**UNIVERSIDAD AUTÓNOMA DE GUERRERO**
**UNIDAD ACADÉMICA DE CIENCIAS QUÍMICO BIOLÓGICAS**

**DOCTORADO EN CIENCIAS BIOMÉDICAS**

# "Participación de las GTPasas Rac1 y Rho en la migración e invasión de células de cáncer de mama en respuesta a leptina"

# T E S I S

**para obtener el grado de**

***Doctor en Ciencias Biomédicas***

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# UNIVERSIDAD AUTÓNOMA DE GUERRERO
## UNIDAD ACADÉMICA DE CIENCIAS QUÍMICO BIOLÓGICAS
## UNIDAD DE INVESTIGACIÓN ESPECIALIZADA EN MICROBIOLOGÍA
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ACTA DE APROBACIÓN DE TESIS

En la ciudad de Chilpancingo, Guerrero, siendo los 11 días del mes de diciembre del dos mil doce, se reunieron los miembros del Comité Tutoral designado por la Academia de Posgrado del Doctorado en Ciencias Biomédicas, para examinar la tesis titulada **"Participación de las GTPasas Rac1 y RhoA en la migración e invasión de células de cáncer de mama en respuesta a leptina"**, presentada por el alumno Miguel Ángel Mendoza Catalán, para obtener el Grado de Doctor en Ciencias Biomédicas. Después del análisis correspondiente, los miembros del comité manifiestan su aprobación de la tesis, autorizan la impresión final de la misma y aceptan que, cuando se satisfagan los requisitos señalados en el Reglamento General de Estudios de Posgrado e Investigación Vigente, se proceda a la presentación del examen de grado.

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**“Participación de las GTPasas Rac1 y Rho en la migración e invasión de células de cáncer de mama en respuesta a leptina”**

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## RESUMEN

El sobrepeso y la obesidad son considerados como factores de riesgo para el desarrollo y progresión del cáncer de mama. Se ha observado que la leptina, una hormona producida por el tejido adiposo, puede promover la proliferación y sobrevivencia de células de cáncer de mama y además puede inducir la migración e invasión de varias líneas celulares de cáncer en cultivo. Las GTPasas Rho son reguladores clave de la remodelación del citoesqueleto de actina y están involucradas en la regulación de la migración celular. Estas proteínas se encuentran sobre expresadas y sobre activadas en varios tipos de cáncer, desempeñando un papel importante en la proliferación, adhesión y migración de células cancerosas. Hasta el momento, no se ha descrito el papel de proteínas Rho en el proceso de migración e invasión inducido por leptina en células de cáncer de mama. **Objetivo:** Evaluar si la leptina induce migración e invasión de células de cáncer de mama de manera dependiente de las GTPasas Rac1 y RhoA. **Materiales y métodos:** Se utilizaron células de cáncer de mama MCF-7 y MDA-MB-231. La migración celular se evaluó mediante ensayos de cierre de herida y ensayos en cámara de Boyden. La invasión celular se evaluó en cámaras de Boyden. Se realizaron ensayos Pull-Down y Western blot para evaluar la activación de Rac1 y RhoA. La activación de la vía PI3K se evaluó midiendo la fosforilación de Akt por Western blot. Se utilizó Faloidina rhodaminada para evaluar cambios en el citoesqueleto de actina. **Resultados:** Leptina induce la activación de Rac1, RhoA y la activación de PI3K en células de cáncer de mama. El tratamiento con leptina estimuló la migración e invasión de células MCF-7 a través de una vía dependiente de PI3K/Rac1/Rho y mediante una vía dependiente de PI3K/Rho en células MDA-MB-231. La leptina indujo la actividad de MMP-2 en células MDA-MB-231 a través de una ruta dependiente de Rho, pero independiente de Rac1 y PI3K. Además, el tratamiento con leptina estimuló la migración de células no tumorales MCF1-0A de manera dependiente de PI3K/Rac1 pero independiente de Rho. **Conclusión:** La leptina estimula la migración e invasión de células de cáncer de mama a través de una vía dependiente de PI3K/Rac1-Rho.

# INTRODUCCIÓN

## Cáncer de mama

El cáncer de mama es una de las neoplasias con mayor prevalencia a nivel mundial. Aunque México no se encuentra entre los países con mayor incidencia de cáncer de mama, este padecimiento es la primera causa de muerte de mujeres por neoplasias en el país (GLOBOCAN, 2008), contribuyendo con 13.3% de las defunciones por neoplasias, superando al cáncer cérvico uterino. El número de nuevos casos de cáncer de mama ha incrementado a nivel mundial y aunque las tasas de mortalidad en Europa y Estados Unidos han disminuido en los últimos años (Morimoto et al., 2008), en otros países como en México las tasas de mortalidad han aumentado (SINAIS, 2010).

El cáncer de mama es una enfermedad maligna en la que la proliferación acelerada, desordenada y no controlada de células pertenecientes a distintos tejidos de la glándula mamaria forman un tumor que invade los tejidos vecinos y puede invadir a órganos distantes del cuerpo. El cáncer de mama puede originarse en lóbulos o ductos de la mama y de acuerdo al tamaño del tumor y la invasión a los nódulos linfáticos en la mama, éste puede clasificarse como cáncer ***in situ*** (etapa 0), **invasivo**, el cual a su vez se divide en etapas tempranas (estados I, II y IIIA) o avanzadas (IIIB/C) y finalmente **metastásico** (etapa IV) (NCI, 2012, Polyak, 2007).

Algunos de los factores de riesgo para el desarrollo del cáncer de mama incluyen edad tardía al primer embarazo, periodos cortos de lactancia, sedentarismo, consumo de alcohol, dietas altas en carbohidratos y menopausia tardía (Barnett et al., 2008). Además, existen factores genéticos de alto riesgo asociados con la susceptibilidad a desarrollar cáncer de mama, como son la presencia de mutaciones en los genes BRCA1, BRCA2 y p53 (Tan et al., 2008). Por otro lado, estudios epidemiológicos muestran una correlación entre la obesidad y la susceptibilidad a desarrollar cáncer (Reeves et al., 2007).

## Obesidad y cáncer de mama

Se ha reportado que en mujeres post-menopáusicas, el incremento de la masa corporal (obesidad) está relacionada con un aumento en el riesgo de desarrollar cáncer de mama y morir por esta patología (Montazeri et al., 2008, Reeves et al., 2007). Aunque el índice de masa corporal es un indicador adecuado de sobrepeso y obesidad en estudios clínicos, este no refleja qué cambios metabólicos inducidos por la obesidad pueden estar involucrados en la carcinogénesis. Se ha observado que el síndrome metabólico, que incluye obesidad abdominal, hipertensión, hipertrigliceridemia, colesterol alto e hiperglicemia, se asocia con el desarrollo de cáncer de mama triple negativo, en el cual las células tumorales no expresan receptor a estrógenos (ERα), receptor a progesterona (PR) ni receptor del factor de crecimiento epidermal (HER2), dificultando su tratamiento (Davis and Kaklamani, 2011). Por otra parte, se sabe que el tejido adiposo produce una gran variedad de hormonas y citocinas, conocidas como adipocinas, por lo que, la disfunción del tejido adiposo provoca un aumento en los niveles de estas adipocinas como insulina, leptina, factor de crecimiento del endotelio vascular (VEGF), interleucina 6 (IL-6) y factor de necrosis tumoral alfa (TNF-α), las cuales favorecen procesos como proliferación y sobrevivencia celular, angiogénesis y metástasis (van Kruijsdijk et al., 2009), además, este aumento de adipocinas es acompañado de la disminución de adiponectina, la cual funciona como un regulador negativo de la sobrevivencia celular (van Kruijsdijk et al., 2009), de hecho se ha observado un antagonismo entre leptina y adiponectina en cáncer de mama, en donde la leptina incrementa la proliferación celular mientras que, la adiponectina muestra un efecto anti-proliferativo y pro-apoptótico (Jarde et al., 2011). Por lo anterior, se ha propuesto que la leptina podría jugar un papel importante en la correlación entre obesidad y cáncer de mama.

La leptina es miembro de una familia de más de 50 adipocitocinas que participan en la señalización hormonal del tejido adiposo, está constituida por 16 aminoácidos y contiene un puente disulfuro necesario para su actividad biológica

(Yang and Barouch, 2007). El principal papel fisiológico de la leptina es comunicar al sistema nervioso central (SNC) la abundancia en las reservas de energía, limitar la ingesta de alimento e inducir el gasto de energía (Coll et al., 2007, Li, 2011). Por lo tanto la ausencia de leptina debería incrementar el apetito y la ingesta de alimento resultando en obesidad mórbida. Sin embargo, sólo algunos casos de obesidad severa en niños se han relacionado con deficiencia de leptina; la población obesa típicamente presenta niveles elevados de leptina, lo que puede ser explicado por el fenómeno de resistencia a la leptina (Yang and Barouch, 2007). Esta resistencia puede ser de tipo periférica o central y puede deberse a alteraciones a tres niveles: el transporte de leptina a través de la barrera sangre-cerebro (periférica), anormalidades del receptor de la leptina (periférica/central) y alteraciones en la señalización río abajo del receptor de leptina (central) (Shimizu et al., 2007).

**Señalización celular de la leptina**

La leptina es una hormona multifuncional, de la familia de las citocinas, que está involucrada en procesos como el metabolismo y la respuesta inmune. La señalización celular de la leptina inicia una vez que ésta se ha unido a su receptor. Los receptores de leptina (Ob-R) pertenecen a la familia de receptores de citocinas clase I, son producidos como isoformas derivadas del *splicing alternativo* de los transcritos de RNA del gen de leptina (*db)* y son llamados Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re y Ob-Rf. Todas las isoformas tienen un dominio extracelular común de más de 800 aminoácidos, un dominio transmembranal de 34 aminoácidos y un dominio intracelular característico de cada isoforma: 301 aminoácidos para Ob-Rb, y 34, 32 y 40 aminoácidos respectivamente para las isoformas Ob-Ra, Ob-Rc y Ob-Rd (Procaccini et al., 2009). Otra clasificación de estas isoformas es en tres clases: formas cortas (Ob-Ra, Ob-Rc, Ob-Rd, y Ob-Rf), forma larga (Ob-Rb) que es la única capaz de desencadenar la señalización río abajo, y la forma secretada (Ob-Re) que actúa como un receptor soluble y contribuye en la regulación de los niveles de leptina en el plasma, uniéndose con alta afinidad a la leptina (Sánchez et al., 2005). Debido a su amplia acción, la vía

de señalización que desencadena la leptina al unirse a la forma larga del receptor (ObRb) puede activar vías de transducción de señales intracelulares en diferentes tipos de células y/o promover comunicación cruzada con otras vías de señalización celular (Procaccini et al., 2009, Garofalo and Surmacz, 2006). La unión leptina/ObR provoca la activación de proteínas Jak2 lo cual resulta en la fosforilación de residuos de tirosina (Tyr985, 1138 y 1077) del ObR, generando sitios de acoplamiento para moléculas de señalización río abajo. Notablemente, los tres residuos de tirosina intracelulares del receptor presentan diferentes capacidades de activar la señalización río abajo. El residuo $Tyr^{985}$ se requiere para la activación de la vía Ras/Raf/ERK que favorecen la expresión de genes del ciclo celular. Los residuos $Tyr^{1077}$ y $Tyr^{1138}$ son necesarios para la fosforilación de STAT5, el residuo $Tyr^{1138}$ es necesario para la activación de STAT1 y STAT3. La vía Jak/STAT inducida por leptina es regulada negativamente por la familia de proteínas supresoras de señalización de citocinas (SOCS), la cual consta de 8 miembros: proteína con homología 2 similar a Src inducible por citocinas (CIS) y SOCS1 a SOCS7 (Procaccini et al., 2009).

Una vez unida la leptina a su receptor, la unión de STAT3 al receptor provoca la activación de STAT1, STAT5 y STAT6, la subsecuente disociación del receptor lleva a la formación de homo- o heterodímeros de STAT y su translocación al núcleo para inducir la expresión de genes como c-fos, c-jun, entre otros. Jak2 activo también puede fosforilar al sustrato del receptor de insulina 1/2 (IRS 1/2) provocando la activación de PI3K/Akt (Procaccini et al., 2009, Fruhbeck, 2006) que participa en la regulación del crecimiento y sobrevivencia celular, además de la migración celular a través de la activación de algunos miembros de la familia de GTPasas Rho (Figura 1) (Garofalo and Surmacz, 2006, Fruhbeck, 2006).

## GTPasas Rho, migración e invasión celular

La motilidad celular direccional es un proceso necesario para el desarrollo normal de un tejido así como para la respuesta inmune, sin embargo, este proceso también favorece la diseminación, invasión y metástasis de células cancerosas. La migración celular requiere de una remodelación dinámica del citoesqueleto de

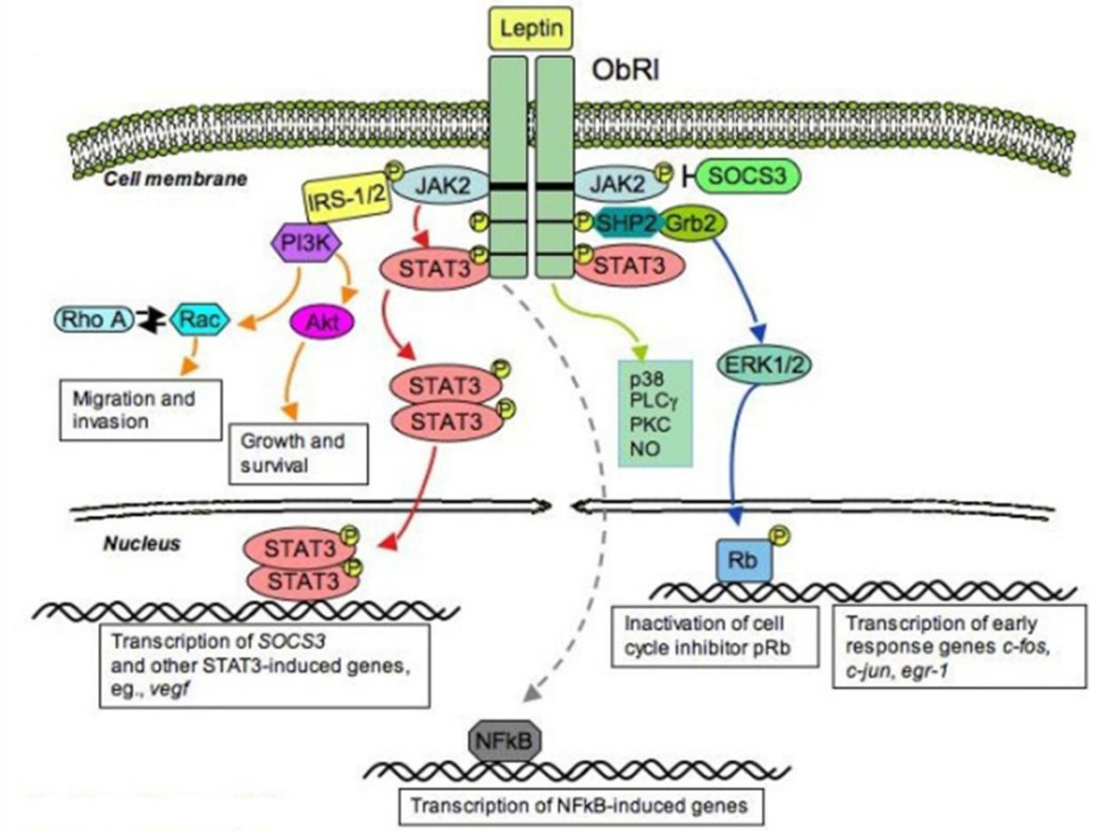


Figura 1. **Vía de señalización de la leptina.** La amplia acción de la leptina puede afectar a distintas vías intracelulares favoreciendo diferentes procesos celulares como migración, crecimiento y supervivencia, expresión de genes involucrados en respuesta inmune, entre otros (Garofalo and Surmacz, 2006).

actina y de los sitios de adhesión focal de la célula con la matriz extracelular (Jiang et al., 2009), asociado con cambios rápidos en la morfología de la membrana y la formación de estructuras de actina necesarias para que la célula pueda moverse: filopodios y lamelipodios (Insall and Jones, 2006, Parri and Chiarugi, 2010). Los lamelipodios, son amplias protrusiones de membrana, similares a hojas, los filopodios son proyecciones delgadas de membrana, ambos en el frente de avance de la célula (Jiang et al., 2009, Parri and Chiarugi, 2010). Se ha observado que la polimerización de filamentos de actina y la formación de estas protrusiones de membrana son reguladas de manera importante por GTPasas de la familia Rho. La familia de proteínas Rho son un grupo numeroso dentro de la superfamilia de proteínas Ras y consta de 5 miembros: RhoBTB, Rac, Rho, Cdc42, y Rnd-Miro, de las cuales, las mejor estudiadas estructural y funcionalmente son Rac1, RhoA y Cdc42 (Parri and Chiarugi, 2010, Spiering and Hodgson, 2010). Rac induce el

ensamble de complejos focales durante la formación de lamelipodios. Rho induce la formación de fibras de estrés, mientras que Cdc42 induce la formación de filopodios (Jiang et al., 2009). Las GTPasas funcionan como interruptores moleculares que fluctúan entre la forma inactiva (unidas a GDP) y la forma activa (unidas a GTP). El recambio entre las formas activa e inactiva de las GTPasas es controlado por dos clases de proteínas reguladoras: las proteínas Activadoras de GTPasas (GAPs: GTPase Activating Proteins) que favorecen la hidrolisis del GTP lo cual resulta en la inactivación de la GTPasa unida a GDP y las proteínas de intercambio de nucleótidos de guanina (GEFs: Guanine nucleotide Exchange Factors) que incorporan GTP activando a la GTPasa (Bos et al., 2007).

Las células tumorales exhiben dos tipos diferentes de movilidad celular: el movimiento tipo mesénquima que requiere orientación celular y el tipo ameboide que es un movimiento no orientado, la capacidad de cambiar de un tipo de movimiento a otro se debe a la plasticidad estructural de las células (Sanz-Moreno et al., 2008, Guck et al., 2010). Se ha demostrado que el movimiento tipo mesénquima es predominantemente regulado por Rac1, mientras que el movimiento tipo ameboide es controlado por RhoA, por lo que existe una regulación recíproca entre las GTPasas Rho y Rac1; cuando Rac1 se encuentra activo favorece la formación de especies reactivas de oxígeno las cuales inducen la fosforilación de p190RhoGAP el cual inactiva a RhoA. A su vez, cuando RhoA se encuentra activo es capaz de inhibir a Rac1 a través de la proteína ARHGAP2 (Parri and Chiarugi, 2010, Sanz-Moreno et al., 2008). Se ha observado que con frecuencia las células tumorales de mama exhiben transición epitelio mesénquima (TEM) (Radisky and Radisky, 2010). El modelo aceptado para el mecanismo de migración celular tipo mesénquima se resume en cuatro pasos: 1) Formación de lamelipodios en la dirección del movimiento (borde líder), 2) anclaje de la membrana celular al sustrato subyacente a través de adhesiones focales en el borde líder, 3) contracción de la red de fibras de estrés, la cual genera suficiente tensión para arrastrar a la célula hacia adelante, 4) desensamble de las adhesiones focales en el borde posterior de la célula, permitiendo a la célula retraerse y desplazarse en la dirección de la migración (Figura 2).

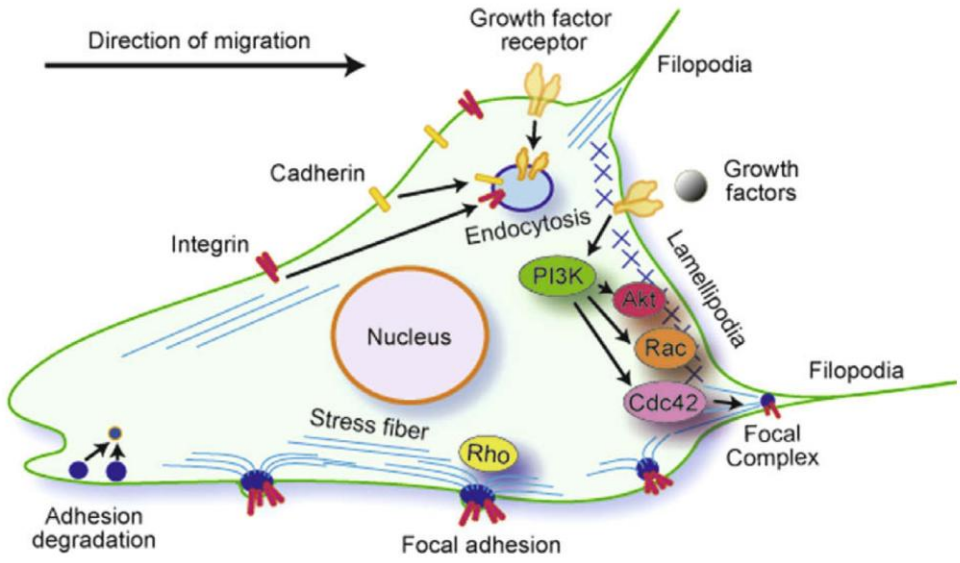


Figura 2. **Esquema del proceso de migración en células de cáncer de mama.** La formación de lamelipodios, filopodios y fibras de estrés necesarios para el proceso de migración celular es regulado por proteínas GTPasas Rho (Jiang et al., 2009).

