Identification of novel mechanisms in human breast cancer lung metastasis and chemoresistance

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Doctoral Thesis 2017

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Doctoral Thesis
UNIVERSITAT DE BARCELONA 2016
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Growth Control and Metastasis Laboratory
Institute for Research in Biomedicine

Biomedicine Programme
University of Barcelona

Thesis defended by Enrique Javier Arenas Lahuerta in order to obtain the doctorate grade from the University of Barcelona

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ABSTRACT

Breast Cancer (BC) is one of the major causes of cancer deaths in women. BC is a heterogeneous disease, and within the heterogeneity of the tumor, **tumor-initiating cells (TICs)** have been reported as important drivers in tumor initiation and progression. On the basis of these observations, we hypothesized that TICs contribute to BC metastasis, as well to **chemoresistance.** Through genetic, transcriptomic, molecular and therapeutic analyses of tumor xenografts, BC cell lines, and/or human tumors, in this thesis we first demonstrate that ***RARRES3***, a lung metastatic suppressor gene, prevents adhesion to the lung parenchyma and the initiation of metastatic lesions by enforcing the retention of **differentiation** features and that this retention is driven by its $PLA_{1/2}$ catalytic activity. These results indicate that *RARRES3* genetic activity blocks both **tissue-specific metastasis** to the lung and metastatic initiation properties. Next, we dissected functional interplay between stem cell (SC)-like master regulator genes (***EVI1*** and ***SOX9***) and resistance to **mTOR inhibitors**, which leads to an aggressive metastatic cancer phenotype.

Collectively, the data shown in this thesis depict novel evidence regarding the role of tumor initiation properties in: I) BC progression and metastasis; II) how cancer progression is functionally linked to resistance to mTOR; and III) how resistance to therapy driven by tumor initiation properties may eventually produce an aggressive cancer phenotype.

Keywords: Tumor-initiating cells, Breast Cancer, Metastasis, Chemoresistance.

RESUMEN DE LA TESIS

El cáncer de mama (BC, de sus siglas en inglés) es una de las mayores causas de muerte por cáncer en mujeres. El cáncer de mama es una enfermedad heterogénea, y en la heterogeneidad del tumor, las células iniciadoras tumorales (TICs, del inglés) han sido asociadas como importantes responsables en la iniciación y progresión tumoral. Basándonos en estas observaciones, nuestra hipótesis es si estas células contribuyen en la metástasis y quimioresistencia en el cáncer de mama. A través de análisis genéticos, transcriptómicos, moleculares y terapéuticos en xenoinjertos tumorales, líneas celulares y tumores humanos, en esta tesis hemos revelado en primer lugar que *RARRES3*, es un gen de supresión metastática en pulmón, que previene la adhesión al parénquima pulmonar y la iniciación de lesiones metastáticas, imponiendo las características de diferenciación, y cuya retención es causada por la actividad catalítica $PLA_{1/2}$. Estos resultados indican que la actividad genética de RARRES3 bloquea específicamente la metástasis a pulmón y las propiedades de iniciación metastática. Además, hemos descrito una relación entre dos genes con características de autorenovación (*EVI1* y *SOX9*) y resistencia a inhibidores de mTOR, que termina con una fenotipo más agresivo y metastático.

Conjuntamente, los datos mostrados en esta tesis demuestran evidencias novedosas relacionadas con las propiedades de iniciación tumoral en distintos contextos: I) progresión y metástasis en el cáncer de mama; II) como la progresión tumoral está funcionalmente relacionada con la resistencia a la inhibición de mTOR; y III) como la resistencia a terapia que está desencadenada por estas propiedades de iniciación tumoral pueden al final producir un fenotipo más agresivo y metastático.

ACKNOWLEDGEMENTS

Estos 4 años han pasado volando. Todavía recuerdo ese verano del 2012 cuando llegué al Metlab y a la ciudad de Barcelona. Vine desde Madrid gracias a la beca de la Caixa para realizar el doctorado en el laboratorio de Roger Gomis. Parece que fue ayer. Sin embargo, ya estamos en el 2017, a punto de defender la tesis. Uno no llega aquí sólo, si no gracias a múltiples factores y múltiples personas y decisiones que nos hacen llegar a donde estamos cada uno. Así es la vida, ya sabéis, nunca terminamos donde pensábamos que íbamos a terminar, incluso la mínima decisión puede cambiar el trayecto de toda tu vida. Dicha la reflexión, ahora voy a ir a por los agradecimientos.

En primer lugar, querría agradecer a mi supervisor y director de tesis, **Roger Gomis**. Primero, por haberme elegido después de todo el proceso de selección de la Caixa. En segundo, por haberme dado la oportunidad de realizar mi tesis doctoral en su laboratorio, y por haberme dado grandes oportunidades que me van a ayudar en mi futuro. Y tercero, por todo el apoyo y comprensión en cualquier tipo de problema que he podido llevarle a su despacho. Muchísimas gracias Roger. Espero que en el futuro por supuesto, mantengamos el contacto, y que sepas que te estaré siempre muy agradecido.

En segundo lugar, me gustaría agradecer a las personas, sin las cuales, yo no estaría aquí, ni en ningún lugar, y esos son mis padres. Desde el fondo de mi corazón, ellos mismos saben lo infinitamente agradecido que les estoy y lo mucho que les quiero. Mis padres. A mi **madre María Pilar Lahuerta Chueca**, por ser el corazón de mi familia, y la que incondicionalmente ha demostrado lo mejor de ella por mí, y ser la madre más orgullosa del mundo. A mi **padre Pedro Gilberto Arenas Chumpitaz**, por ser el padre que más orgulloso está de su hijo pequeño. Además, me gustaría agradecer a las personas con las que crecí, nada más y nada menos que mis 8 hermanos. Porque, sin ellos, no sería lo que soy hoy en día, y porque no cambiaría mi infancia junto a ellos en nada. En orden ascendente: **Yolanda Beatriz, Fernando Raúl, David, Carlos Eduardo, Sergio, Christian, Jean Mitchael y Christopher**. Gracias a todos y cada uno de ellos. Además, me gustaría agradecer a toda mi restante familia (que no es poca), desde prim@s, tí@s, sobrin@s, etc. Especialmente a mis sobrinos: **Alejandro Daniel, Andrea Noemi, Elan, Ian, Lara, Eidan y Ainhoa**. También quiero dedicar unas palabras a dos de mis tías. Primero, a mi **tía Ángela**, por tener sólo buenas palabras y mucho mucho cariño, y por estar tan orgullosa como una madre sólo puede estarlo. Y segundo, a mi **tía Rosaura**, que me recibió como un hijo en mis años de universidad en la ciudad del Turia (Valencia), de allí que la llame mi madre de Valencia. Gracias por el apoyo que he recibido durante todos estos años, y que, pese a la distancia, podamos tener una relación tan cercana sobrino-tío. Como este es el párrafo de la familia, y ya muchos me conocen soy un gran amante de los animales, es imposible que no escriba unas palabras a mis adorables perritas, que me han marcado en mi vida. Primero, a mis dos mascotas actuales,

mi perrita **Sandry** y mi perrita **Luna**, por todo su cariño que me han dado a pesar de vivir en otra ciudad. Muchas veces me apetece estar en Zaragoza, y darles un paseo y estar con ellas en el sofá. Y segundo, a mi antigua mascota, mi perrita **Tais**. Aunque sea complicado de decirlo y entenderlo, siempre me acordaré de ella, porque durante sus 6 años que estuvo con nosotros, nunca pude pensar que pudiera querer tanto a un animal, gracias por todos esos momentos vividos, y de allí, que nunca la olvidaré.

En tercer lugar, agradecer especialmente a **Marina**, que ha sido parte fundamental de mi vida en estos dos últimos años. Muchas gracias por todo tu apoyo, tiempo, momentos vividos en este tiempo, que no son pocos. Muchísimos viajes, como Galicia, Alicante, Mallorca, Andorra, esquiadas, en los que he disfrutado muchísimo, y espero que sean muchos más. Devoción y amor por los animales (cuidado de perros—Linda, Maiah ; salvamento de Palomas—Gavi y Kebi; salvamento de caracoles—Croby); muchas fiestas allá donde vamos; muchas comilonas y cenas (sobre todo sushi a cantidad), con lo que nos gusta comer!; mucha ludopatía en el Bingo (que ha merecido la pena $$); y viciadas a la consola y al Crash Bandicoot. Podría escribir folios de todos los momentos vividos, porque han sido muy intensos estos años. Esta etapa no hubiese sido igual sin ti, no lo dudes. Gracias por haber ido a ese viaje a Lloret de Mar, y por haber terminado esta etapa conmigo. Con tres palabras resumo esto, Te quiero, y Gracias!.

Me gustaría dedicar especiales palabras a la gente con la que he pasado más tiempo durante estos cuatro años, y esos son la gente del MetLab! Por todas esas horas mano a mano en el laboratorio, por todo ese buen rollo, y todas esas risas. No solamente hemos sido compañeros, si no amigos, y así siempre da gusto ir a trabajar. No olvidaré esas cenas de navidad, bolera, cervezas, bailoteos, o bien pasar el rato cualquier día en el lab. Gracias por todo. Sólo os digo una cosa, que os voy a echar de menos y.... **A FREGAR!!!** Lo sé, lo necesitabais. Y otra cosa, El Azúuuucar!! Bueno, en primer lugar a mi compañera y mucho más que eso, a mi amiga **Anna Bellmunt** (o Twerking Sama). Por todos esos momentos y por ser la mejor compañera de laboratorio que he conocido y que uno puede tener. Por esos momentos de fiesta dándolo todo bailando, en el karaoke, en fiestas de gràcia, de sants, en welcome parties, Cool-offs, etc. No sólo fiesta, sino miles de quedadas, cafés, comidas, cenas. Nunca olvidaré esos grandes momentos de confidencias y bailando poniendo pachanga pachanga en la sala de cultivos (sí, era nuestro secreto.... Jajajajaja). Sé que echarás de menos mis Sinyas. Sin ti, indudablemente, estos 4 años no hubiesen sido iguales, y es que se nota cuando dos personas son amigos y se lo pasan bien. Lo que no echaré de menos es tu mirada de odio cuando voy a molestarte cuando estás bien concentrada jajaja. Sabes que te deseo lo mejor, y espero que no haya sido más que el principio de una laaarga amistad. Espero que no te quejes de las palabras, aunque me habías pedido tres folios jaja. A **Marc**, por ser la alegría del laboratorio y por tener una gran paciencia conmigo en todos los momentos, y por ser el primero que me recibió cuando llegué al IRB, y estar siempre dispuesto a ayudar. A

Esther, por todo su apoyo en todos los aspectos, e ir con una sonrisa siempre allá por donde va. A **Fernando**, por ser la "estrella" del laboratorio, y por todas las risas compartidas, y por ser un gran compañero en la bench. Menos mal que no nos importa mucho que nos toquen las cosas, si no sería un problema ;), lo celebramos en la gala de Tu Cara me Suena!. A **Jelena**, por estar siempre como apoyo, y ser la mami que siempre tenga que decir: niñooooooos! A **Juanmi**, que ha demostrado un gran compañerismo y transmitido buen rollo en este último año, y también, por transmitirnos su infinita sabiduría, con su léxico tan culto. A **Ali**, por estar siempre dispuesta a echar unas risas y hacer el bobo en el lab, cosas que siempre se agradecen, y también por alimentarnos constantemente a todos. Te debo mínimo una cena o algo, después de todas las galletas que me he comido, jaja. A **Sara**, que a pesar de que solo hayamos compartido unos meses en el lab, ha traído buen ambiente y nunca tiene una mala cara para nadie. To **Sylwia**, for all her advices and support in all these years, it was a pleasure for me working with you in your project. A **Milica**, por ser única en su especie, y por todas las pullitas que en realidad me han hecho reír mucho. A **Vanya**, por ser un gran compañero, y dar siempre su particular perspectiva positiva de las cosas, incluso a altas horas de la madrugada,

Por supuesto, mi estancia en el IRB no habría sido la misma sin unos grandes amigos con los que he compartido grandísimos momentos en estos 4 años. A los VIPshhh, por todos esos buenos momentos, quedadas, viajes, locuras, fiestas, etc. Nunca olvidaré por ejemplo, ese gran fin de semana en Vilopriu, celebraciones de cumpleaños haciendo locuras en el centro de Barcelona (que vaya tela), etc. Gracias por compartir momentos tan divertidos y únicos. A **Anna**, por ser amiga y compi de lab. A **Ivan**, por no solo ser un gran compañero de cafés, si no un gran amigo, con un gran sentido del humor. Por esos lunes espontáneos mano a mano en Apolo en Nasty Mondays, esos cafés, esas quedadas para running, cenas en casa y muchas cosas más; espero poder seguir compartiendo grandes momentos. A **Clara Suñer**, por contagiar esa risa a carjada limpia a todo el mundo, y siempre estar dispuesta a pasarlo bien y conocer gente. No olvidaré esas primeras cool-offs a lo loco, acabando en el Karaoke y cantando mi música es tu voz, y cuando empezamos a bailar salsa! **A Nuri**, por ser simplemente, Nuri, con su particular visión de la vida y de las cosas, tan happy y tan positiva, y que me hace reir tanto. A **Berta** (o Berra), por ser la sonrisa, y simplemente transmitir buen rollo y felicidad a todo el mundo. A **Clara Morral**, por ese entusiasmo que pone en todo lo que se propone. A **Victor**, por las ganas de pasarlo bien con sus amigos. Aparte me gustaría agradecer a el resto de amigos del IRB. A **Pol**, por esos momentos de running y risas. A **Sonia**, por ser la parte sensata, y con la que se puede contar siempre. A **Rosa**, por ser la alegría de la huerta, y siempre estar dispuesta a pasarlo bien (Al abismó). A **IreeenÉ** (voz de David Jones), por todas esas risas, a pesar de siempre ir con cara de mal genio por los pasillos (con cariño). A **Anna Casas** (O Ninja, o Bucu), porque a pesar de estar desaparecida de vez en cuando, al final siempre está allí. A **Dan**, por compartir tan buenos momentos y risas

durante nuestros primeros años en el IRB, mano a mano. Aparte de todos ellos, y perdón si me he dejado a alguien importante, me gustaría agradecer a tod@s los que habéis formado parte de esta parte tan importante de mi vida, no solo a nivel profesional, si no también personal. A todos los que he compartido momentos como el PhD symposium 2015, el retreat, esquidas, cool-offs, cenas, welcome-parties, y tantas cosas que se han podido compartir en el IRB, un sitio único para realizar el PhD.

A los estudiantes que he tenido durante mi estancia: a Anna Perdrix, Neus, Carla, Anna Sagrera, Noemí, Guler, y Miquel. A **Barbara**, por ser una gran compañera durante su estancia en el laboratorio. Por haberme dado la oportunidad de ser su supervisor y encargarme de su trabajo en el laboratorio. Me gustaría agradecer también a todo el equipo del IRB Barcelona, a todos los equipos, y en especial a los equipos de microscopía, histología, y citometría. Además, me gustaría agradecer a colaboradores, como la gente del Instituto Catalán de Oncología (ICO), por hacer posible el último artículo publicado. A **Miquel Àngel Pujana**, por hacerme partícipe del proyecto, y a **Francesca Mateo** por ser una gran colaboradora en todos los experimentos que hemos llevado a cabo.

También, y no menos, a los amigos que he encontrado estos cuatro años en Barcelona, algunos de ellos ya antes de cuando vine a la ciudad condal, como **Xarly**, **Salva** y **Lucre**, a los que ya he dedico unas palabras después. Aún así, Xarly, muchas gracias por ser el primero en integrarme en esta ciudad, aún recuerdo cuando llegué a mediados de agosto y fuimos mano a mano a las fiestas de gràcia! También por lo mismo gracias a **Jorge**. A **Sergio**, porque a pesar de que nos conocimos en Zaragoza unos meses antes, nos hicimos amigos de verdad en Barcelona. Gracias por compartir ese año y medio conmigo en el piso. A mis antiguos compis de piso **Hector** e **Irantzu** (la vesina, a la que le deseo lo mejor en Oslo). A mi **Anchel**, el mejor compañero de piso que he tenido nunca, no por ser ya antes grandes amigos en Valencia, si no por ser una gran persona, amigo, siempre dispuesto a echar unas risas con los colegas. Sin ti, ese tiempo que viviste en Barcelona, no hubiese sido lo mismo, esas fiestas constantes en Razz, en casa, o simplemente mandando audios en el sofá mientras veiamos la tele. ¡Eres un grande! A **Alba**, porque aunque nos conocimos en Tenerife, en esa semana única de la UIMP, nos conocimos de verdad estos cuatro años. Gracias por estar siempre disponible para cualquier evento, ya sea ir a Amsterdam, Zaragoza, Jaca, etc, y ayudarme también a sentirme más cómodo en la ciudad, cuando no conocía a mucha gente todavía. A mis amigos de la chupi, con los que he pasado momentos muy pero que muy divertidos estos cuatro años, muchas fiestas cualquier día a cualquier hora, muchos viajes, barbacoas, etc, y que han participado de que esta etapa haya sido tan singular: **Roger**, **Sonia Lianta Mala**, **Fran** (o Fruñón), **Lily** (o Loly), **Dani**, **Anna** (o Rafaela), y en especial a **Lau** (Killer) y **Cristina** (Cia), por haber demostrado ser grandes amigas durante estos 4 años, estando presentes en todo tipo de celebraciones/eventos, quedadas, etc. De Lau, las quedadas a comer

cuando estaba en el IRB, risas, cenas, sushi etc, a pesar de llamarme Gus. De Cia, por sus ganas de pachanguear siempre que se pueda, por esos días de playika, y supuesto, haberme presentado a Marina. También me gustaría dedicar palabras a **Georgina**, por ser una gran compañera ese poquito tiempo que estuvimos en el MetLab, y por seguir viéndonos a menudo, y permitirme disfrutar de la bonita de Laila. A **Ginés**, por sus visitas a Barcelona, Londres y por ser ahora un buen amigo que ha venido a vivir a la ciudad condal hace poco. También, durante esta época, he podido disfrutar de una de las cosas que más me gusta, y es bailar, salsa y bachata. Gracias Clara Suñer e Iván por ser los iniciadores de ello. También me gustaría agradecer a toda la gente que he conocido bailando, y aunque no nos veamos como cuando empezamos a bailar, me habéis aportado muchos buenos momentos, risas y compañerismo. Además, me gustaría agradecer a todo el mundo que he conocido en Barcelona, ya que uno y cada uno de ellos hacen que me sienta así, y pueda escribir estas palabras. Y por último, a **Esteve**, que sin él esta tesis no hubiese sido igual, gracias por esa paciencia tio!.

Unas especiales palabras también a la gente que conocí en mi año en Madrid, justo antes de llegar a Barcelona. A esa gente del Master de Biomedicina, especialmente a la gente de #FAN #FUN. Nunca olvidaré esas celebraciones de cumple, fines de semana en Madrid, y fiestas en el independance o el B12. A mi Bro **Carlos**, por ser un grandísimo amigo, con el que he pasado muy buenos momentos, y en el que me ha apoyado estos cuatro años. Muchas gracias por estar siempre allí, por ser de lo mejor que he conocido, por todos esos viajes: Pilares Zaragoza, Amsterdam, Arenal Sound; todas esas parrandas; y por saber que tengo una casa en Madrid, y ser siempre bien recibido. Has demostrado ser uno de los mejores amigos que alguien puede tener. A mi mareadora repija, **Lidia**, por ser tan paciente y haberme acogido tantas veces en su hogar. A la moderner **Palomalopa**, por haberme enseñado tanto de los modernos y siempre estar riendo. Además, me gustaría agradecer a **Alejandra**, por haber sido una buena amiga todo este tiempo, y permitirme compartir sus momentos conmigo, y no olvidarse de su hus (algún día tendremos que repetir momentos tan buenos como esos findes locos en Madrid en la B12). Además, me gustaría mencionar a la gente que conocí en Tenerife durante ese año, y que hicimos una buena amistad durante ese año en Madrid y estos cuatro años de mi tesis. Gracias por esos viajes y cervezas y cenas cuando estoy en Madrid: **Elena, Patri, Hayk, Fausto y Marta**. Y aunque haya pasado el tiempo y ya no haya tanto contacto me gustaría agradecer a la gente que conocí ese año como **Amparo, Sandra, Ane, Ángel, Cristina, Mike, Elvira, Silvia, Beus, Eva, Alejandro y Alberto.**

Especiales palabras también a mis grandísimos amigos de Valencia, que no solo han demostrado ser únicos durante mis 4 años de la carrera, si no durante todos estos años. Gente que siempre puedes estar seguro que van a estar allí, y con los que me gustaría siempre ver un poquito más. Primero, a la gente de la resi. No olvidaré nunca todos los momentos vividos durante esa gran época, y que por supuesto se ha mantenido durante estos años. A **Jose Miguel**, por ser un amigo

único, con el que he compartido todo, y con el que he contado para cualquier ocasión. No olvidare nunca todos los años vividos, viajes, risas, confidencias, apoyo mutuo, y muchísimas cosas más, y me quedaría corto. Está bien verse al menos un par de veces al año pese a la distancia. A **Fran** (Llombi), por venir a Barcelona siempre que puede, y estar siempre allí. Eres un amigo de verdad, con el que se puede contar siempre. A **Laura Moratón**, que voy a decir de mi hija, pues que gracias por ser tan buena y querer tanto a tu gente (este año nos vemos en Fallas, no lo dudes). A mi morritos Karmenxu, por ser tan única, y definirse como **Karmenxu** morritos, gracias por esos momentos de pura risa, mojitos, party loca, y ganas de pasarlo bien. A **Salva**, por ser mi Salvita, y siempre preocuparse por cada etapa de mi vida. A **Xarly**, por estar siempre allí, un incondicional de la amistad. A **Nachito Manrique**, por esas cervezas que nos echamos siempre que coincidimos en un sitio. A **Lucrecita**, no solo por haber sido mi compañera de piso en mi año en Barcelona, si no por ser una amiga con la que siempre se puede contar. No olvidaré esas celebraciones y esas quedadas donde nos contamos todo en Barcelona. A **Inma** (o más bien Miss Látigo), no solo por ser una de las personas más prudentes que conozco, si no porque ha demostrado ser una gran amiga. No olvidaré esos momentos de risas con Jamón, ya sea en Valencia, Barcelona o Teruel. A los hermanos **Conrado y Antonio**, porque, a pesar de vernos casi nada, uno puede ir a Almería a verles, y ver como no ha pasado el tiempo. En segundo lugar, me gustaría agradecer a mis amigos de la carrera de Biotecnología. Primero, a mis grandes cracks **Pablo** (o Reina Mora), **Fernando** (o a RAE), Y **Jesús** (el paternero loco). Siempre dispuestos a sacar, aunque sea difícil, su tiempo para ir a cualquier lugar del mundo, y simplemente con estar nosotros, aunque sea debajo de un puente, pasarlo en grande. Por todos esos viajes, momentos únicos, y los que nos quedan. A **Pablo**, por estar en uno y cada uno de los momentos importantes, ya sea el master en Inglaterra, y todos estos años con sus visitas esporádicas a Barcelona. A **Fernando**, por ser único, y sacar lo mejor de mí en cada momento. A **Jesús**, por siempre estar dispuesto a reir y pasarlo bien y darlo todo. También me gustaría mencionar a **Miriam, Marta, Vicente y Fran**. Además, también a **Ángela**, por haber demostrado ser una muy buena amiga durante estos años. Gracias por ese tiempo sacado cada vez que vienes a Barcelona, por estar siempre allí, y por permitir ir a Málaga siempre que quiera.

A mis amig@s de Zaragoza. A mis mejores amigas de Zgz **Elisabet** y **Leticia**. A mi amiga Leti, por ser mi mejor apoyo en el mundo de la Jota. Todavía recuerdo aquél día que nos conocimos cuando fui a Daroca, y esas noches locas en el Tras (oyoyoyoy), y más que todo eso, por buscar cualquier momento por vernos a pesar de vivir en Perú. En todas las ciudades que he vivido, siempre has estado allí para venir a visitarme y pasarlo en grande! Tengo una visita pendiente allá, no dudes que te iré a ver! **Eli**, por estos más de 10 años conociéndonos y soportándonos, y pese a las pullitas, ser aquellos gitanillos que pasaron por el instituto de San Valero. Todavía me acuerdo aquellas risas en los pasillos, esas fiestas bailando a lo loco en el pícaro (que nos sabíamos hasta que canción iba a sonar!), y esas explicaciones de última hora para aprobar

matemáticas, física y química. No he de olvidar también a mis amigos **Luis, Dani, Javi, Sergio y David**, por estar siempre allí, dispuestos a ofrecer su tiempo y ganas de pasarlo bien. También a mi gran amiga, y compañera de Jotas **Virginia**, por ser tan buena persona. No olvidaré nuestras rondas bailando Albalate, y tus visitas a Londres y Barcelona.

Y por último, primero **gracias a todos** aquellos que dediquen su tiempo en leer esta tesis, que la he intentado hacer lo mejor que he podido. Y segundo, gracias a todos aquellos que habéis compartido este momento de mi vida. Gracias.

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4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
A	Adenine
ABC	ATP-binding cassette
AKT/PKB	Protein kinase B
ALDH	Aldehyde dehydrogenase
AML	Promyelocytic leukemia
ANGPTL4	TGFB inducible factor angiopoietin-like 4
APC	Antigen presenting cell
ASR	Age standardized rates
ATP	Adenosine triphosphate
ATRA	All-trans retinoic acid
BC	Breast cancer
BCSC	Breast cancer stem cell
BMDC	Bone marrow derived cell
BMP	Bone morphogenic protein
C	Cytosine
CAF	Cancer associated fibroblast
CD	Cluster of differentiation
COX2	Cytochrome c oxidase polypeptide II
CR	Cumulative Risk
CRC	Colorectal cancer
CSC	Cancer stem cell
CTC	Circulating tumor cells
CTL	Cytotoxic T cell
DCIS	Ductal carcinoma in situ
DMBA	dimethylbenz(a) anthracene
DNA	Deoxyribonucleic acid
DNER	Delta/Notch-like EGF-related receptor
DTC	Disseminated tumor cell
E	Estrogen
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
ER	Estrogen receptor

EVI1	Ecotropic virus integration site 1 protein homolog
EZH2	Enhancer of zeste homolog 2
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FISH	Fluorescent in situ hybridization
FKP12	FK506 binding protein 1 A 12kDa
FOXA1	Forkhead box protein A1
G	Guanine
GATA	Transcription factor family, binds to DNA sequence GATA
GH	Pituitary-derived growth hormone
GSK3B	Glycogen synthase kinase 3 β
H&E	Hematoxylin and eosin
HCC	Hepatocellular carcinoma
HER2	Human epidermal growth factor receptor 2
HRT	Hormone replacement therapy
ID	Inhibitor of differentiation
IGF	Insulin growth factor
IHC	Immunohistochemistry
K	Cytokeratin
LN	Lymph node
M	Metastasis
MAPK	Mitogen-activated protein-kinase
MaSC	Mammary stem cell
MDSC	Myeloid-derived suppressor cell
MET	Mesenchymal to epithelial transition
MIC	Metastasis-initiating cell
MiRNA	micro RNA
MMP	Metaloproteinase
MPA	Medroxyprogesterone acetate
mTOR	Mammalian target of rapamycin
NGS	Nottingham grading system
NK	Natural killer
OC	Ovarian cancer
ODX	Oncotype DX™
P	Progesterone
PCR	Polycomb protein complex
PDPK1	3-Phosphoinositide Dependent Protein Kinase 1
PFS	Progression free survival

PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIKK	PI3K-related protein kinases
PIP3	Phosphatidylinositol-3,4,5
PKB	Protein Kinase B
PPAR	Peroxisome proliferator-activated receptor
PR	Progesterone receptor
pS6	Ribosomal protein phosphor serine 235/236
PTEN	Phosphatase and tensin homolog
PTM	Post-translational modification
RA	Retinoid acid
RAI2	Retinoic acid-induced 2
RANK	Receptor activator of NF-kB
RANKL	RANK ligand
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RARRES3	Retinoic acid responder 3 (tazarotene 3)
RB	Retinoblastoma
RFS	Relapse-free survival
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
RXR	Retinoic X receptor
SC	Stem cell
SERM	Selective estrogen-receptor modulator
SFRP1	Secreted frizzled related protein 1
SMA	Smooth muscle actin
SOX2	Sex determining region Y-box 2
SOX9	Sex determining region Y-box 9
SP	Side population
SPARC	Secreted protein, acidic, cysteine-rich/osteonectin
STAT3	Signal transducer and transcription factor 3
T	Thymidine
TA	Transit-amplifying
TEB	Terminal end bud
TGF	Transforming growth factor
TIC	Tumor-initiating cell
TNBC	Triple negative breast cancer
TNC	Glycoprotein tenascin C
TP53	Tumor protein p53

TSC	Tuberous sclerosis protein complex
TSG	Tumor suppressor gene
TSP-1	Thrombospondin-1
VEGF	Endothelial growth factor
WHO	World health organization
Wnt	Wingless-related integration site// wingless-type MMTV integration

With love to all people who live in my heart

You cannot design your life like a building.It doesn't work that way.
You just have to live it.... and it will design itself

CHAPTER 1. INTRODUCTION

1.INTRODUCTION

1.1 Cancer, a disease increasing worldwide

The oldest description and surgical treatment of cancer dates back to 1600 *b.c* in Egypt, when eight cases of breast cancer (BC) treated by cauterization were described on a papyrus. The picture nowadays is completely different. Cancer is one of the leading causes of death worldwide. In 2012, 14.1 million new cases were diagnosed and 8.2 million died from this disease. Global cancer cases are higher in developed countries. In **Figure 1**, the global numbers of cancer incidence and mortality are shown. These data were obtained from 27 major cancers, available in the GLOBOCAN series of the international Agency for Research on Cancer (http://globocan.iarc.fr). The most common cancers diagnosed were lung, breast and colorectal cancer (CRC), and the most common cause of death were lung, liver and stomach cancers. In addition, there are differences in prevalence depending on gender. In men, lung and prostate cancer are more frequent, whereas in women BC is the most common type. These numbers are expected to increase markedly, mainly due to an aging population and also an increase in the number of cancer cases in low and middle income countries. In 2030, 22 million new diagnoses are expected per year (Bray *et al.*, 2012). In this scenario, in 2011 the World Health Organization (WHO) estimated more cancer-related deaths than those caused by stroke or coronary heart disease (Ferlay *et al.*, 2015). Mortality statistics for cancer are available on the WHO webpage (http://www.who.int/es/). Therefore, this public health problem calls for a better understanding of the disease and strategies that allow earlier detection, better stratification of tumors to allow personalized medicine, and the development of better and less toxic therapies.

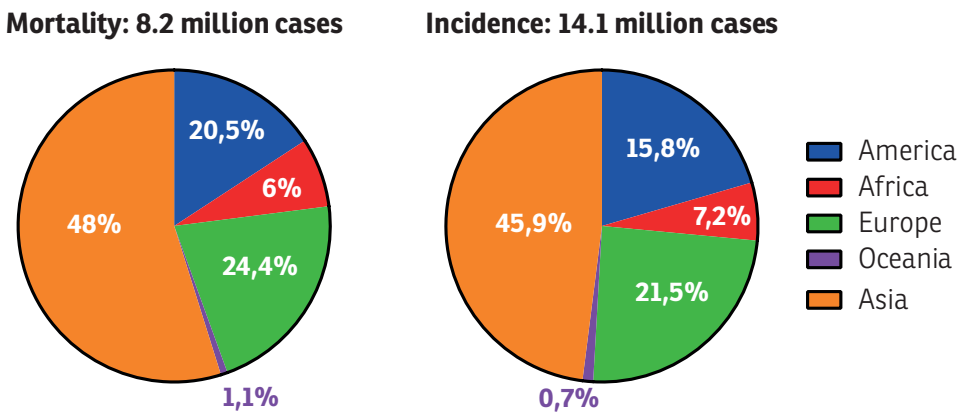


Figure 1: **Epidemiology of cancer incidence and mortality worldwide,** depicted in a pie chart. As observed in the two charts, the greater incidence, the greater the mortality. Modified from (Ferlay *et al.*, 2015).

Cancer is an extremely complex disease and represent a challenge for researchers and clinicians. Cancer shows marked heterogeneity in several features such as cellular morphology, proliferative index, genetic aberrations and therapeutic response and it is therefore not a singular disease but the a collection of hundreds of diseases. One of the main challenges and questions regarding cancer biology is the molecular and cellular mechanisms underpinning tumor heterogeneity. Key findings regarding cancer stem cells (CSCs) and/or tumor-initiating cells (TICs), the interaction with the microenvironment, and the development of targeted therapy shed light on the complexity within a tumor.

The hypothesis that cancer is driven by TICs has recently attracted a great deal of attention for the treatment of haematopoietic and solid malignancies. This thesis is focused on the identification of novel mechanisms in human BC metastasis and chemoresistance, giving particular importance to the contribution of tumor initiation features and identifying potential gene candidates regarding these two phenomena. To this end, we will first define a TIC or CSC. TICs are tumor cells that have the ability to regenerate the tumor from which they were isolated (Zhou *et al.*, 2009). Thus, this represents a reductionist definition based on experimental evidence *in vivo* (Pardal *et al.*, 2003). Generally, TICs are considered to be those cells at the apex of the tumor hierarchy (**Figure 2**), reflecting that aberrations in differentiation and stem cell (SC)-like features are key for tumorigenesis. The multipotency of lineage differentiation is likely to be frequent, but not a necessary property of TICs. In addition, TICs are not necessarily rare populations (Kelly *et al.*, 2007; Quintana *et al.*, 2008), and importantly the microenvironment affects their behavior. In contrast, the term CSC does not imply that the cell is derived from a normal SC (Clarke and Becker, 2006; Clarke *et al.*, 2006; Clarke and Fuller, 2006). Actually, tumors do not need to match the phenotype of the eventual TIC with respect to self-renewal[1]. In fact, the cell of origin, defined as the first cell that acquires cancer-promoting mutations cannot be a CSC (Visvader, 2011). A CSC, by definition, is only the cell that sustains malignant growth. Therefore, CSCs exhibit cancer-propagating functions, and the "cell of origin" refers to cancer-initiating functions (**Figure 3**), and their phenotype may differ. Importantly, the cell of origin model differs from the genetic mutation model, in which mutations determine the tumor phenotype. In contrast, in the cell of origin model, phenotype is determined by mutations in the different cell populations. Below, the principal features of cancer cells will be described and the hallmarks of cancer defined.

1: Self renewal: the ability of a cell to reproduce without losing developemental potential, characterized by cell division in which differentiation is blocked in at least one daughter cell.

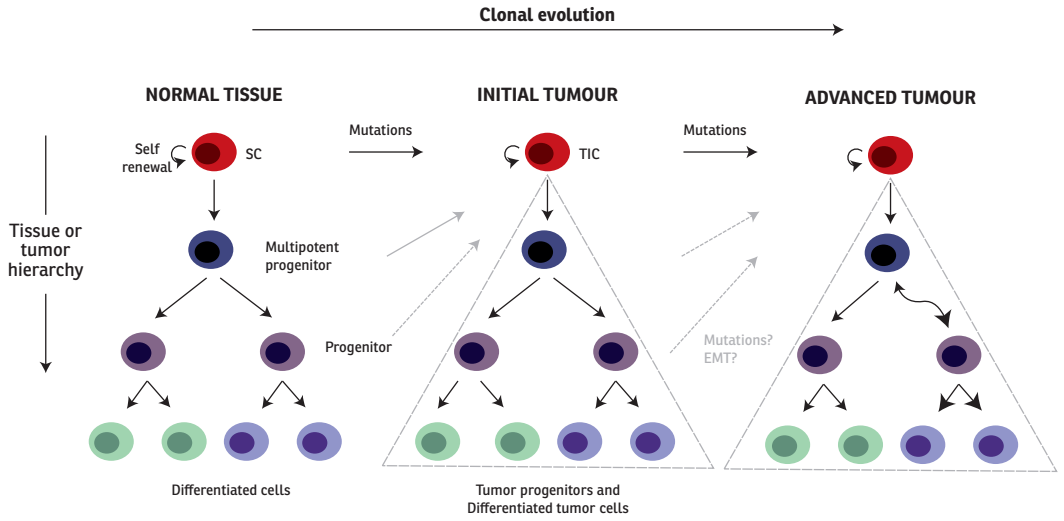


Figure 2: Hierarchical model of TICs and clonal evolution. Several lines of evidence support that oncogenic hits may happen on SCs, due to their greater longevity. These cells acquire tumor initiation properties, and clonal evolution occurs at the level of the SC compartment. Within the tumor there are different stages of differentiation, which resemble those of normal tissue. Therefore, heterogeneity might be the result from aberrations of the different progenitors and cells. Consequently, cancer is not only a proliferation end up disease, it is also a differentiation disease, in which the clonal evolution and CSC model are not mutually exclusive. Importantly, due to genetic instability, TICs from advanced tumors differ from the cell of origin. Modified from (Zhou *et al.*, 2009).

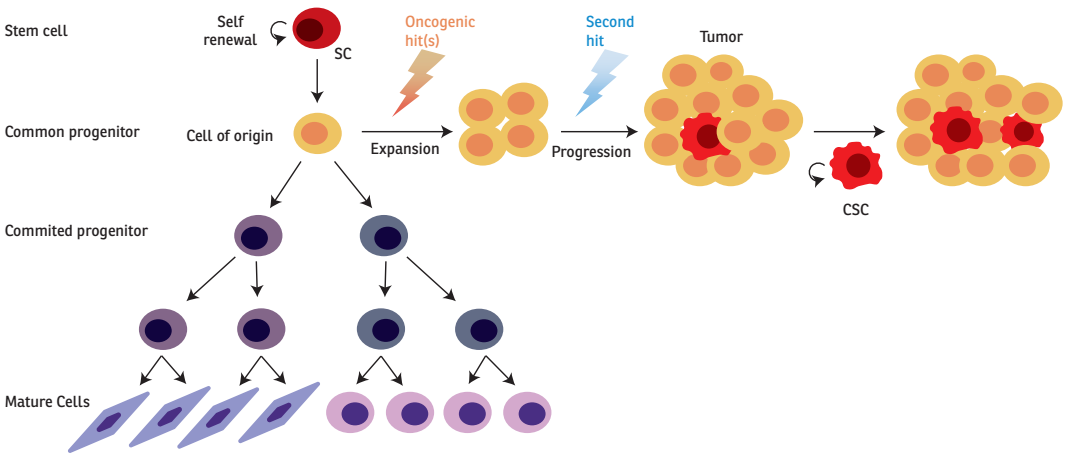


Figure 3: **The cell of origin model.** A CSC might not be the cell of origin of cancer. It is plausible that the first oncogenic hit occurs in a precursor, and further oncogenic aberrations lead to the emergence of a CSC, which has the ability to reinitiate and sustain tumorigenesis. Modified from (Visvader, 2011).

1.2 Hallmarks of cancer

Cancer cells were used to be considered an homogeneous mass of proliferative cells with random and chaotic functions and fate. However, today we know that a cancer is a tightly regulated heterogeneous disease with complex heterotypic interactions with the microenvironment and the metastatic niche. The hallmarks of cancer are distinctive, and the complementary capacities of tumor cells enables them to grow and disseminate throughout the whole body (Hanahan and Weinberg, 2000). In 2000, Hahanan and Weinberg described these essential characteristics of cancer cells. However, in the last decade, research efforts have been channelled and further understanding of the complex biology of cancer shed light on the key abilities of these particular cells. This research effort has led to the enumeration and extension of the first hallmarks (Hanahan and Weinberg, 2011). Here we will provide an overview of these.

1. Sustaining Proliferative Signaling

Cancer cells are able to deregulate proliferative signals and mitogenic pathways, regulating their progression through the cell cycle, their growth, survival and energy metabolism (Hynes and MacDonald, 2009; Lemmon and Schlessinger, 2010; Witsch *et al.*, 2010). Cancer cells can sustain proliferative signaling in various ways: i) by triggering autocrine proliferative stimulation through the synthesis of growth factor ligands; ii) by modulating the microenvironment through paracrine signaling (Bhowmick *et al.*, 2004; Calon *et al.*, 2012; Cheng *et al.*, 2008); iii) by increasing levels of endogenous receptor or structural conformation changes which make them hyperresponsive to stimuli ; iv) by activating the constitutive pathway through the alteration of downstream signaling components. Therefore, mutations in proliferative pathways are crucial for cancer cells. In fact, 40% of human melanomas hold a mutation on the B-RAF protein, leading to constitutive activation of the mitogen-activated protein (MAP)-kinase pathway (Davies and Samuels, 2010). Furthermore, negative feedback mechanisms ensure active signal transmission to safeguard homeostatic regulation (Sudarsanam and Johnson, 2010). This phenomenon ensures the proliferative capacity of cancer cells. However, it is important to bear in mind that excessive proliferative signaling can trigger cell senescence (*i.e* with *RAS*, *myc* or *Raf* oncogenes[2]) (Collado and Serrano, 2010).

2: Oncogene: gene in which a mutation promotes cancer.
3: Tumor supressor gene: that protect cells from oncogenic transformation.
4: Apoptosis: programmed cell death.
5: Authopaghy: mechanism that allows the degradation and recycling of cellular components.
6: Necrosis: non-programmed cell death induced by external factors(*i.e.* infecions, toxins.

2. Evading Growth Suppressors

Cancer cells have the ability to negatively regulate cell proliferation. This regulation is driven by tumor suppressor genes (TSG)[3] such as *RB* (Retinoblasoma) or tumor protein 53 (*TP53*), which are central controllers of cell cycle checkpoints (Burkhart and Sage, 2008; Sherr and McCormick, 2002), and by contact inhibition mechanisms (Martinez-Delgado *et al*., 2013), such as loss of the LKB1 epithelial polarity protein (Shaw, 2009; Vaahtomeri and Makela, 2011).

3. Resisting Cell Death

Cancer cells have the capacity to bypass cell death by evading apoptosis[4] (Adams and Cory, 2007; Lowe *et al*., 2004) or regulating a DNA sensor that functions via the loss of *TP53* (Junttila and Evan, 2009). A high number apoptosis evasion mechanisms reflects the high capacity of cancer cells to adapt to a variety of stressors. Furthermore, cancer cells are able to overcome the autophagy[5] barrier (Lu *et al*., 2008b; White and DiPaola, 2009). In contrast to apoptosis or autophagy, cancer cells can benefit from the tumor-promoting potential of pro-inflammatory-mediated necrosis[6], from pro-inflammatory mediators, and from the recruitment of inflammatory cells (Antonioli *et al*., 2013; Grivennikov *et al*., 2010; Shalapour and Karin, 2015).

4. Replicative Immortalization

In contrast to normal cells, which enter senescence by telomere shortening, cancer cells do not show replicative senescence and they acquire unlimited proliferation capacity, which is driven mainly by the overexpression of the DNA polymerase telomerase (Blasco, 2001; Blasco, 2005; Shay and Wright, 2000). In fact, telomerase, which is absent in normal cells, is expressed in SCs and human cancer cells.

5. Angiogenesis

Angiogenesis is the formation of new blood vessels, and like all tissues, cancer cells require nutrients to grow and also to detoxify metabolic residues. These requirements of cancer cells are covered through angiogenesis, which is induced by hypoxia (Michieli, 2009). Inducers such as endothelial growth factor A (VEGF-A) (Carmeliet, 2005; Ferrara, 2010) or fibroblast growth factor (FGF) (Baeriswyl and Christofori, 2009), and inhibitors such as thrombospondin-1 (TSP-1) (Kazerounian *et al*., 2008) are key players in this process. In contrast to normal angiogenesis, which is tightly regulated, this process in tumors is aberrant and marked by precocious capillary sprouting, excessive vessel branching, and erratic blood flow (Nagy *et al*., 2010). In contrast to what was believed years ago, angiogenesis is induced during early stages of carcinogenesis

(Raica *et al.*, 2009). The microenvironment is a key player in tumor neovasculature. For instance, pericytes are markedly present in the vasculature of almost all tumors (Raza *et al.*, 2010), as well as in bone marrow-derived cells (BMDCs), such as macrophages (Qian and Pollard, 2010), neutrophils (Zumsteg and Christofori, 2009), myeloid cells (Lewis *et al.*, 2007; Murdoch *et al.*, 2008), and monocytes (De Palma *et al.*, 2007; Lewis *et al.*, 2007).

6. Metastasis Dissemination

Metastasis is the ability of cancer cells to colonize secondary organs, and it is achieved by the acquisition of multiple properties along a multistep process (**Figure 4**). Metastasis is a tremendously complex process, and it is the final stage of cancer progression and it is the main process responsible for cancer deaths. Metastasis is chiefly a Darwinian selection. Cancer cells with different traits need to overcome various obstacles (bottlenecks) and are selected genetically and epigenetically within the tumor heterogeneity (Greaves and Maley, 2012; Massague and Obenauf, 2016; Naxerova and Jain, 2015). Metastatic colonization is the final step of the stochastic events within the "metastatic cascade", which is divided into the following steps and acquisition of cancer and metastatic traits:

- **Local invasion**: cancer cells break down the extracellular matrix (ECM) by inducing extracellular proteases such as matrix metalloproteinases (MMPs) and cathepsin. Therefore, cells are able to trigger the release of inflammatory factors that favors growth and invasion, and they overpass the confinement in the primary organ parenchyma (Sevenich and Joyce, 2014, Kessenbrock *et al.*, 2010). Tumors are not homogeneous and are exposed to a gradient of different molecules and oxygen. In fact, in the invasive front, the more invasive cells are selected, and they interact closely with stromal inflammatory and suppressive cells (Joyce and Pollard, 2009; Kalluri and Zeisberg, 2006; Nguyen-Ngoc *et al.*, 2012; Wyckoff *et al.*, 2007).

- **Intravasation**: cells acquire tumor initiation features through epithelial to mesenchymal transition (EMT), and therefore epithelial cells polarize to mesenchymal cells, losing intercellular adhesion (Wan *et al.*, 2013). In addition, EMT provides self-renewal features, invasiveness and motility. Angiogenesis and the formation of new vessels are essential in this step.

- **Dissemination** in the blood stream and arrest at the metastatic site: already in the blood stream, cancer cells can intravasate alone or in clusters with non-tumorogenic cells (*i.e.* platelets), which enhance their survival and dissemination (Aceto *et al.*, 2014; Nash *et al.*, 2002; Nguyen-Ngoc *et al.*, 2012). At the metastatic site, cancer cells reach the capillaries and become dormant. Rapid physical trapping is crucial in this process (Gupta and Massague, 2006), as well as the intrinsic features of cancer cells themselves, such as the ability to adhere to a particular vasculature/parenchyma (Wang *et al.*, 2004).

- **Extravasation**: cells can rupture blood vessels or extravasate straight into the parenchyma. Each organ exhibits particular characteristics. For instance, the brain and the lung have a particular

physical barrier. Brain vasculature is surrounded by the blood-brain barrier and reinforced by pericytes and astrocytes, while lung vasculature is surrounded by a tight basement membrane surrounded by alveolar cells. In contrast, the vasculature of bone and liver is fenestrated. (Nguyen *et al.*, 2009; Valastyan and Weinberg, 2011). Therefore, in organs with protective barriers, cells need to acquire additional specialized functions in order to invade and colonize (Bos *et al.*, 2009).

- **Colonization**: This is the final and most critical step of the metastatic cascade. The few cells that survive may have metastasis-initiating features, becoming metastasis-initiating cells (MICs) (Baccelli *et al.*, 2013). Therefore, disseminated tumor cells (DTCs) or circulating tumor cells (CTCs) that are able to survive may reinitiate a secondary tumor at the metastatic site due to self-renewal and tumor initiation properties. When these MICs reach and extravasate into the parenchyma, they may enter a period of dormancy and remain latent for months to decades. In fact, in ER^+ BC bone metastasis, cells may be dormant for decades (Sosa *et al.*, 2014). The mechanisms underlying BC dormancy remains unknown, and few driver genes have been reported. However, after a latency period, MICs are able to reinitiate a new tumor and establish a full-fledged metastasis by the expansion of transit-amplifying (TA) cells. In this thesis, we will show how these metastatic initiation features are key for the metastatic process in BC lung metastasis.

Cancer cells are challenged to different barriers, and once established, metastasis is devastating. Importantly, cells need to acquire epithelial traits through mesenchymal to epithelial transition (MET) (Ocana *et al.*, 2012). The tumor cells use various mechanisms to invade organs with particular niches. Of note, the organ affected depends on the type of cancer (Budczies *et al.*, 2015; Disibio and French, 2008; Nguyen *et al.*, 2009; Steeg, 2006; Valastyan and Weinberg, 2011). The physical barrier of the organ, microenvironment, and architecture of blood vessels are determinant. In addition, the intrinsic capacity of cancer cells determine organ-specific metastasis (*i.e.* survival, evasion of the immune system and microenvironment interaction). Therefore, both the intrinsic compatibilities of the metastatic site (soil) and cancer cells (seed) are crucial. This is what was described as the "seed and soil hypothesis[7]" more than 100 years ago by (Paget, 1989), and confirmed by Hart and Fidler in melanoma (Hart and Fidler, 1980). Consequently, there is an organ tropism. The liver, bone, brain and lung are more frequently invaded than the ovaries, spleen or skin (Budczies *et al.*, 2015). In addition, some cancers seed one specific organ, such as the bone in prostate cancer. In contrast, BC, melanoma and lung cancer spread to different sites. Other cancers such as CRC exhibit sequential organ-specific colonization, first to the liver, and afterwards to the lungs (Urosevic *et al.*, 2014). In BC, variation can be seen across cancer subtypes. Luminal estrogen receptor $(ER)^+$ BC exhibits tropism to the bone, $HER2^+$ BC frequent disseminates to the liver and brain, and basal ER^- BC metastasizes to the lungs (Kennecke *et al.*, 2010; Smid *et al.*, 2008; Soni *et al.*, 2015). Therefore, it is important to understand why these phenomena occurs.

7: Cancer cells exhibit an organ preference thanks to favourable interaction between metastatic tumor cells (the "seed") and their organ microenviroment("the soil"). this theory has been confirmed by an extensive amount of clinical and research data.

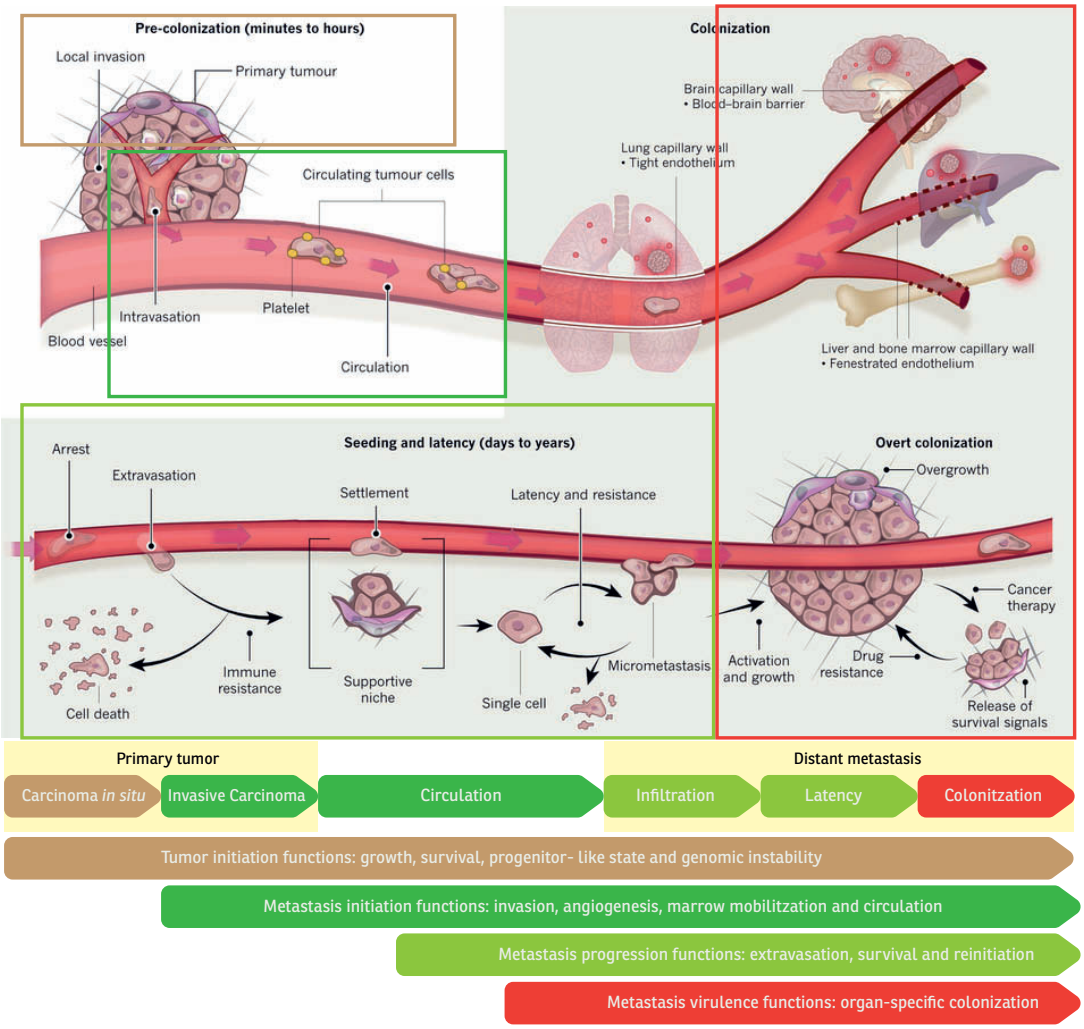


Figure 4: The "metastasis cascade" is divided into different steps. In order to colonize, cancer cells must acquire several features to overcome all the barriers encountered between the primary site and a secondary organ. First, cells need to acquire tumor-initiating functions in order to establish a primary tumor. They then need to intravasate into the blood stream and invade (through EMT) a secondary organ. After, they must extravasate and survive the immune system in a supportive niche. Cells remain latent and in response to growth stimuli, they are able to reinitiate and colonize a secondary organ. Although chemotherapy is able to kill highly proliferative cells, metastatic cells become resistance. The cause of chemoresistance might be explained by the presence of TICs with a slow-cycling dividing profile. Interactions with stromal cells and the microenvironment are essential for metastasis progression. Modified from (Massague and Obenauf, 2016) and (Nguyen *et al.*, 2009).

7. Genomic instability

Cancer cells acquire the aforementioned capacity through several enabling characteristics. The first is the acquisition of genomic instability, which leads to hoard mutations and drives global genomic rearrangements. The acquisition of random mutations confers a selective advantage to certain subclones of cancer cells, allowing their outgrowth. In addition, inactivation of TSGs through epigenetic mechanisms such as DNA methylation and histone modifications is another source of cancer selection (Berdasco and Esteller, 2010; Esteller, 2007). Another key mechanism of genomic instability is the loss of telomeric DNA (Artandi and DePinho, 2000; Artandi and DePinho, 2010).

8. Inflammation

The second enabling feature is the inflammatory state. Inflammatory cells are important drivers of tumor progression. For instance, every neoplasic lesion contains infiltrating immune cells (Pages *et al.*, 2010). These cells secrete growth factors to the microenvironment, thereby enhancing proliferation, survival, angiogenesis and aggressiveness (DeNardo *et al.*, 2010; Grivennikov *et al.*, 2010; Karnoub *et al.*, 2007; Karnoub and Weinberg, 2006). Notably, inflammation fosters growth in early stages of cancer progression (Qian and Pollard, 2010).

9. Metabolic reprogramming

The third enabling characteristic is the reprogramming of energy metabolism in order to support continuous proliferation. Almost 100 years ago, the differences in the metabolic status of cancer cells were reported. Surprisingly, the energy for a cancer cell was provided by aerobic glycolysis (Warburg, 1956a; Warburg, 1956b). These cells use glucose and secrete lactate through the "Warburg effect", and other subpopulations of cells use this lactate to obtain energy through the citric acid cycle (Kennedy and Dewhirst, 2010; Semenza, 2008).

10. Evasion of the immune system

The last enabling characteristic is the evasion of the immune system, which is the main defence barrier in a mammals. Immune cells destroy cancer cells. Both the innate and adaptive cellular arms contribute to tumor eradication (Kim *et al.*, 2007; Teng *et al.*, 2008). For instance, patients with CRC and ovarian cancer (OC) with infiltrating cluster of differentiation (CD)8^+ lymphocytes (CTLs) and natural killer (NK) cells show better prognosis (Nelson, 2008; Pages *et al.*, 2010). However, cancer cells can modulate the microenvironment to benefit from immune cell signals. One example is the transforming growth factor β (TGF-β), which is secreted by aggressive cancer cells, blocking CTLs and NKs (Massague, 2008; Yang *et al.*, 2010). In addition, cancer cells are

able to recruit and activate immunosuppresive immune cells from both the innate system, such as myeloid-derived suppressor cells (MDSCs), cancer Associated Fibroblast (CAFs) and M2-macrophages, and the adaptive system, such as regulatory $CD4^+$ Th cells (Tregs) (Mougiakakos *et al.*, 2010; Ostrand-Rosenberg and Sinha, 2009). All these observations reflect the pivotal role of the microenvironment. Below, we explain the key roles of the microenvironment and intratumoral heterogeneity in cancer progression.

The tumor microenvionment & tumor heterogeneity

The microenvironment of the tumor refers to all specialized cells within a tumor. Given the importance of these cells in tumor progression, the biology of the tumor cannot be understood without the effect of the tumor microenvironment. In addition, intratumoral heterogeneity is one of the main players in tumor progression and interplays with the microenvironment. Regarding intratumoral heterogeneity, the concept of CSCs or TICs is highly relevant. In contrast to what was described many years ago, neoplasic lesions are not homogeneous but have many diverse clonal subpopulations. CSCs was first described in hematopoietic malignancies (Reya *et al.*, 2001), but more recently their impact on tumor progression was described in solid tumors, such as in the breast (Al-Hajj *et al.*, 2003; Gilbertson and Rich, 2007), brain (Barker *et al.*, 2007; Gilbertson and Rich, 2007) and colon (Barker *et al.*, 2007). These populations share transcriptomic programs with SCs, and therefore they are called SC-like. However, the origin of these cells is unclear. They may come from an oncogenic mutation on SCs, or transformation of differentiated TA cells or a transdifferentiation of differentiated cancer cells (Velasco-Velazquez *et al.*, 2012). Regarding this last phenomenon, cancer cells can transdifferentiate through EMT (Mani *et al.*, 2008; Singh and Settleman, 2010; Zavadil, 2010), a process that allows them to acquire not only dissemination properties but also SC-like features, the latter essential for clonal expansion at the metastatic site (Brabletz *et al.*, 2005). Therefore, the interplay between stromal cells and heterotypic signals that triggers EMT is essential for maintaining TIC identity.

The notion of a unique phenotypical CSC pool is still controversial (Boiko *et al.*, 2010; Gupta *et al.*, 2009). In contrast, it is plausible to speculate that there is a dynamic bidirectional interconversion between CSCs and non-CSCs. (**Figure 5**) (Velasco-Velazquez *et al.*, 2012). CSCs provide heterogeneous complexity within a tumor and exhibit several features. First, the hallmark feature of this cell population is self-renewal. Second, these cells are highly resistant to cell cycle inhibition (Buck *et al.*, 2007; Creighton *et al.*, 2009; Singh and Settleman, 2010). Third, they play a key role in metastasis dissemination as MICs and dormant tumor cells. Therefore, cells that exhibit a SC-like phenotype might be crucial in tumor dormancy (Sosa *et al.*, 2014) and may cause relapse as they are resistant to therapy and able to regenerate the whole tumor. Therefore, differentiation programmes, or its absence, are pivotal for tumorigenesis. In addition, clonal selection and genetic heterogeneity are also important as they provides genetic diversification.

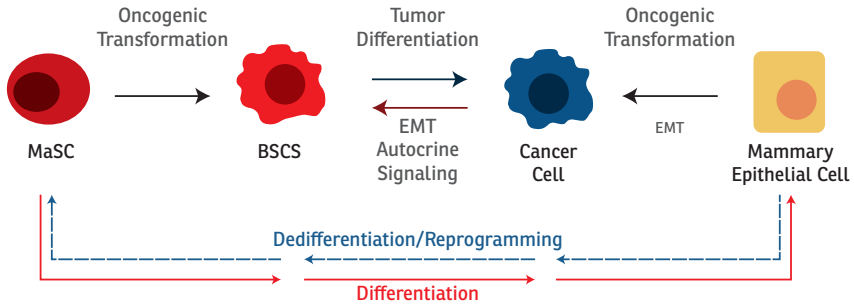


Figure 5: **Origin of a CSC.** A CSC can emerge from a mammary SC (MaSC) that undergoes oncogenic transformation. In addition, differentiated cancer cells can transdifferentiate and acquire tumor initiation potential, through autocrine signaling or EMT. Therefore, EMT is crucial in different stages of cancer progression. Modified from (Velasco-Velazquez *et al.*, 2012).

The cellular heterogeneity of the surrounding microenvironment within a tumor is also important. Endothelial cells (Ahmed and Bicknell, 2009; Nagy *et al.*, 2010; Pasquale, 2010), pericytes (Betsholtz *et al.*, 2005; Mantovani, 2010; Pietras and Ostman, 2010), inflammatory cells (Mantovani, 2010; Mantovani *et al.*, 2008), CAFs (Bhowmick *et al.*, 2004; Calon *et al.*, 2015) and BMDCs (Bergfeld and DeClerck, 2010; Wang *et al.*, 2015b) are essential players in tumor evolution (**Figure 6**). There is heterotypic signalling within the tumor microenvironment, meaning that both tumorogenic cells and stromal cells evolve and change progressively during cancer progression. In addition, there are what is defined as metastatic niches, understood as tissue microenvironments that are more permissive to cancer cell growth. This organ specificity process can be explained by the intrinsic properties of the niche (Talmadge and Fidler, 2010), by released molecules, or by factors carried through exosomes from the primary tumor (Peinado *et al.*, 2011). Understanding these dynamics is crucial to develop targeted therapies and diagnosis for metastatic disease. A rational approach should be taken as cancer cells acquire resistance, and therefore a combination of drugs emerges as the best strategy (Bergers and Hanahan, 2008; Ebos *et al.*, 2009; Grepin and Pages, 2010). In **Figure 7** shows an example of how mechanism-guided targeted therapies might be useful in the context of the aforementioned hallmarks. This approach will result in more effective and durable therapies to defeat cancer.

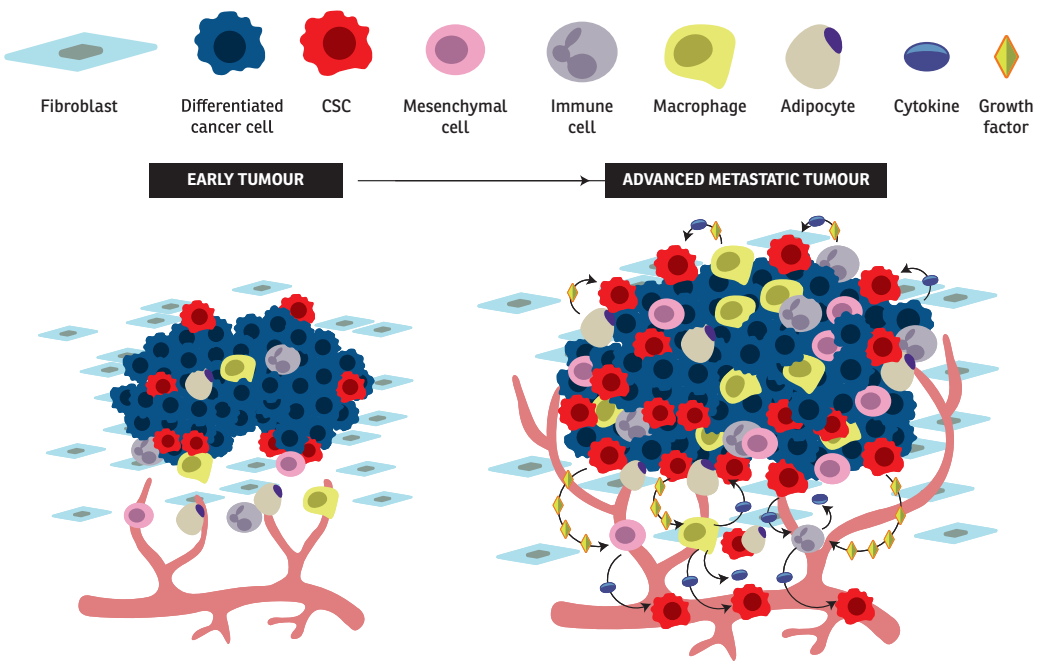


Figure 6: **Intratumor heterogeneity and the microenvironment are essential in cancer progression.** During cancer progression differentiated cancer cells proliferate, and CSCs sustain the malignant growth. Cells are able to survive and proliferate thanks to paracrine signals and heterotypic interactions with stromal cells such as CAFs, macrophages, or inflammatory immune cells. Cancer cells also secrete factors to modulate the environment and stromal cells (immunosuppression) and autocrine factors to survive and proliferate. In addition, it has been speculated that CSCs are responsible for metastatic initiation through the blood stream. Modified from (Korkaya *et al.*, 2011).

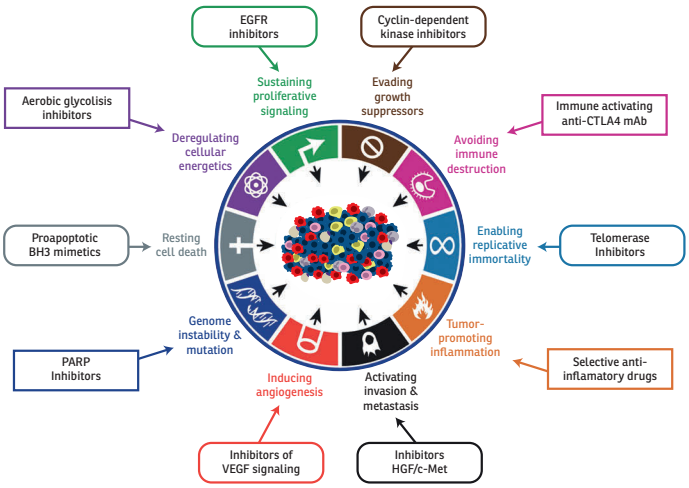


Figure 7: **Targeted therapy approaches based on the hallmarks of cancer.** Modified from (Hanahan and Weinberg, 2011).

1.3 The breast and the mammary gland: structure and functions in mammals

The breast serves the unique function of feeding offspring. It comprises three components, namely skin, adipose tissue and the mammary gland (**Figure 8**). The breast contains 15-20 lobules, each one generating a branching duct system that lies on a fat pad and drains into a nipple. The duct system leads from collecting ducts to the terminal duct-lobular units, or terminal end buds (TEBs). These milk-producing sites are crucial as mammary SCs (MaSCs) are localized in these regions. Apart from adipose tissue, lobes contain connective tissue. **Figure 9** shows the histological structure of the breast. The mammary gland is a remarkably adaptive organ and highly dynamic. It is composed of two main cellular lineages organized into these branching network of ducts and lobualveolar structures—secretory luminal cells that surround a central lumen and highly elongated myoepithelial cells that are located in a basal position adjacent to the basement membrane, with contractile functions (Daniel and Smith, 1999; Hennighausen and Robinson, 1998; Hennighausen and Robinson, 2005). Pregnancy is marked by remodelling of the mammary gland and branching and alveoli development. In addition, the mammary gland undergoes remarkable changes during the morphogenetic cycle (see **Figure 8**). In mice, luminal cells are characterized by the expression of cytokeratins (K) 8 and 18 and myoepithelial/basal cells by the expression of K5, K14, p63 and α- smooth muscle actin (SMA) (Smalley *et al.*, 1998). This means that the mammary epithelium relies on various cell subpopulations and dissect a hierarchy.

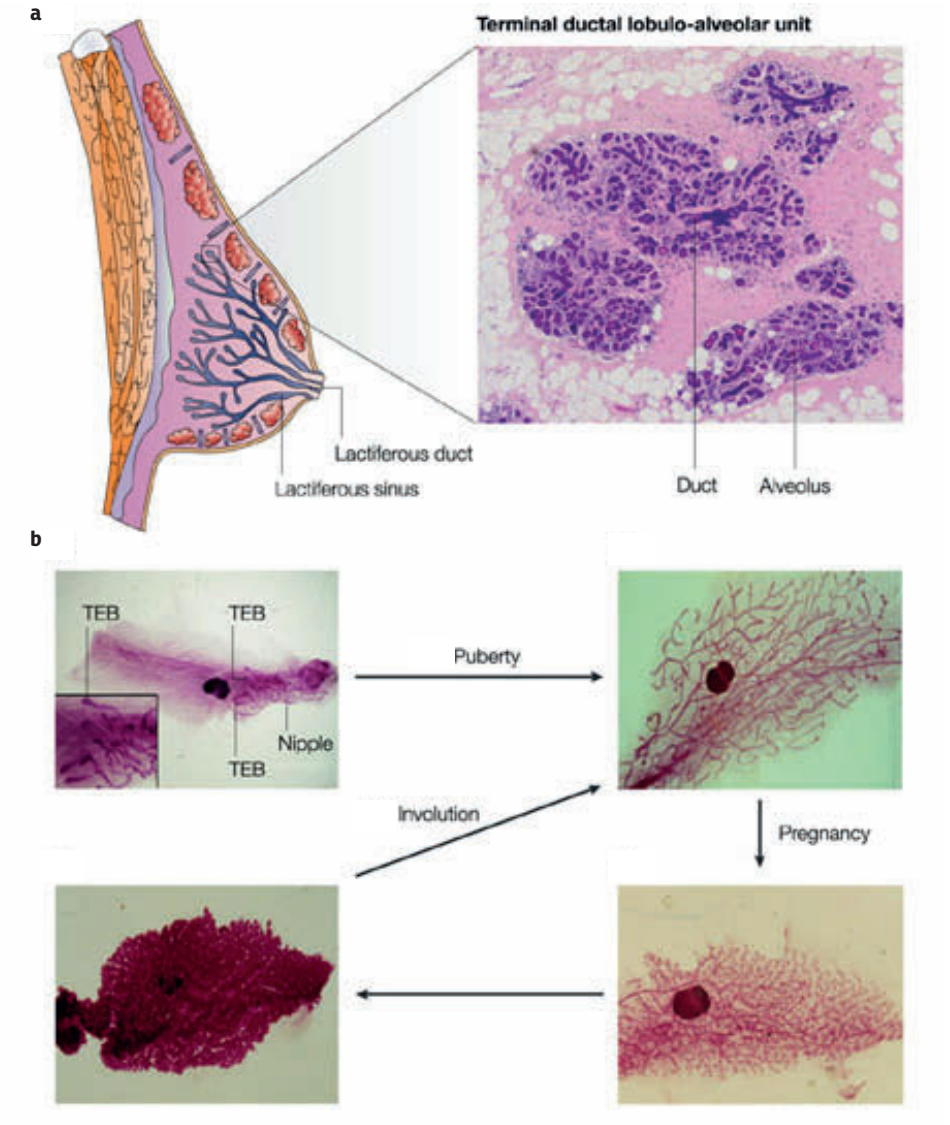


Figure 8: **a) Representation of the human breast epithelium** with hematoxylin- and eosin- stained cross-sections (H&E) through a TEB; **b) Rodent mammary gland development.** In puberty, TEBs generate ducts, and during pregnancy de novo alveolar structures are generated. During lactation, alveolar cells start to secrete milk. After pregnancy, the mammary gland is remodelled with apoptosis of epithelium and then returns to the mature virgin state (involution). Images are carmine-stained whole mounts of mammary gland (Smalley and Ashworth, 2003).

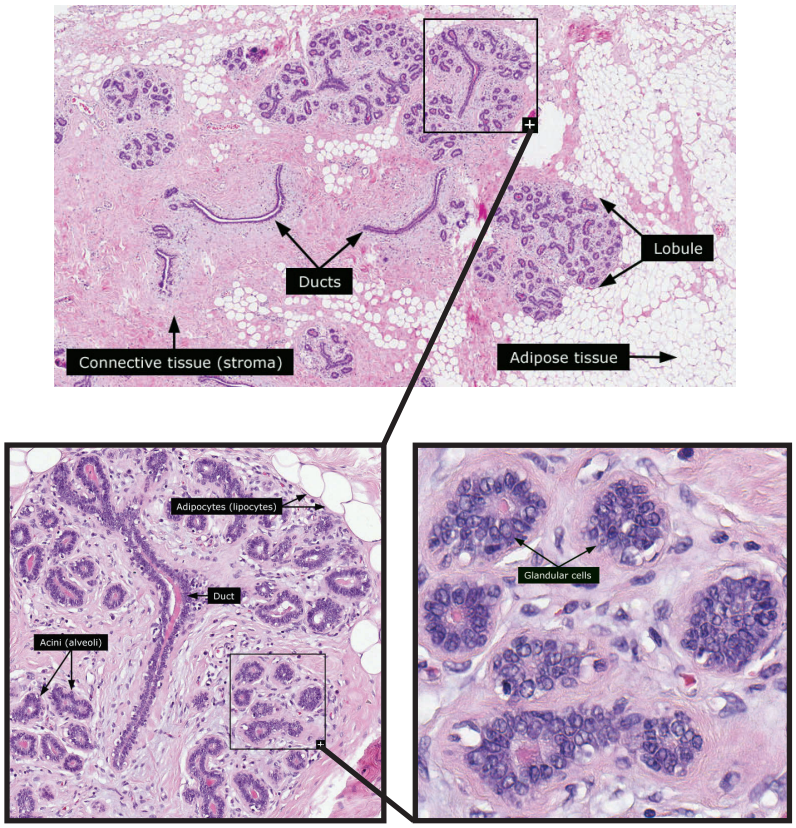


Figure 9: **Histological representations of human breast tissue.** H&E stainings are depicted. Breast tissue is composed of lobules and ducts, surrounded by adipose and connective tissue. Alveoli and granular cells reside within lobules. BC is most frequent in ducts, followed by lobules. Granular cell tumor or acinic-cell carcinoma are less frequent (taken from http://www.proteinatlas.org).

1.3.1 Dynamics and hierarchy of the mammary gland from a mammary stem cell perspective

In each menstrual cycle, the mammary gland undergoes remarkable growth (Potten *et al.*, 1988). During pregnancy, the *de novo* formation of lobules occurs by lateral budding from TEBs (Russo and Russo, 2004). Therefore, these cellular dynamics lead to the postulation of the presence of MaSCs as precursors. In addition, there is functional evidence that confirms the existence of these cells. A mammary gland can be regenerated in mice by transplantation of different epithelial fragments, which is the gold standard assay for MaSC assessment (Kordon and Smith, 1998). In addition, progenitor-enriched populations have been reported in the mouse mammary gland (Alvi *et al.*, 2003; Welm *et al.*, 2002), and in human breast tissue. However, in humans, only cells from terminal ducts, and not lobules, form multipotent colonies and can generate ductal lobular

structures (Villadsen *et al.*, 2007). Therefore, SCs reside in TEBs, however this evidence comes from *in vitro* studies, and *in vivo* validation should be performed.

Importantly, there is an increasing amount of data supporting the notion that MaSCs are key drivers in BC initiation, progression and chemoresistance. In recent years, research efforts have been channelled into identifying MaSCs, and a panel of surface markers have been identified to study the contribution of these cells to breast development. A whole functional mammary gland has been generated from a single cell lineage negative (Lin^-) $CD29^{high}CD24^+$ population (Shackleton *et al.*, 2006), thereby confirming that these cells exhibit MaSC features with self-renewal properties. In addition, the vast majority of these cells also express $CD49f^{high}$ $Sca1^{low}$ (Liu *et al.*, 2004; Smalley *et al.*, 2012). $CD49f^+$ cells were identified as a bipotent epithelial progenitor cells in humans (Stingl *et al.*, 2001) in the basal position of the mammary epithelium. In fact, the $CD24^+/CD49f^{high}$ subpopulation identifies a proliferative basal-like MaSCs population that expresses the myoepithelial $K14^+$ (Stingl *et al.*, 2006). There is some evidence that progenitors are not restricted to the basal compartment in humans (Keller *et al.*, 2012). **Figure 10** shows a schematic hierarchy of the human mammary gland. For more information regarding the subpopulations that have been identified in the mouse mammary gland see Figure 2 from (Visvader and Stingl, 2014). However, there are conflicting data. For instance, the activity of aldehyde dehydrogenase (ALDH-1) has been associated with human epithelial ER^- cells in humans (Ginestier *et al.*, 2007) and malignant features (Charafe-Jauffret *et al.*, 2009). In contrast, the $ALDH1^{low}$ epithelial subset leads to more outgrowths (Eirew *et al.*, 2012). These conflicting observations are attributable to the different approaches used to dissociate breast tissue and the methods used to assess stemness. In this regard, a proper method to assess initiation is essential (Guiu *et al.*, 2015).

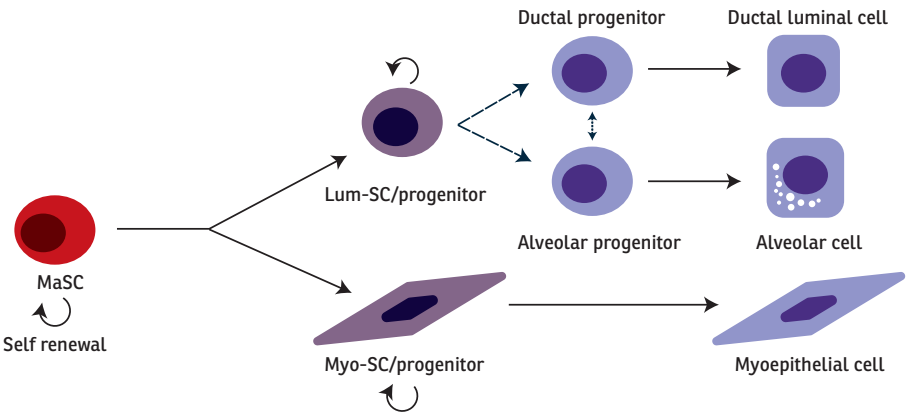


Figure 10: Schematic model of the mammary gland. In the SC compartment, bipotent MaSCs are at the top of the hierarchy and give rise to unipotent luminal and basal/ myoepithelial progenitors. These multipotent luminal cells are able to give rise to two different luminal progenitor subtypes (ductal and alveolar), thus determining their terminal different state (ductal luminal and alveolar cells, respectively). Several lines of evidence suggest that these cells are long-lived progenitors. Modified from (Visvader and Clevers, 2016).

MaSCs are scarce within the epithelium and exhibit particular features. The hallmark property of these cells is self-renewal. Furthermore, they are larger (>10 μm) and have the capacity to reconstitute a mammary gland (Machado *et al.*, 2013). MaSCs seem to be splited account for multiple SC populations: quiescence MaSCs (Boras-Granic *et al.*, 2014; Cicalese *et al.*, 2009) and proliferative MaSCs (Asselin-Labat *et al.*, 2010). Quiescence SCs, those that are hypothetically at the top of the hierarchy, are in a dormant state (G_0) and exhibit a slow-cycling profile and metabolic state in order to protect the integrity of the genome (dos Santos *et al.*, 2013). Therefore, they are termed as long-lived-label-retaining cells with heightened longevity (Shackleton *et al.*, 2006). In addition, they exhibit increased regenerative potential (Orford and Scadden, 2008) and decreased cell cycle progression (Cheung and Rando, 2013). Furthermore, these cells are able to retain DNA strands during mitosis (Cicalese *et al.*, 2009; Pece *et al.*, 2010; Smith, 2005). When dormant SCs are activated, they become proliferative, driving morphogenesis during puberty, as well as alveologenesis (Bai and Rohrschneider, 2010). In fact, retaining EdU^+ cells colocalize with the SC basal K5 in the cap cell layer of TEBs (Rios *et al.*, 2014). Another particular feature is asymmetric division, which gives rise to differentiated cells and cells that maintain their regenerative potential. However, how do they respond to injury or a stimulus? In these conditions, quiescence SCs enter the cell cycle (becoming proliferative-active SCs) with self-renewal capacity, and by asymmetric divisions, they give rise to the different cell lineages (**Figure 11**). However, to date, we cannot reliably identify a *bona fide* quiescent MaSC subpopulation.

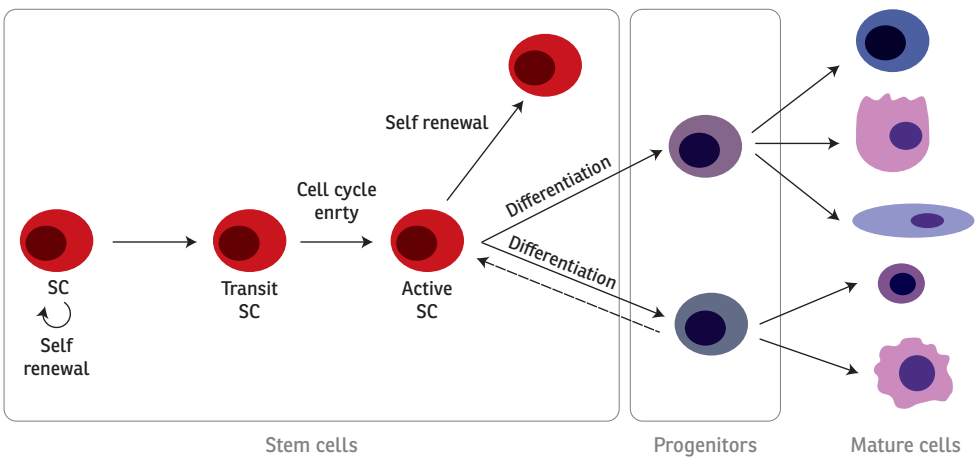


Figure 11: Hierarchical model of MaSCs. Upon a stimulus, a bipotent/multipotent SC in a quiescence/dormant state become a TA cell and enters the cell cycle as an active SC with self-renewal capacity, giving rise to the different cell lineages. When homeostasis is balanced, active SCs become dormant again. Therefore, the cycle of self-renewal/differentiation is pivotal for homeostasis and cell transformation. Modified from (Visvader and Clevers, 2016).

Based on the combination of 3D imaging, which allows single cell resolution, and lineage tracing studies, long-lived bipotent and unipotent SCs can be identified *in vitro* and *in vivo* (Visvader and Stingl, 2014). Both bipotent (Rios *et al.*, 2014; Tao *et al.*, 2014) and unipotent SCs have been tracked using basal-restricted gene promoters such as *K5*, *K14* and *SMA* (Prater *et al.*, 2014; Rios *et al.*, 2014; van Amerongen *et al.*, 2012; Van Keymeulen *et al.*, 2011; Wang *et al.*, 2015a). These studies confirm the existence of bipotent MaSCs, which account for 5-10% of basal cells, and can reconstitute an entire ductal tree, giving rise to alveolar and ductal committed luminal progenitor cells (Visvader and Stingl, 2014). Therefore, bipotent MaSCs are placed at the apex of the epithelial hierarchy (Shackleton *et al.*, 2006; Stingl *et al.*, 2006). In contrast, luminal progenitors are unipotent (Shackleton *et al.*, 2006; Stingl *et al.*, 2006) and may drive ductal or alveolar expansion during pregnancy. Thus, they are at a lower hierarchical position than bipotent MaSCs. In addition, unipotent SCs have been identified both in luminal (Lafkas *et al.*, 2013; Rios *et al.*, 2014; Rodilla *et al.*, 2015; Tao *et al.*, 2014; Van Keymeulen *et al.*, 2011) and basal compartments (de Visser *et al.*, 2012; Prater *et al.*, 2014; van Amerongen *et al.*, 2012; Van Keymeulen *et al.*, 2011). However, it is important to highlight that sorting and lineage tracing approaches have limitations. For example, a negative result does not imply that these particular subpopulations do not exist *in vivo*. A drawback of using cell-specific promoters is that they represent only a small subset of basal/luminal cells and they play a role at a particular time point within the morphogenic cycle. Therefore, more stochastic approaches already used in gut tissue (Kozar, Morrissey *et al.* 2013) should be implemented in order to overcome this limitation. For instance, continuous clonal labeling based on cell barcoding to track *in vivo* differentiation (Nguyen *et al.*, 2014) demonstrated the existence of basal bipotent MaSCs within primary ductal outgrowths.

In summary, MaSCs contribute to morphogenesis stages in the postnatal gland and to the maintenance of the ductal tree throughout adult life (Rios *et al.*, 2014). Bipotent MaSCs coordinate ductal morphogenesis, expansion during pregnancy, as well as remodelling during involution and ductal homeostasis during ageing. In contrast, unipotent alveolar progenitor cells are required for alveologenesis. In addition, basal-restricted cells show more longevity and orchestrate remodelling of the epithelial tree during involution. Such longevity led to the postulation that these cells are prime targets for BC initiation. In fact, it is noteworthy that there is a heightened risk of BC when women are exposed to ionizing radiation, therefore implying the maintenance of long-lived cells (Land and McGregor, 1979). It is remarkable to mention that the MaSC niche is unique because it is under the control of a particular stroma and environment, with the pivotal role of steroid hormones.

1.3.2 The mammary stem cell niche

The MaSC niche has a particular microenvironment in which hormones play a pivotal in the maintenance and activity of these cells (Joshi *et al.*, 2012).. This niche has been localized in mammary ducts and lobules, with progenitor cells residing in the TEBs (Villadsen *et al.*, 2007), and active MaSCs may reside in the cap cell layer during puberty (Bai and Rohrschneider, 2010). Data reveal the presence of a quiescence dormant SC zone in the ducts enriched in SSEA-4^{high} /K5^{+} /K6a^{+} / K15^{+} /Bcl-2^{+} cells. In contrast, proliferative progenitors reside outside of this region and are bordered by laminin-2/4. Another SC zone is defined by myoepithelial/basal K19^{+} /K14^{+} cells, which are able to generate lineage-restricted progenitors (Petersen and Polyak, 2010). The MaSC niche is particularly important in the maintenance of the mammary gland through its dynamic burst. The observation that a single cell can regenerate an entire mammary gland demonstrates that other stimuli participate in this maintenance. These signals, apart from hormones, come from the fat-pad stroma, which comprises fibroblasts, adypocytes, BMDCs, and endothelial cells. **Figure 12** shows a composite of the MaSC niche. Macrophages are essential, as macrophage-deficient fat pads are unable to regenerate the mammary gland and therefore to preserve the MaSC niche (Gyorki *et al.*, 2009). In puberty, morphogenesis/proliferation is driven by the effect of pituitary-derived growth hormone (GH) on stromal cells through insulin-like growth factor-1 (IGF-1). In addition, estrogen acts on MaSCs, inducing their proliferation. Epidermal growth factor (EGF) receptor (EGFR) is expressed in the basal MaSC population and interacts with EGFR^{+} stromal cells (Asselin-Labat *et al.*, 2006).

Steroid hormones, 17 β-estradiol and progesterone, trigger extensive expansion and involution of the mammary epithelium in the reproductive cycles (Fata *et al.*, 2000a; Joshi *et al.*, 2010). 17β-estradiol is pivotal for morphogenesis during puberty, pregnancy and oestrus cycles, inducing progesterone receptor (PR) expression (Beleut *et al.*, 2010). In pregnancy, progesterone causes expansion of MaSCs, leading to a side branching within the mammary gland, in a process known as lobuloalveologenesis, which differentiates into milk secretor cells upon birth and lactation (Asselin-Labat *et al.*, 2010; Oakes *et al.*, 2006). Consequently, alveoli represent a niche for harboring active MaSCs during pregnancy. Interestingly, MaSCs are ER^{-}/PR^{-} cells, thereby suggesting that there is an activation indirect mechanism through paracrine signalling (**Figure 12**). Progesterone is essential for the expansion of basal stem CD24^{+}/CD49f^{high} (Joshi *et al.*, 2010) and human progenitor cells (Graham *et al.*, 2009). Importantly, progesterone regulates essential players in mammary gland development and BC such as receptor activator of NF-kB ligand (RANKL) and Wnt4 (Beleut *et al.*, 2010; Brisken *et al.*, 2000; Duheron *et al.*, 2011; Fata *et al.*, 2000b; Fernandez-Valdivia and Lydon, 2012; Mukherjee *et al.*, 2010). *In vivo*, only RANKL was identified (Asselin-Labat *et al.*, 2010). The Wnt signalling coreceptor Lrp5 (Badders *et al.*, 2009) and RANK have been demonstrated to be required for the maintenance of MaSC activity. Therefore, this network reflects the complexity

of the MaSC niche and the particular importance of this cell population in mammary gland development. In this regard, this population is essential for homeostasis, and its disruption might lead to tumorogenesis. MaSCs can accumulate mutations due to their greater longevity, thereby acquiring tumor initation features, which can trigger BC initiation, progression, and the establishment of a BC SC (BCSC) niche. The interest of this particular population has heightened recently, since they have been implicated in BC initiation (Al-Hajj *et al.*, 2003).

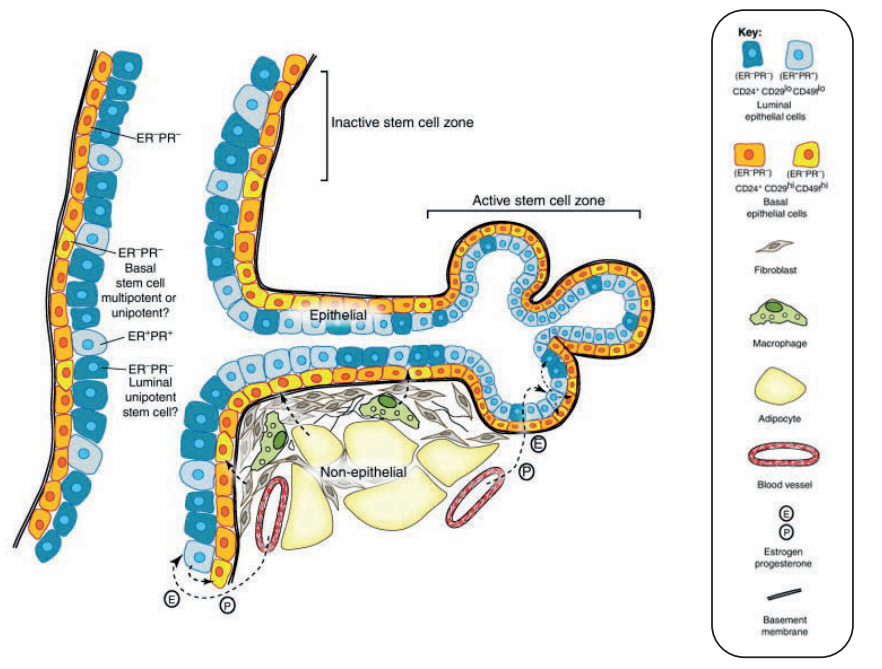


Figure 12: The MaSC niche is unique, regulated by estrogen (E) and progesterone (P). It is comprised of epithelial and non-epithelial cells (fibroblasts, macrophages and adipocytes) and a ECM with structural functions. Hormones act directly on ER^+ luminal epithelial cells, which secrete paracrine factors that activate either ER^+ or basal ER^- epithelial cells. There are two distinct pools of SCs, namely an inactive quiescence SC zone with regenerative potential (devoid for branching), and an active SC zone with self-renewal and expansion capacity, which has been restricted to TEBs (Joshi *et al.*, 2012).

1.4 Breast cancer

BC is a malignant neoplasm originated in the mammary gland (Smalley and Ashworth, 2003). It affects mainly women but also men, although male BC is infrequent. BC occurs usually in the ducts and lobules. It is the most common non-skin cancer and has a higher incidence in Caucasian ethnical background than in Asians and Hispanics. BC is the second most common cancer worldwide, and will on average affect up to one in eight women in the United States and Europe (Jemal *et al.*, 2009). In developed countries 370,000 new cases are diagnosed each year

(Jemal *et al.*, 2011), which represent 25% of all cancers. **Figure 13** shows maps indicating the geographical distribution of all cancers worldwide. Despite medical advances, BC is the leading cause of cancer-related death in women in the western world (up to 14% of cancer deaths in 2008 worldwide), although since 2000 the death rate has dropped. Of all cancers, BC ranks as the fifth cause of death (522,000 in 2012, 29.7%), the major cause of death in women in less developed countries (15.4%), and the second cause of death in more developed regions (198,000 deaths, 15.4%) after lung cancer (Ferlay *et al.*, 2015). Therefore, BC is a major public health problem, and there is need for a better understanding of the disease.

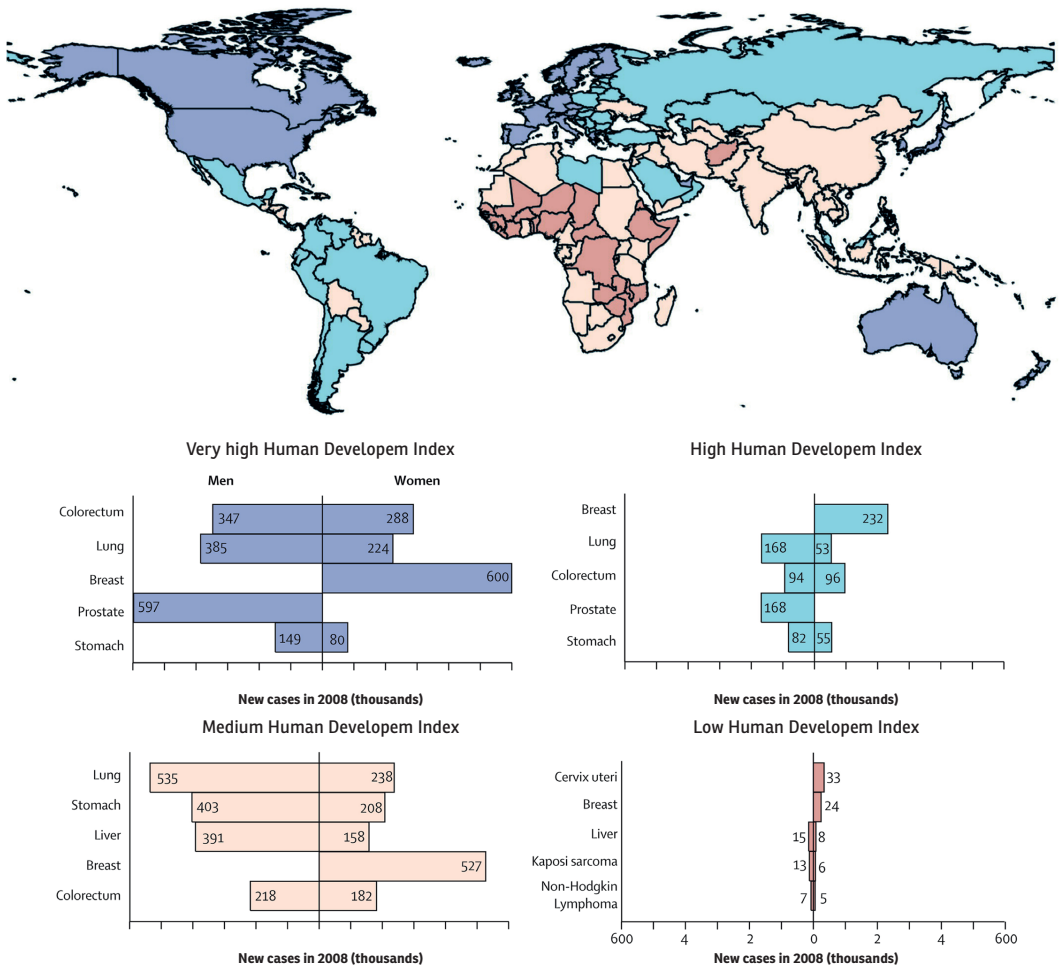


Figure 13: World-wide distribution of the most frequent cancers. BC is the most common cancer among women in developed countries and Caucasians. In contrast, the incidence of BC in women is lower in less developed countries, due to lower longevity. Modified from (Bray *et al.*, 2012).

Like most cancers, BC arises from sporadic mutations and genomic rearrangement in either oncogenes or TSGs. Nevertheless, in around 5-10 % of BC cases, the disease is hereditary (Narod and Salmena, 2011), with specific mutations in particular genes such as the TSG breast cancer (*BRCA1/2*). The aetiology of inherited BC differs from that of the spontaneous type, and the characteristics, diagnosis and driver genes also differ. In this thesis, we will focus on spontaneous BC, which affects women over 45 and is associated with age and sporadic mutations. In contrast, hereditary BC is linked to germline mutations in various genes and family susceptibility and it accounts for less than 25% of all BC cases (Balmana *et al*., 2011). To date, *BRCA1/2* is the only gene used in clinics to identify predisposition to BC. This gene has high penetrance and explains around 20-25% of all cases of hereditary BC (Balmana *et al*., 2011; King *et al*., 2003; Lux *et al*., 2006), and women born with *BRCA1/2* mutation are at a significantly higher risk (King *et al*., 2003). Risk factors for hereditary BC are shown in (Lux *et al*., 2006). In addition, hereditary BC exhibits particular clinicopathological features (Table 4 (Lux *et al*., 2006). Of note, *BRCA1/2*-linked BC is more aggressive, has a higher proliferation index (Adem *et al*., 2003), higher grade and is associated with triple negative BC (TNBC) (Lakhani *et al*., 2002). *BRCA1/2*-linked BC is also associated with higher risk of OC (Lux *et al*., 2006). In contrast, the frequency of BC in unselected patients for family history or age at onset is generally low (less than 7%) (Balmana *et al*., 2011). Remarkably, higher prevalence has been associated with family history of BC or OC, young age at onset, male BC or multiple tumors (bilateral BC, or BC and OC in the same patient). However, despite many efforts, the underlying cause of more than 70% of hereditary BC cases remains unexplained. Chemoprevention with tamoxifen and prophylactic bilateral mastectomy or prophylactic bilateral salpingo-oophorectomy are the approaches used in clinics. Interestingly, loss of BRCA expression seems to be predictive of chemosensitivity, especially to DNA-damaging treatments such as platinum-based chemotherapy (Balmana *et al*., 2011). In addition, the PARP inhibitor olaparib (Lynparza), which blocks DNA repair enzymes, is lethal for *BRCA1/2* deficient cells (Polyak and Garber, 2011). These drug is approved by the FDA for the treatment of advanced hereditary OC (Audeh *et al*., 2010; Kaufman *et al*., 2015; Kaye *et al*., 2012) and is under clinical trials with promising results in advanced hereditary BC (Arun *et al*., 2015; Livraghi and Garber, 2015; Polyak and Garber, 2011; Tutt *et al*., 2010).

An understanding of the aetiology of BC is essential to advance in its diagnosis and treatment. Although huge efforts have been made to elucidate the molecular mechanisms and its high complexity, people continue to die from this condition because of limitations in timely diagnoses and resistance to therapy. Consequently, the following need to be improved: i) prevention (who needs it and when); ii) diagnosis (specificity, sensitivity to detect even a few cells); iii) understanding tumor initiation, progression and recurrence; iv) prediction (who is going to suffer); v) treatment based on personalized medicine (better understanding of the complexity of the tumor and patient stratification); vi) chemoresistance (origin, target identification and how to overcome it).

1.4.1 Classification of BC

BC is highly heterogeneous and is classified on the basis of: i) histology into ductal or lobular carcinomas; ii) subtypes defined by histopathological features ($ER^{+/-}$, $PR^{+/-}$, human epidermal growth factor receptor $(HER2)^{+/-}$); iii) gene expression based classifier (PAM50; Basal, HER2, Luminal A, Luminal B and normal- subtypes) (Perou *et al.*, 2000), or iv) using the new integrative classification based on genomic and transcriptomic data (iClust 1-10) (Curtis *et al.*, 2012; Dvinge *et al.*, 2013). Human breast carcinomas are heterogeneous pathologies defined by molecular profiles, and in this respect it is plausible that BC might be more similar to hematological malignancies than to other common epithelial cancers. Indeed, unlike colon cancer or pancreatic cancer, in which most of mutations occurs within a single pathway and have a dominant role during tumor progression (Kinzler and Vogelstein, 1996; Segditsas and Tomlinson, 2006; Stingl and Caldas, 2007), in breast tumors no single dominant pathway or histological presentation has emerged. Remarkably, BC poses a huge challenge because it has a variety of morphologies, behaviors, and responses to therapy.

1.4.1.1 BC histopathological classification

BC is classified by clinicians into ductal or lobular carcinoma, depending on the morphological features and site of origin. Histological grade is scored using the Nottingham Grading System (NGS), which evaluates tubular differentiation, nuclear pleomorphism, and mitotic activity. The higher the grade, the higher proliferative index (Ki67) and the worse prognosisthe worse the prognosis and the higher the proliferative index (Ki67). The grade of differentiation is established compared to healthy tissue: grade 1 well differentiated: low; grade 2: intermediate; and grade 3 poorly differentiated: high. **Figure 15** shows some representative BC lesions. In addition, BC is classified on the basis of local or distant dissemination stages. In early-stage BC, three main characteristics are evaluated, namely Lymph Node (LN) status, tumor size, and histological grade. Stage I is localized and accounts for 60% of all cases, whereas stage II and stage III LN dissemination is detected in 35% of them. In advanced BC, which accounts for 5% of all cases, there is dissemination to other organs (stage IV). Frequently, well defined lesions termed intraductal carcinoma are localized adjacent to the invasive neoplasia, thus leading to ductal carcinoma in situ (DCIS).

In addition to BC staging, the status of various markers (ER, PR, HER2) is assessed by immunohistochemistry (IHC), a technique that provides key information regarding prognosis and clinical outcome before and after treatment. These proteins are crucial in BC progression. Another important consideration is menopausal status and the patient's general health. ER and PR reflect hormonal status, and are considered positive when exceed 1% of cells by IHC quantification (see **Figure 14** to see representative stainings). HER2 is evaluated either by IHC at four levels ($0,1^{+},2^{+}$

and 3+) or by fluorescent in situ hybridization (FISH) to assess gene amplification. In addition, proliferation status by Ki-67 IHC is used to assess BC aggressiveness. On the basis of the expression of ER, PR and HER2, BC is classified into subtypes such as ER^+/PR^+, $HER2^+$ and TNBC (ER^-,PR^-, $HER2^-$). $HER2^+$ and ER^+ tumors exhibit luminal features, whereas TBNCs show significant but not complete overlap with the basal like subtype. This type of stratification has led to the development of personalized medicine such as antihormonal therapies or HER2- targeted therapy. Furthermore, BC can be classified on the basis of the luminal or basal origin of cytokeratin expression.

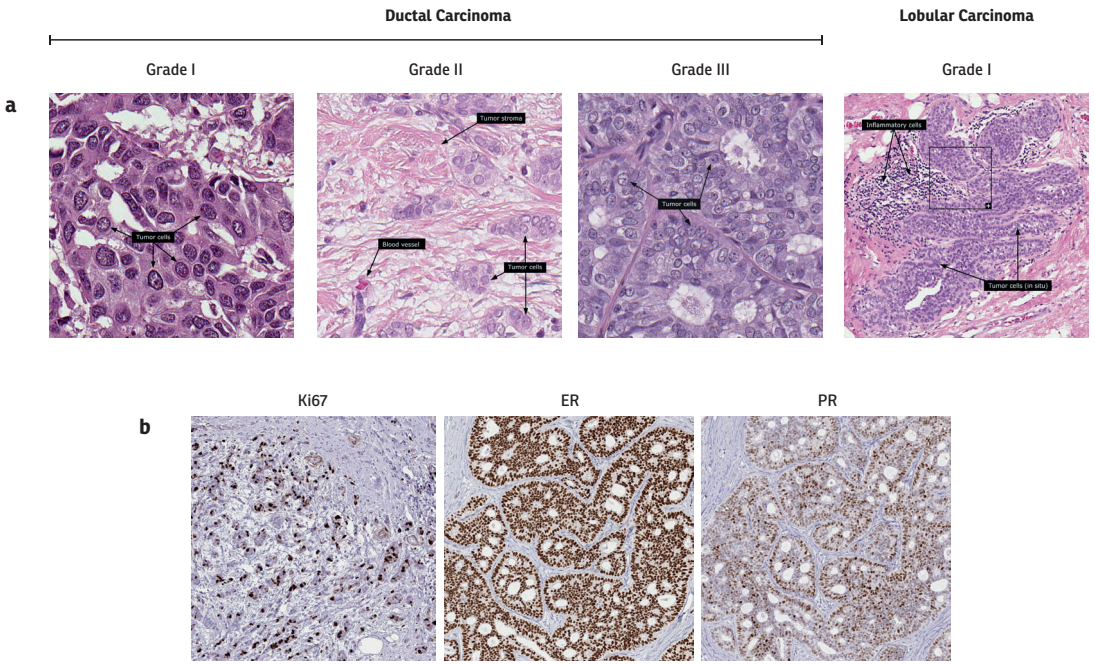


Figure 14: Representative histological images of BC. a) H&E stainings, from left to right: i) ductal carcinoma grade I; ii) ductal carcinoma grade II; iii) ductal carcinoma grade III; and iv)lobular carcinoma grade I. Well differentiated tumors are observed in grade I tumors. In contrast, poorly differentiated and highly invasive tumors are observed in grade III tumors. **b)** IHC stainings from left to right: i) proliferation index (Ki67) in a grade II ductal carcinoma; ii) ER nuclear staining in a grade I ductal carcinoma; and iii) PR nuclear staining in a grade I ductal carcinoma. (IHC pictures taken from the PGA webpage).

1.4.1.2.1 Estrogen Receptor in BC

The ERα nuclear hormone receptor, encoded by the *ESR1* gene, is a ligand-dependent transcription factor. Upon estrogen binding, ERα directly and indirectly modifies a variety of genes involved in proliferation and differentiation (Polyak and Metzger Filho, 2012). ER is expressed in only a small subset of cells in normal breast epithelium, but surprisingly, 65-70% of breast tumors display high ERα levels and ERα dependency. Interestingly, BC tumors exhibit a gradient of ER expression,

thereby showing a high heterogeneity (Harvey *et al.*, 1999). ER status is extremely relevant in the clinical setting because defines the clinical outcome of the disease. ER^+ BC exhibits a longer metastatic latency period with tropism to the bone, whereas ER^- BC is more aggressive, with shorter latency periods and a preference to colonize the lung. Importantly, ER^+ tumors respond to endocrine therapy, including competitive antagonists, downregulators of ER protein levels, and inhibitors of the aromatase enzyme, which produces estrogens. First line treatment for pre-menopausal ER^+ BC patients is tamoxifen, which improves overall survival (1983) (1988; Anampa *et al.*, 2015), reduces recurrence in the adjuvant setting (Baum *et al.*, 1983) and exerts protective action (Fisher *et al.*, 1998). Taxomifen is classified as a selective estrogen-receptor modulator (SERM). In contrast, first line treatment for postmenopausal ER^+ BC patients comprises aromatase inhibitors (*i.e* letrozole or exemestane (Goss *et al.*, 2003; van de Velde *et al.*, 2011), which abrogate the synthesis of estrogen (Baum *et al.*, 2003; Breast International Group 1-98 Collaborative *et al.*, 2005; Coombes *et al.*, 2004; Howell *et al.*, 2005). In fact, the aromatase inhibitor exemestane has been used to prevent BC in postmenopausal patients (Goss *et al.*, 2011).

1.4.1.2.2 Progesterone Receptor in BC

PR is encoded by the *PGR* gene and is expressed in luminal BC subtypes. Progesterone is a major regulator of the mammary gland development. It regulates cell proliferation, expansion and maintenance of MaSCs, angiogenesis and ECM remodeling. In addition, it regulates key mediators such as cyclin D1, RANKL, Wnt4, ID4, and calcitonin and modulates ER expression and its effector functions. Consequently, PR and progesterone are major drivers of BC development (Brisken, 2013).

1.4.1.2.3 HER2 status in BC

HER2 is a member of the EGFR family encoded by the *ERBB2* proto-oncogene. This receptor tyrosine kinase (RTK) is a major driver in tumorogenesis and is amplified in 20-25 % of BC patients (Slamon *et al.*, 1987; Slamon *et al.*, 1989). $HER2^+$, together with basal-BC show the most aggressive phenotype, with metastatic tropism to visceral organs, such as the liver and brain. The number of HER2-targeted therapies has increased markedly in recent years. Examples include the use of blocking antibodies and small molecule inhibitors of RTK activity specific for HER2. These personalized medicine approaches improve the overall survival of patients with HER2 amplification. The gold standard therapy to treat such patients is trastuzumab.

1.4.1.2.4 Keratins in the classification of BC: Luminal and basal-BC

The fibrous structural proteins, keratins, are important markers to determine the origin of epithelial cancers (Cooper *et al.*, 1985). Luminal and myoepithelial epithelia can be distinguished by different keratin expression patterns (Livasy *et al.*, 2006). In addition, keratin assessment can be used to distinguish between BC subtypes and has been used as a prognostic indicator (Shao *et al.*, 2012). For instance, K7, K8, K18 and K19 are expressed in luminal cells, and match with well differentiated tumors and good prognosis (Debus *et al.*, 1982; Nagle *et al.*, 1986). In contrast, myoepithelial/basal cells are characterized by the expression of K5 and K14 (Perou *et al.*, 1999; Perou *et al.*, 2000; Shao *et al.*, 2012). In addition, basal cells also express K17 (Abd El-Rehim *et al.*, 2004), SMA, and p63 (Livasy *et al.*, 2006), and they usually express vimentin, p-cadherin and EGFR (Reis-Filho and Tutt, 2008). High basal K5 or low K7/8 are indicators of poor prognosis, in contrast to low K5 and high K7 (Abd El-Rehim *et al.*, 2004).

1.4.1.2 Molecular classification of BC

Gene expression profiling opened the window to a high amount of opportunities for diagnosis, prevention and treatment, particularly in BC (Sorlie *et al.*, 2001). Genomic and transcriptomic platforms have revealed ten molecular subtypes (iClust 1-10), with distinct features and clinical outcomes (Curtis *et al.*, 2012; Dvinge *et al.*, 2013). However, here, only the five main molecular subtypes (PAM50) and the claudin-low molecular subtype will be described (Perou *et al.*, 2000). **Figure 15** shows the five main molecular subtypes and their clinical outcomes.

1.4.1.2.1 Luminal A BC

Luminal A is the BC subtype with the best prognosis, characterized by low proliferation index (low Ki67 marker), enrichment of the differentiation genes *GATA3*, *FOXA1*, and mutations in *PIK3CA* and *MAP3K1*. Interestingly, luminal tumors correlate with the expression of ER (Sorlie *et al.*, 2001; Sorlie *et al.*, 2003) and expression of luminal K8/18.

1.4.1.2.2 Normal-like BC

Normal-like BC resembles normal breast tissues. The prognosis is worse than luminal A. Normal-like BC shows high expression of basal and adipose tissue-related genes (and other non-epithelial cells), and low expression of luminal differentiated genes (Perou *et al.*, 2000). One signature of these tumors is the expression of genes related to fatty-acid synthesis, peroxisome proliferator activated receptor delta (*PPARγ*) (Perou *et al.*, 2000), and expression of genes such as *PIK3R1*, *AKR1C1* and *FACL2*.

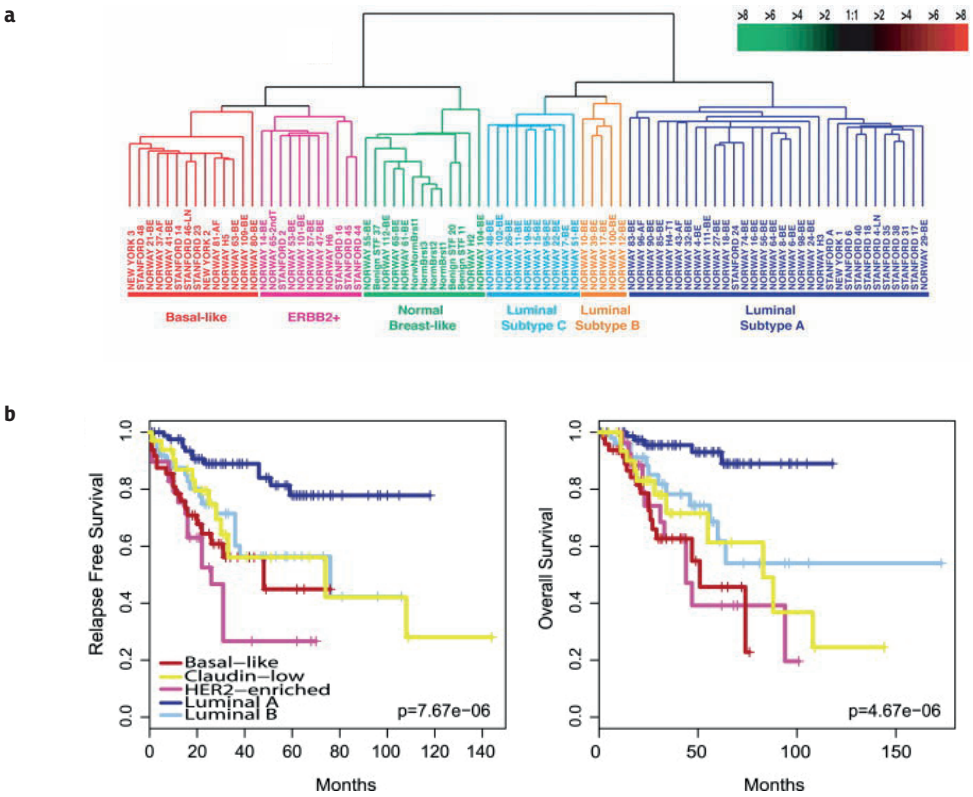


Figure 15: **BC is classified on the basis of the molecular portraits, which can be used as prognosis indicators. a)** Molecular subtypes based on gene expression profile (85 experimental samples). BC is classified in 5 molecular subtypes: luminal A and B/C cluster with luminal epithelial genes and ER, ERBB2^{+} clusters with HER2 amplicon, and basal-like subtype cluster with myoepithelial/basal ER^{-} cell clusters. **b)** Overall and relapse-free survival (RFS) Kaplan-Meier curves for the molecular subtypes. Claudin-low, basal-like and HER2^{+} BC exhibit a more aggressive phenotype with a decrease in overall survival and RFS in comparison to luminal subtypes. In addition, luminal A is the molecular subtype with the best prognosis and it is associated with well-differentiated BC tumors. Luminal B is characterized by an increased proliferation and worse prognosis (Prat *et al.*, 2010; Sorlie *et al.*, 2001).

1.4.1.2.3 Luminal B BC

Luminal B BC is characterized by expression of moderate or low ER and luminal epithelial genes. However, Luminal B BC exhibits worse prognosis than luminal A or normal-like BC, and show greater proliferative capacity. For instance, Ki67 stainings are stronger (Sorlie *et al.*, 2003). Another luminal subtype (C) has been identified with high expression of various clusters of genes and worse prognosis.

1.4.1.2.4 Basal-like BC

Basal-like BC clusters with basal/myoepithelial genes and basal *K5/6*, *K14* and *K17*, and account for up to 15% of BC cases (Livasy *et al.*, 2006). In addition, *BRCA* mutations are associated with basal tumors. Most of these failed to express ER, PR and HER2 (Perou *et al.*, 2000) and are highly proliferative and poorly differentiated. Therefore, most basal-BCs are TBNC (Montagna *et al.*, 2013), although a minor proportion express low levels of these receptors (Kreike *et al.*, 2007; Rakha *et al.*, 2009), and there is no total overlap. Importantly, basal-BC, together with $HER2^+$ BC, is the most aggressive BC. A typical feature of these cancers that differs from luminal A or B is their *TP53* status. In basal-BC, *TP53* is frequently mutated (80%), in contrast to luminal A BC (13%). Interestingly, Basal-like BC also shows EMT (Sarrio *et al.*, 2008; Tang *et al.*, 2016) and SC-like enriched signatures.

1.4.1.2.5 $HER2^+$-like BC

$HER2^+$ BC accounts for 30% of all BCs, and is characterized by the amplification of the *ERBB2* gene. These cancers are highly proliferative and aggressive, and *TP53* is also frequently mutated (Sorlie *et al.*, 2003).

1.4.1.2.6 Claudin-low BC

Claudin-low subtype are TNBCs, with low expression of luminal genes, strong expression of EMT markers, immune response genes, and tumor initiation properties (Prat *et al.*, 2010). These tumors are highly aggressive, poorly differentiated and with poor prognosis, and they resemble the MaSC gene signature (Prat *et al.*, 2010). For instance, these tumors show the highest content of the $CD44^+/CD24^-$ TIC subpopulation (Creighton *et al.*, 2009; Hennessy *et al.*, 2009). Therefore, it is hypothesized that the origin of claudin-low BC is an aberrant mutation on MaSCs or luminal progenitors. Consequently, the tumor-initiating capacity of each molecular subtype differs. For instance, basal-like, claudin-low, and $HER2^+$ BC are poorly differentiated and are enriched in tumor initiation features. Cell lines such as the basal/claudin-low TNBC MDA-MB-231 are highly tumorogenic and enriched in $CD44^+/CD24^-$ (Prat *et al.*, 2010). These molecular subtypes, apart from being a powerful predictive tool, allow better treatment of the disease. Furthermore, the new "omics" era, which has brought about technologies such as RNA profiling, epigenomics, exome sequencing, will help to confirm the molecular portraits of BC. Importantly, luminal and basal cells have been identified and isolated thanks to sorting-based approaches. For instance, $EpCAM^{high}$ $CD49f^{low/med}$ cells are luminal, whereas $EpCAM^{low}$ $CD49f^{high}$ ones are basal (Stingl *et al.*, 2001).

Heterogeneity is present within these molecular subtypes (Lehmann *et al.*, 2011), thereby reflecting the tremendous diversity of BC. In fact, in a whole-genome analysis of 560 BC patients, together with mutational signature analysis and genome rearrangement assessment, verified that there is no dominant activated pathway that confers clonal advantage (Nik-Zainal *et al.*, 2016). In this last study, they found some potential driver mutations, although these mutations rarely act as single dominant. The top 10 most frequently mutated genes were: *TP53*, *PIK3CA*, *MYC*, *CCND1*, *PTEN*, *ERBB2*, *FGFR1*, *GATA3*, *RB1* and *MAP3K1* (62% of drivers). Interestingly, there was a substantial difference between these mutations depending on the ER status. For instance, genes involved in luminal differentiation (*GATA3*) or the PI3K pathway such as *PIK3CA*, *MAP3K1* or FGFR signaling were more prevalent in ER^+ BC, whereas *TP53* mutation was particularly enriched in ER^- BC (more than 75%). As expected, the driver mutation in the $HER2^+$ BC subtype was *ERBB2* amplification. Therefore, the drivers' mutations and the mechanisms underlying BC progression are completely different depending on the BC subtype. The identification of pivotal molecular drivers and signaling pathways for BC progression is essential to develop therapeutic agents to tackle BC, as well as biomarkers to select patients who will benefit from a particular therapy. In **Table 1**, targeted therapies are listed depending on the features of the BC subtype (Polyak and Metzger Filho, 2012).

Hormonal therapy-ER	HER2	PI3K pathway		IGF	Angiogenesis		PARP inhibitors	Others
Anastrozole	Afatinib	AZD8055[2]	INK117	BMS-754807	Aflibercept	Olaratumab	BMN-673	Cabozantinib[5],Foretinib[5],Onartuzumab (MET)
Estradiol	Canertinib	BEZ235 [3]	INK128[2]	Cixutumumab	Axitinib	Pazopanib	CEP-9722	AZD4547,BGJ398,Dovitinib,E-3810[4],HGS1036 (FGFR)
Exemestane	Dacomitinib	BGT226	MK2206[2]	Dalotuzumab	Bevacizumab	Ponatinib	E7016	AUY922, Retaspimycin,Tanespimycin (HSP90)
Fulvestrant	Lapatinib	BKM210	PF-04691502[3]	Figitumumab	Brivanib	Sorafenib	INO-1001	Ruxolitinib (JAK)
Megestrol	MM-121	BLY719	PKI-587	Ganitumab	Lenvatinib	Sunitinib	MK4827	Denosumab (RANKL)
Letrozole	Meratinib	Everolimus[2]	PX-866	Linsitinib	MEDI-575	Semaxanib	Olaparib	
Raloxifene[1]	Pertuzumab	GDC-0032	Temsirolimus[2]	MEDI-573	Motesanib	Vandetanib	Rucaparib	
Tamoxifen	Trastuzumab	GDC-0068 [4]	XL147		Nintedanib	Vatalanib	Veliparib	
Toremifene	T-DM1	GDC-0941	XL765 [3]					
		GDC-0980 [3]	Sirolimus[2]					

Table 1: List of therapeutic agents used to treat BC, based on subtypes and molecular pathways affected. [1]Raloxifene is used for BC prevention, not treatment; [2]mTOR inhibitor; [3]dual PI3K/mTOR inhibitor; [4]AKT inhibitor; [5]also inhibits VEGFR.

The aforementioned information reflects that a different "cell of origin" may give rise to the distinct BC subtypes (Lim *et al.*, 2009; Prat *et al.*, 2010). The claudin-low subtype derives from MaSCs (Prat *et al.*, 2010), whereas the basal-like BC subtype originates from $ALDH^+/ER^-$ luminal progenitors (Shehata *et al.*, 2012). In **Figure 16**, a schematic model of human BC hierarchy and potential "cells of origin" are shown, together with the most frequent mutations present in each subtype.

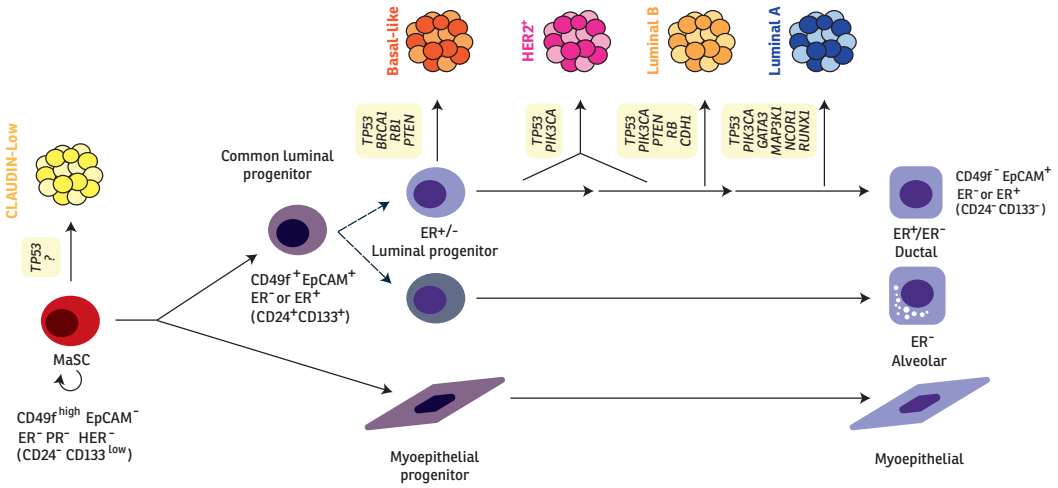


Figure 16: The cell of origin defines BC subtypes. The less differentiated claudin-low BC arises from mutations in MaSCs. Basal-like tumors carry mutations in *TP53*, *BRCA1*, *RB1* and *PTEN* and arises from ER$^-$ luminal progenitors. HER2$^+$ BC is commonly mutated in *TP53* and *ERBB2* amplification and might derive from ER$^{+/-}$ luminal progenitors. Luminal B carries mutations in *TP53*, *PTEN* and *PI3KCA*, and luminal A in *GATA3* and *PI3KCA*. Modified from (Visvader and Stingl, 2014).

1.4.1.2.7 Moving from basic research into the clinic: molecular platforms as a tool to classify and tackle BC

The molecular characterization of subtypes led to the development of the platforms MammaPrint and the Oncotype DX™ (ODX), which are currently used in clinical practice. These gene expression profile tests can provide prognostic and predictive information for patients that are ER$^+$, LN$^-$ and early stage BC (Buyse *et al.*, 2006; Cardoso *et al.*, 2016; Kok *et al.*, 2009; Marchionni *et al.*, 2008; Paik *et al.*, 2006). These tests facilitate more accurate prognosis to select patients for adjuvant systemic therapy. The MammaPrint assay uses paraffin-embedded or fresh tissues from BC patients to predict the risk of BC recurrence (Glas *et al.*, 2006; van 't Veer *et al.*, 2002; van de Vijver *et al.*, 2002). It has been validated in almost 1,600 patients (de Snoo *et al.*, 2009). The 70 genes that make up the MammaPrint signature were selected from genome-wide expression data using a data-driven approach. This test allows physicians to identify younger BC patients at low risk for distant metastasis, who might consequently be spared systemic treatment. Mammaprint scores low risk as 10% risk of relapse within 10 years without any treatment after surgery, and with hormonal therapy alone this risk can be reduced to 5%. A high risk score means more than 29% risk of relapse, and for these patients chemotherapy and hormonal therapy is recommended. On the other hand, the ODX assay is the most common gene expression profile test used in the US. ODX evaluates the expression levels of 21 genes, among them 16 associated with tumor proliferation, invasion and ER signaling (Sparano and Paik, 2008). ODX can predict the likelihood

of a patient benefiting from chemotherapy after BC surgery (Sparano *et al.*, 2015), as well as DCIS, distinguishing those patients that can benefit from radiation therapy after surgery (Carlson and Roth, 2013). Lately, a novel platform has been developed by Prosigna (PAM50), in order to stratify the patients in the 5 molecular subtypes: luminal A, luminal B, HER2-enriched, basal-like, and normal-like. PAM50 predicts risk of recurrence with improved stratification, particularly for ER^+ LN^- BC patients at high/intermediate risk of relapse (Dowsett *et al.*, 2013).

1.4.2 BC progression and metastasis

Despite all the research efforts devoted to understand metastasis, it continues to be the major cause of cancer deaths (>90%) (Weigelt *et al.*, 2005). Several hypotheses suggest that metastases are seeded by exceptional cells with SC-like properties and able to propagate and reinitiate a new lesion at the metastatic site (Massague and Obenauf, 2016; Oskarsson *et al.*, 2014). Surprisingly, metastasis is an extremely inefficient process, and few cells are able to succeed and reinitiate a metastatic tumor. Cancer cells require extensive "training" in order to overcome the enormous number of obstacles along the metastatic cascade. Intrinsic cell autonomous functions of cancer cells are pivotal for organ specificity. Furthermore, the host-niche and physiological barriers are determinants for cancer colonization. Therefore, cancer cells interact with the metastatic niche, activating autocrine and paracrine cytokine signaling loops, and remodeling the microenvironment and ECM structures in order to expand. In addition, these cells need to acquire important features in order to evade the immune system and amplify survival pathways. Recently, these survival features have been associated with SC-like properties driven by sex determining region Y box (SOX)2 and SOX9 in BC bone metastasis (Malladi *et al.*, 2016). BC exhibits a particular variable metastatic course. In this thesis, we focus on TNBC, which preferentially seeds the lungs. In BC, it is widely known that ER^+ BC cells exhibit tropism to the bone, liver and brain, whereas ER^- BC has preference to the lung and a short latency period. In contrast, HER2-enriched BC show tropism to visceral organs such as liver and the brain. Interestingly, ER^+ BC bone and brain metastasis relapse occurs up to decades after initial diagnosis. Below we explain only the BC lung metastasis niche. Bone metastasis and brain metastatic niches can be explored in (Obenauf and Massague, 2015).

1.4.2.1 BC lung metastasis

Lung metastasis is frequent in several cancer types, such as BC, CRC, melanoma, sarcoma, lung and renal carcinomas (Paget, 1989). Lung is a particular organ, in that the capillaries are lined with endothelial cells delimited by alveolar cells and a basement membrane (Nguyen *et al.*, 2009). BC cells express various mediators such as FSCN1 (Minn *et al.*, 2005a) and SPARC (secreted protein, acidic, cysteine-rich/osteonectin), ANGPTL4 (TGF-β inducible factor angiopoietin-like 4), and the secreted C-terminal fibrinogen-like domain of AGNPTL4 (Huang *et al.*, 2011; Padua *et al.*, 2008; Tichet *et al.*, 2015), in order to overcome the cell-cell junctions formed by endothelial cells and extravasate into the lung. Epiregulin, COX2 (cytochrome c oxidase polypeptide II), MMP1 and MMP2 are other factors that foster seeding (Gupta *et al.*, 2007a; Minn *et al.*, 2005a; Minn *et al.*, 2005b). Therefore, in primary tumors, metastatic traits required in the early steps of the metastatic cascade are already selected (Obenauf and Massague, 2015). After lung parenchyma extravasation, cells interact with the tumor stroma, and tumor initiation features are essential in order to survive the suppressor mechanisms. Indeed, cancer cells secrete periostin, stimulating Wnt ligands in TICs (Malanchi *et al.*, 2012). Periostin interacts with the hexameric glycoprotein tenascin C (TNC) in the ECM, and consequently survival programmes are enhanced through the Wnt and notch signalling pathways (O'Connell *et al.*, 2011; Oskarsson *et al.*, 2011). Therefore, there are constantly interactions between BC cells and the metastatic niche. Furthermore, cell-autonomous mechanisms are also pivotal for metastasis colonization. For instance, expression of inhibitor of differentiation 1 and 3 (*ID1* and *ID3*) promotes metastasis initiation after lung parenchyma infiltration (Gupta *et al.*, 2007b). Furthermore, cells need to overcome dormant signals such as those from bone morphometric protein (BMP) (Gao *et al.*, 2012; Sosa *et al.*, 2014) (**Figure 17**).

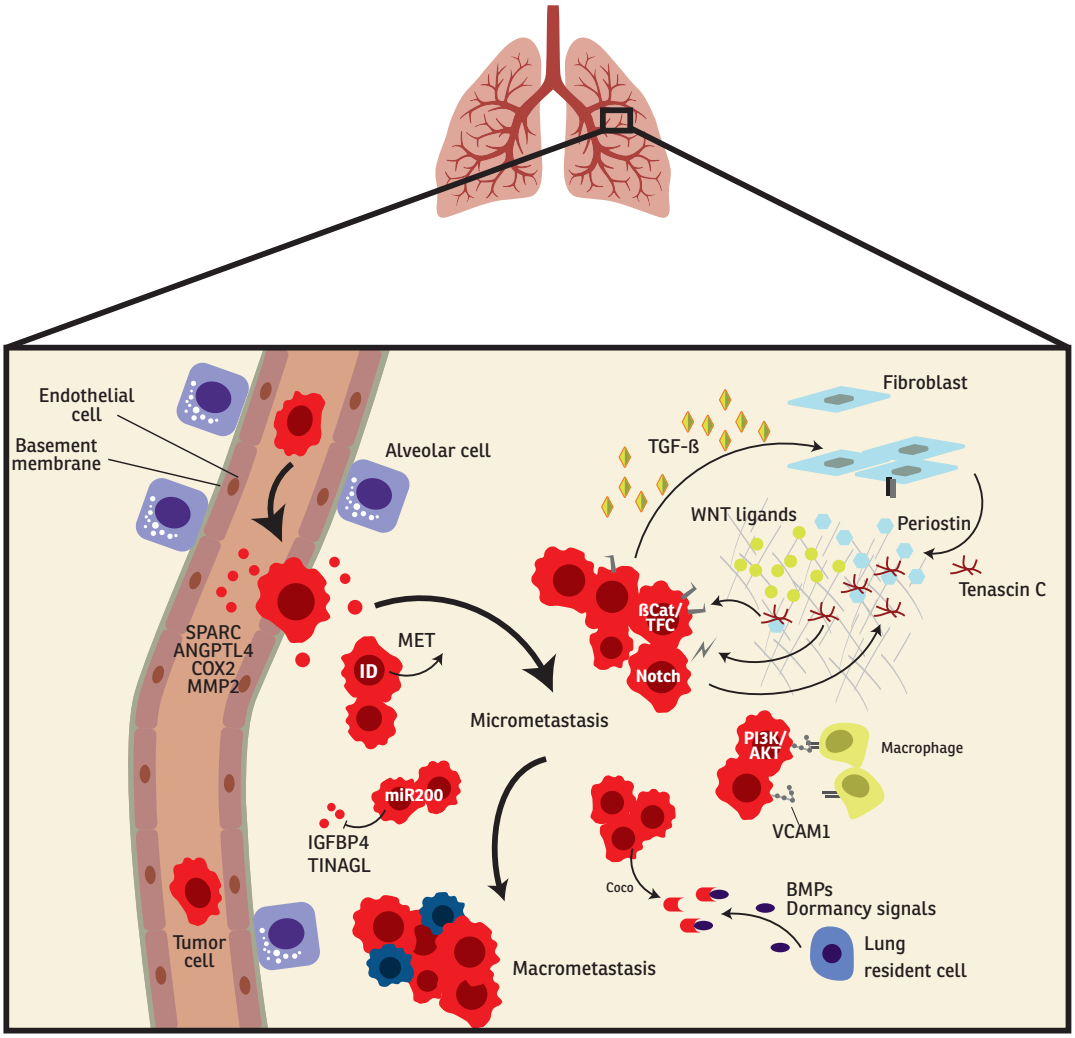


Figure 17: BC lung metastasis niche. The lung capillaries are lined with a basement membrane, and BC cells extravasate thanks to various mediators (SPARC, ANGPTL4, COX2, MMP2). In addition, ID proteins induce MET, allowing them to survive and grow. BC cells interact with the stroma, and ECM POSTN and TNC proteins stimulate survival by allowing Wnt access to their receptor (periostin) or by amplifying notch and Wnt signaling itself. Protein kinase B (AKT or PKB) cell survival signaling is also activated by VCAM1 expression. Finally, BC cells need to overcome dormant signals (BMPs) from resident lung cells by overexpressing Coco. Modified from (Obenauf and Massague, 2015).

1.5 Tumor initiation features and BC

TICs are defined by their ability to efficiently seed new tumors upon inoculation into recipient host mice (Cho and Clarke, 2008). Therefore, TICs are based on a functional definition (Al-Hajj *et al.*, 2003). Fractionation of cancer cells on the basis of displayed cell surface markers has allowed the identification of populations with heightened tumorigenic capacity. In human, $CD44^{+}/CD24^{low}$ is a bona fide TIC subpopulation (Al-Hajj *et al.*, 2003; Zhang *et al.*, 2012). In addition, $CD49f^{+}$ (Cariati *et al.*, 2008), Notch ligand Delta/Notch-like EGF-related receptor (DNER) (Pece *et al.*, 2010), ABCG2 (Doyle *et al.*, 1998), PKH26high (Akrap *et al.*, 2016) as well as ALDH-1 (Balicki, 2007; Charafe-Jauffret *et al.*, 2006; Ginestier *et al.*, 2007) exhibit increase tumorigenic potential. In mouse models other subpopulations such as $CD29^{high}/CD24^{+}$ (Asselin-Labat *et al.*, 2010; Schramek *et al.*, 2010; Shackleton *et al.*, 2006), $CD49f^{high}/CD24^{+}$ (Joshi *et al.*, 2010; Schramek *et al.*, 2010; Stingl *et al.*, 2006) have been identified.

The heat-stable antigen CD24 mediates cell-cell and cell-ECM interactions, and its absence characterizes human TICs (Stingl *et al.*, 2006). It is associated with proliferation and adhesion in cancer cells (Kristiansen *et al.*, 2003; Zheng *et al.*, 2011) with and is less expressed in progenitors (Fang *et al.*, 2010).

CD44, the lymphocyte homing receptor, mediates communication and adhesion between cells and the ECM, which is essential in tissue integrity, and it has been widely associated with tumor initiation features (Louderbough and Schroeder, 2011), EMT, chemoresistance and metastasis (Xu *et al.*, 2016; Zoller, 2011). Epigenetic factors and tumor microenvironment are essential for CD44 regulation (*i.e* TGF-β) (Chaffer *et al.*, 2013).

CD49f is an integrin associated with basal/progenitor subpopulations (Pece *et al.*, 2010)

DNER is an epigenetically modulated gene important in cancer cell fate and differentiation (Sun *et al.*, 2009).

ABCG2 is a multidrug resistance transporter protein (Dean *et al.*, 2005); PKH26 is a lipophilic fluorescent dye which allow to purify long-labeling retaining cells (Huang *et al.*, 1999; Lanzkron *et al.*, 1999; Pece *et al.*, 2010).

ALDH is a family of enzymes that oxidize aldehydes to carboxylic acids, such as ALDH1A1/2/3, which convert vitamin A to RA (Black and Vasiliou, 2009). ADLH1A3 has been associated with CSCs, aggressiveness (Marcato *et al.*, 2011), and chemoresistance (Croker and Allan, 2012) in BC. In fact, *ALDH1A3* expression correlates with poorer survival in TNBC (Marcato *et al.*, 2015).

TICs can be also isolated by Hoeschst-negative side population (SP) cells (Patrawala *et al.*, 2005) and enriched by a mammosphere assay (an *in vitro* assay to assess tumor initiation) or by *in vivo* serial transplantation (Ponti *et al.*, 2005). Interestingly, the RANK/RANKL axis has been associated with the maintenance of MaSCs and BCSCs, leading to tumorigenesis (Gonzalez-Suarez *et al.*, 2010; Tarragona *et al.*, 2012), and to metastasis (Schramek *et al.*, 2011). Of note, these cells are not static, and there is a continuous interconversion between non-TICs and TICs, a process regulated by the

environment and the chromatin status (Chaffer *et al.*, 2013). Therefore, the interplay between TICs and the SC niche is essential to understand breast tumorigenesis, which occurs in a particular environment with the presence of steroid hormones and stromal cells. Self-renewal and SC-like features have been extensively associated with tumorigenesis and poor prognosis. In contrast, differentiation attributes have been associated with good prognosis (Albergaria *et al.*, 2009).

In addition, tumor initiation properties (*i.e* self-renewal and slow cycling profile) have been considered the main cause of chemoresistance. For instance, metastatic dormancy initiation has been associated with SC-like signatures (Malladi *et al.*, 2016). Metastatic niches play an essential role in these contexts (Kii *et al.*, 2010; Kim *et al.*, 2009; von Holst, 2008). However, the distribution and function of BCSC populations in adults remains to be fully elucidated. Therefore, these clinically important matters lead to the following: i) identification of TICs; ii) identification of specific metastatic initiator genes (Guiu *et al.*, 2015); iii) identification of chemoresistance mechanisms driven by SC-like features; and iv) the design of targeted therapies.

1.5.1. The BCSC niche

Earlier we explained the singularity of the MaSC niche, which has several implications in BC. Steroid hormones are key players in BC and its niche. Women are continuously exposed to hormones, and this has been linked to BC risk. In fact, the more menstrual cycles, the higher risk of BC in humans (Kelsey *et al.*, 1993). Surprisingly, an increased number of menstrual cycles increases BC risk for all types of BC, reflecting that in early stages of TNBC, there might be a subset of hormone-sensitive BC cells. In this regard, cessation of cycles by oophorectomy imposed in *BRCA1/2* hereditary BC reduces BC risk (Kauff *et al.*, 2002). Furthermore, pregnancy also increases the risk of BC (Lambe *et al.*, 1994), due to expansion of MaSCs (Asselin-Labat *et al.*, 2010), but early pregnancy and lactation are beneficial (Chung *et al.*, 2013; Ritte *et al.*, 2013). For instance, a pregnancy before 20 years of age is highly protective, which reflects that pregnancy is the most important BC risk factor. Furthermore, hormone replacement therapy (HRT) used for menopausal women increases BC risk (Anderson *et al.*, 2014; Bao *et al.*, 2011; Ritte *et al.*, 2012; Setiawan *et al.*, 2009; Tamimi *et al.*, 2012). The key effectors of this evidence are the steroid hormones 17β-estradiol and progesterone. 17β-estradiol is one of the main drivers in BC progression, and it regulates proliferation, survival, and cell cycle progression by activation of cyclin D1 (Frasor *et al.*, 2003). Estrogen is a major physiological modulator, affecting bone density and adipocyte synthesis. Importantly, clinical treatment against estrogen function in ER^+ BC patients causes dramatic reduction in bone density, which increases the risk of osteoporosis (Clemons and Goss, 2001).Progesterone is pivotal for BC initiation and metastasis, and it regulates key molecules in these processes such as Wnt4 (Tanos *et al.*, 2013), RANKL (Schramek *et al.*, 2010), and ID4 (Wen *et al.*, 2012) (**Figure 18**). Clinical evidence supports these observations. For instance, estrogen

plus synthetic progesterone agonist such as progestins (medroxyprogesterone acetate-MPA or dimethylbenz(a)anthracene-DMBA) substantially increase BC risk (Chlebowski *et al.*, 2010; Rossouw *et al.*, 2002), due to increased epithelial cell proliferation (Hofseth *et al.*, 1999). The effect of progestins is mediated in part by RANKL, and overexpression of its receptor RANK increase proliferation in mouse models (Gonzalez-Suarez *et al.*, 2007). In fact, these effects are abrogated by RANKL ablation (Gonzalez-Suarez *et al.*, 2010; Schramek *et al.*, 2010). Therefore, although in the last 20 years, hormonal therapy was devoted on targeting estrogen effector functions (*i.e.* tamoxifen), targeting PR signaling and its downstream effector molecules RANKL and Wnt could be a novel strategy to treat BC. For instance, Wnt inhibitors are in clinical trials (Takebe *et al.*, 2011); and denosumab, an antibody that blocks RANKL, is approved by the FDA for postmenopausal BC bone metastatic patients (Gnant *et al.*, 2015; Stopeck *et al.*, 2016; Stopeck *et al.*, 2010). **Figure 18** shows the role of estrogen and progesterone in BC progression.

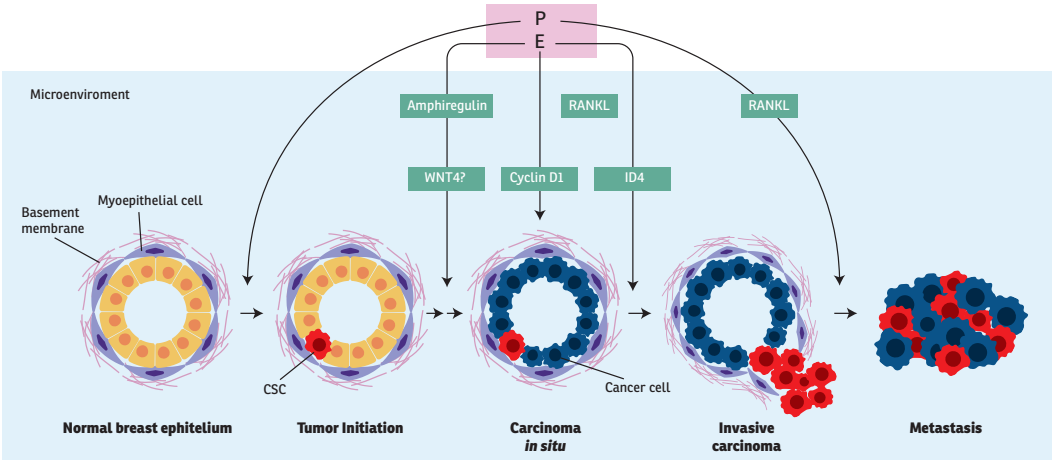


Figure 18: **Estrogens (E) and progesterone (P) impinge on the different stages of BC and increase BC risk.** Progesterone is pivotal for BC initiation and metastasis and it modulates the microenvironment by regulating various pathways, such as RANKL Wnt, cyclin D1 and ID4. Estrogens are pivotal for BC progression and proliferation. Therefore, an increased number of menstrual cycles promotes BC. Modified from (Brisken, 2013).

1.5.2 Self-renewal pathways in mammary gland development and BC initiation

Self-renewal properties are regulated by a variety of pathways. Perturbation on these pathways leads to tumorigenesis, metastasis, and chemoresistance. Therefore, knowledge of how these pathways are regulated is essential in the context of tumor initiation. In BC, pathways such as Wnt, Notch, ERK, p38, PI3K/AKT or retinoid signaling have been described as key drivers of tumor initiation and more recently, of metastasis. To date, developmental pathways and SC-like reprogramming have been studied in depth.

1.5.2.1 The Wnt pathway in mammary gland development and BC initiation

Wnt proteins are a family of secreted, glycosylated and palmitoylated peptides that mediate a wide range of processes during embryogenesis by regulating SC division, migration and integrity of the SC niche (Pardal *et al.*, 2003; van Amerongen and Nusse, 2009), and they are central to sustain pluripotency *in vitro* (Sato *et al.*, 2004) and *in vivo* (Kielman *et al.*, 2002). Defects in Wnt signaling lead to developmental deficiencies in human cancer. In fact, in those tissues where Wnt regulates SCs, cancer ensues upon dysregulated activation of this pathway. This is the case of medulloblastoma with mutations in Wnt components such as β-catenin (Zurawel *et al.*, 1998), and AXIN1 (Baeza *et al.*, 2003; Dahmen *et al.*, 2001) in CRC and epidermal cancer (see Table 1 from (Espada *et al.*, 2009; Reya and Clevers, 2005). In breast, Wnt is essential for development and maintenance of progenitor cells, as well as tumorigenesis. For instance, transgenic Wnt1 mice develop tumors and exhibit increased tumor initiation features (Li *et al.*, 2003; Liu *et al.*, 2004).

In addition, Wnt/β-catenin signaling is pivotal for the maintenance of tumor initiation features in various cancer models (Eaves and Humphries, 2010; Nusse, 2008). In breast, MaSCs with high levels of Wnt exhibit greater tumorigenic potential than their counterparts with low levels (Monteiro *et al.*, 2014). This is triggered by the action of Wnt negative regulators. For instance, the Wnt inhibitor secreted frizzled related protein 1 (SFRP1), which competes with FZD receptor, is frequently down-regulated in BC and associated with poor prognosis together with low therapeutic response (Klopocki *et al.*, 2004; Ugolini *et al.*, 1999; Veeck *et al.*, 2006). In addition, high levels of Wnt components increase BC bone (Chen *et al.*, 2011) and lung metastasis (Bilir *et al.*, 2013; Dey *et al.*, 2013; Pacheco-Pinedo *et al.*, 2011). Therefore, Wnt is a potential target to treat BC. Indeed, blockade of Wnt/β-catenin supresses BC lung metastasis by suppressing the expansion and self-renewal of TICs *in vivo* (Jang *et al.*, 2015). Interestingly, in that study, Wnt inhibition impaired $CD44^{+}/CD24^{-}$ expansion, mammosphere capacity formation (an *in vitro* assay to assess tumor initiation) and invasiveness. Recent data suggest a model whereby Wnt signaling inhibition through the negative regulator DKK1 imposes quiescence in latent competent bone metastatic

cells (Malladi *et al.*, 2016). These quiescence metastatic cells exhibit an increase in SC features and a slow cycling profile. This SC program also confers BC cells the ability to evade the immune system attack and remain latent for extended periods. In conclusion, Wnt/β-catenin signaling is a critical regulator of tumor initation features in BC, and is associated with survival and metastasis dissemination/dormancy.

1.5.2.2 The Notch pathway

Notch signaling pathway is a key mediator in cell-cell communication (Lai, 2004; Stylianou *et al.*, 2006), apoptosis (Stylianou *et al.*, 2006), proliferation (Politi *et al.*, 2004), and self-renewal (Bouras *et al.*, 2008; Dontu *et al.*, 2004). In fact, Notch signaling is pivotal for breast development and MaSC activity (Bouras *et al.*, 2008), and it is dysregulated in invasive BC (Gallahan *et al.*, 1996; Jhappan *et al.*, 1992; Smith *et al.*, 1995). Several findings links notch molecules to differentiation and BC tumor initiation. For instance, *Notch4* overexpression prevents the differentiation of epithelial cells *in vitro* (Uyttendaele *et al.*, 1998) and triggers mammary gland transformation. In addition, Notch molecules are increased in mammospheres and $CD44^{+}/CD24^{-}$ TIC subpopulation (Ponti *et al.*, 2005; Shipitsin *et al.*, 2007), contributing to early aberrant activation of BC (Farnie and Clarke, 2007). Therefore, notch may represent a novel therapeutic target to block tumor initiation features, thereby preventing BC recurrence.

1.5.2.3 The ERK and the JNK pathways

The ERK and JNK signaling pathways are essential for survival, proliferation and differentiation in various types of cancers (Roberts and Der, 2007; Shields *et al.*, 2000). *MAP3K1* and *MAP2K4*, which regulate the ERK-JNK axis, are frequently mutated in BC (Polyak and Metzger Filho, 2012). Importantly, ERK signaling has been described to be pivotal for BC initiation and progression (Shin *et al.*, 2010). For instance, ERK induces EMT and confers tumor initiation features to BC cells *in vitro* and *in vivo* (Liu *et al.*, 2009; Shin *et al.*, 2010). In addition, ERK is pivotal for BCSC maintenance and expansion (Chang *et al.*, 2011; Luo *et al.*, 2015). Several ERK inhibitors showed promising results in BC models. For instance, the MEK/ERK inhibitor AZD6244 blocks BC tumors by abolishing $CD44^{+}/CD24^{-}$ subpopulation and decreasing the EZH2 epigenetic regulator (Chang *et al.*, 2011).

1.5.2.4 The polycomb protein complex regulation

The epigenetic regulators polycomb protein complexes 1 (PCR1) and PRC2 are essential for stem cell maintenance, survival and self-renewal. These proteins repress transcription of tissue-specific genes and differentiation genes (Juan *et al.*, 2011; Pal *et al.*, 2013) through post-translational modifications (PTMs) in chromatin histones (Simon and Kingston, 2009). Methylation is exerted by the PRC2 enzymatic subunits EZH1/2. In addition, PRC2 comprised the following three components: SUZ12, EED, and RbAp46/48. In contrast, PCR1 is formed by two main components, namely RinG1A/B and BMI1 (Whitcomb *et al.*, 2007). These components are essential for pluripotency maintenance and cell-specification/differentiation (Boyer *et al.*, 2006), and they maintain the repression of several key developmental regulators such as Wnt. For instance, EZH2 ablation prevents lineage differentiation (Lee *et al.*, 2006; Wang *et al.*, 2010). Furthermore, these proteins are essential for the maintenance of adult SCs. For instance, BMI1 regulates self-renewal in MaSCs (Liu *et al.*, 2006). Interestingly, these two groups of proteins are essential in carcinogenesis, especially for TIC activity/identity and TSG repression (Sparmann and van Lohuizen, 2006). For instance, *BMI1* is critical for MaSC malignancy (Al-Hajj *et al.*, 2003) and is associated with poor prognosis (Glinsky *et al.*, 2005). *SUZ12* overexpression is present in BC, CRC, and liver cancer (Kirmizis *et al.*, 2003), and it regulates Wnt signaling components. Importantly, *EZH2* expression is highly correlated with metastasis and poor prognosis in BC (Kleer *et al.*, 2003; van 't Veer *et al.*, 2002) and other cancers such as melanoma, lymphoma and prostate cancer (Sparmann and van Lohuizen, 2006). Therefore, *EZH2* is an important oncogene (Bracken *et al.*, 2003) which regulate tumor initiation features in BC. In this context, the microenvironment regulation on epigenetic changes is key (Hu and Polyak, 2008). Importantly, EZH2 induces RAF/ERK and β-catenin signaling pathways which enhance survival, expansion and maintenance of BCSC, leading to a high-grade BC (Chang *et al.*, 2011). Therefore, these components might be a potential therapeutic target to block the transition to metastatic disease initiated by TICs.

1.5.2.5 The PI3K/Akt pathway

PIK3CA encodes a 100-KDa catalytic subunit of a class I phosphatidylinositol 3-kinase (PI3K). PI3K generates phosphatidylinositol -3,4,5 (PIP3), which regulates signaling involved in proliferation, growth and survival. This effector function is mediated by the recruitment of PH-domain containing proteins (including AKT1 and PDPK1) to the cell membrane. Importantly, AKT activates the mammalian target of rapamycin (mTOR), a pivotal kinase for BC. In fact, mTOR is implicated in advanced metastatic BC disease (Baselga *et al.*, 2012). In this thesis, an example of how mTOR inhibition chemoresistance is driven by tumor initiation features in BC will be described. Therefore, a full section is dedicated to explain mTOR implication in BC and its potential as therapeutic target. PI3K is the most frequent mutated pathway in several cancers (*i.e.* BC), which lead to constitutive

activation of PI3K/AKT components. Interestingly, PI3K is antagonized by PTEN tumor suppressor. PTEN suppress AKT kinase activity, and its inactivation is common in BC. 50% of BC exhibit activated PI3K signaling pathway, and regulates MaSC gene programs through GSK3β phosphorylation and activating Wnt signaling (Korkaya *et al.*, 2009). Importantly, PTEN and signal transducer and transcription factor 3 (STAT3) are pivotal for BCSC maintenance and viability (Zhou *et al.*, 2007). For instance, the AKT inhibitor perifosine impairs BC tumorogenesis in xenografts by depleting BCSCs (Korkaya *et al.*, 2009). Therefore, PI3K is an attractive therapeutic target to tackle BC. Small molecule inhibitors targeting PI3K, AKT, mTOR or their combination are in various phases of clinical development, such as the PI3K inhibitors alpesinib (BLY719) (phase IIb, in combination with the aromatase inhibitor letrozole) (Mayer *et al.*, 2016) and taselibib (GDC-0032) (phase III, in combination with fulvestrant).

1.5.2.6 HER2 signaling

HER2 gene amplification has been associated with tumor aggressiveness and metastasis, where HER2 regulates mainly PI3K/AKT MAPK pathways. In the last years, due to its central role in a subset of BC patients, significant efforts to its blockade have been taken. The gold standard example is the antibody trastuzumab or Herception (Beuzeboc *et al.*, 1999; Carter *et al.*, 1992), which improves disease-free and survival of $HER2^+$ BC patients in the adjuvant setting (Slamon *et al.*, 2011). Nowadays a dual tyrosine kinase inhibitor of EGFR and HER2 has been used to treat $HER2^+$ BC (de Azambuja *et al.*, 2014; Geyer *et al.*, 2006; Kim and Murren, 2003; Sonnenblick *et al.*, 2016; Zhang *et al.*, 2008). However, trastuzumab response is modest (Gajria and Chandarlapaty, 2011), due to either primary/inherently (<35% of patients respond to therapy) (Narayan *et al.*, 2009; Wolff *et al.*, 2007) or secondary/acquired resistance (70% of patients who responded to therapy experience progression to metastatic disease within a year) (Gajria and Chandarlapaty, 2011; Miller, 2004; Seidman *et al.*, 2001). Among other mechanisms, resistance occurs due to the expansion of TICs, which drive tumorigenesis and aggressiveness through the HER2 kinase pathway. Besides, HER2 signaling regulates the tumor initiation features and drive carcinogenesis (Korkaya *et al.*, 2008). Other resistance mechanisms include steric effects (*i.e.* structural mutation or proteolysis of HER2 extracellular domain with HER2 constitutive kinase activity) (Scaltriti *et al.*, 2007; Scott *et al.*, 1993); compensatory mechanisms such as activation of IGR-1R (Lu *et al.*, 2001) and c-Met (Shattuck *et al.*, 2008), or hyperactivation of the PI3K pathway due to *PTEN* deficiency (Berns *et al.*, 2007; Kataoka *et al.*, 2010; Nagata *et al.*, 2004; Vu and Claret, 2012; Zhang *et al.*, 2011b).

1.5.2.7 Retinoid signaling and cancer

Retinoids have been studied extensively and associated to differentiation attributes. Retinoid acid (RA) regulates the expression of more than 500 genes through its interaction with the retinoic acid receptors (RAR) and retinoic X receptors (RXR) (Collins, 2002; Zechel, 2005), binding to RA response elements (RAREs) (Balmer and Blomhoff, 2002). These genes are involved in differentiation, apoptosis, cell cycle arrest and proliferation (Tang and Gudas, 2011). Therefore, RA triggers two opposite functions. Cell cycle arrest function is driven by RARs activation, whereas proliferation is triggered by activation of the nuclear hormone receptors PPARβ/γ (Schug *et al.*, 2007; Schug *et al.*, 2008) or by epigenetic silencing of TSG (Tang and Gudas, 2011). Interestingly, RAR binding colocalize and cooperates with ERα binding. For instance, there is a crosstalk between RA and estrogen signaling. Importantly, luminal differentiation genes such as *FOXA1* and *GATA3* coincides with *RAR* and *ERα* expression (Hua *et al.*, 2009), and these genes have been associated extensively with well differentiated and good prognosis features in BC (Albergaria *et al.*, 2009). A key enzyme in RA metabolism is ALDH, which oxidize intracellular aldehydes and have a role in early differentiation of SCs though conversion of retinol to RA (Sophos and Vasiliou, 2003). Therefore, RA inducers are potential candidates to treat cancer. In fact, all-trans-retinoic acid (ATRA) is considered the first example of targeted cancer therapy since 1997 , for the treatment of acute promyelocytic leukemia (AML) (Tallman *et al.*, 1997), and has been further validated (de The and Chen, 2010; Huang *et al.*, 1988; Sanz and Lo-Coco, 2011).

In addition, ATRA treatment impairs BC tumor initiation features by inducing differentiation (Ginestier *et al.*, 2009), blocking expansion/proliferation and invasiveness (Mangiarotti *et al.*, 1998; Van heusden *et al.*, 1998). Importantly, retinoid signaling is a major regulator of different pathways in BC. ATRA can modulate SC gene expression programs by regulating self-renewal remodeling PRC2 EZH2 network (Ginestier *et al.*, 2009). Additionally, ATRA modulates AKT/ β-catenin activity (Ginestier *et al.*, 2009), arrest BC cells by modulating EGF pathway (Tighe and Talmage, 2004), impairs HER2 phosphorylation (Paroni *et al.*, 2012), (Fontana *et al.*, 1992; Valette and Botanch, 1990) and IGF1/2 growth activity (Fontana *et al.*, 1991; Oh *et al.*, 2010), and modulates Wnt function (Mulholland *et al.*, 2005) to the same extend than estradiol (Easwaran *et al.*, 1999). In addition, ATRA reduce invasiveness of TBNC cell line MDA-MB-231 through notch and PI3K inhibition (Farias *et al.*, 2005), and impose long term inhibition of AKT (Paroni *et al.*, 2012). Besides, ATRA effector function can be enhanced by TGFβ1 activity (Fontana *et al.*, 1992; Valette and Botanch, 1990). Interestingly, ATRA and HER2 signalling pathways are interconnected. *HER2* overexpression causes ATRA resistance via AKT (Tari *et al.*, 2002), and trastuzumab increases RARs/RXRs binding via AKT stimulation (Siwak *et al.*, 2003). Recently, a synergistic effect of ATRA and lapatinib (HER2 antibody) was observed.

ATRA modulates survival and motility of BC cells through microRNA networks (Fisher *et al.*, 2015). ATRA resistance is also associated to *PTEN* suppression (Stefanska *et al.*, 2012). Furthermore, ATRA inhibits aggressiveness of TBNC cell lines through the p38 MAPK pathway (Wang *et al.*, 2013a). In fact, RARα, RARγ are downstream targets of p38-MAPK (Alsayed *et al.*, 2001; Gianni *et al.*, 2012), and ATRA regulates p38 functions (Alsayed *et al.*, 2001). Importantly, dual application of p38 inhibitors and ATRA has synergestic activity in AML models (Garattini *et al.*, 2014), and given the importance of p38 in BC dormancy (Sosa *et al.*, 2011), it would be relevant to extend this observation in BC. Dual therapy using p38 inhibitors (Garattini *et al.*, 2014), AKT inhibitors or HER2 antibodies (Fisher *et al.*, 2015) could be a useful strategy to treat ATRA resistance BC. However, ATRA may be detrimental in resistant BC cells, as ATRA stimulates the growth of BC cells by activating Wnt and PPARβ/γ (Schug *et al.*, 2007; Schug *et al.*, 2008), and ALDH inhibition may expand HSC (Chute *et al.*, 2006).

Recently, the mechanism underlying ATRA inhibition on ER^+, $HER2^+$ and TNBC human cells was identified, with no toxicity on normal breast cells (Wei *et al.*, 2015). In this report, ATRA ablates prolyl isomerase Pin1 function, which regulates the expression of different pivotal oncoproteins such as ERα, HER2, cycling D1 and AKT (see supplementary Figure 11 from (Wei *et al.*, 2015). Since Pin1 regulates BCSC expansion (Luo *et al.*, 2014; Rustighi *et al.*, 2014), and ATRA inhibit Pin1, ATRA effect on BC growth could be explained by tumor initiation features abolishment. Interestingly, Pin1 activates ERK phosphorylation leading to BCSC expansion and poor prognosis in BC patients (Luo *et al.*, 2015).

It is widely known the impact of retinoid activity on the primary growth. However, few studies regarding metastatic disease have been reported. Interestingly, an association between RA signalling and BC metastasis has been explored (Werner *et al.*, 2015). Here, RA-induced 2 (*RAI2*) downregulation gene was associated with early bone metastasis in ER^+ BC due to loss of epithelial differentiation, being a key event for early steps of the metastatic cascade by inducing cellular plasticity, dissemination and dedifferentiation. RAI2 sustains differentiation of luminal breast cells, controlling the gene expression of the bona fide luminal genes *FOXA1*, *ERα* and *GATA3*. Interestingly, RAI2 loss of function activates AKT signalling.

In summary, retinoid signalling is essential in the maintenance of SC biology, and further investigation regarding its link with tumor initiation features and metastasis is required. Indeed, the bone marrow is an enriched RA microenvironment (Ghiaur *et al.*, 2013; Purton *et al.*, 2006) and in fact, RA regulates differentiation of HSC in this niche (Purton, Dworkin *et al.* 2006). Importantly, ATRA has beneficial impact against advanced BC patients in clinical trials (Budd *et al.*, 1998; Connolly *et al.*, 2013), suggesting that differentiation therapy is useful to tackle cancer disease. In addition, differentiation therapies might be useful to overcome chemoresistance, as

they stop different cancer-driving molecules. In this thesis, an example of how retinoid signal is key for BC lung metastasis will be described. Particularly, retinoic acid responder (tazarotene 3) (*RARRES3*), also known as *TIG3* or *RIG1*, regulates differentiation and adhesion of ER^- human BC cells, blocking lung metastasis.

1.6 Tumor initiation and metastasis

Tumor initiation features are an increasing subject of interest, particularly in dormant and advanced metastasic disease. TICs are malignant cell subsets organized as unidirectional cellular hierarchies, with banner capacity to perpetuate tumor growth indefinitely (Husemann *et al.*, 2008; Psaila and Lyden, 2009; Visvader and Lindeman, 2012). However, TICs are not restricted to participate in the primary site. In fact, tumor initiation properties have been extensively related to metastatic aggressive phenotypes (Oskarsson *et al.*, 2014) in different cancers such as colon (Hermann *et al.*, 2007; Pang *et al.*, 2010), breast (Oskarsson *et al.*, 2011), pancreatic (Dieter *et al.*, 2011) , haematological cancer (Visvader and Lindeman, 2008), brain cancer (Guo *et al.*, 2011) and melanoma (Rappa *et al.*, 2008). However, little is known about the importance of metastasis initiation, and few suppressor genes have been reported. Previously we explained that several populations/markers have been identified (*i.e.* $CD44^+/CD24^{-/low}$, a bona fide subpopulation).

Several reports identified RANK/RANKL axis as a player in the maintenance of BC tumor initiation (Gonzalez-Suarez *et al.*, 2010) and metastasis (Schramek *et al.*, 2011). In addition, hormones play a key role, driving tumorogenesis and regulating the BCSC niche (Brisken, 2013). In **Figure 19**, an example of how steroid hormones and the environment can promote neoplasia and metastasis is depicted. Therefore, metastatic niches are essential in the maintenance of TICs effector functions.

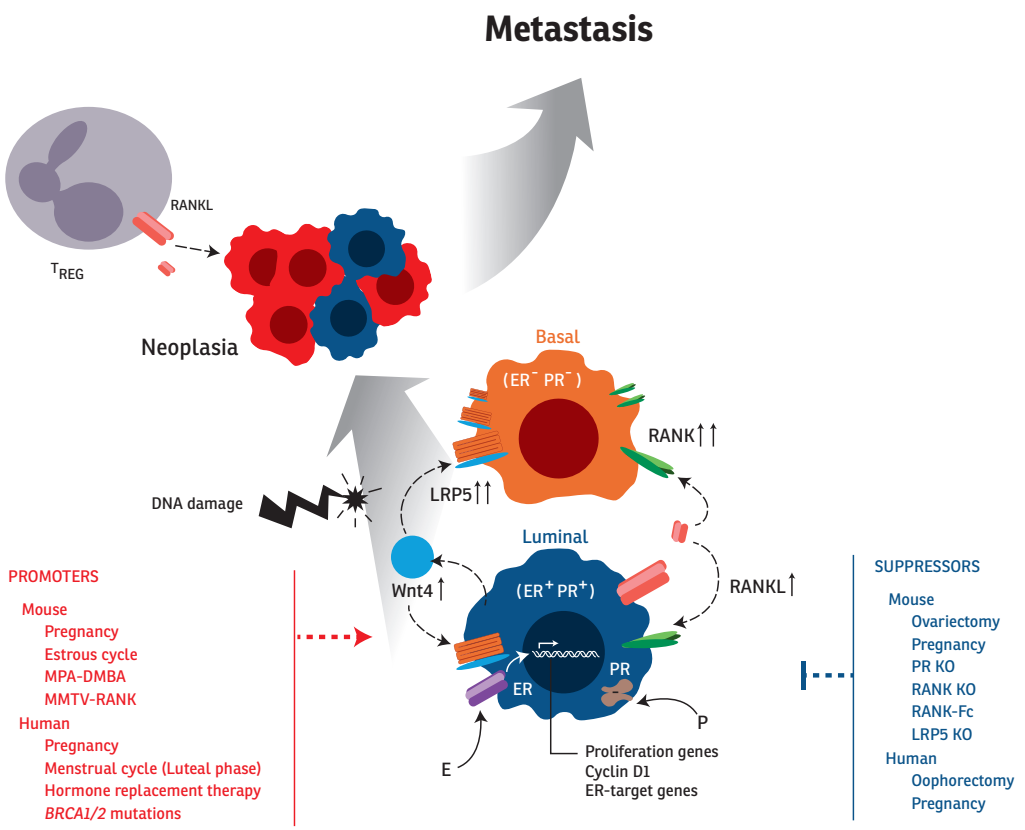


Figure 19: **The hormone-driven niche in BC**. Progesterone (P) acts on ER⁺ luminal cells, which secrete the mitogenic effectors RANKL and Wnt4. RANKL binds to RANK and Wnt4 to Lrp5-Frizzled complex on basal SCs, triggering expansion required for reproductive cycles and pregnancy. Estrogen (E) act on ER⁺ cells, activate cyclin D1, triggering proliferation. However, mutations in MaSC trigger neoplastic events. In advanced BC, RANKL secreted by Tregs acts on highly RANKhigh TICs. (In red: promoters / In blue: suppressors). Modified from (Luo *et al.*, 2015).

Furthermore, SC-like features have been associated with EMT in normal and neoplastic human breast. EMT generates cells with SC-like properties (Mani *et al.*, 2008), and $CD44^{+}/CD24^{-}$ BC cells exhibit an EMT signature (Mani *et al.*, 2008). In addition, EMT has been associated with poorly differentiated genes (basal-BC) (Pece *et al.*, 2010), enhanced motility and invasion (Visvader and Lindeman, 2008), which demonstrate the importance of SC-like features in EMT driven metastasis. Recently, a population of CTCs ($CD44^{high}$, $CD47^{high}$, MET^{high}) was detected in BC patients with the ability to initiate metastasis in a xenograft assay (Baccelli *et al.*, 2013). In addition, the existence of a SC program in metastatic BC initiation was observed. The authors proposed that metastasis are initiated by SC-like that proliferate and differentiate to produce advanced metastasis (Lawson *et al.*, 2015). These low-burden metastatic cells encompass a SC, EMT, pro-survival and basal dormant associated program, while high-burden metastatic cells dissect a proliferative, differentiated and

luminal-like signature. These evidences support a hierarchical model for metastasis, in which metastasis is initiated by TICs with SC-like features that differentiate and upon a certain stimuli, expand and proliferate, triggering macrometastasis (**Figure 20**). Supporting these observations, tumor initiation features are determinant in BC dormancy/latency, by the expression of the SC-like master regulator genes *SOX2* and *SOX9* (Malladi *et al.*, 2016). These findings suggest that MICs may represent circulating metastatic SCs. Interestingly, TICs are less sensitive to cell cycle inhibition than the bulk of the tumor, in part due to either asymmetric division or slow cycling profile. As a result, upon treatment mammary tumors will apparently be eradicated but in a subset of patient's recurrence will happen years or decades post primary tumor excision, a process that is suggested to be driven, in part by the rare populations of TICs that survived. Interactions of the TICs and their microenvironment (metastasis niches) may also contribute to resistance to the treatment and disease dissemination. These clinical important matters call for methodologies to identify TICs and functional analysis to assess tumor initiating capabilities, and their role in metastasis and chemoresistance. Therefore, identifying these cells and understanding their function in the metastatic cascade will help in order to eradicate metastatic disease.

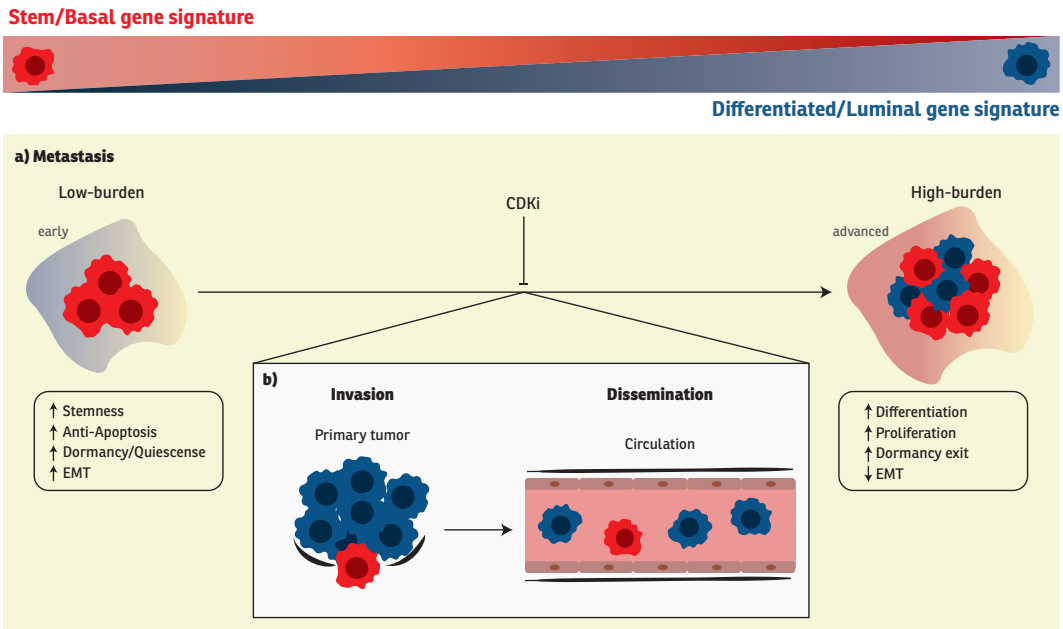


Figure 20: **Metastasis is initiated by cells with tumor initiation features. a)** Metastatic cells (red) exhibit increased SC-like features, EMT, pro-survival/anti-apoptotic programmes and quiescence/dormant associated genes. Once metastatic initiation is established, cells (blue) acquire proliferative capacity, and activate MET and differentiation programmes, leading to the exit of dormancy and metastatic advance disease. Therefore, metastasis is derive from TICs. High-burden metastasis is associated with MYC expression, and therefore may be abolish with CDK inhibitors, which induce apoptosis in MYC^{high} cancer cells (Huskey *et al.*, 2015); **b)** In the primary tumors, metastatic SC-like cells represent around 1,4% and only 16,7% of CTCs exhibit tumor initiation features. Therefore, TICs might be the origin of metastasis. Modified from (Lawson *et al.*, 2015).

1.7 Drug chemoresistance as the main cause of cancer relapse

1.7.1 Tumor initiation features and chemoresistance in BC

The identification of molecular drivers in cancer has paved the way for targeted therapy. However, incomplete responses and relapse on therapy remain the biggest problem for improving patient survival. Evidence suggests that a tumor consists of a majority of cells that are sensitive to targeted therapy while few cells that are intrinsically resistant or poised to quickly adapt to drug treatment already pre-exist within this heterogeneous tumor population. Although a multitude of resistance mechanisms have been described, it is largely unknown how resistant cells behave in a heterogeneous tumor during treatment and whether a regressing tumor microenvironment could influence disease relapse. Several drugs have been developed in order to tackle advanced BC disease, particularly to block different pathways such as RAS, MEK, AKT, PI3K, etc. However, cancer cell bypass the effect of these drugs and acquire resistance. Interestingly, TICs are less sensitive to cell cycle inhibition that the bulk of the tumor, in part due to either asymmetric division or quiescence/ slow cycling nature (Zhou *et al*., 2009). In addition, TICs exhibit other chemoresistance mechanisms such as high expression of ATP-binding cassette (ABC) drug pumps (Schatton *et al*., 2008; Yu *et al*., 2007; Zhou *et al*., 2001), pro-survival and anti-apoptotic programs (Reya *et al*., 2001), resistance to DNA damage and oxidation and heightened DNA repair efficiency (Bao *et al*., 2006; Diehn *et al*., 2009; Ito *et al*., 2004). As a result, upon treatment mammary tumors will apparently be eradicated, but in a subset of patients recurrence will happen years or decades post primary tumor excision, a process that is suggested to be driven, in part by the rare populations of TICs that survived. Interactions of the TICs and their microenvironment (metastasis niches) may also contribute to resistance to the treatment and disease dissemination. Recently another resistant mechanism based on therapy-induced secretomes, which stimulates dissemination and metastasis of drug-resistant cancer cell clones has been described (Obenauf *et al*., 2015).

Plenty of reports demonstrate the clinical implication of tumor initiation features in resistance to radiation and chemotherapy in BC (Korkaya and Wicha, 2007). For instance, by neo-adjuvant conventional cytotoxic chemorapy administration, CSCs expand (Li *et al*., 2008b). In addition, the higher number of TICs, the more aggressive and refractory the cancer is (Al-Hajj *et al*., 2004; Pece *et al*., 2010; Singh *et al*., 2004). These evidences are observed in the clinic. If the tumor is well differentiated, the cancer usually does not relapse. However, in poorly differentiated cancers with larger number of immature cells (including TICs), the cancer is likely to relapse (Clarke and Becker, 2006). In BC, $CD44^{+}$ gene expression signature correlates with SC markers, basal phenotype, EMT and decreased patient survival (Shipitsin *et al*., 2007; Xu *et al*., 2016; Zhou *et al*., 2009). In addition, the *bona fide* TIC population $CD44^{+}/CD24^{Low}$ cells are expanded after chemotherapy. Furthermore, generation of tamoxifen resistance cell lines exhibit properties of tumor initiation

with an increase of $CD44^+/CD24^-$ subpopulation (Piva *et al.*, 2014; Wang *et al.*, 2012). Therefore, $CD44^+/CD24^-$ are associated with chemoresistant properties. In fact, the more $CD44^+/CD24^-$ content, the higher aggressiveness of BC cells. These cells have higher regenerative potential, and with a small number of cells, a whole tumor can be regenerated. This implies that therapy has to efficiently target these subset of cells to avoid relapse (Huff *et al.*, 2006). Consequently, even if a cancer treatment kill 95% of cancer cells, TICs can eventually relapse. More evidences show the importance of TICs in chemoresistance. Several findings indicate that trastuzumab efficiency is due to TICs ablation (Korkaya *et al.*, 2008; Magnifico *et al.*, 2009). However, these cells are highly plastic and evolve as a function of therapeutic challenges and progression. Therefore, most of $HER2^+$ patients treated with trastuzumab, develop resistance (Huang *et al.*, 2013; Nahta *et al.*, 2006; Wilken and Maihle, 2010).

The existence of TICs offers novel therapeutic opportunities rather than anti-proliferative drugs, and will allow for the design of new strategies to counter drug resistance (**Figure 21**). This is particularly important in the metastatic setting, in which anti-neoplasic drugs are inefficient. Before, we explained the importance of self-renewal/survival signalling pathways for tumor initiation features maintenance. Targeting these pathways is one approach to block tumor initiation properties. Another approach to block these particular subset of cells is blocking their cell surface markers through antibody-based approaches (*i.e* P245, an antibody which target CD44 used in preclinical models to treat BC) (Marangoni *et al.*, 2009). Targeting the particular niche which maintain TICs identity may be also useful in order to block their expansion and induce differentiation. Particularly, induction of tumor cell differentiation has been considered useful in aggressive cancers, particularly in BC, in order to inhibit developmental pathways or epigenetic programmes. One example is ATRA, which have been used to induce BC differentiation, blocking different self-renewal pathways such as Wnt, Notch, or the EZH2 network. In this thesis, an example of how chemoresistance (in this case to mTOR inhibitors) is driven by tumor initiation features is showed. Therefore, mTOR signaling pathway and resistance associated mechanisms will be described below.

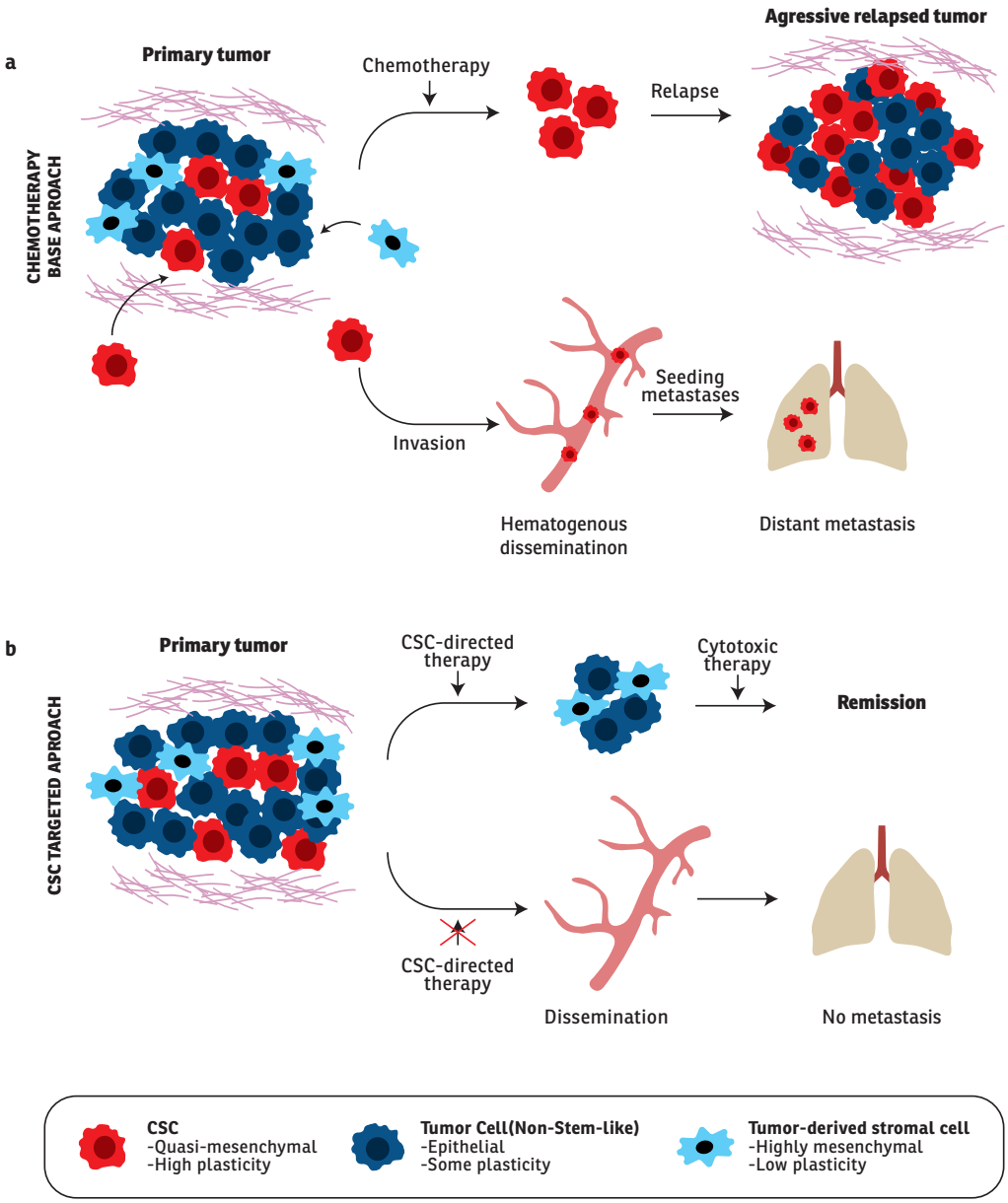


Figure 21: TICs are responsible for resistance to chemotherapy. a) These cells, with enhanced SC-like features, are less sensitive to anti-proliferative agents, and upon treatment, tumors eventually relapse, and acquire an invasive and metastatic phenotype, invading distant organs. In addition, cancer cells which acquire mesenchymal features through EMT also exhibit self-renewal properties and resistance. **b)** Therefore, if TICs are blocked, the invasive phenotype and the ability to reinitiate new lesions are impaired. Therefore, based on the role of tumor initiation in BC progression, we hypothesized that tumor initiation may contribute to BC metastasis and chemoresistance. Modified from (Pattabiraman and Weinberg, 2014).

1.7.2 The mTOR pathway & chemoresistance

Little is known about survival pathways which maintain TICs identity and functions. However, blocking these pathways may be useful to kill cancer cells. One pivotal driver in BC is the mTOR signalling pathway, which is required for survival and expansion of BCSCs (Zhou *et al.*, 2007). The mTOR signalling pathway is a master regulator of growth, glucose and lipid metabolism (*i.e.* HIFα, SREBP and PPARγ). mTOR is a serine-threonine intracellular kinase whose deregulation is implicated in those diseases where growth is deregulated such as cancer, metabolic diseases and ageing. Indeed, dysregulated mTOR signalling fuels the destructive growth of cancer (Zoncu *et al.*, 2011). mTOR belongs to the PI3K-related protein kinases (PIKK) and is composed of two complexes termed mTOR complex 1 (mTORC1) and (mTORC2). In addition, mTOR activation activates PI3K/AKT and lead to PTEN loss in a large fraction of BC patients (Campbell *et al.*, 2004; Dancey, 2010; Liedtke *et al.*, 2008; Perez-Tenorio *et al.*, 2007), and is correlated with poor prognosis (Meric-Bernstam and Esteva, 2005). In fact, mTOR hyperactivation is present in all BC subtypes (Yu *et al.*, 2001). In **Figure 23**, the mTOR signalling network with the downstream effector molecules and regulators are depicted.

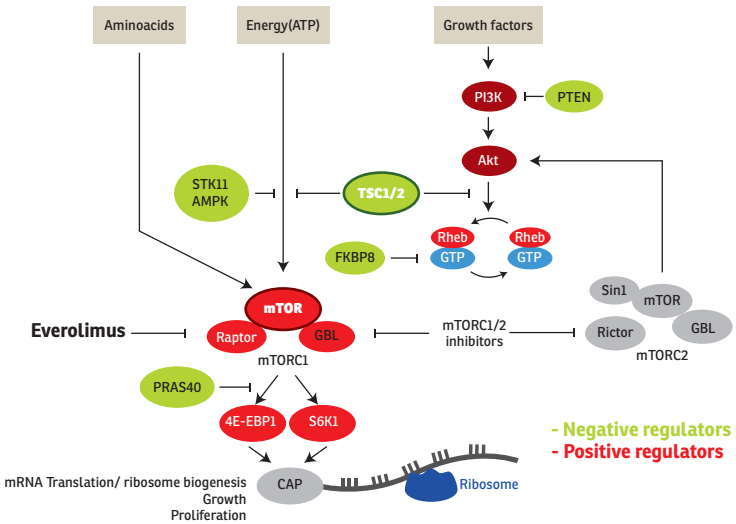


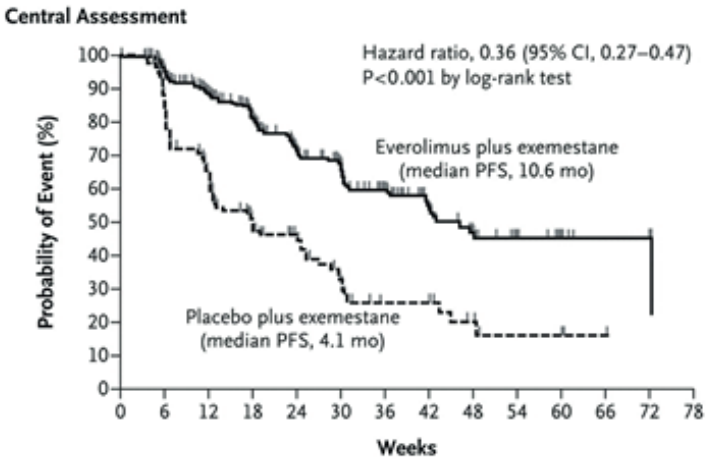
Figure 22: **The mTOR signaling pathway network**. mTOR is activated by nutrients (*i.e.* aminoacids and ATP), and activates a translational program through downstream targets ribosomal protein phosphor serine235/236 (pS6) and the Eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), promoting growth, survival and proliferation. mTOR pathway is composed of two complexes, distinguished by their adaptor proteins RAPTOR (mTORC1) and RICTOR (mTORC2). Furthermore, mTOR is positively regulated by PI3K through AKT signalling pathway, and pAKT activation triggers mTOR activation through the positive regulator RHEB. The negative regulator tuberous sclerosis protein complexes (TSC1/2) is pivotal for mTOR activity, and mutations in these complex lead to mTOR hyperactivation, which is frequent in cancer. TSC1/2 controls RHEB activity and is modulated by the energy level, stressors, growth factors and cytokines.

Because mTOR is essential for cancer growth, an extensive work has been done to develop inhibitors of this pathway. The first described mTOR inhibitor and currently under several clinical trials to treat cancer was the macrolid rapamycin (Brown *et al.*, 1994; Heitman *et al.*, 1991; Sabatini *et al.*, 1994). The mTOR inhibitors **everolimus** (RAD001) or sirolimus, which bind to the intracellular receptor immunophilin FK506 binding protein 1 A 12kDa (FKP12) (O'Donnell *et al.*, 2008), have been used to impair BC growth. Interestingly, everolimus administration is a promising therapeutic agent in the metastatic setting (*i.e.* renal carcinoma) (Bodnar *et al.*, 2015). These inhibitors impair mTORC1 kinase activity, blocking substrate recruitment (Yang *et al.*, 2013), and after long exposure, mTORC2 is inhibited (Sarbassov *et al.*, 2006). The main difference between them rely on their pharmacokinetic characteristics (Waldner *et al.*, 2016). Other inhibitors have been developed with promising results, termed as TORKinibs, which inhibit both mTOR complexes (*i.e.* AZD-2014) (Huo *et al.*, 2014).

Importantly, mTOR activation has been associated with resistance to different treatments such as chemotherapy, hormone therapy and trastuzumab (Berns *et al.*, 2007; Zhou *et al.*, 2004). For instance, a synergistic/additive effect of mTOR inhibitors and chemotherapeutic agents (*i.e.* paclitaxel, vinorelbine) was reported (Mondesire *et al.*, 2004). Besides, everolimus is currently used to treat advanced metastatic ER^+ BC (Baselga *et al.*, 2012) to overcome resistance to hormone therapy (exemestane) (**Figure 23a**). Furthermore, mTOR drives $HER2^+$ BC progression. mTOR activation is responsible of trastuzumab resistance (Margariti *et al.*, 2011), and mTOR inhibitors exhibit a synergestic effect with trastuzumab in preclinical studies (Lu *et al.*, 2007). Interestingly, in a phase III clinical trial (breast cancer trials of oral everolimus 3) (BOLERO-3), everolimus in combination with trastuzumab and vinorelbine had beneficial effects in advanced metastatic BC patients (Andre *et al.*, 2014) (**Figure 23b**).

a

BOLERO-2 TRIAL



b

BOLERO-3 TRIAL

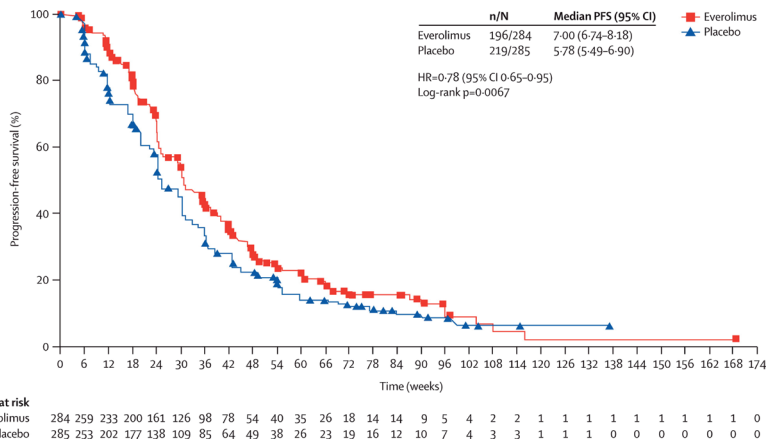


Figure 23: **a) mTOR inhibition increases progression free-survival (PFS) in advanced metastatic ER⁺ BC patients.** Resistance to endocrine therapy is associated with mTOR activation, and treatment with everolimus (RAD001) in a phase III clinical trial (**BOLERO-2)** indicated promising results (PFS from 4.1 months to 10.6 months). However, within a relatively short term, patients acquired resistance to mTOR inhibitors (Baselga *et al.*, 2012). **b)** Everolimus improves PFS in a phase III clinical trial (**BOLERO-3**). Trastuzumab-resistant HER2+ advanced BC patients who had previously received taxane therapy were selected for the study. Patients were stratified by previous lapatinib use. All patients received weekly trastuzumab and vinorelbine, and divided in placebo and everolimus groups during 3-weeks. Patients treated with everolimus had increased PFS (median from 5.78 months in placebo control to 7 months in everolimus treated group). However, patients become resistance in a short period of time, similarly what occurs in BOLERO-2 trial. This trial is registered in ClinicalTrials.gov (NCT01007942) (Andre *et al.*, 2014).

Furthermore, mTOR is pivotal in the regulation of both the innate and the adaptive immune system (Thomson *et al.*, 2009), particularly on T-cell and antigen-presenting cell (APC) differentiation and functions (Powell *et al.*, 2012). In fact, mTOR inhibitors strongly modulate the immune system. mTOR inhibitors exhibit strongly immunosuppressive effects (Powell *et al.*, 2012), and since 1977 rapamycin immunosuppressive effects are well reported (Martel *et al.*, 1977). These immunosuppressive functions are mediated by the expansion of $Foxp3^{+}$ Tregs (Battaglia *et al.*, 2005; Kang *et al.*, 2008; Kim *et al.*, 2015) and inhibition of T-cell differentiation (*i.e.* Th1, Th2 17) (Kopf *et al.*, 2007). In addition, mTOR suppress antigen presenting cells activity and maturation, and induces their apoptosis (Hackstein *et al.*, 2003; Haidinger *et al.*, 2010; Monti *et al.*, 2003); impairs NKs survival (Wai *et al.*, 2008), differentiation and cytokine production (Zhang *et al.*, 2014), and supress neutrophils (Gomez-Cambronero, 2003). Besides, mTOR activates MDSCs, which induces T-cell suppression (Nakamura *et al.*, 2015), and stimulates endothelial cells, displaying an activation of allogenic Tregs (Wang *et al.*, 2013b). mTOR inhibition also decrease antibody functions (Boor *et al.*, 2013) and alters B-cells (Benhamron and Tirosh, 2011). Therefore, mTOR inhibitors have been used as immunosuppressive agents for bone marrow and solid organ transplantation (Weir *et al.*, 2010), inducing long-term tolerance (McMahon *et al.*, 2011). Promising results regarding allogenic transplantion were obtained. mTOR inhibitors expand Tregs *ex vivo* (Badell *et al.*, 2010; Hippen *et al.*, 2011; Nanji *et al.*, 2004), and show clinical implications in Type I diabetes patients (Piemonti *et al.*, 2011; Putnam *et al.*, 2009), kidney (Fernando *et al.*, 2014; Kahan, 2000), heart and lung transplantation (Ghassemieh *et al.*, 2013). Interestingly, mTOR inhibition decrease tumor recurrence in transplanted patients with hepatocellular carcinoma (HCC) (Toso *et al.*, 2010; Zimmerman *et al.*, 2008).

Unfortunately, BC cells become resistant to these inhibitors in a short period of time (see **Figure 24**), and the link between cancer progression and resistance to allosteric inhibition of mTOR is not fully understood. Several reports indicate that this resistance is driven by a positive feedback loop through the PI3K and MEK MAPK signalling pathways (**Figure 24**). Other studies reveal a link between resistance to mTOR inhibition and cancer progression, which lead to an aggressive cancer phenotype (Chandarlapaty, 2012). Furthermore, mTOR activity is linked to BC tumor initiation features (Lawson *et al.*, 2015; Ruiz de Garibay *et al.*, 2015) and its inhibition lead to Notch activation and increase SC-like properties in TBNC cell lines (Bhola *et al.*, 2016). Interestingly, increased SC-like features and survival upon mTOR inhibition was observed in animals models in different cancers (Johnston *et al.*, 2008; Yang *et al.*, 2011), regulating differentiation (Ma *et al.*, 2010).

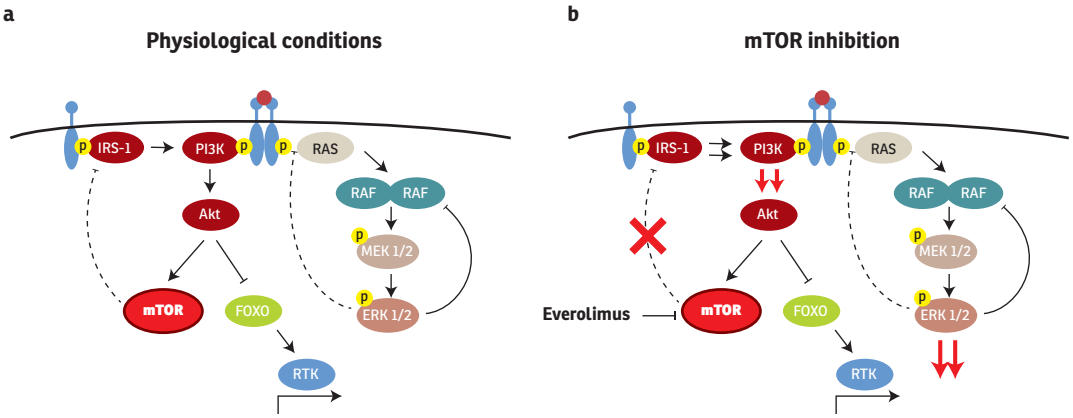


Figure 24: The mTOR network regulation and chemoresistance feedback mechanisms. a) In physiological conditions mTOR negative regulates insuling receptor substrates (IRS), and consequently PI3K and AKT activation. In this situation, RAF/MEK/ERK signaling is unalterated. **b)** By contrast, upon mTOR inhibition (i.e. Rapamycin, everolimus or sirolimus), the IRS negative feedback loop is suppressed, therefore IRS and PI3K/AKT are hyperactivated. Besides, RAF/MEK/ERK and STAT3 signalings are hyperactivated. These compensatorial mechanisms lead to resistance to mTOR inhibitors.

Following these observations, and giving the importance of tumor initiation features in chemoresistance, a key question emerges as to whether resistance to mTOR inhibitors is driven by SC-like or tumor initiation features, and eventually, metastasis. The work presented in this thesis, show how blocking tumor initiation properties prevents metastasis. This work dissected a functional interplay between SC-like master regulator genes (*EVI1* and *SOX9*) and resistance to mTOR inhibitors which lead to an aggressive cancer phenotype. This study is based on genetic, transcriptomic, molecular and therapeutic analyses in tumor xenografts, breast cancer cell lines, and/or human tumors. Collectively, the data depicts a novel mechanistic link between resistance to mTOR inhibition and cancer metastatic potential, thus enhancing the understanding of mTOR targeting.

CHAPTER 2.
AIMS AND OBJECTIVES

2. AIMS AND OBJECTIVES

Despite major advances in understanding the molecular and genetic basis of cancer, metastasis remains the cause of >90% of cancer-related mortality. Understanding metastasis initiation and progression is critical to developing new therapeutic strategies to treat and prevent metastatic disease. Prevailing theories hypothesize that metastases are seeded by rare tumor cells with unique properties, which may function like SCs in their ability to initiate and propagate metastatic tumors. However, the identity of metastasis-initiating cells in human BC remains elusive, and whether these cells are the cause of metastasis, relapse and the consequence of chemoresistance is still under investigation. Several reports show that early stage metastatic cells possess a distinct stem-like gene expression signature. These findings support a hierarchical model for metastasis, in which metastases are initiated by stem-like cells that proliferate and differentiate to produce advanced metastatic disease.

HYPOTHESIS: Based on the role of tumor initiation in BC progression we hypothesized that tumor initiation may contribute to human BC metastasis and to chemoresistance.

Objective 1: Identification of metastatic suppressor genes associated with differentiation attributes in human BC cells and in patients.

Objective 2: Functional validation of *RARRES3*, a novel human metastatic suppressor gene in experimental models.

Objective 3: To demonstrate how resistance to mTOR inhibitors is driven by tumor initiation features, which leads to an aggressive cancer phenotype.

Objective 4: To demonstrate the implication of the master gene regulators *EVI1* and *SOX9* in mTOR chemoresistance.

CHAPTER 3. RESULTS

GROUP LEADER REPORT

Chapter 3.1

Paper published in **EMBO MOLECULAR MEDICINE (2014)**

TITLE: **RARRES3 suppresses breast cancer lung metastasis by regulating adhesion and differentiation**
This paper was highlight in Nature Reviews Clinical Oncology
Reference: Errico, A. (2014). "Breast cancer: RARRES3-suppressing metastases to the lung in breast cancer." Nat Rev Clin Oncol 11(7): 378

Impact Factor: **9.547**
Enrique Javier Arenas Lahuerta: Co-authorship (together with Mònica Morales)

I state that Enrique performed experiments, analyzed data, and wrote the paper. The experiments he performed were:
Cell biology and molecular experiments (cell culture, adhesion, oncosphere formation assays, organotypic 3D formation assays, RT-qPCRs)
In vivo (lung colonization assays, mammary fat pad injections, intrapulmonary limiting dilution injections).

Chapter 3.2

Paper published in **ONCOGENE (2016)**

TITLE: **Stem cell-like transcriptional reprogramming mediates metastatic resistance to mTOR inhibition**

Impact Factor: **7.932**

Enrique Javier Arenas Lahuerta: Co-authorship (together with Francesca Mateo and Helena Aguilar)

I state that Enrique performed experiments and analyzed data. The experiments he performed were:
Cell biology and molecular experiments (cell culture, RT-qPCRs and toxicity assays)
In vivo (lung colonization assays)

Signature (Roger Gomis Cabré)
Date:

CHAPTER 3.1
RARRES suppresses breast cancer lung metastasis by regulating adhesion and differentiation

Research Article

TRANSPARENT PROCESS OPEN ACCESS

EMBO Molecular Medicine

RARRES3 suppresses breast cancer lung metastasis by regulating adhesion and differentiation

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Abstract

In estrogen receptor-negative breast cancer patients, metastatic relapse usually occurs in the lung and is responsible for the fatal outcome of the disease. Thus, a better understanding of the biology of metastasis is needed. In particular, biomarkers to identify patients that are at risk of lung metastasis could open the avenue for new therapeutic opportunities. Here we characterize the biological activity of *RARRES3*, a new metastasis suppressor gene whose reduced expression in the primary breast tumors identifies a subgroup of patients more likely to develop lung metastasis. We show that *RARRES3* downregulation engages metastasis-initiating capabilities by facilitating adhesion of the tumor cells to the lung parenchyma. In addition, impaired tumor cell differentiation due to the loss of RARRES3 phospholipase A1/A2 activity also contributes to lung metastasis. Our results establish *RARRES3* downregulation as a potential biomarker to identify patients at high risk of lung metastasis who might benefit from a differentiation treatment in the adjuvant programme.

Keywords breast cancer; lung metastasis; metastasis suppressor
Subject Categories Cancer; Stem Cells
DOI 10.15252/emmm.201303675 | Received 18 November 2013 | Revised 3 April 2014 | Accepted 23 April 2014

Introduction

Breast cancer (BC) is a highly heterogeneous disease, and there is clinical evidence of distinct patterns of disease relapse (Kennecke *et al*, 2010). In fact, the capacity of metastatic BC cells to grow in diverse environments may give rise to metastatic speciation, as indicated by the coexistence of tumor cells with distinct organ tropisms (bone, lung, liver, and brain) in patients with advanced BC (Nguyen *et al*, 2009). Analysis of gene expression profiles in experimental models of estrogen receptor-negative (ER^-) BC contributed to identifying potential genes regulating or initiating lung metastasis (Minn *et al*, 2005; Eckhardt *et al*, 2012). Among the set of genes whose expression in breast tumor is associated with lung relapse, several encoded cytokines or secreted products that supported transendothelial migration from circulation into the lung parenchyma (Gupta *et al*, 2007; Padua *et al*, 2008). Additional genes, such as the extracellular matrix protein TNC, support the critical stem and progenitor cell pathways NOTCH and WNT and the viability of metastatic cancer cells in the lungs (Oskarsson *et al*, 2011). Interestingly, gene signatures associated with poor prognosis or site-specific metastasis indicate that relevant rearrangements in aggressive tumors and metastatic cells may also involve gene silencing (van't Veer *et al*, 2002; Minn *et al*, 2005; Lo *et al*, 2010; Cancer Genome Atlas Network, 2012). The silenced genes may encode several potential metastasis suppressors, responsible for the inhibition of overt metastasis at a secondary organ without affecting tumor growth at the primary site (Horak *et al*, 2008). *RARRES3*, a member of the lung metastasis gene signature (LMS) previously described (Minn *et al*, 2005), was identified in this group of genes as a potential metastasis suppressor.

The description of metastasis as an orderly sequence of basic steps—local invasion, intravasation, survival in circulation, extravasation, matrix remodeling, reinitiation, and colonization—has helped to rationalize the complex set of biological properties that must be acquired in order for a particular malignancy to progress toward overt metastatic disease (Vanharanta & Massague, 2013). In addition to acquiring motility properties, adapting adhesion

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capacity, and remodeling the new microenvironment to enable metastasis, cancer cells also turn off differentiation programmes (Yang & Weinberg, 2008) and secure stemlike properties (Mani *et al*, 2008). In mouse models of BC and in patient samples, the loss of expression of differentiation markers correlates with tumor progression and metastasis (Kouros-Mehr *et al*, 2008a; Yu *et al*, 2012). GATA3 transcription factor determines luminal epithelial cell differentiation in the mammary gland (Kouros-Mehr *et al*, 2008b). Additionally, reduced GATA3 expression is strongly predictive of BC poor prognosis (Mehra *et al*, 2005) due to decreased cellular differentiation (Kouros-Mehr *et al*, 2008a) and increased tumor-initiating capacity (Asselin-Labat *et al*, 2011). However, it is unknown whether genes that promote the maintenance of differentiation attributes in basal-like ER^- BC tumors restrain the malignant phenotype and metastatic dissemination by limiting metastasis-initiating capacity.

RARRES3 is a small protein with phospholipase $A_{1/2}$ ($PLA_{1/2}$) activity, responsible for producing signaling lipid secondary messengers in the form of arachidonic and eicosanoid derivatives (Han *et al*, 2010). Interestingly, *RARRES3* was identified as a retinoic acid responder gene, and its expression was proposed to cause G_0 growth arrest in BC cells (DiSepio *et al*, 1998). Retinoic acid, a regulator of gene transcription and an inducer of cellular differentiation, has long been associated with differentiation patterns in both normal and cancer cells, with particular impact on certain hematopoietic malignancies (Grimwade *et al*, 2010). In this context, high expression of aldehyde dehydrogenase ALDH1A1, an enzyme that catalyzes the oxidization of retinol to retinoic acid (Marchitti *et al*, 2008), has been linked to retinoid metabolism and the attenuation of self-renewal capacity in normal hematopoietic stem cells (Chute *et al*, 2006). Similarly, it has been suggested that BC cells that retain tumor-initiating capacity select for the loss of expression of ALDH1A1 (Ginestier *et al*, 2007, 2009). Given the putative condition of *RARRES3* as a responder gene to retinoic acid and its intrinsic catalytic activity (DiSepio *et al*, 1998; Han *et al*, 2010), the association of *RARRES3* silencing in primary tumors with an increased lung metastatic activity is intriguing.

On the basis of these lines of evidence, we investigated whether cancer cells expressing *RARRES3* have a selective disadvantage for metastasis, in particular in the lung microenvironment. Using BC cells, here we show that RARRES3 protein inhibits lung metastasis at two levels. First, RARRES3 blocks adhesion to the lung parenchyma and, second, the phospholipase activity of RARRES3 stimulates differentiation attributes, thus blunting metastasis-initiating functions at the lung required for the ER^- BC cells to establish a lesion.

Results

RARRES suppression in breast tumors

RARRES3 is among the lung metastasis gene set whose mRNA expression level in breast tumors is associated with relapse to the lungs (Minn *et al*, 2005). In particular, in highly metastatic populations to the lung, *RARRES3* mRNA is downregulated (Minn *et al*, 2005), thereby suggesting a potential metastasis suppressor function. To study this relationship, we confirmed the inverse association of *RARRES3* expression with lung metastasis previously described in the MSKCC primary breast cancer set ($n = 82$) and, particularly, in those tumors defined as positive according to the lung metastasis signature (LMS) (Minn *et al*, 2005) (Fig 1A). Furthermore, our analysis was increased to cover a primary BC set including 560 patient samples with annotated clinical follow-up (MSK/EMC BC tumor dataset) (Bos *et al*, 2009) (details on the dataset in Supplementary Materials and Methods). The reduced expression of *RARRES3* in primary tumors was significantly associated with the risk of lung metastasis (Fig 1B). Since low expression of *RARRES3* strongly correlates with a higher propensity to develop lung metastasis (Fig 1B), and because *RARRES3* levels vary widely between ER^+ versus ER^- samples, we analyzed the effect of *RARRES3* separately in the two tumor sets. This was particularly relevant given that ER status is a strong determinant of lung metastasis-free survival in BC patients (Supplementary Fig S1A). On the basis of ER status, we show that the inverse association of *RARRES3* expression with high probability of lung metastatic disease is specific for the ER^- tumor set (Fig 1C). Moreover, within the ER^- subgroup, *RARRES3* expression levels were exclusively inversely associated with risk of lung metastasis, but were not associated with the risk of bone or brain colonization (Supplementary Fig S1B and C). To date, compelling evidence associates high risk of BC relapse only with loss of expression of the metastasis suppressors *PEBP1*, *NM23-H1*, and *IRF5*. *NM23-H1* has been proposed to act as a general metastasis suppressor in various tumor types (Marino *et al*, 2013), while *PEBP1* and *IRF5* have been described as *bona fide* metastasis suppressor genes in BC (McHenry *et al*, 2008; Li *et al*, 2009; Bi *et al*, 2011). Interestingly, *PEBP1* expression levels are decreased in primary tumors (MSK/EMC dataset) that relapse to brain and lungs, thereby confirming the accuracy of our analysis, while *RARRES3* levels in these clinical samples have prognostic value exclusively for the prediction of lung metastasis (Supplementary Table S1). In summary, these analyses highlighted *RARRES3* as a putative key lung metastasis suppressor whose expression is reduced in primary BC tumors.

RARRES3 prevents breast cancer lung metastasis

We studied the functional role of *RARRES3* in experimental models of BC metastasis to lung. We used the metastatic BC cell line MDA-MB-231-LM2 (LM2), which was selected *in vivo* on the basis of a high capacity to colonize the lungs in mice, and the corresponding parental cell line MDA-MB-231, namely parental cells (Minn *et al*, 2005). LM2 cells showed a fivefold lower *RARRES3* expression than their parental counterparts (Supplementary Fig S2A and B) and have been described to rapidly colonize the lungs when inoculated orthotopically in the mammary fat pad of immunodeficient mice (Padua *et al*, 2008). We examined how RARRES3 overexpression (Supplementary Fig S2A, B and C) modified the capacity of LM2 cells to colonize the lungs (Fig 2A, B and C). Of note, modulation of *RARRES3* levels did not significantly alter the expression of any other LMS gene in parental or LM2 cell derivatives (Supplementary Fig S2A and B). In detail, Mock and *RARRES3*-overexpressing LM2 cells were injected into the mammary fat pad (MFP) of BALB/c Nude mice, and tumors were allowed to grow until they reach 300 mm^3. The tumors were then surgically resected, and lung colonization was allowed to develop (Fig 2A). Seven days after

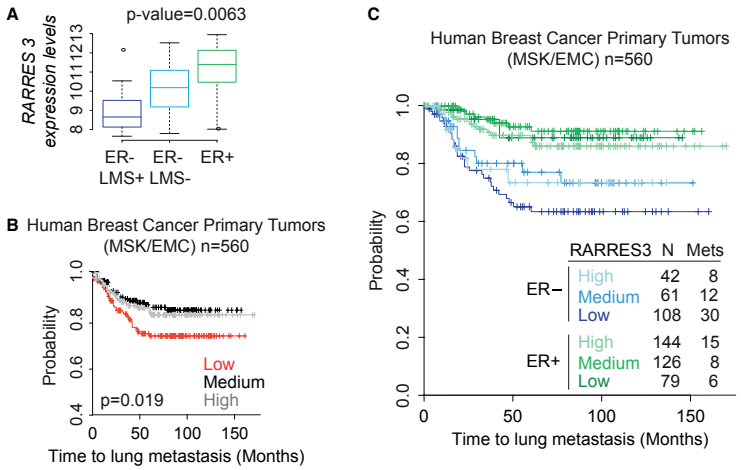

Figure 1. *RARRES3* suppression in breast tumors.

A Box plot of *RARRES3* expression levels in the MSKCC (n = 82) breast cancer tumor dataset according to ER and lung metastasis signature (LMS) status.

B Kaplan–Meier representation of the probability of lung metastasis-free survival in the MSK/EMC breast cancer tumor dataset (n = 560) according to *RARRES3* levels of expression. Low, medium, and high represent *RARRES3* expression levels in the following way: low (< mean ± SD), medium (≥ mean ± SD and ≤ mean ± SD), and high (> mean ± SD).

C Kaplan–Meier representation of the probability of lung metastasis-free survival in 560 breast cancer cases according to the ER status and *RARRES 3* expression levels according to (B).

mastectomy of the primary tumor, we assayed metastatic activity by bioluminescence imaging (BLI) of luciferase-transduced LM2 cells both in live animals and in the lungs *ex vivo* (Fig 2B). While six out of eight mice inoculated with LM2-Mock cells presented luciferase activity in the lungs, bioluminescence was detected in only two out of nine animals injected with LM2-RARRES3 cells (Fig 2B). Moreover, the amount of luciferase detected differed significantly, as shown in representative *in vivo* and *ex vivo* images of the lungs (Fig 2B). The resulting metastatic lesions showed positive staining for human Vimentin by immunohistochemistry (IHC), which specifically stains human MDA-MB-231 cells (Fig 2C). Several metastatic foci were observed throughout the lungs of mice bearing LM2-Mock tumors, while these were hardly observed in mice bearing LM2-RARRES3 tumors. Interestingly, *RARRES3* expression did not provide any growth advantage to cells when implanted at the MFP, as tested in an independent experiment (Fig 2D), or *in vitro* (Supplementary Fig S3A). RARRES3-expressing tumors did not display any change in vascular permeability, measured as effusion of intravenously injected rhodamine-conjugated dextran into the tumor or changes in VEGF expression levels (Supplementary Fig S3B and C). In addition, RARRES3 expression in primary tumors did not lead to differences in the number of circulating tumor cells, as measured by relative levels of human GAPDH to murine B2M (Supplementary Fig S3D). This observation suggests that the early steps of metastasis, including tumor vascularization and intravasation, were not under the influence of *RARRES3* expression.

Next, we focused on the late steps of metastasis with an emphasis on lung colonization. To this end, we examined the effect of RARRES3 restoration or depletion on lung metastatic colonization in LM2 or parental MDA-MB-231 cells, respectively (the latter, by means of two independent short hairpin RNAs (Supplementary Fig S2A and B). We injected 2×10^5 cells into the lateral tail vein (TV) of BALB/c Nude mice and monitored lung colonization over time. Five days after cancer cell inoculation, the lung colonization signal was reduced in cells expressing high levels of *RARRES3*, compared to their counterparts, while growth upon this point was paralleled in all groups (Fig 3A and B). The overexpression of *RARRES3* greatly reduced the photon flux in the lungs of mice injected with LM2 cells (Fig 3A); this effect correlated with decreased lung colonization, as observed in H&E sections (Fig 3A). Concurrently, RARRES3-depleted parental cells exhibited enhanced capacity to colonize the lungs (Fig 3B). No differences in proliferation were observed among different groups, as measured by Ki-67 staining (Fig 3A and B), thereby suggesting that proliferation did not account for the differences observed at the metastatic site. Similarly, although apoptosis was diminished in lung lesions arising from LM2 cells when compared to parental ones, the modulation of *RARRES3* expression levels did not affect the amount of activated Caspase-3 in lung lesions or at the primary tumor site (Supplementary Fig S4A). Next, to generalize our findings, we validated the contribution of RARRES3 to lung colonization in patient-derived CN37 cells (Gomis *et al*, 2006) and in 4T1 mouse-spontaneous ER^- metastatic BC cells (Aslakson & Miller, 1992). CN37 and parental MDA-MB-231 cells show similar *RARRES3* expression, which we effectively downregulated (Supplementary Fig S4B). CN37 cells showed a low metastatic propensity to colonize the lungs. Control mice remained free of disease for 24 weeks (Fig 3C). However, RARRES3-depleted CN37 cells were able to initiate new lesions after a long latency period, and by week 20, lung colonization was observed in half the animals injected with CN37 shRARRES3 #1 and #2 cells (Fig 3C). Lesions

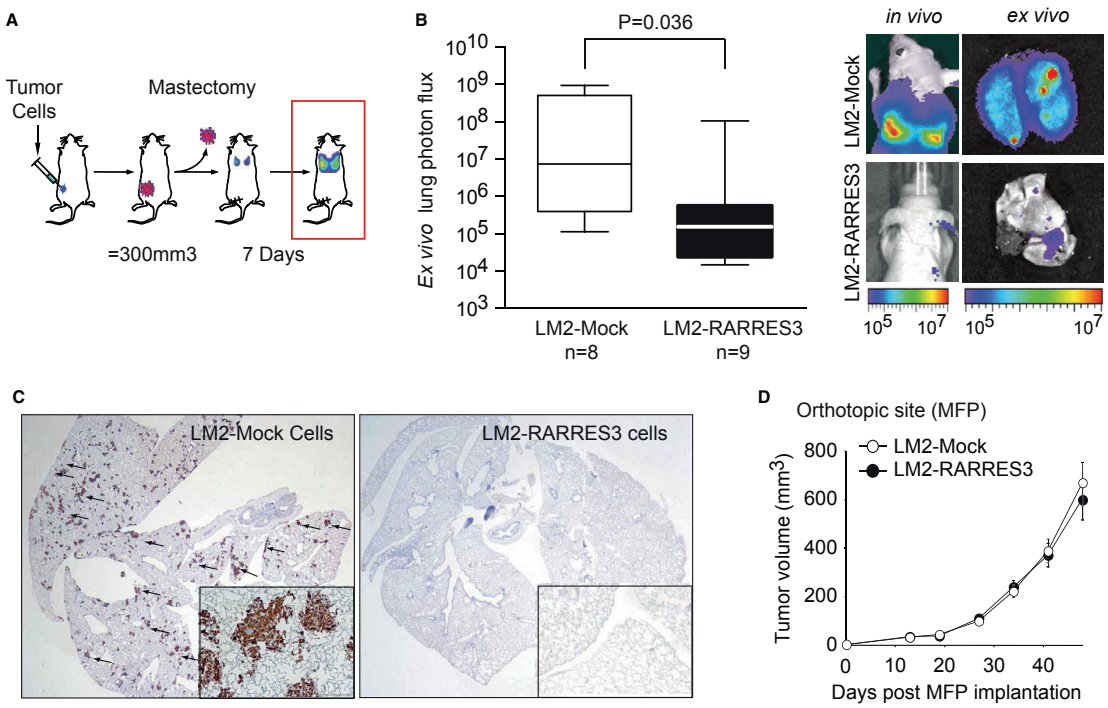

Figure 2. RARRES3 prevents breast cancer lung metastasis.

A Schematic representation of *in vivo* experimental procedure to evaluate lung metastatic potential from the orthotopic site. The indicated cell lines (5×10^5 cells) were injected contralaterally into the fourth mammary fat pad of mice. Tumors reaching 300 mm^3 were surgically removed. Seven days post-mastectomy, lung metastasis burden originated from size-matched tumors was quantified.

B (Left panel) Quantification of *ex vivo* bioluminescent signal at the lungs in each experimental group subjected to the tumor growth/resection scheme described in (A) at end point. $n = 8$ and $n = 9$ mice per group were used. Whiskers plots from min–max values were used. (Right panel) Representative bioluminescence images of *in vivo* and *ex vivo* lung colonization of the mice are shown.

C Representative human Vimentin IHC staining of whole lung sections to highlight metastatic tumor lesions from (B) are shown. Inset panels (4× magnification) reflect the size and multiple metastatic foci detected in the LM2-Mock group.

D LM2 cells (5×10^5) expressing an empty vector (LM2-Mock) or a RARRES3-expressing vector (LM2-RARRES3) were injected contralaterally into the fourth mammary fat pad of mice, and tumor growth was measured over time. $n = 20$ per group. Data are averages ± SEM.

caused by *RARRES3*-depleted CN37 cells continued to grow until week 24 (Supplementary Fig S4C). As observed in parental MDA-MB-231 cells, RARRES3 levels did not affect the capacity of CN37 cells to grow at the primary site, determined 10 weeks post-inoculation (Supplementary Fig S4D). In contrast, 4T1 cells show reduced *RARRES3* expression compared to parental MDA-MB-231 cells, which we increased by means of exogenous expression (Supplementary Fig S4E). 4T1 cells showed a high metastatic propensity to colonize the lungs in syngeneic BALB/c mice, which developed overt lung metastasis 20 days after inoculation. RARRES3-expressing 4T1 cells displayed a significant reduction in the capacity to colonize the lung 20 days post-injection (Fig 3D).

Interestingly, human Vimentin IHC revealed significant increase in lung metastatic foci when cells with low *RARRES3* expression were inoculated (LM2 cells or RARRES3-depleted parental cells) compared to populations expressing high levels of *RARRES3* (parental cells and RARRES3-expressing LM2) (Fig 3E). Although the area of the lesions, as expected, was larger for LM2 cells than parental ones, no differences were observed in LM2 or parental cells expressing different *RARRES3* levels in comparison with their respective controls (Fig 3A and B). This observation was consistent with the lack of differences in proliferation or apoptosis, as measured by Ki67 or caspase-3 activity, caused by variations in *RARRES3* expression (Fig 3A and B and Supplementary Fig S4A). The above results suggest that *RARRES3* expression prevents lung colonization initial steps.

RARRES3 suppresses metastatic cell adhesion to the lung parenchyma

Next, we addressed the mechanism by which RARRES3 may prevent lung metastasis. We initially investigated whether RARRES3 regulates apoptosis in the circulation or at the metastatic site. Under the former scenario, cell death may occur by anoikis due to the absence

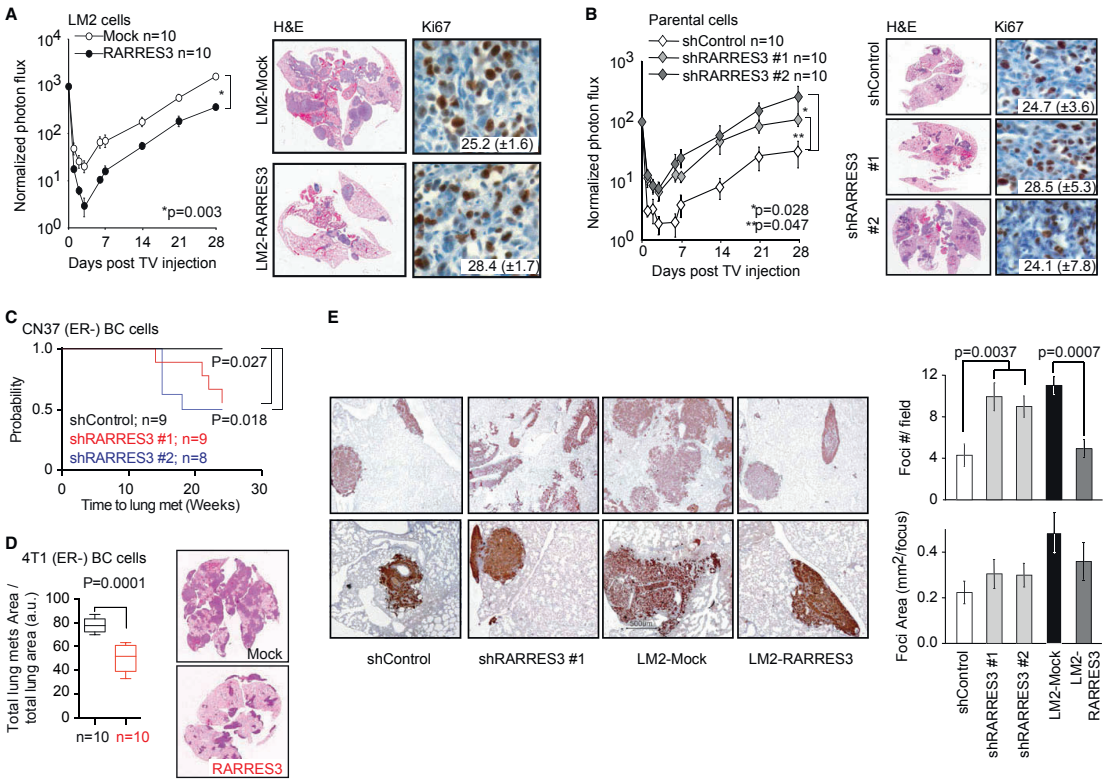

Figure 3. RARRES3 depletion facilitates lung colonization.

A LM2-Mock and LM2-RARRES3 cells (2×10^5) were injected into the tail vein of mice. Lung colonization was assayed by weekly bioluminescence imaging. Plots show normalized photon flux in the lung over time (n = 10 per group). Representative H&E and Ki-67 staining of lung sections 4 weeks after engrafting are shown. Data are averages ± SEM.

B Parental MDA-MB-231 cells (2×10^5) transduced with a control vector (shControl) or two independent *RARRES3* shRNA vectors (shRARRES3 #1 and #2) were injected into the tail vein of mice. Lung colonization was assayed by weekly bioluminescence imaging. Plots show normalized photon flux in the lung over time (n = 10 per group). Representative H&E and Ki-67 staining of lung sections 4 weeks after xenografting are shown. Data are averages ± SEM.

C CN37 patient-derived metastatic breast cancer cells (2×10^5) transduced with a control vector (shControl) or two independent *RARRES3* shRNA vectors (shRARRES3 #1 and #2) were injected into the tail vein of mice (n = 8, 9 and 9 per group, respectively). Lung colonization was assayed by weekly bioluminescence imaging. Kaplan–Meier curve of the probability of lung metastasis-free survival for CN37 shControl, shRARRES3#1, and #2 is presented. Log-rank test was used to establish statistical significance.

D 4T1 mouse-derived breast cancer cells (2×10^5) transduced with a Mock or *RARRES3* vector were injected into the tail vein of mice (n = 10 per group). Lung colonization was assayed by calculating the total area of lung metastasis lesion normalized per the total area of the lungs (H&E). Three sections were analyzed per lung. Data are averages of 10 lungs (mice) per group ± SEM. Wilcoxon test was used to establish statistical significance. Representative H&E sections are shown.

E Vimentin IHC of lung sections from animals inoculated with the indicated lines. Upper panels: 2× magnification. Lower panels: 4× magnification. Quantification of the number of foci per field and of the average area per foci is shown in the right panels. Data are averages ± SEM.

of cell attachment (Nagaprashantha *et al*, 2011). Anoikis, tested *in vitro* by culturing cells in suspension, was reduced in LM2 cells compared to the parental MDA-MB-231 line; however, RARRES3 downregulation or overexpression did not alter the fraction of cells that succumbed to the lack of cell attachment (Fig 4A). Similarly, we tested apoptosis *in vivo* 6 h post-injection, when cells were trapped at the lung vasculature but had not yet extravasated. Apoptosis was assessed by injection of a luciferase ZVAD-protected prosubstrate susceptible to be activated only upon Caspase-3/7 activation in apoptotic cells. In concordance with the results obtained *in vitro*, LM2 cells exhibited lower levels of apoptosis than parental cells, but RARRES3 did not modify the intensity of apoptosis in parental or highly metastatic populations (Fig 4A).

In the absence of a direct cellular pro-apoptotic effect, we hypothesized that RARRES3 instead controls the metastatic lung colonization steps of extravasation/adhesion and/or metastatic lesion initiation. First, we tested the contribution of RARRES3 to lung extravasation and adhesion *in vivo* using LM2 cells with and without RARRES3 overexpression. These cellular populations were injected into mice and 2 days later the number of cells extravasated

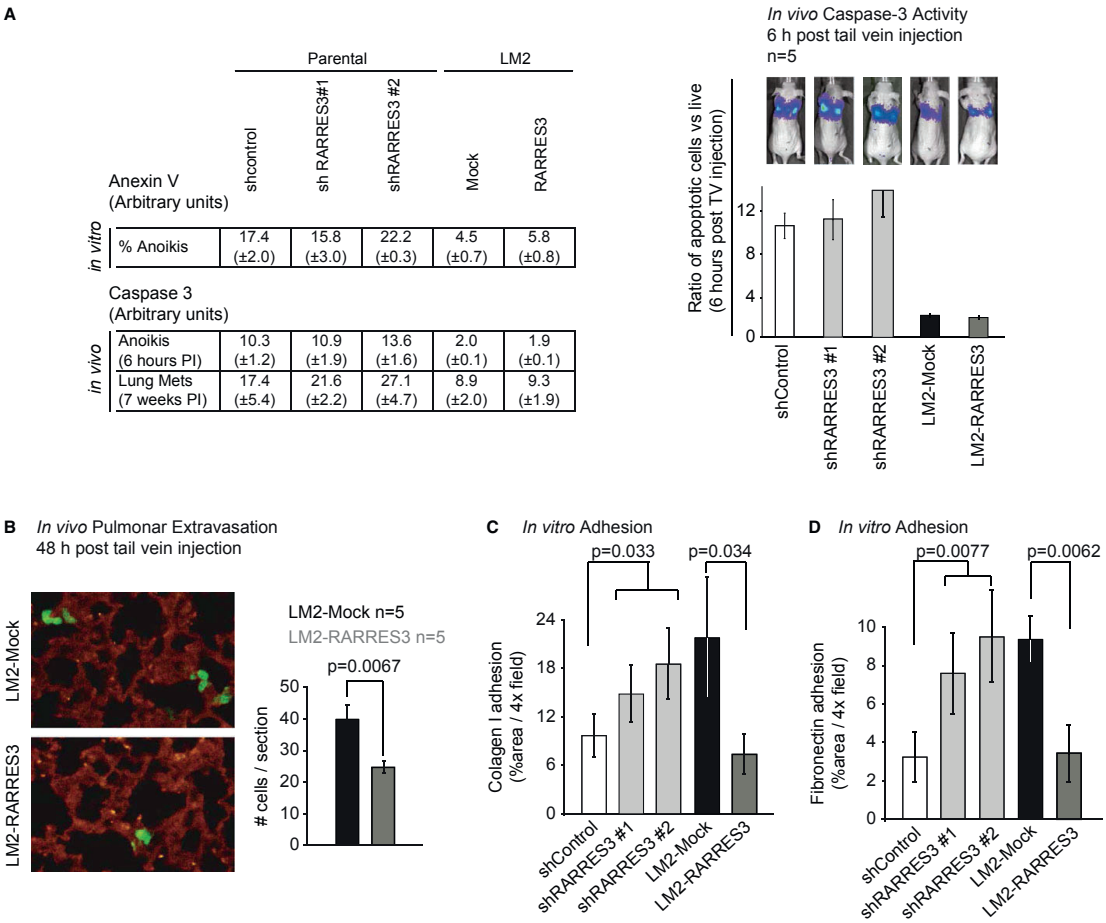

Figure 4. RARRES3 impairs metastatic cell adhesion to the lung parenchyma.

A (Left panel) Percentage of apoptotic cells under anoikis conditions, as determined by FACS measurement of Anexin V staining. Same number of cells of the indicated cell lines were plated, and measurements were taken at the indicated time point. Data are averages of three independent experiments ± SD. (Right panel) Apoptotic human luciferase activity of the indicated cell lines was measured *in vivo* using a Caspase-3 luciferin pro-substrate and normalized to total luciferase activity at the indicated time points after injection (n = 5 per group). