in humans (Howard, Chen, & Zecevic, 2006; Mo et al., 2007). Several transcription factors regulate the differentiation of NSC in both embryo telencephalon, and adult NSC population at subventricular zone such as Pax6 or Gsx2 in mice (Brill et al., 2008; Lopez-Juarez et al., 2013). Besides, it has been proposed that a quiescent state of the adult NSC is promoted by differences between neurogenic niches during embryo life (Fuentealba, Obernier, & Alvarez-Buylla, 2012).

1.4 Brain plasticity during childhood and adulthood

In the brain at the moment of birth, each neuron has 7500 connections, and these connections will reach the maximal number at 2 years old. Half of these synapses are pruning through programmed cell death. This phenomenon is explained by the Hebb Rule, which says that only synapses that have been constantly stimulated will be preserved (Mundkur, 2005). During childhood, BP is present through several mechanisms, including the persistence of neurogenesis, elimination of some neurons, refinement of synaptic connections, and proliferation and pruning of synapses (Johnston, 2004). During several years, BP had been seen only as an event predominantly in childhood, but nowadays, thanks to improvements in the field of imaging techniques is clear that BP takes place throughout the entire life (Pauwels, Chalavi, & Swinnen, 2018).

In a newborn, the brain is very immature, and in humans, this organ is entirely mature at 20 years old. All this period is known as "critical period" because all experiences have a significant impact on learning and development, which results in a healthy behavior accordingly to a particular environment in which an individual is exposed. For example, in the case of language acquisition, the critical period is during the first 6 years of life, this ability decreases drastically after 12 years old (Vaegan & Taylor, 1979).

Although BP is maximal in childhood, it does not disappear in adulthood, it rather decreases and is activated in a more specific context (Mundkur, 2005). The most convincing data about BP in adults comes from the fact that stroke patients with severe damage have shown the highest recovery after continuous therapy (Ramanathan, Conner, & Tuszynski, 2006).

Myelination is a mechanism of BP that has been underappreciated. The first steps in myelination begin at the last period of fetal development and continue during the first days of life and into early adult life. In the same way, the environment influences BP, and evidence of improvement of myelination in a context-dependent way has been documented (Fields, 2005).

1.5 Abnormalities in brain plasticity

BP is a fundamental event for the healthy development of cognitive and social activities. Dysfunctions in any of its molecular or structural elements lead to some neurological and psychiatric impairments. Neurodegenerative diseases such as Alzheimer's (AD), Parkinson's, and Huntington's disease or neuropsychiatric disorders like schizophrenia or major depression are closely related to synaptic dysfunction (Martella, Bonsi, Johnson, & Quartarone, 2018).

AD is the principal cause of dementia in older people, and it is characterized by the loss of cholinergic neurons in the basal forebrain. Although an effective treatment for AD is not yet available, some treatments consist are using agents that enhance the action of endogenous glutamate, nonsteroidal anti-inflammatory drugs, and estrogens (Gooch & Stennett, 1996).

Tuberous sclerosis, neurofibromatosis, Fragile X syndrome, or cerebral palsy are among neurological disorders that are a concern of pediatrics with frequency. Signaling pathways associated with processes like learning and memory are linked with most of these disorders. For example, Fragile X syndrome has been associated with a deficiency in the FMRP protein, implicated in the regulation of activity-dependent protein translation during synapsis. In mouse with Fragile X syndrome, dendritic spines present abnormalities in shape and size. These mice also show alterations in signaling through AMPA receptors and a more perdurable long-term depression (Comery et al., 1997).

2. Sex hormones and brain

2.1 General mechanisms of sex hormone actions

The sex hormones (SH) progestins, estrogens, and androgens exert their actions through a classic mechanism that involves the activation of nuclear

receptors, progesterone receptor (PR), estrogen receptor (ER) and androgen receptor (AR), respectively. Also, there are non-classic mechanisms that can be activated by SH modulating different signaling pathways. These mechanisms are mediated by specific membrane progesterone receptors (mPRs), membrane estrogen receptors (mERs), membrane androgen receptors (mARs), or some neurotransmitters receptors (Camacho-Arroyo, Hansberg-Pastor, Vázquez-Martínez, & Cerbón, 2017; Lai, Yu, Zhang, & Chen, 2017) (Fig. 1).

2.2 Actions of progesterone, estradiol, and testosterone in the brain

Actions of SH in the brain can be observed at different levels, such as cell differentiation, synaptogenesis, axon guidance, myelination, neurogenesis, cell migration, among others. Together, these actions result in changes in the number of cells, the cytoarchitecture, cellular activity, synaptic connectivity, and the content of neurotransmitters (Catenaccio, Mu, & Lipton, 2016; McEwen & Milner, 2017).

Progesterone (P4), estradiol (E2), and testosterone (T) are SH derived from cholesterol mainly synthesized in the adrenal gland and the gonads, also are synthesized within the brain, and their levels vary in different areas of the brain of males and females (Kato et al., 2013; Konkle & McCarthy, 2011). Changes in BP occur as a result of the action of SH produced by the gonads and adrenal glands, as well as by the local synthesis in the brain (Do Rego et al., 2009; Mellon & Griffin, 2002).

The effects of SH in the brain can be classified into two main groups: reproductive and non-reproductive. In the case of the reproductive effects, it is known that SH levels are differentially modified throughout life, resulting in various changes in the anatomy of the brain as well as in its physiology, and consequently in sexual behavior (Hillerer, Jacobs, Fischer, & Aigner, 2014; Nugent et al., 2012). Among the non-reproductive effects are neuroprotection, learning, and memory, sleep, the course of different mental and neurological diseases in response to physical damage, or the modulation of the immune system (Colciago, Casati, Negri-Cesi, & Celotti, 2015; Klein & Flanagan, 2016).

3. Sex hormones and brain plasticity

3.1 Progesterone in brain plasticity

P4 is known for its essential role in the reproductive process and the establishment and maintenance of pregnancy. However, since the discovery of P4

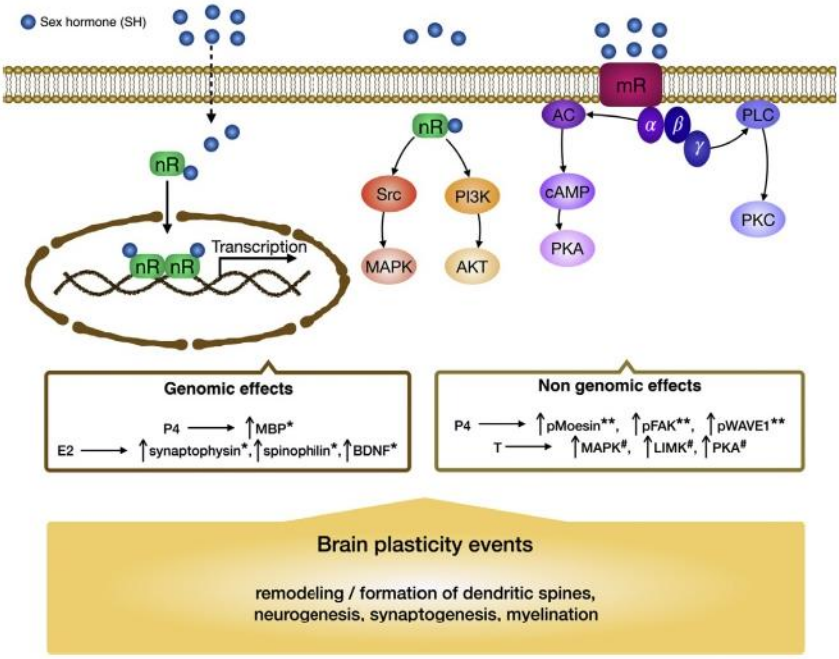

Fig. 1 General mechanism of sexual hormones action and brain plasticity. Sexual hormones such as progesterone (P4), estradiol (E2), and testosterone (T) can be synthesized de novo in the brain and exert a group of actions through their nuclear or membrane receptors (nR, mR, respectively). Once within the cell, these hormones can interact with their nR and activate the transcription of a group of genes related with specific processes of brain plasticity, for example, E2 induce the transcription of synaptophysin (synapse formation), spinophilin (formation and function of the dendritic spine) or BDNF (enhancement of dendritic spines and synaptogenesis). P4 induces the transcription of myelin basic protein (MPB; the protein component of the myelin sheat). Once activated by their respective hormones, nRs can also induce non-genomic effects through the activation of Src-MAPKs or PI3K-AKT signaling pathway. Besides, these hormones induce non-genomic effects trough their mRs, which are G-protein-coupled receptors, and therefore induce the activation of PKC or PKA. The activation of all these kinases finally conduces to the enhancement of FAK, Moesin, and WAVE phosphorylation (pFAK, pMoesin, pWAVE). All of these events lead up to the remodeling and formation of dendritic spines, neurogenesis, synaptogenesis, or myelination (* enhancement of expression, ** enhancement of phosphorylation, # signaling pathway activation).

metabolism in the CNS, plenty of studies determining its participation in BP processes has been made. Neurons and glial cells are capable of synthesizing P4 from cholesterol (Schumacher et al., 2012; Tsutsui, Ukena, Sakamoto, Okuyama, & Haraguchi, 2011). Several authors have reported the expression of PR-A/B receptors, mPRs, and PGRMC1 in neurons and glia

(Cabrera-Muñoz et al., 2009; Camacho-Arroyo, Hansberg-Pastor, Cabrera-Muñoz, Hernández-Hernández, & González-Arenas, 2012; González-Agüero, Ondarza, Gamboa-Domínguez, Cerbón, & Camacho-Arroyo, 2001; González-Orozco & Camacho-Arroyo, 2019; Valadez-Cosmes et al., 2015). The expression of PR has been associated with changes in the size and or formation of new dendritic spines, cytoskeleton reorganization, myelination, and neurogenesis (Ghoumari et al., 2003; Kato et al., 2013; Montes et al., 2019; Sanchez, Flamini, Genazzani, & Simoncini, 2013; Zhang et al., 2010).

Different research groups have studied the effect of hormones on dendritic spines. One of the most evident effects of P4 on dendritic spines has been observed during the estrous cycle in which a positive correlation between the levels of P4 in the hippocampus of rats and the density of dendritic spines has observed. Thus, when P4 locally reaches the highest levels, the highest density of dendritic spines is observed (Kato et al., 2013). Although the mechanisms by which these effects occur have not been described in detail, authors such as Sakamoto and collaborators have shown that in cerebellar Purkinje cells of newborn rats, P4 increases the number of dendritic spines in a dose-dependent manner, and the administration of a PR antagonist (RU486) blocked the effect of P4. These findings suggest that the mechanism by which these events occur is the genomic one (Sakamoto, Ukena, & Tsutsui, 2002). Besides, P4 administered for 2 h in hippocampus slices of adult male rats significantly increases the density of dendritic spines (Murakami et al., 2017), suggesting the participation of non-genomic mechanisms.

Morphological changes in dendritic spines depend on the cytoskeletal rearrangements, which can be dynamically regulated by hormones (Hansberg-Pastor, González-Arenas, Piña-Medina, & Camacho-Arroyo, 2015; Kaech, Parmar, Roelandse, Bornmann, & Matus, 2001). Although most of the SH effects on actin cytoskeleton remodeling have been observed with estrogens, P4 also induces cytoskeleton changes. Sanchez and collaborators demonstrated that P4 increases the phosphorylation as well as the distribution of the proteins moesin, focal adhesion kinase (FAK), and Wiskott-Aldrich syndrome protein family member 1 (WAVE1). Thus, promoting an increase in the density and the number of dendritic spines in primary cultures of rat cortical neurons (Sanchez et al., 2013) (Fig. 1). Another essential process in BP is myelination, in which P4 has been shown to have effects. In slices of rat cerebellum organotypic cultures, P4 by activating PR promotes in a dose-dependent manner, the expression of myelin basic protein (Ghoumari et al., 2003) (Fig. 1).

Finally, we cannot talk about P4 effects on BP without considering neurogenesis. Different groups have shown that P4 treatment improves neurogenesis during pathological conditions, such as cerebral ischemia. When P4 is administered to male mice after being subjected to an ischemia process, a higher density of new neurons in the dentate gyrus was observed (Zhang et al., 2010). These observations were confirmed by other groups. Recently by Montes and cols, they administered P4 to male rats after their induction of cerebral ischemia. It was observed a significant number of proliferating neurons in the dentate gyrus; additionally, these rats show a better performance in the Morris water maze test compared to rats that were treated with vehicle (Montes et al., 2019).

3.2 Estrogens in the brain plasticity

The first work about presence and activity of aromatase, the enzyme responsible for E2 synthesis, in the brain appeared beginning the 70s (Naftolin, Ryan, & Petro, 1971). Nowadays, it is known that E2 and aromatase have a vital role in physiological events during embryonic development such as sexual differentiation (Bakker & Baum, 2008) and through adulthood, on cognition, by its capacity to regulate synapses formation (Hara, Waters, McEwen, & Morrison, 2015). Also, E2 has been associated with neuroprotective functions in the context of some pathologic conditions such as perinatal asphyxia (Saraceno, Bellini, Garcia-Segura, & Capani, 2018).

E2 is in a sixfold higher concentration in the hippocampus than in plasma (Kretz et al., 2004). The main cells in which have been detected the aromatase expression are neurons (Yague et al., 2008). Some glial cells, including astrocytes in brain areas suffering different types of injuries, also present aromatase immunoreactivity (Azcoitia, Sierra, Veiga, & Garcia-Segura, 2003; Garcia-Segura et al., 1999).

There is a positive correlation between aromatase and expression of estrogen receptor alpha (ERα) and beta (ERβ) (Azcoitia, Yague, & Garcia-Segura, 2011). ERα and ERβ are widely distributed along human telencephalon, despite their different pattern of distribution. They are detected first during the fetal development, and their localization within the cell are different (González et al., 2007).

Neurogenesis has been mainly explored in the dentate gyrus of the hippocampus and the subventricular zone (Spalding et al., 2013). However, there are other areas such as the amygdala and hypothalamus, where newly proliferated neurons have been detected. In the hippocampus, treatments

with specifics ERα (propyl-pyrazole triol) or ERβ (diarylpropionitrile) agonists increased cell proliferation in adult ovariectomized Sprague-Dawley rats (Mazzucco et al., 2006). In the amygdala and hypothalamus of fetal rat, E2 induced the proliferation and survival of new neurons (Chowen, Torres-Alemán, & García-Segura, 1992). Various factors have been attributed to the role of E2 in the enhancement of neuronal proliferation. One of these factors is associated with the reduction of oxidative stress. In an Alzheimer's disease model, mice that received E2 during the initial stage of the disease exhibited a reduction in the levels of nitric oxide and reactive oxygen species. In addition, the treatment of E2 in these mice reduced the activation of the cytochrome-*c*/Bax/Bcl-2/caspase-3 pathway (Zheng et al., 2017). The effect of E2 in the neurogenesis has also been associated with its role through the activation of its nuclear receptors in changing the expression of genes related to different physiological processes (Aenlle, Kumar, Cui, Jackson, & Foster, 2009; Coyoy, Guerra-Araiza, & Camacho-Arroyo, 2016). Other authors have associated the proliferative effects of E2 with the activation of the extracellular ERK/MAPK signaling (Fig. 1) (Wang, Liu, & Brinton, 2008).

Synaptogenesis and synaptic plasticity are processes involving synapse formation, maintenance (stabilization), and activity-dependent refinement and elimination (Cohen-Cory, 2002). In the 90s, several studies demonstrated the role of E2 in synapses remodeling. Ovariectomy in rats for 6 days caused the decrease in density of dendritic spines (Gould, Woolley, Frankfurt, & McEwen, 1990): This effect was reverted by E2 replacement (Woolley & McEwen, 1993). E2 not only changes the density of the spine but also the shape of spines. For example, rats in the proestrus period (when E2 levels are the highest) showed more thin and fewer mushroom spines than rats in the estrus period (González-Burgos, Alejandre-Gómez, & Cervantes, 2005). Interestingly, E2 increased the frequency of multiple synapse boutons in CA1 neurons in the presynaptic terminal. These experiments were conducted in female ovariectomized rats (Woolley, Wenzel, & Schwartzkroin, 1996). Besides, other authors have demonstrated the relationship between E2 and proteins highly enriched in the presynaptic and postsynaptic areas such as synaptophysin I or spinophilin, respectively (Fig. 1). In both cases, immunoreactivity for the proteins was enhanced after E2 treatment, and this increase was reverted by ER antagonists (Rune et al., 2002). Synaptophysin is a synaptic vesicle protein ubiquitously expressed throughout the brain. Some pieces of evidence point to a role in synapse formation since the extent of synapses decreased in synaptophysin-mutant

cultured hippocampal neurons (Tarsa & Goda, 2002). Spinophilin is a dendritic spine protein closely related to the formation and function of the dendritic spine (Feng et al., 2000). E2 promotes the synthesis of neurotrophic factors such as insulin-like growth factor-1 (IGF-1) and brain-derived neurotrophic factor (BDNF) (Fig. 1). Both neurotrophic factors have been associated with the enhancement of dendritic spines (Luine & Frankfurt, 2013) and synaptogenesis (Sato, Akaishi, Matsuki, Ohno, & Nakazawa, 2007). Then, what are the molecular mechanism responsible for the rapid or non-genomic activation during events related to synaptogenesis? After binding to E2, ERs translocate to the plasma membrane. Once in the membrane, they interact with metabotropic glutamate receptors (mGluRs) and activate the ERK/MAPK pathway, which in turn induces the activation of the transcription factor cAMP response element-binding protein (CREB). These events have been associated with the increase in dendritic spines density in the rat somatosensory and prefrontal cortex (Khan, Dhandapani, Zhang, & Brann, 2013). In the hippocampus, non-genomic effects of E2 are associated with the stimulation of NMDA receptors (NMDAR), which activate PKA. Then, PKA induces the phosphorylation of ERK, finally resulting in the modulation of dendritic spines and synaptic functions (Lewis, Kerr, Orr, & Frick, 2008). Another kinase that has been linked to synapse formation by E2 action is mTOR. Once E2 interacts with its receptors, PI3K-mTOR pathway can be activated, inducing protein synthesis and actin polymerization (Briz & Baudry, 2014).

Long-term potentiation (LTP) and long-term depression (LTD) are necessaries events to consolidate BP and, therefore, learning, and short and long-term memories. LTP is characterized by structural changes that let to enlargement of a thin spine while in LTD, the changes result in shrinkage of spines. E2 has been associated with LTP induction but no with LTD. Molecular events associated with LTP involve the activation of the NMDAR, followed by postsynaptic $Ca2+$ influx and actin polymerization. Several studies have proved the central role of E2 in the induction of LTP. Grassi and cols demonstrated that inhibition of aromatase or ER blockage decreases the high-frequency stimulation-induced LTP in male rat brainstem slices (Grassi, Frondaroli, Dieni, Scarduzio, & Pettorossi, 2009). PI3K/Akt pathway has been widely associated with LTP induction (Horwood, Dufour, Laroche, & Davis, 2006; Kelly & Lynch, 2000). In this way, E2 participates in the LTP regulation through the PI3K/Akt signaling and other kinases. Hasegawa and cols found that in the hypothalamus of young adult male Wistar rats, inhibitors of Erk MAPK, PKA, PKC, PI3K, NR2B, and CaMKII blocked the E2-LTP induction (Hasegawa et al., 2015).

In addition, the mechanism by which the E2 induces LTP implicates, first, the interaction with ERα or ERβ, which in turn can activate to PI3K that stimulates ERK through Akt. Then, NMDAR is activated, and the influx of Ca2+ induces the phosphorylation of AMPA receptors and, finally, the enhancement of the LTP (Mannella, 2006). In 2004, it was demonstrated that E2 induces a lateral movement of NR1/NR2B along the synapsis in the aged hippocampus, specifically in the CA1 region of female Sprague-Dawley rats. This change in the distribution of NR1/NR2B was related to a more dynamic profile of this receptor, which is more similar to the physiologic state in a young rat (Adams, Fink, Janssen, Shah, & Morrison, 2004). Therefore, E2 can induce molecular changes not only related to expression or activation of proteins but also with their subcellular localization. As it was mentioned, the actin polymerization is an essential event for the formation and stabilization of LTP. Kramar and cols described one of the possible mechanisms by which E2 regulates the cytoskeletal remodeling in the LTP context. These authors found that latrunculin, a toxin that prevents F-actin assembly, inhibited the LTP induced by E2. Moreover, an antagonist of RhoA kinase was administered to acute hippocampal slices, and the previously plasticity effect induced by E2 was blocked. Besides, E2 induced the phosphorylation (inactivation) of cofilin, a protein that disassembles F-actin. These authors proposed that E2 can induce actin polymerization through the pathway: RhoA-ROCK-LIM kinase-cofilin (Kramar et al., 2009).

All these molecular and plastic changes allow the consolidation of a variety of physiological events fundamental to maintain a healthy status of cognitive processes. For example, in the prefrontal cortex and hypothalamus, modification in the pre-existing synapsis such as strengthening and weakening have been associated with the learning of new information (Bourne & Harris, 2007). Administration of E2 to Long-Evans hooded female rats, results in improvement of the working memory through the increase of NMDAR binding in CA1 neurons (Daniel & Dohanich, 2001). In the case of young and middle-aged females C57BL/6 mice, E2 enhanced the object recognition memory through the PI3K/Akt and ERK signaling (Fan et al., 2010). The enhancement of this kind of memory was observed in young and middle-aged but not in aged mice. What could be the causes of this difference? Aging is associated with the lowest expression of IGF-1, and the interaction between ERα and IGF-1 receptor is implicated in activating PI3K/Akt signaling pathway (Cardona-Gómez, DonCarlos, & Garcia-Segura, 2000). Thus, it has been proposed that young and middle-aged mice have a better response to E2 than aged mice.

The object recognition and memory consolidation are related to the activation of the classical Wnt signaling pathway by E2 (Fortress, Schram, Tuscher, & Frick, 2013). Zhang and cols demonstrated that E2 could stabilize the Wnt pathway through inhibition of dickkopf-1 (Dkk1), a negative regulator of the Wnt/beta-catenin pathway (Zhang, Wang, Khan, Mahesh, & Brann, 2008).

Many neurodegenerative diseases such as AD (Caricasole, 2004), Parkinson (Dun et al., 2012), stroke (Seifert-Held et al., 2011), and temporal lobe epilepsy (Busceti et al., 2007) are closely related to Dkk1 overexpression. As mentioned, E2 can stabilize the Wnt pathway through Dkk1 inhibition. Then, E2 might exert meaningful participation in neuroprotection. In a model of global cerebral ischemia, the treatment with E2 protected the hippocampus CA1 through the decrease in the expression of Dkk1, which induced the elevation of beta-catenin in the nucleus. In this study, E2 induced survivin expression after 24 and 48 h of cerebral ischemia and attenuated tau hyperphosphorylation (Zhang et al., 2008). Taking into account these facts, E2 could prevent the development of a group of neurodegenerative diseases or contribute to the recovery of patients.

3.3 Androgens in the brain plasticity

T is the most abundant male sex hormone, mainly produced in Leydig cells of the testis, in ovaries, and in the adrenal gland cortex. Conversion of T into dihydrotestosterone (DHT) is a clue in the modulation of the effects of androgens. T and its metabolite DHT are important androgens implicated in brain plasticity throughout human life. However, it is crucial to consider that some of the effects of T on learning, memory, and BP could be mediated by T aromatization to E2 by the aromatase enzyme (Bimonte-Nelson et al., 2003). In the previous section, it has been addressed the relevance of aromatase expression and the aromatization of T to E2. Effects of androgens on BP have been experimentally determined by using aromatase inhibitors or using the non-aromatizable androgen metabolite DHT. Other androgen metabolites, such as androstanediol (5α-androstane-3α,17β-diol), are considered neurosteroids (Reddy, 2008) because they are synthesized in the brain, and induce BP through different mechanisms of action. Androstanediol, for example, is a positive modulator of GABA-A receptors (Reddy & Jian, 2010).

The AR regulates the expression of target genes through their classic mechanism of action. AR also could interact with other intracellular

signaling molecules such as MAPKs (Foradori, Weiser, & Handa, 2008). T and DHT play a critical role during prenatal life, due to the activation of AR. Since prenatal life, AR is highly expressed in the hypothalamus, hippocampus, amygdala, olfactory bulb, and cortex (Genazzani, Pluchino, Freschi, Ninni, & Luisi, 2007). During fetal life of rodents, it has been reported that the dimorphic sex area of the preoptic area of females could present male characteristics, such as enhanced volume, due to T administration. This effect may be independent of proliferative events of such zone, since changes in POA/HA morphology begins after neurogenesis and at the same time of the beginning of T release from fetal testis (Clayton, Kogura, & Kraemer, 1970).

As the hippocampus is one of the most dimorphic brain areas in mammals, differences between androgenic and estrogenic effects in BP in both males and females have been broadly studied in this area. In adult male rats, T and DHT but not E2 could maintain neuronal survival in the hippocampus and dentate gyrus (Hamson et al., 2013; Spritzer & Galea, 2007), which suggest that both androgens actively participate in the survival and differentiation of neurons. Besides, AR promotes neurogenesis in the male rodent hippocampus (Okamoto et al., 2012).

Interestingly, there are reports that T or DHT but not E2, increase spine dendritic density in orchidectomized rodent males similar to that observed in intact animals. This finding contrasts with the fact that dendritic spines in females are highly augmented due to the effect of estrogens. In ovariectomized females, dendritic spines are induced by T. However, concomitant administration of T, with the aromatase inhibitor letrozole, abolish dendritic spines formation (MacLusky, Hajszan, Prange-Kiel, & Leranth, 2006; Okamoto et al., 2012). In female canaries, treatment with T increases the volume of the high vocal center (HVC), involved in the learning and control of song due to the enlargement of soma and the number of fusiform morphology of neurons. Interestingly, treatments with DHT or E2 alone did not exert similar results, although they do so when they are simultaneously administered (Yamamura, Barker, Balthazart, & Ball, 2011). All these data suggest that T or DHT actions in females or males are AR-dependent, and AR is highly expressed in the hippocampus. However, afferent inputs to the hippocampus need to be considered. For example, in female ovariectomized rats with contralateral transected fimbria/fornix area whose most afferences are located in the hippocampus, the subcutaneous treatment with E2 did not increase the dendritic spines number (Leranth, Shanabrough, & Horvath, 2000; Yamamura et al., 2011). Other reports demonstrate that

T modifies dendritic spines morphology in hippocampus slices between 0.5 and 2 h of treatment. This effect was due to the non-genomic mechanism of the AR, which involves the activation of signaling cascades of MAPKs, PKA, and LIMK (Fig. 1). DHT displays similar effects, however, within the time-lapse of 2 h, the metabolism of T to DHT or E2 did not contribute to the effects of T alone, as seen when this hormone was concomitantly administered with letrozole or the 5αR inhibitor, finasteride (Hatanaka et al., 2015).

It has been mentioned that the expression of 5αR is elevated in myelin-rich brain zones. T and DHT promote myelination in the cuprizone model of multiple sclerosis in rodents (Hussain et al., 2013). Also, in a ventral spinal cord demyelination model, T induces peripheral myelination through AR, and also promotes the recruitment of astrocytes into the demyelinated lesion and the recruitment and maturation of oligodendrocyte progenitors (Bielecki et al., 2016).

Other androgens such as dehydroepiandrosterone (DHEA) possess several mechanisms of actions additional to AR binding. DHEA is an allosteric modulator of the GABA-A receptor and thus, potentiating the antagonism of GABA in a long-term way in the hippocampus, which is related to learning and memory processes, remarkably in the older population (Wolf, Naumann, Hellhammer, & Kirschbaum, 1998).

4. Conclusions and future directions

After concluding this chapter, it is clear that sex hormones such as P4, E2, and T can be metabolized and exert a variety of functions in the CNS. Once in the brain, these hormones can interact with several kinds of receptors and activate a variety of signaling pathways, which included the regulation of gene transcription through their nuclear receptors or the activation of a plethora of signaling pathways through their mRs.

All of these actions are transcendental to confer the brain the capacity of remodeling and form dendritic spines, as well as regulate neurogenesis, synaptogenesis, or myelination. The specific mode of action of sex hormones on BP depends on sexual, age, and brain area. Based on the important role of sex hormones in the brain, they represent a promising field in the comprehension of BP, as well as in the therapeutic approach to the treatment of neurodegenerative diseases.

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