Por otra parte, se sabe que E-cadherina e integrinas participan en la regulación de adhesiones célula-célula y célula- matriz extracelular (MEC), eventos necesarios en el proceso de migración celular (Figura 2). En carcinomas epiteliales, frecuentemente se ha observado la pérdida de adhesiones célula-célula y célula-MEC, como consecuencia de la disminución en la expresión de E-cadherina que resulta en el aumento de N-cadherina, conocida como cadherina mesenquimal, lo cual disminuye la afinidad por las células vecinas favoreciendo la migración e invasión celular. Otra proteína que se ha asociado con la reorganización del citoesqueleto de actina y motilidad de células de cáncer de mama es Akt, un efector de PI3K, y se ha observado participa en una gran variedad de procesos celulares como sobrevivencia, crecimiento, proliferación, angiogénesis, metabolismo celular, así como migración e invasión celular (Jiang et al., 2009). Como se mencionó anteriormente, PI3K/Akt es una de las vías de señalización que se enciende en respuesta al estímulo con leptina.

Si bien el mecanismo de migración permite el movimiento lateral de las células, las células tumorales pueden adquirir la capacidad de invasión, proceso que involucra la degradación de la MEC creando espacio para el crecimiento y movimiento del tumor. Se ha observado que cortactina, una proteína reguladora de la nucleación

de actina y frecuentemente sobre expresada en cáncer, regula cambios en el citoesqueleto de actina que promueven la formación de estructuras especializadas en la degradación de MEC: invadopodios (Kirkbride et al., 2011). Los invadopodios tienen una elevada actividad proteolítica y se encuentran sobre la superficie basal de las células invasivas, son filamentos de actina que se entrecruzan en una red tridimensional y son los responsables de la invasión en la MEC (Chhabra and Higgs, 2007). Un evento clave para la invasión de células tumorales es la sobreexpresión y secreción de metaloproteinasas de matriz extracelular (MMPs) que rompen la barrera de tejido conectivo que comprende colágenos, lamininas, fibronectina, vitronectina y heparan sulfato proteoglicanos modificando la MEC, así como los contactos célula-MEC y célula-célula facilitando el libre movimiento de las células tumorales en el tejido a través de protrusiones de membrana (Radisky and Radisky, 2010, Yilmaz and Christofori, 2010).

**Leptina y cáncer de mama**

Estudios epidemiológicos muestran que niveles elevados de leptina sérica están correlacionados con un mal pronóstico de cáncer de mama (Cust et al., 2008). Se ha reportado que los niveles de RNAm y proteína de leptina y ObR es más alta en células tumorales y estos se relacionan con un fenotipo más diferenciado de cáncer de mama favoreciendo su proliferación y capacidad invasiva (Revillion et al., 2006, Ishikawa et al., 2004). En otros estudios se ha observado que los niveles de leptina y ObR fueron significativamente más altos en las biopsias de pacientes con cáncer *in situ* que en las de cáncer invasivo, sin embargo la alta expresión de leptina se asoció significativamente con la expresión del marcador de proliferación celular ki-67 (Jeong et al., 2011). Por otro lado, estudios en modelos experimentales muestran que la leptina estimula la proliferación de células derivadas de cáncer de mama en cultivo (Mauro et al., 2007), y promueve el crecimiento tumoral de injertos de células MCF7 en ratones (Gonzalez et al., 2006). Además, se ha demostrado que la leptina sinergiza con otras vías de señalización que regulan procesos celulares como la proliferación, migración, angiogénesis y sobrevivencia (Lawrence et al., 2012, Cirillo et al., 2008, Ando and

Catalano, 2011). En células de cáncer de mama, se ha observado que existe comunicación de la vía de señalización de leptina, IL-1 y Notch favoreciendo la proliferación y migración celular así como la expresión del factor de crecimiento del endotelio vascular VEGF (Guo and Gonzalez-Perez, 2011), también se reportado comunicación cruzada entre leptina y el receptor del factor de crecimiento epidermal Her2, lo cual puede contribuir al aumento de la actividad de Her2 reduciendo la sensibilidad al tratamiento dirigido contra este receptor (Fiorio et al., 2008). En células MCF7 se ha reportado que el tratamiento crónico con leptina sensibiliza a las células al posterior tratamiento con estrógenos, aumentando significativamente la proliferación celular, lo cual se acompaña de un aumento en la expresión del receptor a estrógenos alfa (ER-α), de igual forma, en biopsias de pacientes con cáncer de mama la expresión de ObR se correlacionó significativamente con la expresión del ER-α (Valle et al., 2011a, Fusco et al., 2010).

Por otra parte, mediante estudios de microarreglos se identificaron más de sesenta y cuatro genes regulados por leptina, entre estos, el tratamiento con leptina suprimió la expresión de TGF-β, un represor del ciclo celular, entre los que fueron positivamente regulados por leptina se encuentra la ciclina D1, factores de crecimiento de tejido conectivo *villina 2* y *basignina* que se sabe favorecen el crecimiento del tumor así como los genes antiapoptóticos BCL2 y *survivina* (Perera et al., 2008). Además, utilizando un análisis proteómico, se encontraron treinta proteínas expresadas diferencialmente después del tratamiento con leptina, entre estas estaban proteínas previamente implicadas en cáncer de mama como cathepsina D, hsp27 y proteínas reguladoras de la vía de señalización Ras, así como proteínas involucradas en otras funciones celulares como respuesta a estrés y remodelación del citoesqueleto (Valle et al., 2011b).

**Leptina, GTPasas Rho y migración celular en cáncer de mama**

Se ha demostrado que la leptina en combinación con insulina (Saxena et al., 2008), Notch1 y IL-1 (Guo and Gonzalez-Perez, 2011) induce la migración de células de cáncer de mama, sin embargo no se han descrito los mecanismos que

regulan este proceso. Por otra parte, se ha demostrado que la leptina estimula la migración de células endoteliales HUVEC (Goetze et al., 2002) y de células de condrosarcoma (Yang et al., 2009), e induce la migración e invasión de células de carcinoma hepatocelular, de células de cáncer de próstata (Frankenberry et al., 2004), de células de glioma (Yeh et al., 2008) y de células de carcinoma de colon (Ratke et al., 2010). En células tumorales de riñón y colon se ha demostrado que la leptina induce la migración celular a través de una vía de señalización dependiente de PI3K, GTPasas Rac y Rho (Attoub et al., 2000, Ratke et al., 2010).

Como se mencionó anteriormente, se sabe que la migración celular es regulada por GTPasas de la familia Rho (Jiang et al., 2009). La GTPasa Rac1 ha sido la más estudiada al respecto de su participación en el proceso de migración celular, además de que se ha encontrado sobre expresada en varios tipos de cáncer incluyendo el de mama (Wertheimer et al., 2012). Se ha reportado a Rac1 como una proteína necesaria para la migración de células tumorales de riñón y colon (Attoub et al., 2000, Ratke et al., 2010, Espina et al., 2008) así como de cáncer de mama (Li et al., 2011, Johnson et al., 2010, Morimura and Takahashi, 2011), incluso se ha propuesto a la inhibición de Rac1 como blanco terapéutico en cáncer de mama con el fin de disminuir los niveles del receptor a estrógenos (Rosenblatt et al., 2011), inducir el arresto en la fase G1 del ciclo celular o apoptosis (Yoshida et al., 2010), restablecer la sensibilidad de las células tumorales al tratamiento con trastuzumab (Dokmanovic et al., 2009) así como para bloquear la motilidad e invasión de las células tumorales de mama (Katz et al., 2012). Sin embargo, se necesitan más evidencias que confirmen la efectividad de la inhibición de Rac1 como blanco terapéutico para esta patología.

En cáncer de mama no se ha descrito la participación de GTPasas Rho en la motilidad celular inducida por leptina. El conocer qué proteínas están implicadas en los procesos de migración e invasión celular inducidos por leptina favoreciendo la progresión del cáncer de mama, es crucial para entender los mecanismos de esta enfermedad en pacientes obesas que se sabe presentan niveles elevados de leptina sérica y que se ha reportado presentan tumores más agresivos que una

mujer delgada con cáncer de mama, contribuyendo de esta manera en la búsqueda de nuevos marcadores y/o blancos terapéuticos para esta enfermedad. Por lo anterior, el propósito de este estudio fue evaluar la participación de las GTPasas Rac1 y Rho en la migración e invasión de líneas celulares de cáncer de mama en respuesta al tratamiento con leptina.

## CAPÍTULO I

# Leptin-induced migration and invasion of breast cancer cells is mediated by Rac1/Rho activation.

# Leptin-induced migration and invasion of breast cancer cells is mediated by Rac1/Rho activation.

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## Abstract

Breast cancer is the leading cause of death by malignant neoplasia in women worldwide. It has been suggested that obesity is a risk factor for developing breast cancer. Leptin, an adipocyte-synthetized hormone, stimulates migration of several cancer cell lines in culture, through activation of Rho GTPases. Rho GTPases are key regulators of cell migration and invasion. The aim of this study was to investigate if leptin induces breast cancer cell migration and invasion through activation of RhoGTPases. MCF7 and MDA-MD-231 cells were stimulated with leptin and Rac1 or Rho inhibitors. Cell migration was determined by scratch wound assays or Boyden chambers assays. Invasion was determined using Boyden chambers with matrigel. Actin cytoskeleton was visualized using Rhodamine-coupled phalloidin. We found that leptin induces migration and invasion in both cell lines. Blocking Rac1 activation inhibits leptin-induced migration/invasion in MCF7 cells, whereas Rho inactivation inhibits migration and invasion of both cell lines. In conclusion, Rac1 and Rho proteins play differential roles in leptin-induced migration and invasion of low invasive and highly invasive breast cancer cells.

## Introduction

Breast cancer is the leading cause of death by malignant neoplasia in women worldwide (GLOBOCAN, 2008). Epidemiological data suggest that overweight and obesity are associated with increased risk of developing breast cancer (van Kruijsdijk et al., 2009, Montazeri et al., 2008, Davis and Kaklamani, 2011). Experimental data suggest that leptin, an adipocyte-synthetized hormone (Li, 2011), can promote tumor progression through regulating multiple pathways (Lawrence et al., 2012, Amjadi et al., 2011, Ellerhorst et al., 2010, Fava et al., 2008, Saxena et al., 2007). It has been demonstrated that in breast cancer cells in culture leptin treatment affects gene expression (Valle et al., 2011b, Perera et al., 2008), sensitizes cells to estrogen-induced proliferation (Valle et al., 2011a), promotes tumor

cell proliferation, angiogenesis, cell-cycle progression, and cell survival (Jarde et al., 2011, Ando and Catalano, 2011). Moreover, crosstalk signaling has been demonstrated between leptin receptor and several receptors including HER2 (Fiorio et al., 2008, Soma et al., 2008), Notch-IL1 (Guo and Gonzalez-Perez, 2011) and estrogen receptor-α (Fusco et al., 2010). Additionally, it has been shown that leptin stimulates migration of several cancer cell lines in culture (Huang et al., 2011, Ratke et al., 2010, Attoub et al., 2000, Jaffe and Schwartz, 2008, Barone et al., 2012).

Rho GTPases are molecular switches that regulate a large number of cellular functions such as actin cytoskeletal dynamics, cell polarity, migration, cell transformation, and invasion (Parri and Chiarugi, 2010, Spiering and Hodgson, 2010). RhoA, Rac1 and Cdc42, the best studied members of the Rho family, are well known for their regulatory role on reorganization of the actin cytoskeleton (Spiering and Hodgson, 2010). Overexpression or hyperactivation of these proteins has been reported in several types of human tumors (Karlsson et al., 2009). Moreover, it has been shown that Rac1 and Rho proteins play important roles in tumorigenesis, by regulating cell proliferation, adhesion and migration (Johnson et al., 2010, Wu et al., 2010, Jiang et al., 2009, Parri and Chiarugi, 2010, Wertheimer et al., 2012). In breast cancer cells in culture, Rac1 and RhoA regulate proliferation in response to several stimuli (Burguera et al., 2006, Ogunwobi and Beales, 2008, Soma et al., 2008, Catalano et al., 2009, Lautenbach et al., 2009). Interestingly, it has been demonstrated that leptin-induced migration of kidney and colonic cancer cells in culture is dependent on the activation of Rho GTPases (Attoub et al., 2000, Jaffe and Schwartz, 2008, Horiguchi et al., 2006).

In the present study, we investigated whether leptin promotes cell migration and invasion of breast cancer cells in culture through activation of Rac1 and RhoA GTPases.

## Material and methods

### Reagents

Rhodamine-Phalloidine and ProLong® Gold Antifade Reagent with DAPI were purchased from Invitrogen; The Rac1 inhibitor NSC 23766 was obtained from Santa Cruz Biotechnology; Leptin human recombinant, The Rho inhibitor ADP-ribosyltransferase C3 from *Clostridium botulinum* (C3 transferase), cytosine β-D-arabinofuranoside hydrochloride (AraC), and Dulbecco's modified Eagle´s medium (DMEM)/Ham F12 (DMEM) were purchased from Sigma-Aldrich. Crystal violet and extraction buffer were purchased of Millipore. FBS was purchased from Byproductos SA de CV (Mexico). Penicillin/streptomycin was purchased from Gibco.

### Cell culture

MCF7 and MDA-MB-231 breast cancer cell lines were cultured in Dulbecco's modified Eagle´s medium (DMEM)/Ham F12 (1:1) supplemented with 3.2 g/L sodium bicarbonate, 5% fetal bovine serum (FBS) and 1% antibiotics in a humidified atmosphere containing 5% $CO_2$ at 37°C.

### Rac1 and RhoA activation assays

Rac1 and Rho activation were evaluated by using the Rac1 or Rho Activation Assay Kit (Cytoskeleton, Inc.), respectively, according to manufacturer's instructions. Briefly, MCF7 and MDA-MB-231 cells were grown until 60% confluence in p100 culture dishes (Sarstedt) and treated with leptin for different times. Cells were lysed and 1mg total protein lysates was pulled-down with GST-PAK-PBD beads (Rac1) or GST-Rhotekin-RBD (Rho) followed by Western blot analysis using anti-Rac1 or anti-RhoA antibodies (Cytoskeleton).

### Scratch-wound assay

MCF7 and MDA-MB-231 cells were grown until confluence in 6-well plates and starved for

24 h in serum-free DMEM. After starvation, cells were scratch-wounded using a sterile spatula, detached cells were removed by washing twice with PBS and the remaining cells were incubated for 24 h or 48 h with or without leptin in the presence of Rac1 or Rho inhibitors. 50 µM cytosine β-D-arabinofuranoside hydrochloride (AraC) were added to each well to inhibit cell proliferation. To assess cell migration, five photographs from each well were taken at 0h, 24h and 48h under an Olympus microscope using the 10x objective. Cell migration was evaluated by measuring the migrated distance from initiation of wound to advancing front cells on five different points along the wound on each picture, using National Institute Health ImageJ software.

**Chemotactic migration assay**

Chemotactic cell migration was evaluated by using QCM 24-well Colorimetric Cell migration assay (Millipore, ECM508) according to manufacturer's instructions. Briefly, $25x10^4$ cells were resuspended in 200 µl of serum-free DMEM and added on the top of the chamber. 500 µl of DMEM medium with leptin, with or without Rac1 or Rho inhibitors, were added in the lower chamber. Cells were allowed to migrate for 24 h. Non-migrated cells were removed from the upper side of the chamber (filter) by cotton swabs, migrated cells (cells on the lower side of the filter) were fixed and stained with crystal violet, washed in distilled $H_2O$, crystal violet was eluted using an extraction buffer, and the optical density was measured at 600nm. All experiments were repeated at least three times.

**Invasion assay**

160 µl of DMEM with 0.1 % BSA and leptin were added to the bottom chamber of a 12-well Boyden chamber (Neuroprobe AA12) with polycarbonate filters coated with Matrigel (BD Biosciences). $1x10^5$ cells in 100 µl of serum-free medium with or without leptin plus 25 µM NSC 23766 or 1 ng/ml C3-transferase were placed in the top of the chamber, and incubated

at 37°C during 24 h. Non-invaded cells were removed from the upper side of the chamber (filter) by cotton swabs, and the invaded cells were stained with crystal violet, washed twice with PBS, the dye was eluted using extraction buffer, and the optical density was measured at 600nm. All experiments were repeated at least three times.

**F-actin Staining**

$5x10^4$ MCF-7 or MDA-MB-231 cells were plated on glass coverslips in 6-well culture plates. Cells were maintained in DMEM supplemented with 5% FBS at $37^oC$ in a 5% $CO_2$ atmosphere. Cells were starved in serum-free medium during 24 h and then treated with leptin plus Rac1 or Rho inhibitors. 48 h after plating, cells were fixed in ice-cold acetone, blocked in PBS + 1% BSA for 30 min, and incubated with Rhodamine-phalloidine (1:1,000) for 1 h at room temperature. Samples were mounted using ProLong® Gold Antifade Reagent with DAPI. Cells were visualized with an Olympus BX43 microscope fitted with a cooled-camera (EvolutionMP color, MediaCybernetics) using the Q-capture pro 7 software. Images were processed using ImageJ software.

**Statistical analysis**

Results are expressed as mean. Data were statistically analyzed using t-student test using *GraphPad Prism v5.03* software. A result was considered statistically significant when p value was $<0.05$.

## Results

### Inhibition of Rac1 impairs leptin-induced migration in MCF7 cells, but not in MDA-MB-231 cells.

It has been shown that leptin can induce migration of cancer cells trough Rac1 and RhoA activation. To test if leptin treatment can activate Rac1 in MCF7 and MBA-MD-231 cells, cells were incubated in the presence of leptin, and Rac1 activation was measured as described under materials and methods. As shown in Figure 1, leptin induces Rac1 activation in both MCF7 and MDA-MB-231 cells. In MCF7 cells the maximum activity was observed 15 min after treatment, whereas in MDA-MB-231 cells the maximum activation was observed after 30 min. We next determined the effect of leptin treatment on cell migration using scratch wound-healing assays. We found that leptin induces cell migration in both cell lines in a concentration-dependent manner (Supplementary figure 1). To determine if Rac1 activation is required for leptin-induced migration, we performed scratch assays in the presence of the Rac1 inhibitor NSC23766. As show in Figure 2 (A,B), inhibition of Rac1 results in a decrease in leptin-induced migration of MFC7 cells. In contrast, in MDA-MB-231 cells, inhibition of Rac1 resulted in an increase in leptin-induced migration (Figure 2: C, D).

**Inhibition of RhoA impairs leptin-induced migration in MCF7 and MDA-MB-231 cells.** As Rac1 inhibition resulted in increased leptin-induced migration in MDA-MB-231 cells, we tested whether leptin-induced migration may be dependent on RhoA activation. As shown in Figure 3, leptin induces RhoA activation in MDA-MB-231 cells after 30 min of treatment. Moreover, we found that inhibition of Rho activation using the specific Rho inhibitor, C3 Botulinum neurotoxin, resulted in a decrease on cell migration in both cell lines (Figure 4). To further validate our results, we evaluated the effect of leptin and the Rac1 and Rho inhibitors in cell migration using a chemotactic cell migration assay. In agreement with our data from scratch assays, Rac1 inhibition reduces leptin-induced migration of MCF7 cells,

but not of MDA-MB-231 cells, whereas Rho inhibition reduces leptin-induced migration of both cell lines (Figure 5).

**Effect of Rac1 and Rho inhibition on leptin-induced invasion of MCF7 and MDA-MB-231 cells.**

We next investigated the effect of leptin on the invasive potential of MCF7 and MDA-MB-231 cell lines, and the role of Rac1 and Rho GTPases in this process. As shown in Figure 6, leptin treatment increases invasiveness of both breast cancer cell lines. Similarly to our results from cell migration assays, Rac1 inhibition results in a decrease in leptin-induced invasion in MCF7 cells whereas in MDA-MB-231 cells, Rac1 inhibition results in enhanced invasion. In contrast, Rho inhibition impairs leptin-induced invasion of both MCF7 and MDA-MB-231 cells (Figure 6).

**Leptin induces changes in actin cytoskeleton reorganization through Rac1 and Rho activation.**

Rho GTPases are well known for their role in regulating actin cytoskeleton dynamics and they play important roles in migration and invasion. To investigate the effect of leptin treatment on actin cytoskeleton reorganization in MCF7 and MDA-MB-231 cells, cells were treated with leptin in the presence or absence of Rac1 or Rho inhibitors, and actin cytoskeleton was stained as described under materials and methods. Untreated MCF7 cells exhibited an epithelial morphology with F-actin localized along the cytoplasm, with strong staining at the cell borders. After leptin treatment, the cells acquire an enlarged morphology, exhibiting long membrane protrusions rich in F-actin fibers, resembling lamellipodia (Figure 7). Rac1 inhibition prevents leptin-induced morphological changes and actin relocalization. Similarly, inhibition of Rho impairs leptin-induced lamellipodia formation and interestingly, resulted in redistribution of F-actin fibers along the cytoplasm and perinuclear F-actin was

found. On the other hand, in untreated MDA-MB-231 cells, which exhibit an enlarged fibroblastic phenotype, leptin treatment resulted in the formation of long and thin membrane protrusions. Rac1 inhibition resulted in the formation of membrane protrusion longer and thinner than the ones observed in leptin-treated cells. In contrast, Rho inhibition resulted in rounded cells and an accumulation of F-actin fibers in the cytoplasm and at the perinuclear region.

**Discussion**

Obesity is a very important risk factor for the development and progression of several types of cancer, including breast cancer (van Kruijsdijk et al., 2009, Davis and Kaklamani, 2011). Recently, it has been shown that leptin, an adipocyte-synthetized hormone, promotes progression of breast cancer by inducing cell migration and invasion (Barone et al., 2012). On the other hand, it has been reported that migration and invasion of breast cancer cells is regulated by small GTPases of the Rho family (Wu et al., 2010, Jiang et al., 2009, Parri and Chiarugi, 2010). Interestingly, it has been shown that leptin can induce migration of cancer cells through activation of Rac1 and Rho GTPases (Attoub et al., 2000, Jaffe and Schwartz, 2008). However, the mechanisms by which leptin promotes cell motility on breast cancer cells are not well understood.

Here, using wound-healing assays, we demonstrated that leptin induces cell migration and invasion of breast cancer cells through activation of Rho GTPases. We observed that leptin induced Rac1 activation in both MCF7 and MDA-MB-231 cells. Chemical inhibition of Rac1 abrogated the leptin-induced migration and invasion of poorly invasive MCF7 cells, but increased the migration and invasion of highly invasive MDA-MB-231 cells. These observations are in agreement with the report from Zuo et al (2006), in which they show that blocking Rac1 function (either with a chemical inhibitor NSC23766 or by siRNA) in non-metastatic MCF7 and T47D cells results in a decrease in migration, whereas in highly

metastatic MDA-MB-435, MDA-MB-231 and C3L5 cells, Rac1 inhibition stimulates cell migration (Zuo et al., 2006). In contrast, Moon and Koh et al (2011) found that upon EGF stimulation, invasiveness and Ras/Rac1 activation were significantly increased in MDA-MB-231 and Hs578T cells, but not in MDA-MB-453 and T47D cells (Koh and Moon, 2011). Moreover, Morimura and Takahashi (2011) found that depletion of Rac1 in MDA-MB-231 cells abrogated the IGF-I-induced invasion in these cells (Morimura and Takahashi, 2011). These evidences suggest that, the role of Rac1 in breast cancer migration may be dependent on the initiating stimuli.

On the other hand, we observed that Rho inhibition abrogates the migration of both, poorly invasive MCF7 cells and highly invasive MDA-MB-231 cells. This result is comparable to study by Zuo et al., (2006), in which the RhoA inhibition decreased the migration index of MDA-MB-231 and Hs578T cells (Zuo et al., 2006). It is already known that tumor cells exhibit two different forms of individual cell movement, a mesenchymal-type and an ameboid movement (Parri and Chiarugi, 2010), and that tumor cells can switch from one type of migration to another. It has been demonstrated that in melanoma cells, mesenchymal-type movement is driven by activation of Rac1 through a complex containing NEDD9 (metastasis gene) and DOCK3, a Rac guanine nucleotide exchange factor. Conversely during ameboid movement RhoA/ROCK signaling activates ARH-GAP22, which in turn inactivates Rac1, suppressing the mesenchymal-type movement (Sanz-Moreno et al., 2008). On the other hand, Vega et al (2011), using a 2D and 3D invasion assay observed an increase in invasive behavior of MDA-MB-231 and PC3 cells depleted of RhoA, and the silencing of RhoC reduced the cell invasion (Vega et al., 2011). Furthermore, Deakin et al (2011) using 3D extracellular matrices, demonstrated that paxillin and Hic-5 regulate of breast cancer cell lung metastasis modulating morphology of MDA-MB-231 cells. Interestingly, the cells depleted of paxillin by RNA interference displayed a highly mesenchymal morphology and

Rac1 activity was increased, whereas cells depleted of Hic-5 induced an ameboid phenotype and RhoA activity was increased (Deakin and Turner, 2011). This observation by Deakin et al., and the reciprocal regulation between Rac1 and RhoA during mesenchymal or ameboid motility observed by Sanz-Moreno et al., (Sanz-Moreno et al., 2008) could explain the highly elongated phenotype and the increase in motility of Rac1-depleted MDA-MB-231 cells, perhaps because an increase in the RhoA activity. A disadvantage of our study is that, we performed migration assays in 2D, in which is not possible to observe switch between mesenchymal-type and amoeboid movement. Moreover, we used the Rho-inhibitor C3 transferase, which inactivate the three Rho-isoforms (RhoA, -B and C), making impossible to discriminate a differential role of RhoA and RhoC leptin induced cell invasion.

In conclusion, leptin-induced migration and invasion of MCF-7 cells is dependent of Rac1/Rho activation, whereas in MDA-MB-231, leptin-induced migration depends on Rho activation.

**Author's contributions**

MAMC, performed the experimental procedures and analyzed the data; NNT participated in data analysis and manuscript preparation. ICM, participated in Rac1 activation assay and Boyden chamber migration assays, ECHA, MER, IPR and AAL, participated in data acquisition and analysis and manuscript preparation. ECS conceived the project, analyzed the data, supervised the whole project and wrote the manuscript. All authors read and approved the final version of the manuscript.

**Conflict of interest**

Authors declare no conflict of interest.

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**Figure 1.**

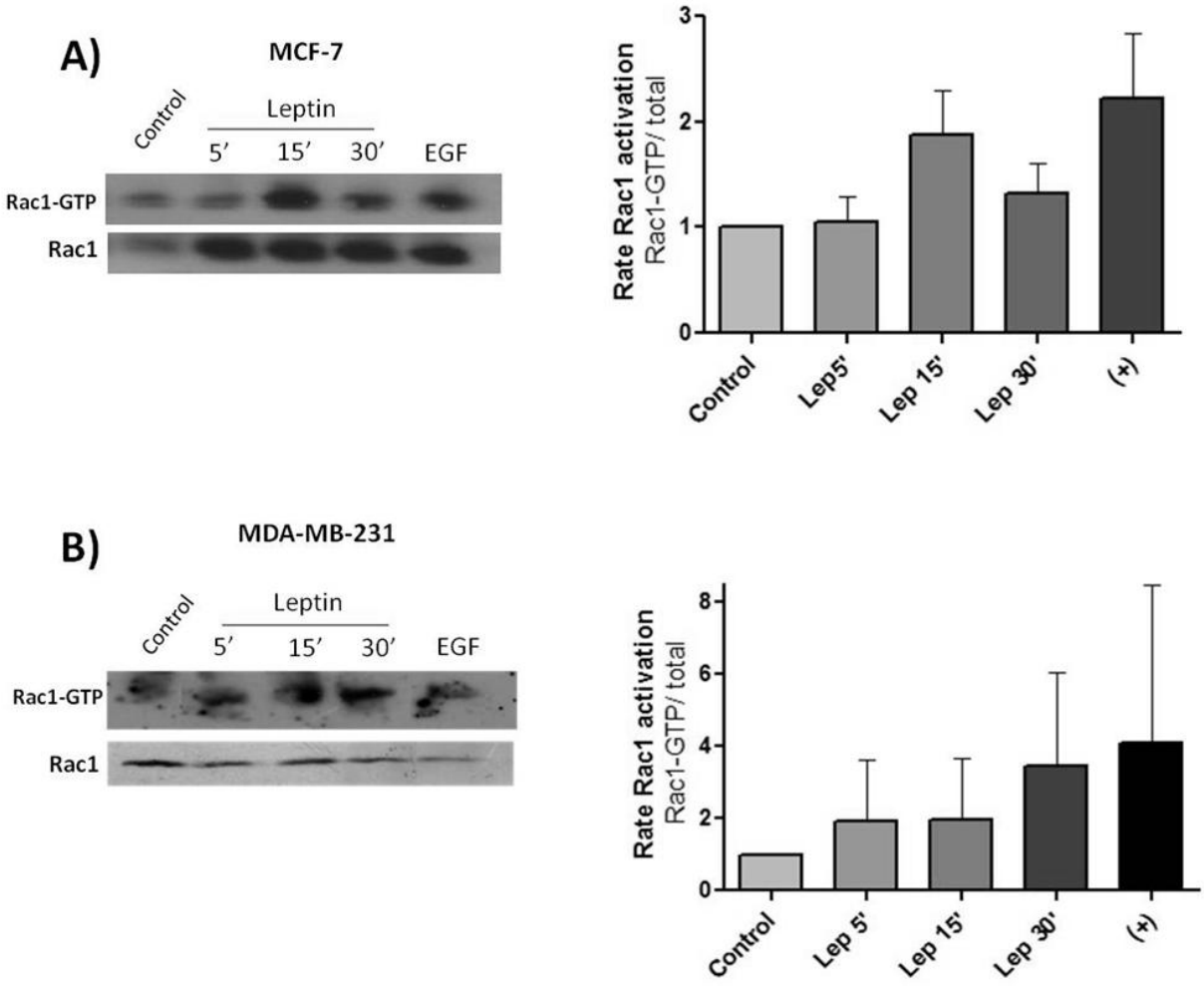



**Figure 1**. **Leptin induces the activation of Rac1 in MCF7 and MDA-MB-231 cells.** Time-course of Rac1 activation in MCF7 (A) and MDA-MB-231 (B) cells in response to 400 ng/ml and 50 ng/ml of leptin, respectively. Rac1 activation was measured by pull down assays for GTP-Rac1. Cells stimulated with 100 ng/ml of EGF were used as positive control. The histograms on the right panels represent the ratios of GTP-Rac1 over total Rac1.

**Figure 2.**

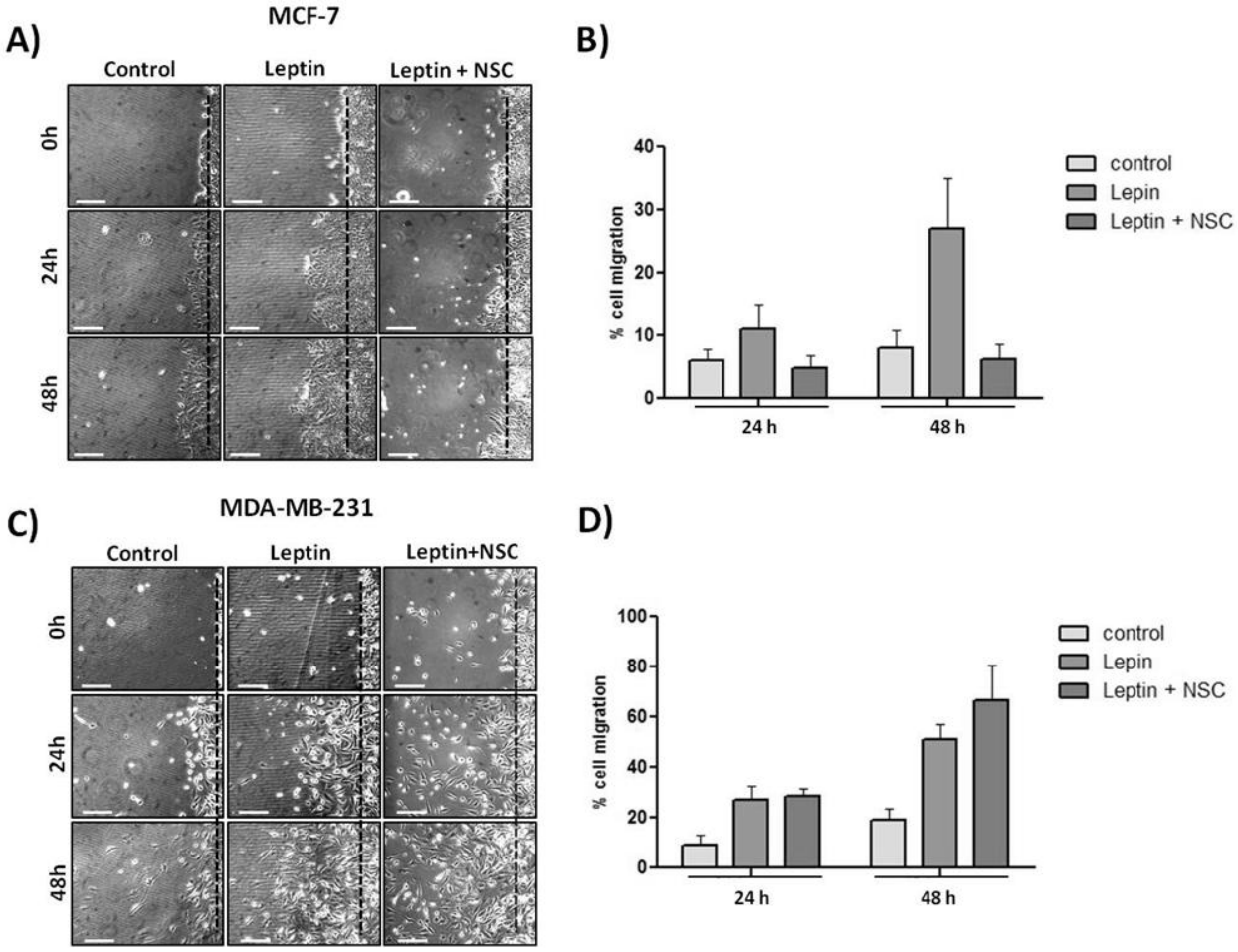



**Figure 2**. **Effect of Rac1 inhibition on cell migration of MCF7 and MDA-MB-231 cells.** A and C: Representative images of the scratch-migration assays. B and D: Histograms represent the average of migration from the scratch-migration assays. Control: cells treated with vehicle. Leptin: cells treated with 50ng/ml (MDA-MB231 cells) or 400 ng/ml (MCF7 cells) of leptin. Lep+NSC: cells treated with leptin plus the Rac1 inhibitor NSC23766, 25µM.

**Figure 3.**

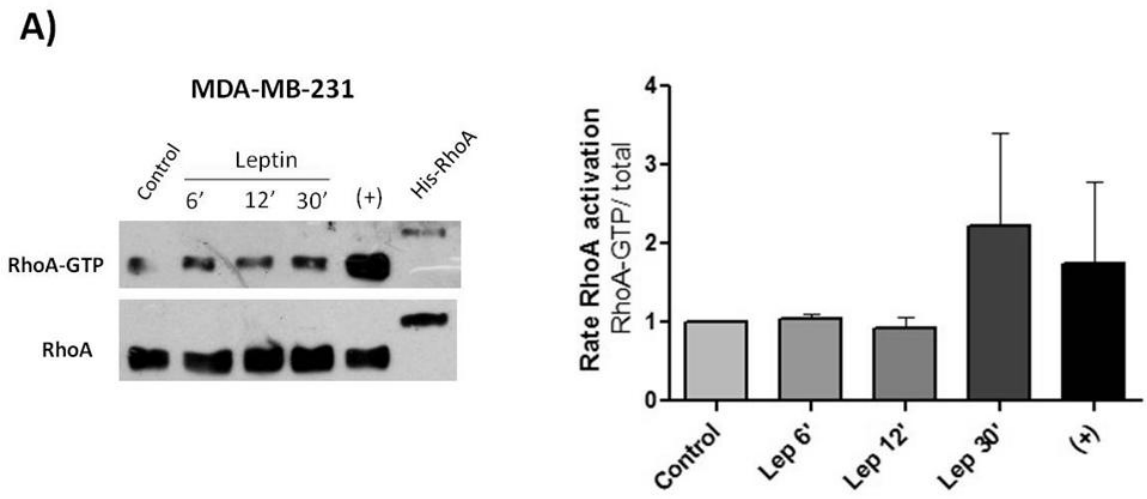



**Figure 3. Leptin induces the activation of RhoA in MDA-MB-231 cells.** Time-course of RhoA activation in MDA-MB-231 cells in response to 50 ng/ml of leptin. Rac1 activation was measured by pull down assays for GTP-RhoA. (+) positive control, in which an excess of non-hydrolysable GTP was added. A His-tagged purified protein was used as a positive control for the WB (His-RhoA). The histograms on the right panels represent the ratios of GTP-RhoA over total RhoA.

## Figure 4.

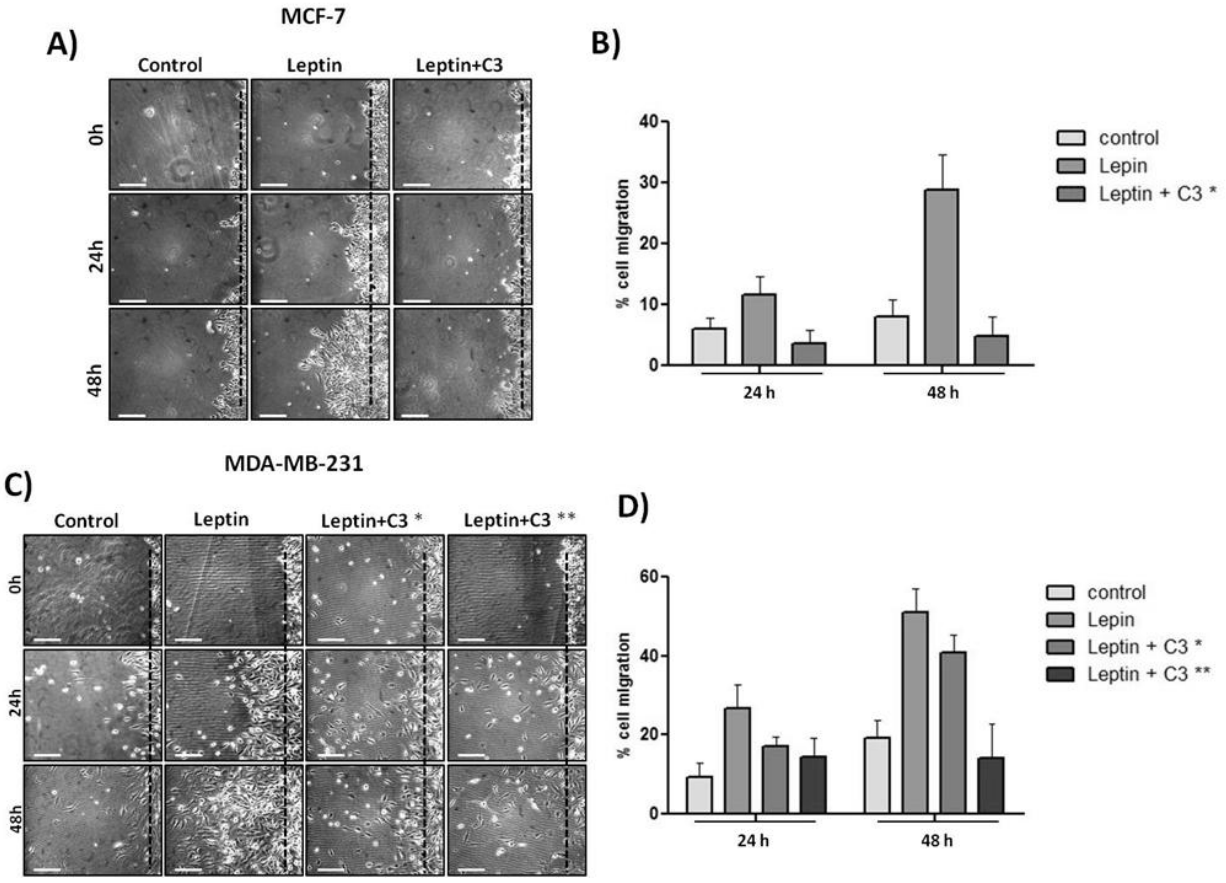



**Figure 4**. **Effect of RhoA inhibition on cell migration of MCF7 and MDA-MB-231 cells.** A and C: Representative images of the scratch-migration assays. B and D: Histograms represent the average of migration from the scratch-migration assays. Control: cells treated with vehicle. Leptin: cells treated with 50ng/ml (MDA-MB231 cells) or 400 ng/ml (MCF7 cells) of leptin. Lep+C3: cells treated with leptin plus the RhoA inhibitor, ADP-ribosyltransferase C3 from *Clostridium botulinum,* 1-2 ng/ml.

**Figure 5.**

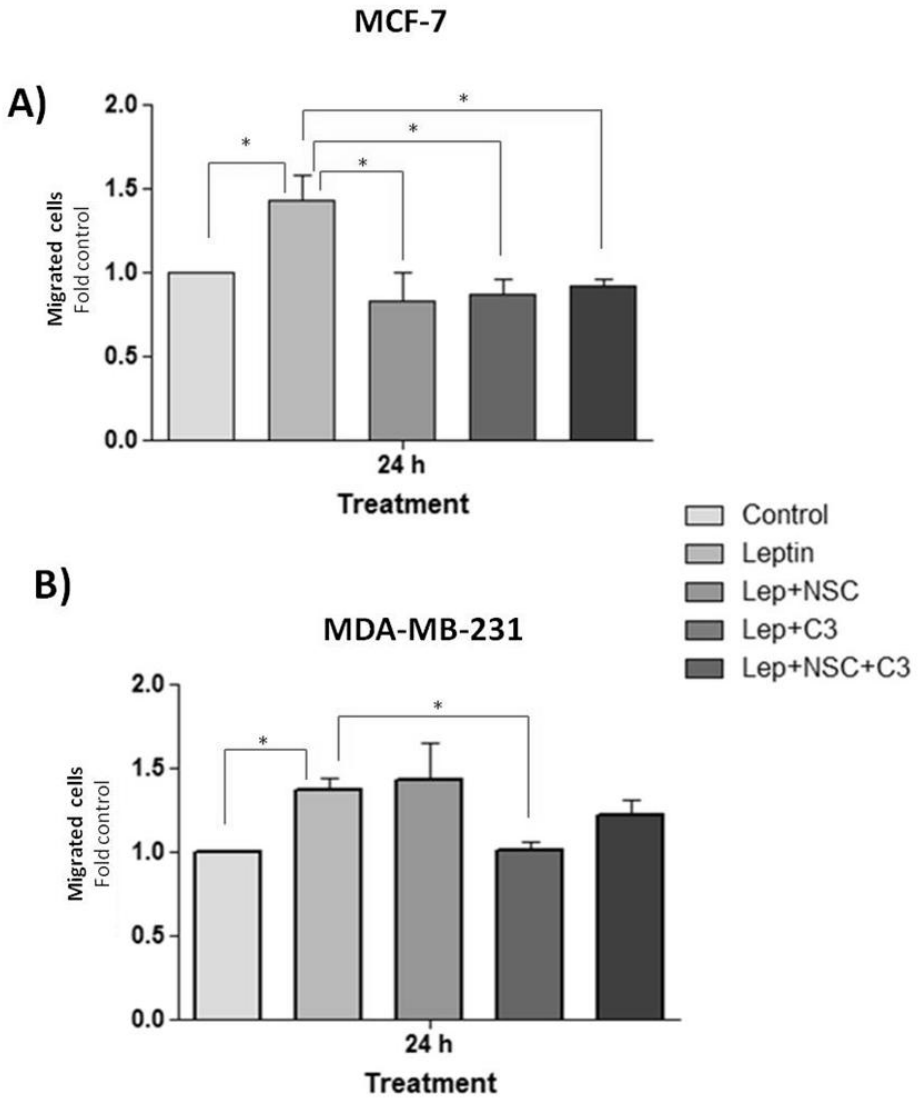



**Figure 5**. **Differential effect of Rac1 and RhoA inhibition on cell migration of MCF7 and MDA-MB-231 cells.** Cell migration was determined by colorimetric migration assays**.** Histograms represent the average fold migration of MCF7 (A) or MDA-MB-231 (B), respect to untreated cells. Error bars represent duplicate experiments. Control: cells treated with vehicle. Leptin: leptin, 50 ng/ml for MDA-MB231 cells or 400 ng/ml for MCF7 cells. NSC: Rac1 inhibitor NSC23766, 25 µM. C3: RhoA inhibitor, ADP-ribosyltransferase C3 from *Clostridium botulinum*, 1ng/ml. * *p<0.05.*

**Figure 6.**

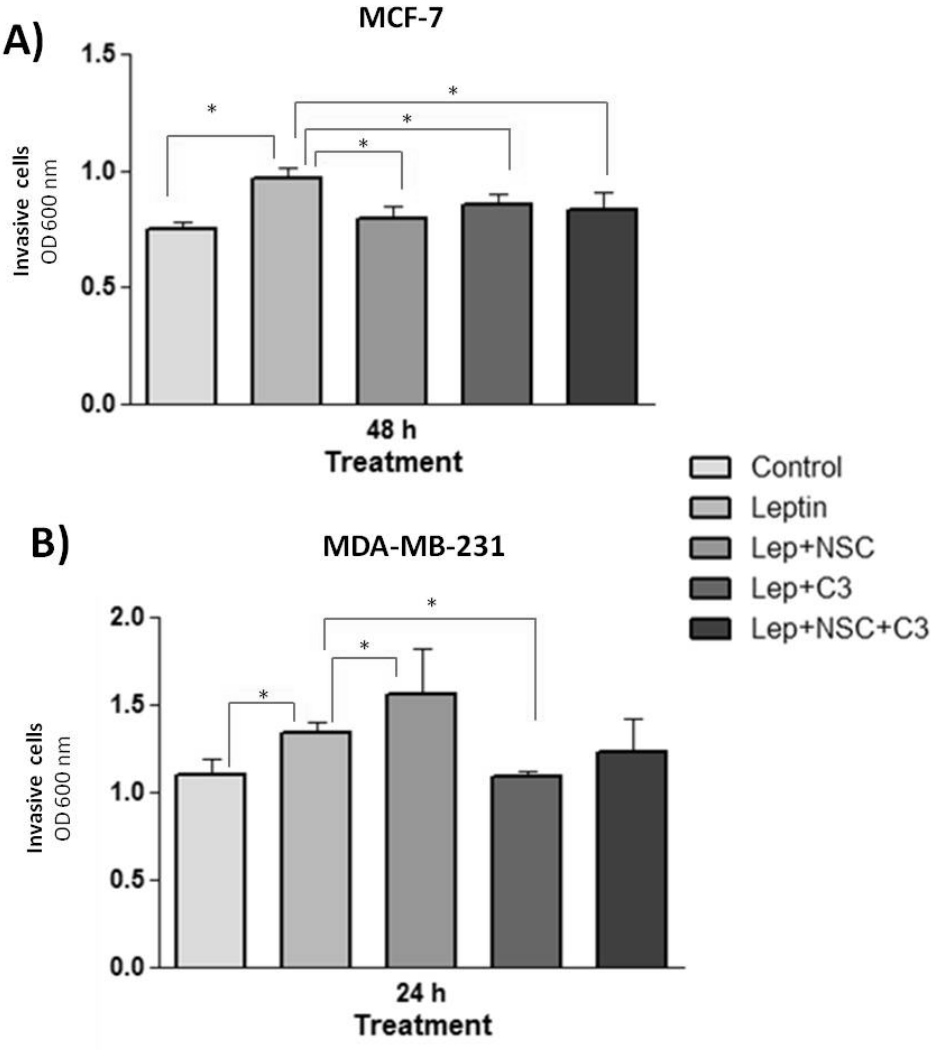



**Figure 6**. **Differential effect of Rac1 and RhoA inhibition on invasiveness of MCF7 and MDA-MB231 cells induced by leptin.** Cell invasion was determined by colorimetric Boyden chamber assays**.** Histograms represent the average fold invasion of MCF7 (A) or MDA-MB-231 (B), respect to untreated cells. Error bars represent duplicate experiments. Control: cells treated with vehicle. Leptin: leptin, 50 ng/ml for MDA-MB-231 cells or 400 ng/ml for MCF7 cells. NSC: Rac1 inhibitor NSC23766, 25 µM. C3: RhoA inhibitor, ADP-ribosyltransferase C3 from *Clostridium botulinum*, 1ng/ml. * *p<0.05.*

## Figure 7.

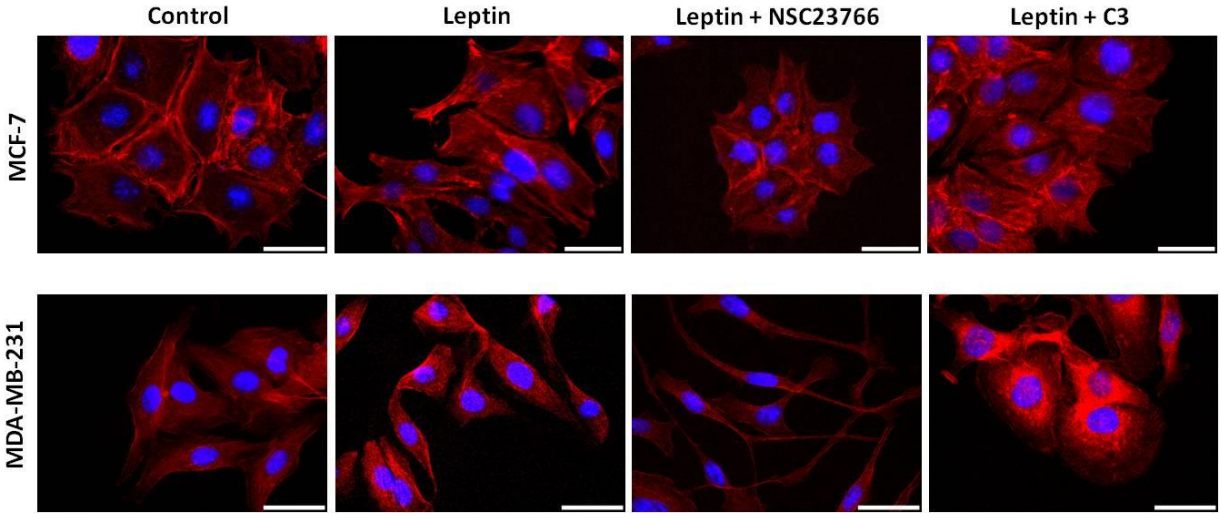
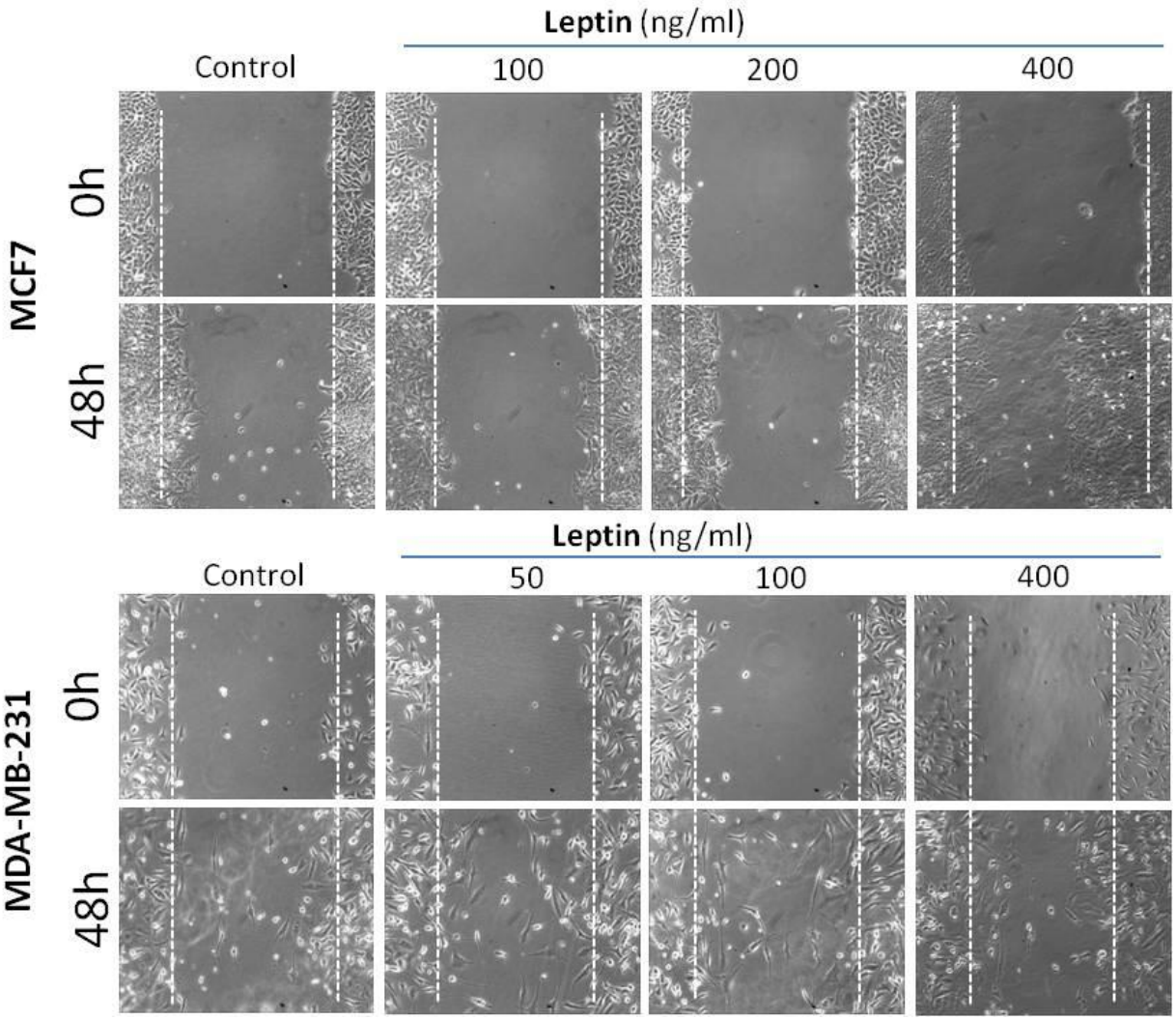



**Figure 7**. **Actin cytoskeleton rearrangements induced by leptin in MCF7 and MDA-MB-231 cells.** Representative images from Rhodamine-phalloidin stained cells. Top panel: MCF7 cells. Bottom panel: MDA-MB-231 cells. Control: cells treated with vehicle. Leptin: 400 ng/ml and 50 ng/ml for MCF7 and MDA-MB-231 cells, respectively. NSC: Rac1 inhibitor NSC23766, 25 µM. C3: RhoA inhibitor, ADP-ribosyltransferase C3 from *Clostridium botulinum,* 1 ng/ml.

**Figure S1.**


Leptin (ng/ml)
Control
100
200
400
MCF7
0h
48h
Leptin (ng/ml)
Control
50
100
400
MDA-MB-231
0h
48h

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CAPÍTULO II

# Leptin-induced migration and invasion of breast cancer cells requires PI3K/Rac1/RhoA activation and involves MMPs secretion

# Leptin-induced migration and invasion of breast cancer cells requires PI3K/Rac1/RhoA activation and involves MMPs secretion

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## Abstract

Overweight and obesity are considered risk factor for developing breast cancer, the leading cause of death by malignant neoplasia in women. It has been demonstrated that leptin affects several intracellular pathways promoting cell proliferation, angiogenesis, cell-cycle progression, and survival of breast cancer cells. We have recently shown that leptin induces migration and invasion of breast cancer cells through Rac1/Rho-dependent pathway. Here, we demonstrated that leptin promotes cytoskeleton rearrangements and cell migration through a PI3K-dependent pathway in MCF-7 and MDA-MB-231 cells, and increases the MMP-2 activity through Rho in MDA-MB-231 breast cancer cells.

Keywords: Breast cancer, leptin, Rho-GTPases, PI3K, MMPs.

## Introduction

Breast cancer is the leading cause of death by malignant neoplasia in women worldwide (GLOBOCAN, 2008). Epidemiological data suggest that overweight and obesity are associated with increased risk of developing breast cancer (van Kruijsdijk et al., 2009, Montazeri et al., 2008, Davis and Kaklamani, 2011). Experimental data suggest that leptin, an adipocyte-synthetized hormone (Li, 2011), can promote tumor progression through regulating multiple pathways. It has been demonstrated that in breast cancer cells in culture leptin treatment affects gene expression (Valle et al., 2011b, Perera et al., 2008), sensitizes cells to estrogen-induced proliferation (Valle et al., 2011a), promotes tumor cell proliferation, angiogenesis, cell-cycle progression, and cell survival (Jarde et al., 2011, Ando and Catalano, 2011). Moreover, molecular crosstalk has been shown between leptin signaling and several receptors including HER2 (Fiorio et al., 2008, Soma et al., 2008), Notch-IL1 (Guo and Gonzalez-Perez, 2011) and estrogen receptor-α (Fusco et al., 2010).

We have recently shown that leptin induces migration and invasion of low migratory (MCF-7) as well as of highly migratory/invasive (MDA-MB-231) breast cancer cell lines in culture. Moreover, we demonstrated that inhibition of the Rho

GTPase Rac1 impairs leptin-induced migration and invasion of MCF7 cells, but not of MDA-MD-231, whereas inhibition of the GTPase RhoA impairs leptin-induced migration and invasion of both cell lines (Mendoza-Catalán, et al 2012. Submitted). Others authors have shown that leptin stimulates migration of cancer cell lines in culture trough activation of PI3K and Rho GTPases (Attoub 2000; Jaffe 2008) (Barone et al., 2012). Furthermore, leptin can induce expression and/or activation of matrix metalloproteinases (Schram et al., 2011, Endocrinology; Bilbao et al., 2011; Park et al., 2001), which play a crucial role in cell migration and invasion.

The aim of this study was to investigate whether leptin-induced migration and invasion in breast cancer cells in culture is dependent on the PI3K pathway, and if it involves MMPs activation.

## Materials and Methods

### Cell culture

MCF7 and MDA-MB-231 breast cancer cell lines were cultured in Dulbecco's modified Eagle´s medium (DMEM)/Ham F12 (1:1) supplemented with 3.2 g/L sodium bicarbonate, 5% fetal bovine serum (FBS) and 1% antibiotics in a humidified atmosphere containing 5% $CO_2$ at 37°C.

### Western blotting

Cells were seeded on Petri dishes and grown until 80% confluence. After, the cells were treated with fresh serum-free DMEM in the presence of leptin for 5, 15 or 30 minutes or with EGF as positive control. Cells were washed with PBS and lysed in 500 µl of RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH8). A total of 50 µg of pooled proteins were separated at 12% SDS-PAGE by electrophoresis and then transferred onto a PVDF membrane. The membranes were blocked with 3% milk TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) and incubated with primary antibodies for *p*-Akt and Akt total (Cell signaling) overnight at 4°C. The immune complexes were visualized with chemiluminescense.

## Scratch-wound migration assays

MCF-7 and MDA-MB-231 cells were grown until confluence in 6-well plates and starved for 24 h in serum-free DMEM. After starvation, cells were scratch-wounded using a sterile spatula, detached cells were removed by washing twice with PBS and the remaining cells were incubated for 24h or 48 h with or without leptin in the presence of Rac1, Rho or PI3K inhibitors. 50 µM cytosine β-D-arabinofuranoside hydrochloride (AraC) were added to each well to inhibit cell proliferation. To assess cell migration, five photographs from each well were taken at 0h, 24h and 48 hours and cell migration was evaluate taking the mean of five measures from initiation of wound to advancing front cell in each picture using National Institute Health ImageJ software.

## Chemotactic migration assay

Chemotactic cell migration was evaluated by using QCM 24-well Colorimetric Cell migration assay (Millipore, ECM508) according to manufacturer's instructions. Briefly, $25x10^4$ cells were suspended in 200 µl of serum-free DMEM and added on the top of the chamber. 500 µl of DMEM medium with leptin plus Rac1 and Rho inhibitors were added in the lower chamber. Cells were allowed to migrate for 24 hours. Non-migrated cells were removed from the upper side of the chamber (filter) by cotton swabs, migrated cells (cells in the lower side of the filter) were fixed and stained with crystal violet (Millipore, catalog no. 90144), washed in distilled $H_2O$, cristal violet was eluted using an extraction buffer (Millipore, catalog no. 90145), and the optical density was measured at 600nm. All experiments were repeated at least three times.

## Zymography assays.

MCF-7 and MDA-MB-231 cells were grown until confluence in 6-well plates and starved for 24 h in serum-free DMEM. After starvation the cells were incubated for 48 hours in fresh medium with or without leptin plus Rac1, Rho or PI3K inhibitors. After of treatment, the medium of treatment was collected and centrifuged at 14,000 rpm for 10 minutes at 4°C and supernatants were collected. The samples

were electrophoresed using 10% geles containing 0.1% gelatin (bovine skin, type B, Sigma-Aldrich, G9391). Following electrophoresis the gel was washed twice 2% Triton-X100-PBS to remove excess SDS and incubated in MMP substrate buffer (50mM Tris-HCl pH 7.5, 10mM $CaCl_2$) during 24 hours. The gel was washed with distilled ultrapure and stained with 0.1% Coomassie Brilliant Blue (Sigma-Aldrich R-250). Proteolysis areas appeared as clear bands against a blue background of gelatin substrate. Stained gels were scanned and band densities were quantified by densitometric analysis using National Institute Health ImageJ software.

## Results

### Leptin activates the PI3K pathway in MCF-7 and MDA-MB-231 breast cancer cells.

To test if leptin can induce the activation of the PI3K pathway, cells were treated with leptin and activation of the pathway was determined by measuring phosphorylation of Akt by Western blotting. We observed the highest increase of Akt phosphorylation after 15' of leptin treatment on MCF7 cells (Figure 1), which corresponds to the time of maximum Rac1 activation observed in these cells in response to leptin (Mendoza-Catalán, et al 2012. Submitted).

### Inhibition of the PI3K pathway impairs leptin-induced migration and invasion of MCF-7 and MDA-MB-231 breast cancer cells.

We next determined if leptin-induced migration and invasion is dependent on the activation of the PI3K pathway. Using scratch-wound assays we observed that PI3K inhibition by Wortmanin (20 nM) abrogated cell migration induced by leptin in both MCF-7 and MDA-MB-231 cells (Figure 2), which suggests that leptin-induced cell migration in breast cancer cells is dependent on PI3K. These results were confirmed by colorimetric migration assays (Figure 3).

## Inhibition of PI3K decreases cytoskeleton rearrangements induced by leptin in MCF7 and MDA-MB-231 breast cancer cells.

Previously we observed that leptin induces changes in the morphology and actin cytoskeleton rearrangements on MCF7 and MDA-MB-231 cells. To evaluate the participation of PI3K in leptin-induced actin cytoskeleton rearrangements, the cells were treated with leptin in the presence or absence of Wortmanin, and F-actin fibers were stained with rhodamine-phalloidin. In both cell lines, we observed that Wortmanin-treated cells showed perinuclear accumulation F-actin (Figure 4). In normal conditions MCF-7 cells show an epithelial phenotype, but the leptin-treated cells changes to mesenchymal phenotype, and the PI3K inhibition abrogated these leptin-induced changes, retaining the cells in an epithelial shape similar to control cells (Figure 4A). In MDA-MB-231 cells, leptin treatment induced the formation of narrow protrusions at the front or rear of the cells, inhibition of PI3K resulted in cells with elongated shape cells by extending narrow protrusions in multiple directions; moreover, Wortmanin treatment increased cell spread area in several cells, which had broad lamellipodia spread at the cell periphery (Figure 4B). These changes are similar to the ones previously observed in NSC23766 or C3 transferase-treated cells (Mendoza-Catalán, et al 2012. Submitted).

## Leptin induces MMP2 activation in MDA-MB-231 cells trough a Rho dependent pathway

To investigate if leptin-induced migration and invasion of MDA-MB-231 cells involves activation/secretion of MMPs, we performed zymography assays. We observed an increased activity of MMP2 when the cells were treated with leptin (200ng/ml) respect to cells without treatment. This increase in MMP2 activity was not affected when the cells were treated with the Rac1 inhibitor NSC 23766 or with Wortmanin. However, when the cells were incubated with the Rho inhibitor C3 transferase, we observed a decrease in leptin-induced MMP2 activation (Figure 5). This observation suggests an important role of MMP2 in leptin-induced invasion, which is dependent of Rho but independent of Rac1 and PI3K activation.

## Discussion

It is already know that leptin induces proliferation, migration and invasion of several types of cancer cells (Amjadi et al., 2011, Attoub et al., 2000, Fava et al., 2008, Jaffe and Schwartz, 2008), including the breast cancer (Barone et al., 2012), and that expression of metalloproteinases is a key event for epithelial-mesenchymal transition and invasiveness of tumor cells (Radisky and Radisky, 2010). Previously, we showed that leptin induces the migratory and invasive potential of MCF7 and MDA-MB-231 breast cancer cells through a Rac1/RhoA-dependent pathway (Mendoza-Catalán, et al 2012. Submitted). However the mechanisms used by leptin to induce cell migration and invasion are still poorly understood. Here, using migration assays we demonstrated that leptin induces cell migration in a PI3K-dependent pathway in MCF7 and MDA-MB-231 breast cancer cells. Moreover, leptin induces activity of MMP2 through a Rho-dependent pathway.

It has been demonstrated that expression of some Rho-GTPases, leptin and leptin receptor is higher expressed in breast cancer tumors respect to normal breast tissue (Ishikawa et al., 2004, Schnelzer et al., 2000, Fritz et al., 2002). Moreover, is already known that leptin promotes invasiveness of kidney and colonic cells through PI3K, Rho and Rac-dependent pathways (Attoub et al., 2000) and PI3K is known to be a key molecule regulating cell migration in breast cancer cells (Bastian et al., 2006). We demonstrated that cell motility induced by leptin in MCF-7 cells is dependent of PI3K, and the PI3K inhibition causes changes in the actin polymerization similar to the ones observed in cells treated with Rac1 or Rho inhibitors, which suggest that leptin induces cell motility through a PI3K-Rac1/RhoA-dependent pathway.

On the other hand, is already know that overexpression of matrix metalloproteinases is a key event for the invasiveness of breast cancer cells (Radisky and Radisky, 2010), and it has been demonstrated that leptin treatment induces the expression of MMP-2 and MMP-9 of HUVEC and HCASMC cells (Park et al., 2001), moreover, it has been reported that leptin regulates the expression and activity of MT1-MMP and MMP-2 through RhoA/ROCK-dependent pathway in

cardiac fibroblasts (Schram et al., 2011). Using zymography, we demonstrated that leptin induces the activity of MMP-2 in MDA-MB-231 cells, and this effect was abrogated by C3 transferase treatment, but not by inhibition of Rac1 or PI3K. This suggests that leptin promotes the cell invasion in a Rho-dependent pathway, and seems PI3K/Rac1-independent pathway.

In conclusion, our results indicates that leptin induces cell migration through a PI3K-Rac1/Rho-dependent pathway and promotes cell invasion inducing the enhanced activity of MMP-2 through a Rho-dependent pathway, suggesting to Rho inhibition as a possible and interesting therapeutic target in breast cancer, a disadvantage of our study is that C3 transferase abrogates the activity of three isoforms of Rho (A, B and C) and we can't determinate the role of each. Further studies are needed for elucidate the role of PI3K/Rac1/RhoA and RhoC in the cell migration and invasion in response to leptin.

## Figure 1.

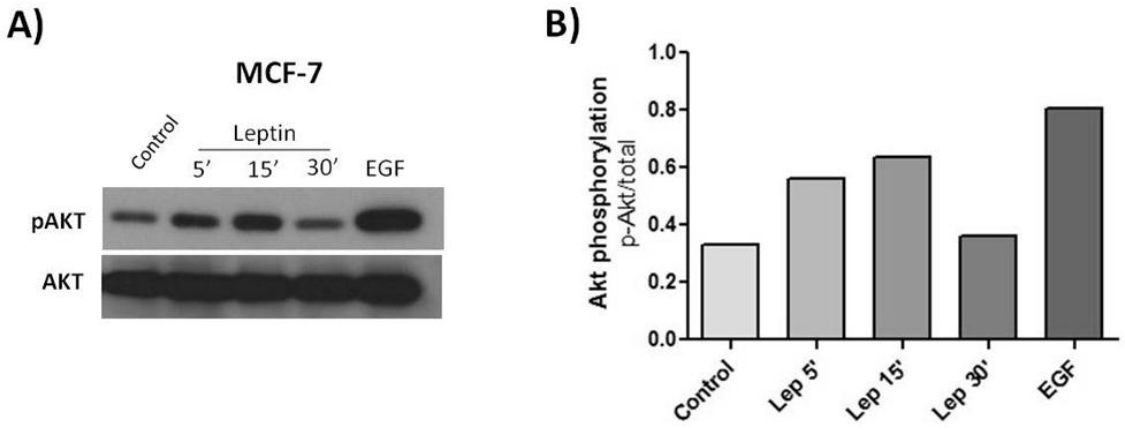


**Figure 1**. **Leptin induces the Akt phosphorylation.** A) Western blotting. Control: cells treated with vehicle (10mM Tris-HCL and 150mM NaCl pH 8.5). Lep: Leptin 400 ng/ml. Cells treated with 100 ng/ml EGF were used as positive control. B) Densitometry of A.

**Figure 2.**

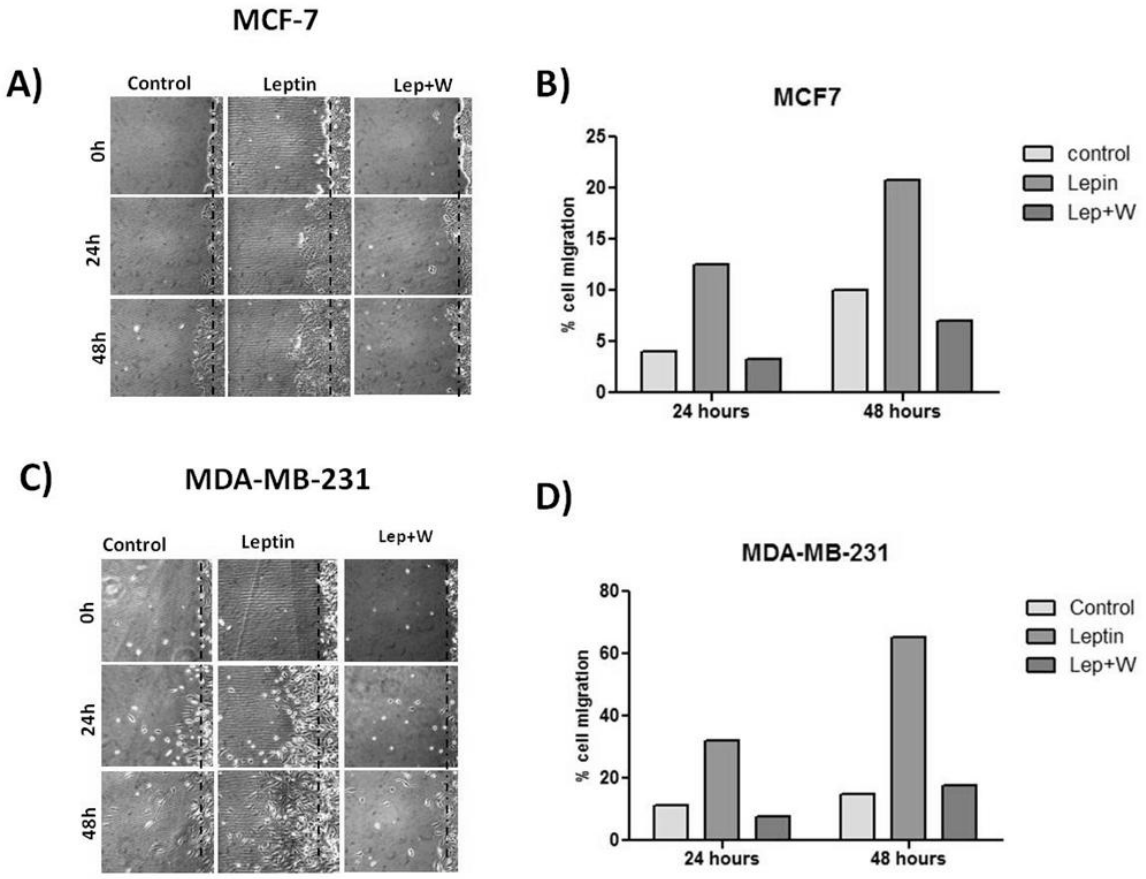


**Figure 2**. **Participation of PI3K in leptin-induced cell migration.** A and B. Scratch-wound assays for MCF7 cells. C and D. Scratch-wound assays for MDA-MB-231 cells. Control: cells treated with leptin diluents. Lep: leptin, 400 ng/ml and 50 ng/ml for MCF7 and MDA-MB-231 cells, respectively. W: Wortmanin, 20nM.

**Figure 3.**

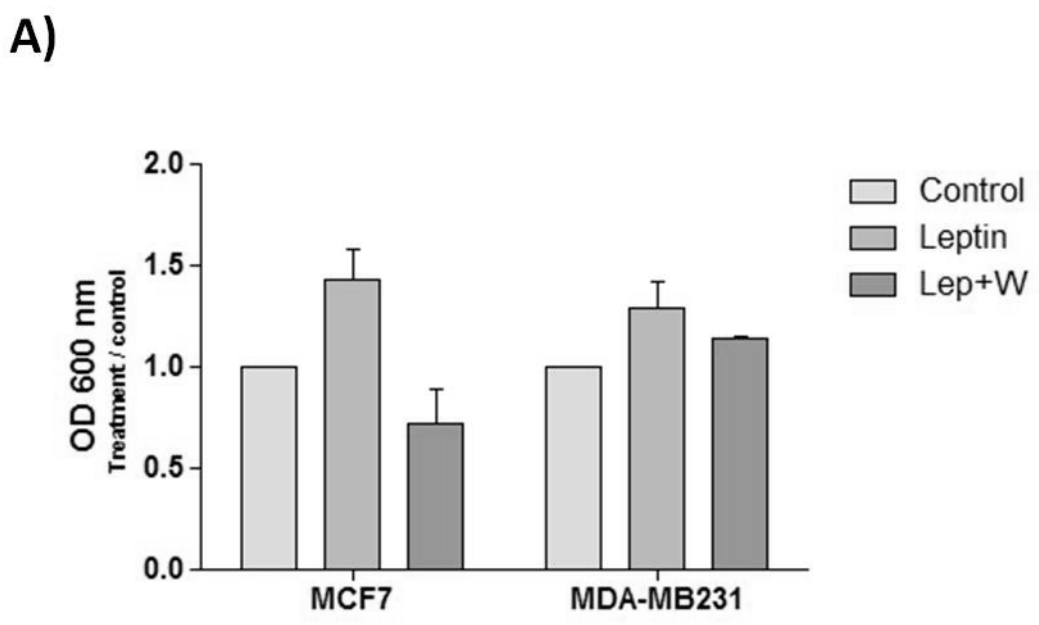


**Figure 3**. **Effect of PI3K inhibition on leptin-induced cell migration.** Colorimetric migration assays. Control: cells treated with leptin diluents. Leptin, 400 ng/ml (MCF7 cells) and 50 ng/ml (MDA-MB-231 cells). W: Wortmanin, 20nM. Error bars represent duplicate experiments.

**Figure 4.**

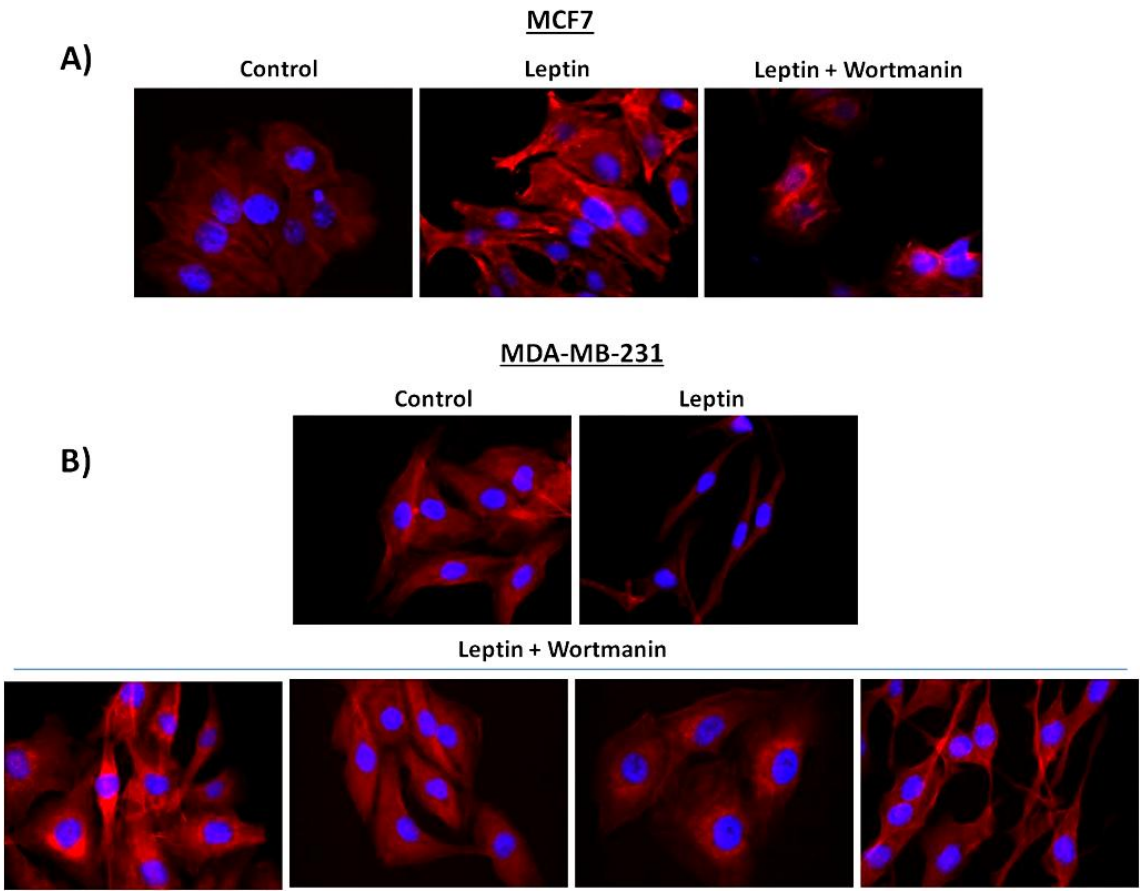


**Figure 4. Effect of PI3K inhibition on leptin-induced cytoskeleton rearrangements.** Cells stained with Rhodamine-phalloidin. Control: cells treated with leptin diluents. Leptin, 400 ng/ml and 50 ng/ml for MCF7 and MDA-MB-231 respectively. W: Wortmanin, 20nM. Red: F-actin. Blue: cell nuclei stained with DAPI.

**Figure 5.**

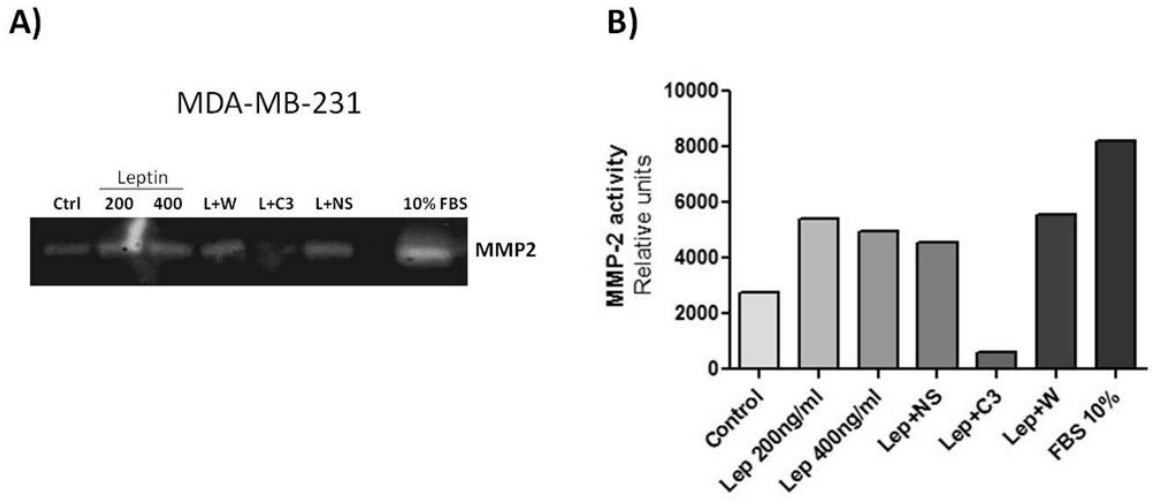


**Figure 3**. **Participation of Rac1, Rho and PI3K on Leptin-induced MMP-2 activity of MDA-MB-231 cells.** Zymography assays. Ctrl: cells treated with vehicle. Leptin, 200 and 400 ng/ml. W: Wortmanin, 20nM. NS: Rac1 inhibitor NSC23766, 25 µM. C3: RhoA inhibitor, ADP-ribosyltransferase C3 from *Clostridium botulinum*, 1ng/ml. 10% FBS: positive control.

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## IV. DISCUSIÓN

Se ha reportado que la obesidad es un importante factor de riesgo para el desarrollo de cáncer de mama (Davis and Kaklamani, 2011, Montazeri et al., 2008) y que la leptina puede promover la progresión tumoral regulando varias vías de señalización que favorecen la proliferación celular, angionénesis, progresión del ciclo celular y sobrevivencia celular (Ando and Catalano, 2011, Jarde et al., 2011). Por otra parte, se ha reportado que la leptina estimula la migración e invasión de células tumorales de riñón y colon de manera dependiente de PI3K y las GTPasas Rac1 y Rho (Attoub et al., 2000) y se sabe que las GTPasas de la familia Rho son los principales reguladores de la motilidad de células de cáncer de mama (Jiang et al., 2009). Además, se ha propuesto a la inhibición de Rac1 como blanco terapéutico para frenar la progresión de tumores de mama (Dokmanovic et al., 2009, Katz et al., 2012, Rosenblatt et al., 2011). Sin embargo, no se ha descrito la participación de GTPasas Rho en la migración e invasión celular inducida por leptina. En este estudio, se evaluó la participación de PI3K y las GTPasas Rac1 y RhoA en los procesos de migración e invasión inducidos por leptina en células de cáncer de mama poco invasivas MCF-7 y altamente invasivas MDA-MB-231.

Los resultados obtenidos muestran que el tratamiento con leptina induce la activación de Rac1 y la migración e invasión de ambas líneas celulares. Al evaluar la participación de Rac1 en este evento, se encontró que la inhibición química de Rac1 disminuye el efecto de la leptina sobre la migración e invasión de células MCF-7, sin embargo, la inhibición de Rac1 potencia aún más el efecto de la leptina en la migración e invasión de células MDA-MB-231. Esta observación coincide con lo reportado por Zuo *et al* en 2006, donde encuentran que la inhibición de Rac1 disminuye la migración de células poco invasivas MCF-7 y T-47D pero la potencia en células altamente invasivas MDA-MB-453, MDA-MB-231 y C3L5 (Zuo et al., 2006). Las células de cáncer de mama migran generalmente adoptando una forma similar a fibroblastos mediante la transición epitelio-mesénquima (Jiang et al., 2009), sin embargo, se ha demostrado que algunas células tumorales pueden cambiar de un tipo de migración mesenquimal a uno de tipo ameboide y se ha

observado que esta plasticidad celular es regulada por la activación e inactivación de Rac1 (Parri and Chiarugi, 2010). En células de melanoma, se ha demostrado que existe una regulación recíproca entre Rac1 y Rho, la cual regula el cambio entre un fenotipo de migración tipo mesénquima a tipo ameboide, la señalización ameboide lleva a la desregulación del movimiento mesénquimal y viceversa (Sanz-Moreno et al., 2008). Rac1 regula la migración celular de tipo mesénquima mediante la vía DOCK3 (un GEF específico de Rac1) y WAVE2, una proteína efectora de Rac1 que promueve la nucleación de actina y que inhibe a RhoA, en contraparte, RhoA activo es capaz de inhibir a Rac1 a través de ARHGAP2, lo cual induce el movimiento tipo ameboide (Sanz-Moreno et al., 2008). Este mecanismo de plasticidad en el tipo de movimiento de células de cáncer de mama, podría ser la explicación del por qué la inhibición química de Rac1 potencia la migración de células MDA-MB-231.

Por otra parte, observamos que el tratamiento con leptina induce la activación de RhoA en células MDA-MB-231, y la inhibición de Rho interrumpió el efecto inducido por leptina sobre la migración en ambas líneas celulares, incluso en las células MDA-MB-231 que recibieron el tratamiento con el inhibidor químico de Rac1, sugiriendo una participación importante de Rho en la migración celular inducida por leptina. Es importante mencionar que la inhibición de Rac1 ha sido recientemente propuesta por varios autores como blanco terapéutico para el cáncer de mama. Se ha observado que la inhibición de Rac1 restaura la sensibilidad a trastuzumab de células SKBR3 resistentes reduciendo los niveles de ErbB2 (Dokmanovic et al., 2009), reduce la sobrevivencia celular desregulando a ciclina D1, survivina y proteínas inhibidoras de apoptosis (Yoshida et al., 2010), además de que reduce la proliferación celular inducida por estrógenos y disminuye los niveles de RNAm y proteína de ERα (Rosenblatt et al., 2011) y se ha reportado también que redujo la diseminación de tumores de mama en cultivo (Katz et al., 2012). Los resultados de este estudio sugieren que la inhibición de Rac1 no es una herramienta confiable para el tratamiento del cáncer de mama, puesto que podría favorecer la agresividad de algunos tumores y afectar la salud de la paciente. La observación de que el tratamiento combinado con los inhibidores de

Rac1 y Rho interrumpió la migración celular en ambas líneas celulares y la dependencia de Rho para el proceso de migración inducido por leptina en ambas líneas celulares, señala a Rho como un interesante blanco terapéutico para cáncer de mama, sin embargo se requieren más estudios que exploren esta posibilidad.

Además, se observó que el tratamiento con leptina promovió la capacidad invasiva en ambas líneas celulares. De manera similar al proceso de migración, la inhibición química de Rac1 disminuyó la invasividad de las células MCF-7, pero potenció la capacidad invasiva de las células MDA-MB-231. En contraste, la inhibición de Rho interrumpió la capacidad invasiva inducida por leptina en ambas líneas celulares. Por otra parte, se encontró que el tratamiento con leptina indujo la actividad de MMP-2 y, solamente la inhibición de Rho disminuyó la actividad gelatinasa de MMP-2 inducida por leptina, mientras que la inhibición de Rac1 y PI3K no mostraron efecto al respecto. Lo anterior sugiere una importante participación de Rho en la invasión celular inducida por leptina a través de la actividad de MMP-2. De acuerdo con estas observaciones, se ha demostrado que la leptina induce la expresión de MMP-2 y MMP-9 en células endoteliales HUVEC y células de músculo liso cardiaco HCASMC (Park et al., 2001), además, se ha reportado que la leptina regula la expresión y actividad de MT1-MMP y MMP-2 a través de una vía dependiente de RhoA/ROCK en fibroblastos cardiacos (Schram et al., 2011). Una desventaja de este estudio es que el inhibidor C3-transferase desregula la actividad de las tres isoformas de proteínas Rho (Rho-A, B y C), por lo que no es posible evaluar la participación de una isoforma en particular.

Aunado a esto, al evaluar los cambios en el citoesqueleto de actina inducidos por leptina, se encontró que varias células MCF-7 tratadas con leptina adquieren un fenotipo mesénquimal lo que favorecería su capacidad migratoria. La inhibición química de Rac1 y Rho rompió el efecto de leptina sobre los rearreglos del citoesqueleto manteniendo a las células MCF-7 en su forma epitelial, lo cual coincide con la disminución de la capacidad migratoria de estas células tras la inhibición de Rac1 y Rho. Por otra parte, en células MDA-MB-231 el tratamiento con leptina provocó la elongación de las células, con protrusiones delgadas

similares a filopodios en el borde frontal y trasero de las células. La inhibición química de Rac1 incrementó la formación de este tipo de estructuras mostrándose aún más elongadas y frecuentemente con protrusiones en dos direcciones en uno de los bordes y, en algunos casos, con formaciones similares a lamelipodios en el extremo de las mismas, lo cual podría favorecer su migración. Además, se observaron células con protrusiones de membrana en uno o ambos bordes laterales de la célula. La inhibición de Rho en células MDA-MB-231 indujo diferentes rearreglos del citoesqueleto de actina, en algunas células provocó la formación de protrusiones de actina en múltiples direcciones, y en algunas otras, indujo la formación de estructuras similares a lamelipodios amplios alrededor de la célula, observándose una acumulación de actina polimerizada en la periferia nuclear, lo cual sugiere que tras la inhibición de Rho la célula sufre anormalidades en la distribución de actina perdiendo su capacidad para iniciar el proceso de migración. Estas observaciones en células MDA-MB-231 coinciden con lo observado por Vega *et al* en 2011, donde reportan que la inhibición de RhoA por ARN de interferencia provoca la elongación y formación de protrusiones de membrana laterales y el bloqueo de RhoC causa la formación de lamelipodios en toda la periferia de células de próstata PC3 (Vega et al., 2011). Hasta el momento no existen reportes sobre rearreglos del citoesqueleto de actina inducidos por leptina de manera dependiente de Rac1 o Rho. Sin embargo, se ha demostrado que la proteína SH2B1β, un sustrato de JAK2 necesario para iniciar la señalización de leptina y otras adipocinas, es una proteína de unión a actina necesaria para la migración en fibroblastos embrionarios y, su inhibición provocó estructuras de actina similares a las observadas en este estudio en células MDA-MB-231 (Rider et al., 2009), además SH2B2 y SH2B3 miembros de la misma familia de proteínas, han sido descritos como reguladores del citoesqueleto de actina y se ha demostrado que SH2B2 puede unirse a VAV3 (Yabana and Shibuya, 2002), un activador de Rac1 y RhoA, lo cual podría favorecer el proceso de migración celular mediado por estas GTPasas. Además, la proteína adaptadora SH2B1β presenta domininios SH2 en su extremo C-terminal de manera conservada (Rider et al., 2009), por lo que es probable que mediante este dominio

pueda unirse a PI3K regulando así la señalización inducida por leptina. PI3K es una proteína canónica en la señalización de la leptina (Procaccini et al., 2009) y se ha demostrado que es necesaria en la migración inducida por leptina en células de cáncer de riñón, colon y carcinoma hepatocelular (Attoub et al., 2000, Saxena et al., 2007). Interesantemente, en este estudio se observó que la inhibición de PI3K bloquea el efecto de la leptina sobre la migración celular, además de que, afectó los cambios inducidos por leptina sobre los rearreglos del citoesqueleto en ambas líneas celulares, observándose células con cambios morfológicos similares a los provocados por la inhibición de Rac1 y Rho en ambas líneas celulares. En conjunto, estos datos sugieren que es posible que la leptina induzca migración celular y rearreglos del citoesqueleto de actina en células de cáncer de mama a través de una vía de señalización en la que estén implicadas las proteínas ObR-JAK2/SH2B1β/PI3K y Rac1/Rho (Anexo 1).

Tomando en cuenta estas observaciones, se evaluó si la leptina induce la migración de células de mama no tumorales MCF10A (Anexo 2). Se observó que la leptina estimula la migración de estas células induciendo la formación de estructuras similares a lamelipodios e interesantemente, algunas células adquieren una forma alargada, lo cual sugiere que la leptina induce transición epitelio mesénquima en células de mama normales, este efecto de la leptina fue dependiente de PI3K y Rac1 pero parece ser independiente de Rho.

En conclusión, la leptina induce la activación de la vía PI3K y de las GTPasas Rac1 y RhoA y estimula la migración de células de cáncer de mama a través de una vía de señalización dependiente de PI3K/Rac1/RhoA en células poco invasivas MCF-7 y a través de PI3K/RhoA en células altamente invasivas MDA-MB-231. La exposición a leptina favorece la invasividad de células de cáncer de mama a través del aumento en la actividad de MMP-2 mediante una vía dependiente de Rho, pero independiente de PI3K y Rac1. Lo anterior y lo observado en células MCF-10A, sugiere que Rac1 podría regular la motilidad de células con un fenotipo más similar al epitelial en tumores tempranos y Rho podría tener mayor importancia en los procesos de migración e invasión de células de

cáncer de mama con un fenotipo mesenquimal en tumores más avanzados. Sin embargo, se requieren más estudios que evalúen la participación de Rac1 y RhoA en diferentes etapas de cáncer de mama y ayuden a elucidar su posible uso como biomarcadores de progresión y/o blancos terapéuticos, así como aporten al mejor entendimiento de esta patología en personas obesas que presenten niveles altos de leptina que como se observó, puedan estar estimulando la progresión del cáncer de mama.

## V. Perspectivas

Para el mejor entendimiento de los mecanismos por los que la leptina puede favorecer la progresión del cáncer de mama, restan varios puntos por estudiar.

- ✓ Evaluar si la leptina promueve invasión de células de cáncer de mama a través de RhoC o RhoA.
- ✓ Evaluar transición epitelio mesénquima en células MCF7 y MCF10A en respuesta a leptina.
- ✓ Evaluar la participación de GEFs de Rac1 y/o Rho en la migración e invasión de células de cáncer de mama en respuesta a leptina (Vav3, P-Rex, Tiam1).
- ✓ Evaluar la participación de efectores (Proteínas de unión a actina) de Rac1 y/o Rho en la migración de células de mama en respuesta a leptina.
- ✓ Evaluar la participación de **Cdc42** (otra GTPasa Rho involucrada en migración celular) en la migración e invasión de células de cáncer de mama en respuesta a leptina.
- ✓ Evaluar el posible uso de la GTPasa RhoA/C como blanco terapéutico en cáncer de mama.

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# VI. ANEXOS

## Anexo 1.

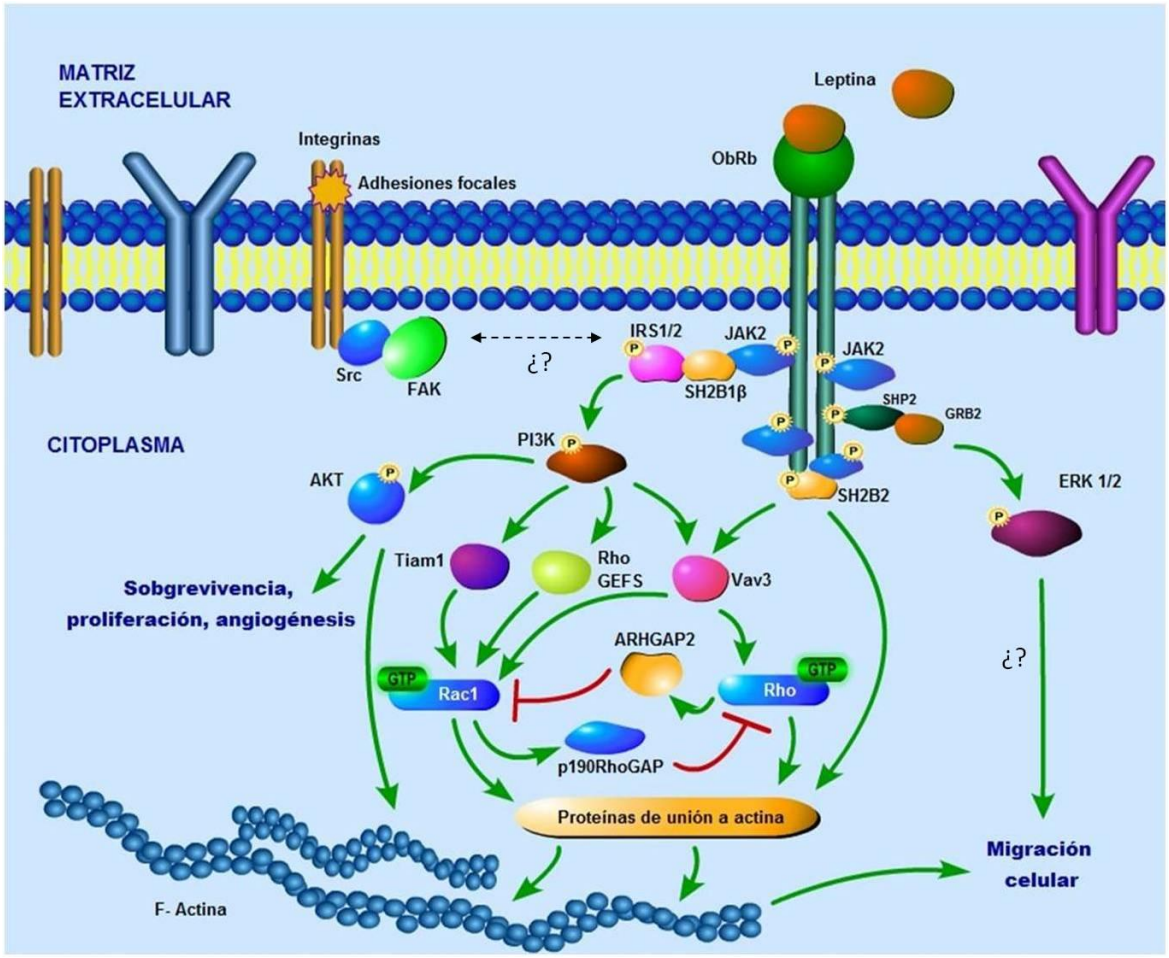


**Figura A1. Modelo hipotético de la señalización inducida por leptina en el proceso de migración de células de cáncer de mama.** Una vez unida la leptina al ObRb, las cinasas JAK2 pueden reclutar y fosforilar a la proteína SH2B1β, la cual funciona como modulador de la señalización del ObRb, ésta puede fosforilar al IRS1/2 e iniciar la cascada de señalización PI3K, que se ha observado puede inducir la Fosforilación de Tiam1, un activador directo de la GTPasa Rac1, así como a otros GEFs que pueden activar a Rac1 o RhoA, los cuales regulan la polimerización de actina a través de sus efectores, proteínas de unión a actina. Por otra parte, JAK2 puede también fosforilar a SH2B2 la cual induce la Fosforilación de VAV3, un activador de las GTPasas Rac1 y Rho. Existe una regulación reciproca entre Rac1 y RhoA que median la migración tipo mesénquima o ameboide respectivamente, la participación de Rac1 pudiera ser importante en la migración células poco invasivas (MCF7) mientras que RhoA podría regular este proceso en células más agresivas (MDA-MB-231). Se ha reportado también la participación de ERk1/2 y Akt en el proceso de migración en respuesta a leptina, sin embargo, se desconocen los mecanismos.

**Anexo 2.**

## La leptina induce la migración de células MCF10A a través de una vía dependiente de PI3K/Rac1

### Material y métodos.

### Cultivo celular y estimulación con leptina.

Las células MCF10A se cultivaron en medio DMEM suplementado con EGF ng/ml, insulina e hidrocortisona a 37°C y 5% de $CO_2$. Para el estímulo con leptina se utilizó leptina recombinante humana (SIGMA) a dosis de 200ng/ml o 400ng/ml durante 24 a 96 horas.

**Ensayos de migración en placa.** Se sembraron células MCF10A en placa de cultivo de 6 pozos hasta confluencia. Posteriormente se marco una línea de referencia y se realizó una herida en la monocapa celular con una espátula de plástico para eliminar las células de un lado de la línea de referencia. Las células fueron lavadas con PBS previamente atemperado y se agregó medio fresco suplementado con leptina en las dosis antes mencionadas más el inhibidor químico de Rac1 NSC 23766 25µM, el inhibidor de RhoA C3-transferase 1ng/ml o Wortmanina 20nM para la inhibición de PI3K. Se tomaron cuatro fotos por cada pozo a las 0, 24 y 48 horas de tratamiento en un microscopio invertido.

**Tinción con Faliodina rhodaminada.** Se sembraron células MCF10A sobre cubreobjetos en placas de 6 pozos y después de 24 horas, las células fueron tratadas con leptina y los inhibidores antes mencionados durante 48 horas. Después del tiempo de tratamiento, las células fueron fijadas con acetona fría durante tres minutos y lavadas con PBS. Posteriormente fueron permeabilizadas con PBS-Triton X-100 0.5%, boqueadas con PBS-BSA 1% durante 30 minutos y después incubadas con faloidina rhodaminada en una dilución 1:1000 durante una hora a temperatura ambiente y montadas con Prolong Reagent Antifade con DAPI (Invitogen, Molecular Probes). Las células fueron visualizadas en un microscopio

Olympus BX-43 equipado con una cámara QImaging pro5.0. Las imágenes fueron ajustadas utilizando el software ImageJ.

## Resultados

Se evaluó el efecto de la leptina sobre la migración de células no tumorales de mama MCF10A mediante ensayos de migración en placa. Se observó que el tratamiento con 200ng/ml y 400ng/ml de leptina indujo la migración de estas células (Figura A1). La dosis de 100ng/ml de leptina no mostró efecto en la migración celular.

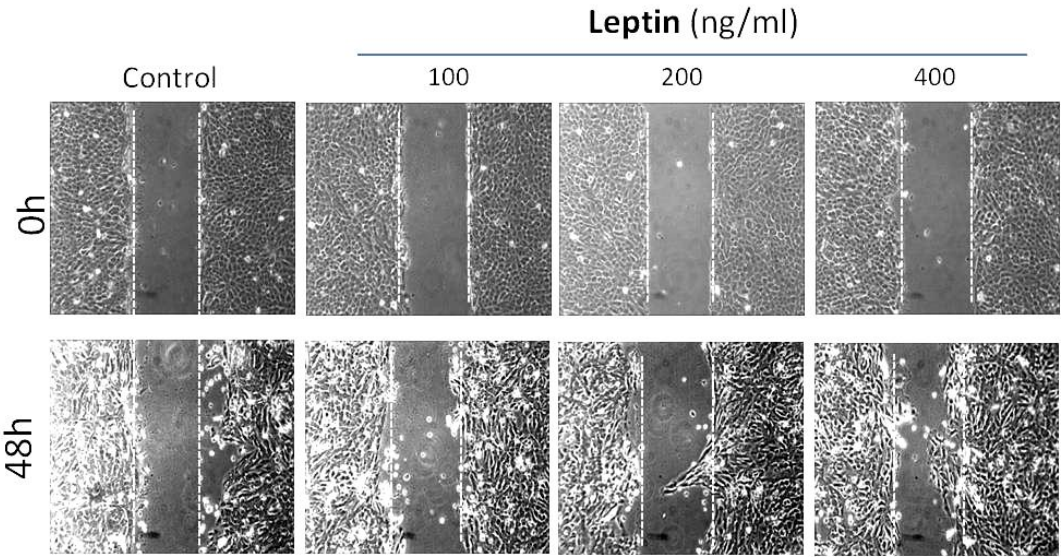


**Figure A1**. **Leptina induce la migración de células MCF10A.** Ensayos de migración en placa. Control: células tratadas con vehículo de leptina (Tris-HCl 10mM, NaCl 150mM, pH 8.5).

Se evaluó la participación de Rac1 y Rho en la migración de células MCF10A inducida por leptina. Se observó que la inhibición química de Rac1 bloqueó el efecto de la leptina sobre la migración celular disminuyendo significativamente las filas de células migrando, por otra parte, la inhibición de RhoA no tuvo un efecto significativo en este evento (Figura A2). Se observó que el tratamiento con leptina provoca que las células adquieran un fenotipo más alargado, similar a fibroblastos, sugiriendo que leptina puede inducir la transición epitelio mesénquima en estas células (Figura A3).

El tratamiento con leptina indujo también la formación de estructuras similares a lamelipodios en el frente de avance de las células migratorias. Este efecto de leptina fue bloqueado en las células tratadas con el inhibidor químico de Rac1 (Figura A3). Por otra parte, las células tratadas con el inhibidor de Rho presentaron también formación de lamelipodios, sin embargo estos se observan en distintas direcciones (Figura A3), lo cual sugiere que Rho es importante para mantener la orientación de células no tumorales MCF10A y Rac1 participa en la polimerización de actina necesaria para el proceso de migración.

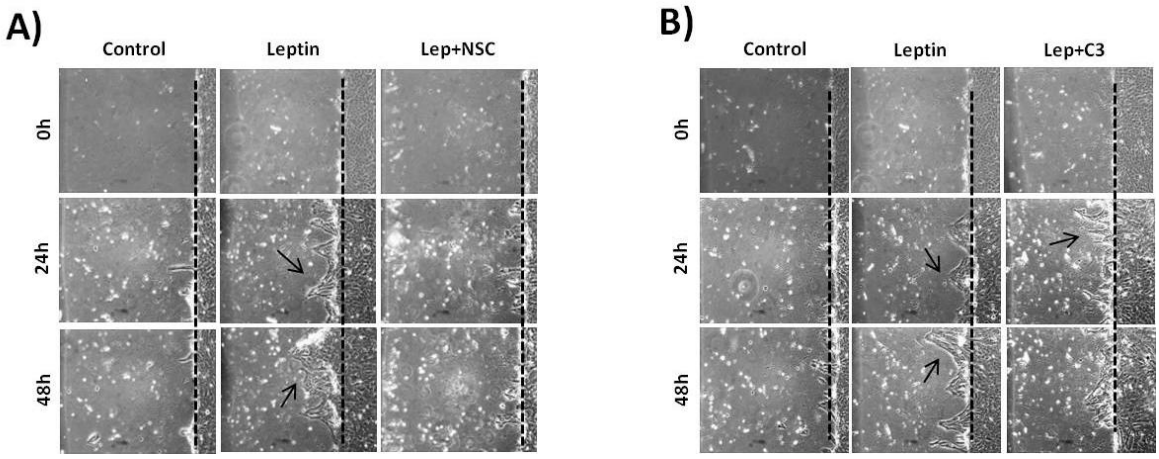


**Figure A2**. **Participación de Rac1 y Rho en la migración de células MCF10A inducida por leptina.** Ensayos de migración en placa. Control: células tratadas con vehículo. Leptin: 200ng/ml. NSC: inhibidor de Rac1, NSC23766 µM. C3: Inhibidor de Rho 1ng/ml.

Se evaluó si la leptina provoca cambios en el citoesqueleto de actina de células MCF10A y la participación de Rac1, Rho y PI3K en este evento. Se observó que el tratamiento con leptina favorece la migración y orientación celular con protrusiones de membrana similares a lamelipodios. La inhibición de Rac1 bloquea la formación de lamelipodios en estas células y las mantiene en un fenotipo epitelial, mientras que la inhibición de RhoA no bloquea la formación de estas estructuras y las células mantienen un fenotipo alargado. El tratamiento con el inhibidor de PI3K, Wortmanina 20nM, provoco alteraciones evidentes en el citoesqueleto de actina, observándose células con protrusiones de actina muy largas, completamente distintas a las células sin tratamiento (Figura A4). Estas observaciones sugieren

que la leptina induce cambios en el citoesqueleto de actina que pueden favorecer la migración de células no tumorales de mama en una vía dependiente de PI3K y Rac1, mientras que Rho parece mediar el proceso de orientación celular.

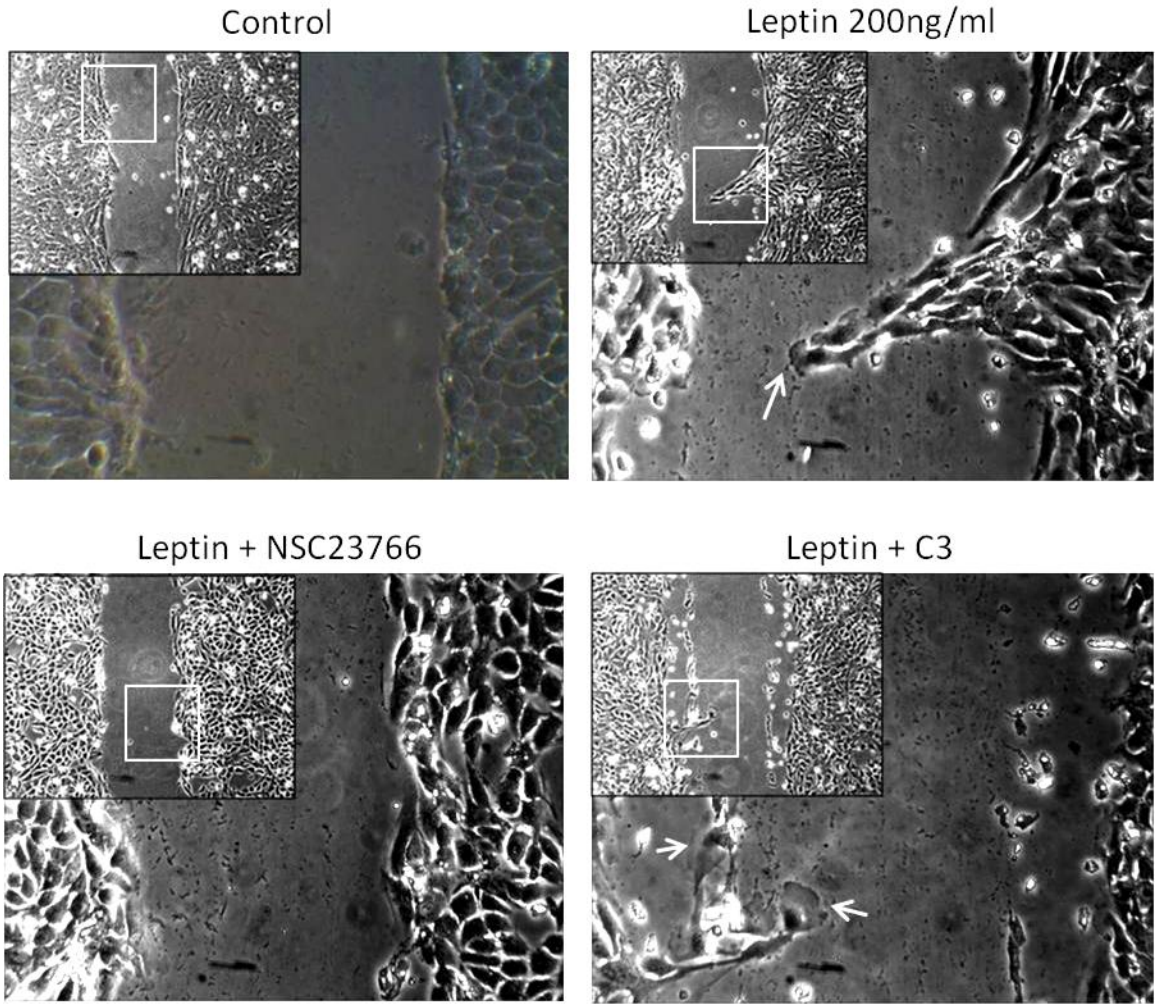


**Figure A3**. **Formación de protrusiones de membrana en respuesta a leptina, participación de Rac1 y Rho.** Ensayos de migración en placa. Cuadro: 10x, aumento a 20x. Flechas: estructuras similares a lamelipodios. Control: células tratadas con vehículo. NSC: inhibidor de Rac1, NSC23766 µM. C3: Inhibidor de Rho 1ng/ml.

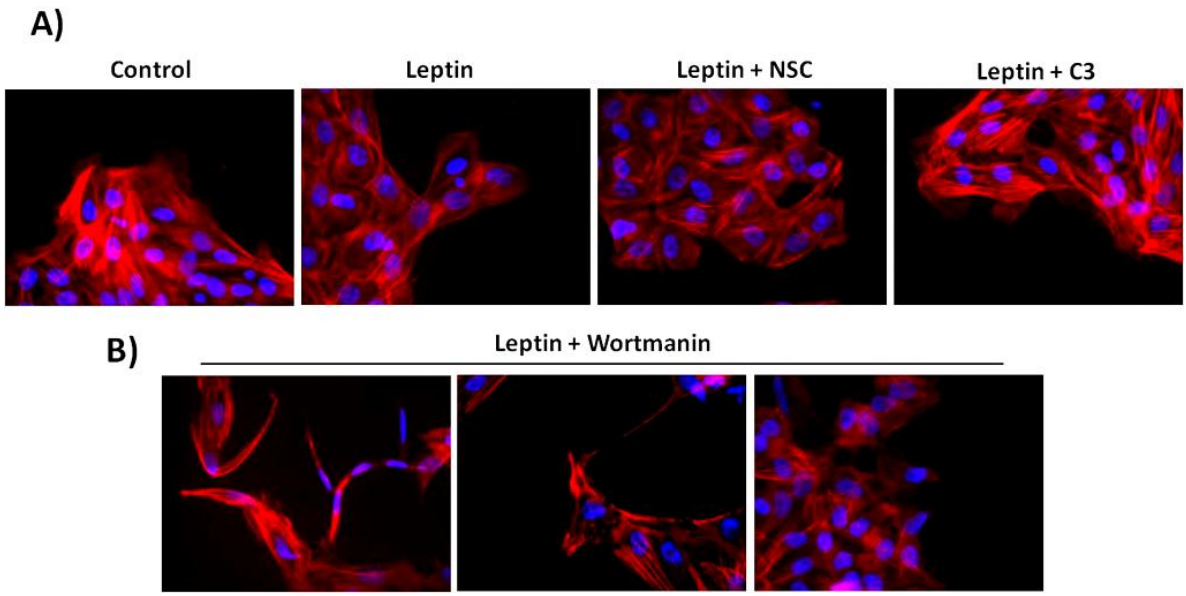


**Figure A4**. **Participación de Rac1, Rho y PI3K en los rearreglos del citoesqueleto de actina inducidos por leptina,.** Ensayos de migración en placa. Control: células tratadas con vehículo. Leptin: 200ng/ml, 48h. NSC: inhibidor de Rac1, NSC23766 25 µM. C3: Inhibidor de Rho 1ng/ml. Wortmanin: inhibidor de PI3K, 20nM

En conclusión, la leptina induce la migración de células no tumorales MCF10A, provoca cambios en el citoesqueleto de actina y parece promover la transición epitelio mesénquima de estas células. Estos cambios fueron dependientes de Rac1 y PI3K. Rho no bloquea la formación de protrusiones de membrana necesarias para la migración celular, sin embargo, parece participar en la orientación de estas células.

## Anexo 3.

En el periodo de la Maestría en Ciencias Biomédicas (2007-2009) se evaluó la expresión de GTPasas Rac1, RhoA, Cdc42 y los GEFs Tiam1 y β-Pix en biopsias de tejido cervical sano así como en lesiones escamosas intraepiteliales de bajo grado (LEIBG) y alto grado (LEIAG). Demostrándose por primera vez en tejido cervical, la sobreexpresión de las GTPasas Rac1 y RhoA y de los GEFS Tiam1 y β-Pix en lesiones premalignas de cérvix en comparación con el tejido sano. Además, de manera importante, se observó la expresión nuclear de Rac1 en lesiones premalignas cervicales, evento que no se observó en células de tejido cervical sano. Esta observación ha dado lugar a varios trabajos de tesis en el Laboratorio de Biología Celular del Cáncer de la Unidad Académica de Ciencias Químico Biológicas.

Durante el primer año del Doctorado en Ciencias Biomédicas se realizaron algunos experimentos y se preparó el manuscrito para la publicación de estas observaciones. El fruto de esta investigación se encuentra publicado en la revista Internacional **BMC cáncer,** factor de impacto 3.02 (2012):

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# Nuclear expression of Rac1 in cervical premalignant lesions and cervical cancer cells

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**Abstract**

**Background:** Abnormal expression of Rho-GTPases has been reported in several human cancers. However, the expression of these proteins in cervical cancer has been poorly investigated. In this study we analyzed the expression of the GTPases Rac1, RhoA, Cdc42, and the Rho-GEFs, Tiam1 and beta-Pix, in cervical pre-malignant lesions and cervical cancer cell lines.

**Methods:** Protein expression was analyzed by immunochemistry on 102 cervical paraffin-embedded biopsies: 20 without Squamous Intraepithelial Lesions (SIL), 51 Low- grade SIL, and 31 High-grade SIL; and in cervical cancer cell lines C33A and SiHa, and non-tumorigenic HaCat cells. Nuclear localization of Rac1 in HaCat, C33A and SiHa cells was assessed by cellular fractionation and Western blotting, in the presence or not of a chemical Rac1 inhibitor (NSC23766).

**Results:** Immunoreacivity for Rac1, RhoA, Tiam1 and beta-Pix was stronger in L-SIL and H-SIL, compared to samples without SIL, and it was significantly associated with the histological diagnosis. Nuclear expression of Rac1 was observed in 52.9% L-SIL and 48.4% H-SIL, but not in samples without SIL. Rac1 was found in the nucleus of C33A and SiHa cells but not in HaCat cells. Chemical inhibition of Rac1 resulted in reduced cell proliferation in HaCat, C33A and SiHa cells.

**Conclusion:** Rac1 is expressed in the nucleus of epithelial cells in SILs and cervical cancer cell lines, and chemical inhibition of Rac1 reduces cellular proliferation. Further studies are needed to better understand the role of Rho-GTPases in cervical cancer progression.

**Keywords:** Rho-GTPases, Carcinogenesis, Risk factors, Rac1

## Background

Cervical cancer is the second most common malignant neoplasia affecting woman worldwide. Infection with High-Risk Human Papillomavirus (HR-HPV) is considered the main risk factor for developing cervical cancer and its precursor lesions [1-3]. Development of cervical Low-grade Squamous Intraepithelial Lesions (L- SIL) and High-grade Squamous Intraepithelial Lesions (H-SIL), and progression to invasive carcinoma, are associated with alterations in the regulation of several cellular processes such as cell cycle progression, apoptosis, and DNA repair [2,4,5]. The HR-HPV oncoproteins E6 and E7 are responsible for many of these alterations, they act by binding to, and/or modifying the expression/activity of a growing number of cellular proteins [6], including p53 [7], pRb [8], p21 [9,10], and p27 [11,12]. Rho-GTPases are small signaling proteins involved in the regulation of crucial cellular functions such as cell shape, cell-cell adhesion, cell proliferation, cell division, migration and invasion [13-15]. Experiments using cell culture and animal models have demonstrated an important role for these proteins in carcinogenesis [16,17]. Moreover, it has been shown that expression of some Rho-GTPases and their regulatory proteins is altered in human cancers such as prostate, colon, lung, and breast cancer [18].

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Cell culture experiments showed that RhoC regulates invasion and motility of cervical cancer cells [19,20]. Furthermore, it has been reported that RhoC is overexpressed in biopsies from squamous carcinoma of the cervix (SCC) and cervical intraepithelial neoplasia (CIN) II/III when compared to normal cervical epithelium and CIN I [21]. However, expression of other Rho-GTPases has not been investigated in cervical cancer or its precursor lesions. The aim of this study was to investigate the alterations on the expression of the GTPases Rac1, RhoA, and Cdc42, and the Rho GEFs Tiam1 and beta-Pix in cervical premalignant lesions.

## Materials and methods

### Sample selection

102 paraffin-embedded cervical tissue specimens were obtained from the Department of Pathology at the "Vicente Guerrero" General Regional Hospital (IMSS), in Acapulco, Mexico. Eighty-two samples corresponded to cervical biopsies or cones with confirmed histological diagnosis of L-SIL (n = 51) or H-SIL (n = 31), and 20 corresponded to cervical tissue specimens without SIL, selected from patients undergoing hysterectomy for benign conditions, without a history of SIL or abnormal Pap results. One pathologist (LSL) reviewed all of the slides to confirm the diagnoses. All SIL cases were additionally reviewed by a second pathologist (JFC) to establish a consensus diagnosis (discrepancies relative to the original diagnoses were resolved by the interpretation of a third pathologist).

Approval to conduct this study was obtained from the Institutional Ethics Comitee at the "Universidad Autónoma de Guerrero". The study was conducted in compliance with the Helsinki Declaration.

#### *Detection of HR-HPV*

The presence of HR-HPV was determined by *in situ* hybridization using the GenPoint tyramide amplification signal kit (DAKO, Carpinteria, CA). Briefly, 3- micron paraffin sections were placed on silanized slides, deparaffinized, and incubated for 5 min at 37°C with proteinase K. Samples were dehydrated, and a mixture containing a pool of biotinilated DNA probes (directed against HPV 16, 18, 31, 33, 39, 45, 51, 52, 56, 58, 59 and 68 types) was added to each section. Sections were covered with a glass coverslip, denatured for 10 min at 95°C, and hybridization was performed for 20 h at 37°C in a humidified atmosphere in a Dako hybridizer (Dako, Carpinteria, CA). The slides were incubated with a streptavidin peroxidase-conjugated primary antibody, followed by incubation with biotil-tyramide, and with streptavidin. The reaction was developed by adding DAB, followed by staining with Mayer's hematoxylin (Merck, Germany), and mounted with Entellan mounting medium (Merck, Germany). The positive reaction was seen as a maroon or brown nuclear signal (Additional file 1: Figure S1).

#### Immunohistochemistry and immunocytochemistry

For immunohistochemistry, 3-micron paraffin sections were deparaffinized and rehydrated, followed by 20 min incubation in sodium citrate buffer (pH 6.0) at 110°C for antigen retrieval, using a pressure cooker (T-FAL Clipso). Samples were incubated for 10 min with immunodetector peroxidase block solution (Bio-SB Inc. Santa Barbara, CA.) to inactivate endogenous peroxidase, blocked with PBS + 1% BSA during 30 min, and incubated with primary antibodies for 1 h at room temperature. For immunocytochemistry, $5 \times 10^4$ HaCat, C33A, or SiHa cells were plated on glass coverslips in 6-well culture plates. Cells were maintained on DMEM medium (Invitrogen, Carlsbad, CA,) supplemented with 10% FBS (Byproductos, Mexico) at 37°C in a 5% $CO_2$ atmosphere. Where indicated, cells were treated with the Rac1 chemical inhibitor NSC23766 (Santa Cruz Biotechnology Inc, CA) at 25 μM or 50 μM. 24 h or 48 h after plating, cells were fixed with methanol-acetone (1:1) for 30 min, washed with PBS and antigen retrieval, blocking and primary antibody incubation were performed as described for immunohistochemistry. Primary antibodies were detected using a Mouse/Rabbit Immunodetector HRPw/DAB kit (Bio-SB Inc. Santa Barbara, CA.), following manufacturer's instructions, samples were counterstained with Harris's hematoxylin and mounted using Entellan mounting medium (Merck, North America Inc). Antibodies used were: Rac1 (C-14), RhoA (C-15), Cdc42 (B-8), Tiam1 (C-16), and beta- Pix (C-19) (Santa Cruz Biotechnology Inc, CA). The intensity of cytoplasmic staining was scored as weak, moderate or strong at 40× magnification (Additional file 2: Figure S2).

#### *Cellular fractionation and western blotting*

Cells were seeded on petri dishes and incubated for 24 h in the presence or absence of the Rac1 inhibitor NSC23766. Cells were washed with PBS and lysed in 500 μl of buffer A (10 mM HEPES, pH 9.7; 10 mM KCl, 0.1 M EDTA, 1 mM DTT; 0.5 mM PMSF plus protease inhibitors) directly on the plate and the protein lysate was transferred to a new microtube and centrifuged at 15 000 g for 3 min at 4°C. The cytoplasmic fraction (supernatant) was recovered in a new microtube and the pellet was resuspended in 150 μl of RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH8). The supernatant (nuclear fraction) was transferred to a new microtube. For total protein extracts, cells were lysed with RIPA buffer (50 mM Tris-HCl pH 7.6, 160 mM NaCl, 0.5 mM EDTA/EGTA, 1% Triton X-100, 10% glycerol, 1 mM PMSF and 1 μg/ml leupeptin). Whole cell, cytoplasmic and nuclear proteins were separated by SDS- PAGE in 10% acrylamide gels, transferred to PVDF membranes and detected by Western blot using antibodies against alpha-tubulin, lamin B, Rac1 (all from Millipore) and Tiam1 (Santa Cruz Biotechnology).

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*Cell proliferation assay*

$8 \times 10^3$ cells were plated on 24-well plates (Sarstedt AG & CO, Germany) and cultured in DMEM medium supplemented with 10% FBS for 24 h. Cells were treated with the Rac1 inhibitor NSC23766 (Santa Cruz Biotechnology Inc, CA) at 25 μM or 50 μM or with vehicle. Cells were fixed after 48 h of treatment, in 4% formaldehyde for 30 min. Cell proliferation was determined using crystal violet assay. The relative number of cells was determined by measuring the optical density of each well at 600 nm in a biophotometer (Eppendorf RS-2312 DH 8.5 mm).

### Statistical analysis

Association between variables was evaluated by Chi squared test or Fisher's exact test, as appropriate. Differences between data were determinate by two-way ANOVA test. A result was considered to be statistically significant when the $p$ value was < 0.05. Statistical analysis was performed using the software STATA *v9.2 or GraphPad Prism v5.03*.

## Results

### Overexpression of rho-GTPases and RhoGEFs in cervical pre-malignant lesions

We first determined HR-HPV infection as described under materials and methods. 20 samples were negative and 62 were positive for HR-HPV. In 20 samples HR-HPV infection could not be determined (Additional file 3: Table S1). Next, we determined the expression of Rac1, RhoA, Cdc42, Tiam1 and beta -Pix in cervical samples. Expression of the five proteins was observed in all cervical samples and the intensity of the signal for Rac1, RhoA, Tiam1, and beta-Pix was stronger in L-SIL and H-SIL, when compared to samples without SIL (Figure 1). As shown in Table 1, in the majority of samples without SIL the immunoreactivity for the five proteins was weak, only 35% and 20% of these samples had moderate/strong signal for Rac1 and Tiam1, respectively. In contrast, 64.7% L-SIL and 74.2% H-SIL showed moderate/strong signal for Rac1, and 80.4% L-SIL and 80.6% H-SIL had moderate/strong reactivity for Tiam1. Similarly, moderate/strong reactivity of RhoA was observed in 40%, 51%, and 71% for samples without SIL, L-SIL and H-SIL, respectively. Cdc42 reactivity was moderate/strong in 40% of samples without SIL, 41.2% L-SIL, and 61.3% H-SIL. For beta-Pix, moderate/strong reactivity was observed in 25% of samples without SIL, 37.2% L-SIL, and 64.5% H-SIL (Table 1). A significant association was found between the immunoreactivity of Rac1 and L-SIL ($p$ = 0.02) and H-SIL ($p$ = 0.005); RhoA and H-SIL ($p$ = 0.03); Tiam1 and L-SIL ($p$ < 0.001) and H-SIL ($p$ < 0.001); and beta-Pix and H-SIL ($p$ = 0.006). No significant association was found between the reactivity of Cdc42 and L-SIL or H-SIL, or between the reactivity of RhoA or beta-Pix and L-SIL. We found that the intensity of Tiam1 immunoreactivity was associated with HR-HPV infection ($p$ = 0.014), whereas no significant association was found between the immunoreactivity of Rac1, Cdc42, RhoA or beta-Pix, and HR-HPV infection (data not shown).

### Nuclear expression of Rac1 in SILs and cervical cancer derived cell lines

We observed a strong nuclear reactivity for Rac1 in a subset of L-SIL and H-SIL samples, whereas no nuclear reactivity was observed in samples without SIL (Figure 2A). Nuclear staining was observed along all layers of the epithelium and was found in 52.9% L-SIL and 48.4% H-SIL (Table 2). In line with these observations, nuclear immunoreactivity for Rac1 was observed in cervical cancer derived cell lines C33A (HPV-negative) and SiHa (HPV-16), but not in immortalized non-tumorigenic keratinocytes (HaCat). A strong perinuclear Rac1 immunoreactivity was also observed in SiHa cells (Figure 2B). These observations were further confirmed using cellular fractionation and Western blot analyses. As shown in Figure 2D, Rac1 was detected in the cytoplasmic fraction of the three cell lines, and in the nuclear fraction of C33A and SiHa cells, but not in the nuclear fraction of Hacat cells (Figure 2C). Using Western blot analysis on whole-cell extracts from HaCat, C33A and SiHa cells, we found that Rac1 protein levels are similar in all cell lines (Figure 2D).

### Chemical inactivation of Rac1 reduces its nuclear immunoreactivity and inhibits cell proliferation

To test whether nuclear Rac1 expression in C33A and SiHa cells is dependent on its activation status, C33A and SiHa cells were treated with 25 μM or 50 μM of the Rac1 inhibitor NSC23766. Imnunocytochemical analyses of NSC23766-treated cells showed an apparent reduction in the nuclear Rac1 immunoreactivity in both cell lines, as well as a reduction of the perinuclear immunoreactivity in SiHa cells (Figure 3A). However, cellular fractionation and Western blot analysis demonstrated that treatment with the Rac1 inhibitor does not affect the nuclear localizacion of Rac1 in these cell lines (Figure 3B). We next tested whether the chemical inhibition of Rac1 has an effect on the proliferation of Hacat, C33A and SiHa cells. We found that tNSC23766 treatment resulted in a significant decrease in the proliferation of the three cell lines (Figure 3C).

## Discussion

Overexpression of Rho-GTPases and Rho-GEFs has been described in various types of human tumors [18], and in some cases overexpression is associated with tumor progression or poor prognosis [22,23]. However, little is known about the role of Rho-GTPases in cervical carcinogenesis.

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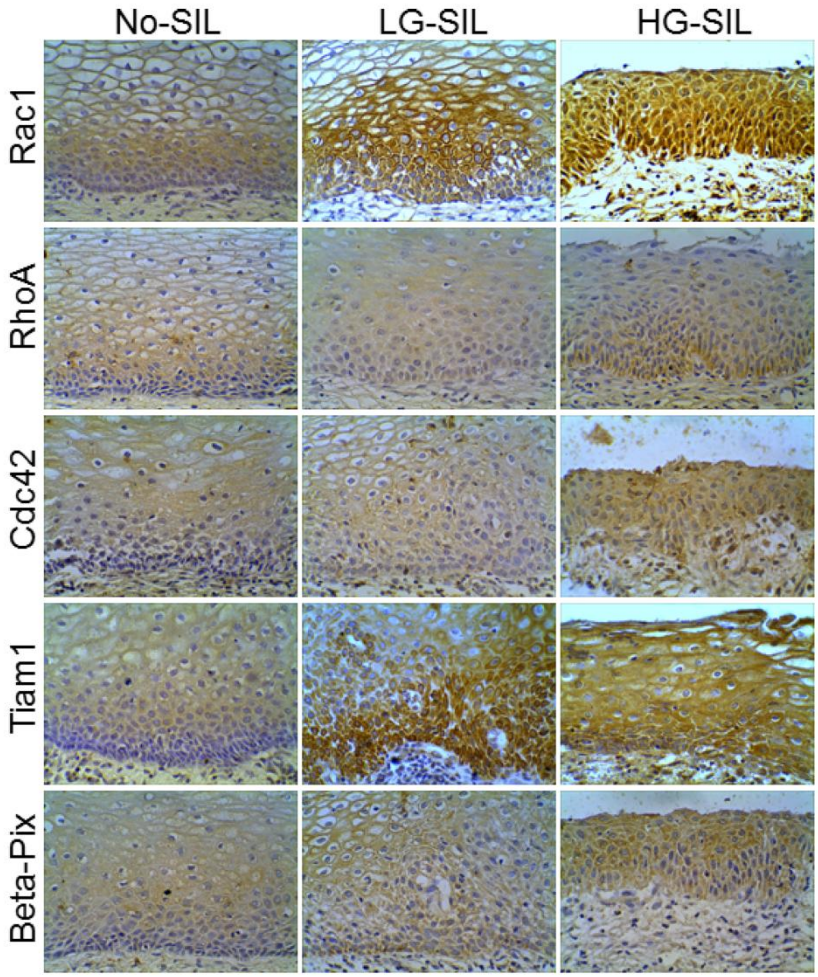


**Figure 1 Expression of Rho-GTPases and Rho-GEFs in cervical epithelium without squamous intraepithelial lesions, low-grade squamous intraepithelial lesions (L-SIL), and high-grade squamous intraepithelial lesions (H-SIL)**. Representative images of immunohistochemical analysis of Rac1, RhoA, Cdc42, Tiam, and beta-Pix expression. Magnification: 40 ×.

Here, using immunohistochemistry, we show that the immunoreactivity of the GTPases Rac1 and RhoA, and the Rho GEFs Tiam1 and beta-Pix, is increased in SILs, compared to cervical epithelium without SIL. Interestingly, we found that Rac1 is present in the nucleus of a subset of L-SIL and H-SIL, but not in samples without SIL. In agreement with these findings, we observed nuclear localization of Rac1 in cancer derived C33A and SiHa cells but not in non-tumorigenic HaCat cells.

Rac1 has a nuclear localization signal (NLS) [24], and it has been recently shown that the importin Karyopherin alpha 2 (KPNA2) mediates Rac1 nuclear import trough the interaction with its NLS, and that KPNA2-mediated nuclear import of Rac1 requires Rac1 activation [25]. Here we show that the nuclear localization of Rac1 in C33A and SiHa cells is not affected by treatment with the Rac1 inhibitor NSC23766. These data indicate that in these cells, the presence of Rac1 in the nucleus is not dependent on its activation. Michaelson et al. (2010) showed that Rac1 translocates to the nucleus during the G2 phase of the cell cycle, and that targeting an active form of Rac1 to the nucleus promotes cell proliferation [26]. We found that chemical inhibition of Rac1 reduces the proliferation of cervical cancer cell lines C33A and SiHa, as well as that of non-tumorigenic HaCat cells. In HaCat cells, in which Rac1 is localized to the cytoplasm, chemical inhibition of Rac1 may reduce its nuclear translocation during the G2 phase of the cell cycle, resulting

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**Table 1 Association between the intensity of Rac1, RhoA, Cdc42, Tiam1 and beta-Pix immunoreactivity, and the histological diagnosis**

| | Histological diagnosis | | | | |
|---|---|---|---|---|---|
| IR* intensity | without SIL % (n) | L-SIL % (n) | p value† | H-SIL % (n) | p value† |
| **Rac1** | | | | | |
| Low | 65 (13) | 35.3 (18) | **0.02** | 25.8 (8) | **0.005** |
| moderate/high | 35 (7) | 64.7 (33) | | 74.2 (23) | |
| **Cdc42** | | | | | |
| Low | 60 (12) | 58.8 (30) | 0.93 | 38.7 (12) | 0.12 |
| moderate/high | 40 (8) | 41.2 (21) | | 61.3 (19) | |
| **RhoA** | | | | | |
| Low | 60 (12) | 49 (25) | 0.41 | 29 (9) | **0.03** |
| moderate/high | 40 (8) | 51(26) | | 71(22) | |
| **Tiam1** | | | | | |
| Low | 80 (16) | 19.6 (10) | **< 0.001** | 19.4 (6) | **< 0.001** |
| moderate/high | 20 (4) | 80.4 (41) | | 80.6 (25) | |
| **Beta-Pix** | | | | | |
| Low | 75 (15 | 62.8 (32) | 0.33 | 35.5 (11) | **0.006** |
| moderate/high | 25 (5) | 37.2 (19) | | 64.5 (20) | |
| ***Total*** | ***20*** | ***51*** | | ***31*** | |

*IR = immunoreactivity; † = Chi squared test

in a reduction in cell proliferation. Buongiorno et al. (2008) showed that an inactive form of Rac1 is present in the nucleus of colorectal cancer cells, where it associates with the transcription factor TCF-4 [27]. Interestingly, these authors demonstrated that activation of the Wnt signaling pathway induced the nuclear translocation of Tiam1, a Rac1-specific activator, in a complex with beta-catenin, and that once in the nucleus a beta-catenin/Tiam1/TCF4/Rac1 complex can be formed, resulting in the activation of Rac1 and transcriptional activation of Wnt target genes [27]. Activation of the Wnt signaling pathway plays an important role during cervical cancer progression [28,29]; therefore nuclear Rac1 may cooperate with this pathway to stimulate proliferation of cervical cancer cells. We found that chemical inhibition Rac1 in C33A and SiHa cells, in which Rac1 localizes both to the cytoplasm and the nucleus, impairs proliferation without affecting Rac1 nuclear localization. In these cells, inactivation of the nuclear pool of Rac1 may impair the interaction of Rac1 with nuclear proteins such as TCF4 and beta-catenin, resulting in a reduction in the expression of proliferation-related genes and therefore the reduction in cell proliferation. However, Rac1 can also regulate proliferation trough the activation of cytoplasmic signaling pathways such as NF-kB [30], MAPK [31], Jak/Stat [32] and Wnt [33] pathways. Therefore, it is possible that inhibition of the cytoplasmic pool of Rac1 in both cervical cancer-derived and non-tumorigenic cells may result in a reduction of cell proliferation, independently of Rac1 nuclear functions. Altogether, these data suggest that nuclear Rac1 may play an important role in regulating cell proliferation and gene expression in cervical cells, and that the presence of Rac1 in the nucleus of cervical epithelial cells from pre-malignant lesions may contribute to cancer progression.

In our study, we observed overexpression of Rac1, RhoA and Tiam1 in L-SIL and H-SIL, and beta-Pix in H-SIL, when compared with epithelia without SIL. In vitro experiments in HeLa cells demonstrate that Rac1 [34] and Rho [35] activation is required for cell growth and migration. Similarly, experiments in CaSki cells showed that inhibition of migration and invasion by the anticancer agent JOTO1007, is associated with a reduction in the expression of RhoA and the Rho downstream effector ROCK-1 [36]. Moreover, experimental evidences indicate that Rho GTPases play a role in cellular transformation. It has been shown that Rac1 and its activator Tiam1 are required for Src-induced transformation [37]. Similarly, it has been demonstrated that Rac1 and Cdc42 are necessary for H-Ras-induced transformation, although overexpression of constitutively active forms of Rac1 or Cdc42 is not sufficient for cellular transformation [38]. It has also been shown that RhoA overexpression can induce pre-neoplastic transformation of primary mammary epithelial cells [39]. These data suggest that overexpression of Rho GTPases in SILs may cooperate with other signaling pathways to promote tumor progression.

We found that the increased immunoreactivity of Rac1, RhoA and beta-Pix correlates with the histological diagnosis but not with HR-HPV infection. In contrast, Tiam1 immunoreactivity was associated with both histological diagnosis and HR-HPV infection. These observations suggest that altered expression of Tiam1, but not that of Rac1, RhoA and beta-Pix may be dependent of HR-HPV infection. However, further studies are needed in order to determine if increase levels of Rho proteins and their GEFs is induced directly by HPV oncoproteins or is the result of a secondary event related to the progression of the malignancy. Our data indicate that nuclear expression of Rac1 in cervical lesions may be independent of HR-HPV infection as not all HR-HPV positive samples have nuclear staining for Rac1. Moreover, both HPV-negative and HPV-positive cervical cancer derived cells have nuclear staining for Rac1. However, as mentioned above, it is possible that infection with other HPV types not detected by ISH technique we used in this work may affect the subcellular localization of Rac1. Moreover, ISH does not allow us to identify which HR-HPV type is present in the samples, and it is possible that infection with some HR-HPVs such as HVP16 and HPV18 will have a more dramatic effect on the expression of these proteins.

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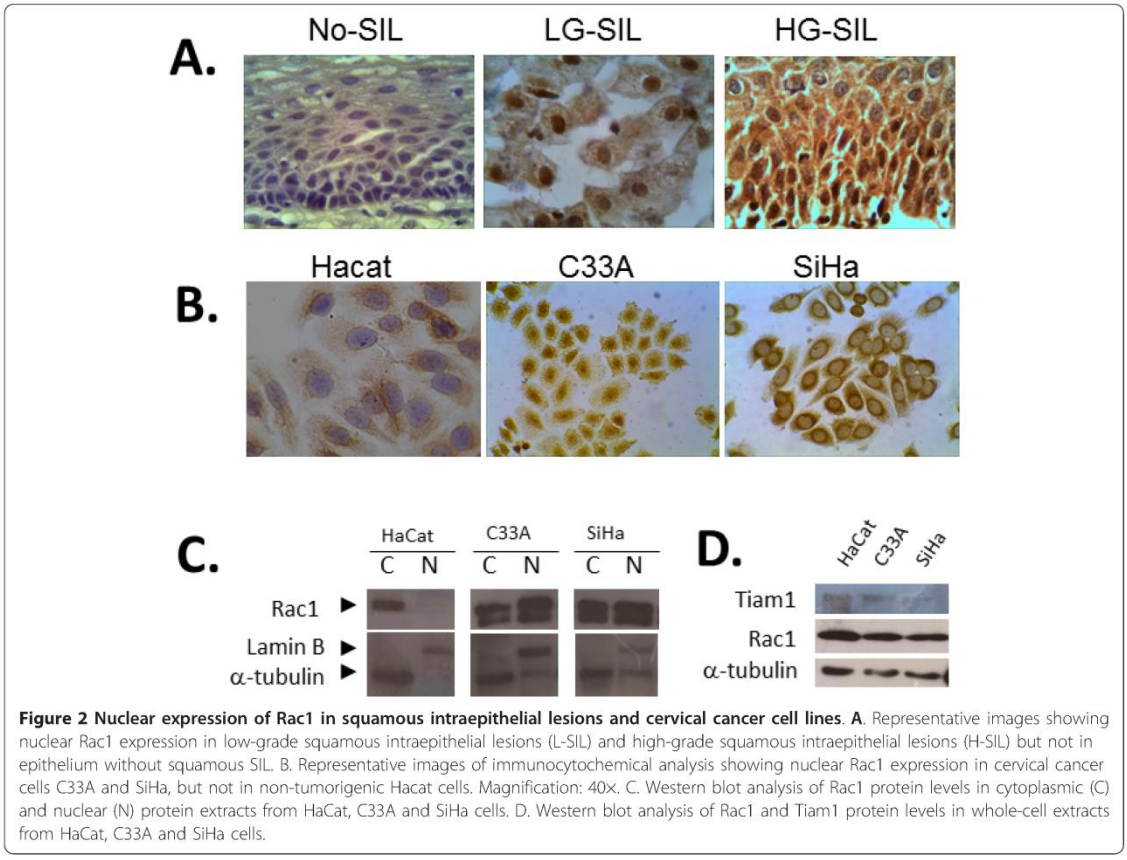


**Figure 2 Nuclear expression of Rac1 in squamous intraepithelial lesions and cervical cancer cell lines**. **A**. Representative images showing nuclear Rac1 expression in low-grade squamous intraepithelial lesions (L-SIL) and high-grade squamous intraepithelial lesions (H-SIL) but not in epithelium without squamous SIL. B. Representative images of immunocytochemical analysis showing nuclear Rac1 expression in cervical cancer cells C33A and SiHa, but not in non-tumorigenic Hacat cells. Magnification: 40x. C. Western blot analysis of Rac1 protein levels in cytoplasmic (C) and nuclear (N) protein extracts from HaCat, C33A and SiHa cells. D. Western blot analysis of Rac1 and Tiam1 protein levels in whole-cell extracts from HaCat, C33A and SiHa cells.

This could be of particular relevance for our study population, as in a recent study performed on women from Guerrero state in the south of Mexico, Illades-Aguiar et al., (2010) reported that whereas HPV16 is the most frequent HPV type present in women with cervical cancer, the most frequent type in women with L-SIL was HPV33 [40]. We also found moderate-strong reactivity for the five proteins in samples without SIL (Table 1). Recent evidences demonstrate infection with HR-HPVs in patients without SILs [40-42]. It is possible that some of the samples without SIL that showed moderate-strong reactivity are positive to HR-HPV. As mentioned above, we used ISH for the detection of HR-HPV infection. However this method has limitations as it detects only a subset of HR-HPV types. Further studies using more sensitive techniques such as PCR-RFLP or sequencing for the detection and typing of HPV infection will be required to answer to this concern. Finally, we could not determine HR-HPV infection in a subset of samples. Further investigation is required to determine the possible association between the overexpression of Rho-GTPases and HR-HPV infection.

**Table 2 Association between the Rac1 nuclear immunoreactivity, and the histopathological diagnosis**

| | Histopathological diagnosis | | | |
|---|---|---|---|---|
| Rac1 nuclear | Without SIL % (n) | L-SIL % (n) | H-SIL % (n) | p value* |
| Negative | 100 (20) | 47.1 (24) | 51.6 (16) | |
| Positive | 0 (0) | 52.9 (27) | 48.4 (15) | < 0.001 |
| ***Total*** | ***20*** | ***51*** | ***31*** | |

* = Fisher's exact test: without SIL versus L-SIL, and without SIL versus H-SIL.

One of the limitations in our study is that expression of the analyzed proteins in cervical biopsies was studied only by immunochemistry. Further studies using Western blotting, as well as analysis of a larger number of samples are required.

## Conclusions

In conclusion, Rac1 and Tiam1 are overexpressed in L-SIL and H-SIL, RhoA and beta-Pix are overexpressed in

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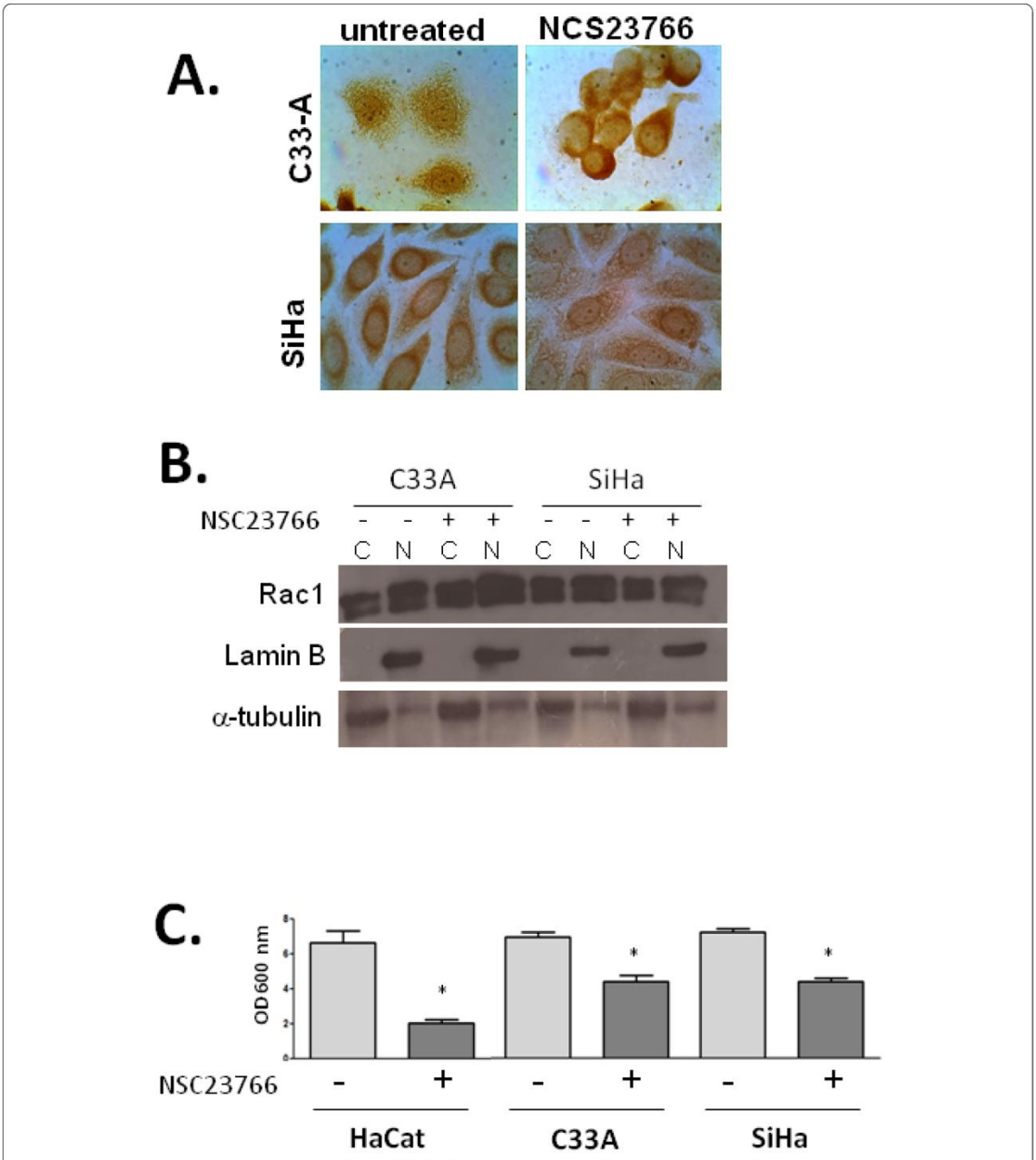


**Figure 3 Effect of the Rac1 chemical inhibitor NSC23677 on the subcellular localization of Rac1, and on cell proliferation**. **A**. Representative images of immunocytochemical analysis for Rac1 in C33A and SiHa cells, treated or not with the Rac1 inhibitor NSC23677. **B**. Detection of Rac1 in cytoplasmic (**C**) and nuclear (N) proteins from HaCat, C33A and SiHa cells treated or not with the Rac1 inhibitor NSC23677. Lamin B was used as a nuclear marker and α-tubilin is a cytoplasmic marker. **C**. Results from crystal violet cell proliferation assays on HaCat, C33A and SiHa cells treated or not with the Rac1 inhibitor NSC23766 for 48 h. Data shown are average optical density values plus standard deviation of experiments performed in triplicate.

H-SIL. Rac1 is expressed in the nucleus of cervical premalignant-lesions and cervical cancer derived cells lines. The chemical inhibition of Rac1 inhibits cell proliferation in Hacat, C33A and SiHa cells. To our knowledge, this is the first report showing abnormal expression of Rho-GTPases in cervical cancer, Further studies are needed to better understand the role of the overexpression of Rho-GTPases, as well as the nuclear Rac1 expression in cancer progression.

## Additional material

**Additional file 1: Figure S1**. HR-HPV detection by ISH. Representative images of (a) L-SIL sample in which HPV-probe set was excluded (negative control); (b-d) HR-HPV positive H-SIL showing strong nuclear staining. (a-c) 40×, (d) 100 ×.

**Additional file 2: Figure S2**. Establishment of the criteria for interpretation of IHC results. To analyze the differences in the intensity in immunoreactivity of the five proteins, we defined four categories based on signal intensity: a) negative, b) low, c) moderate and d) strong.

**Additional file 3: Table S1**. Characteristics of the study population.

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**Authors' contributions**
MAMC, GRCM, JAG and HNVF performed the experimental procedures and analyzed the data; MAMC performed statistical analyses; LSL and JFC performed the histopatological diagnosis and participated in the interpretation of IHC results; LSL and MAR, participated in sample selection and review of patient's files. MAR and LCAR performed detection of HR-HPV. BIA and MAMC participated in interpretation and analysis of data and manuscript preparation. ECS conceived the project, designed experiments, analyzed the data, supervised the whole project and wrote the manuscript. All authors read and approved the final version of the manuscript.

**Competing interests**
The authors declare that they have no competing interests.

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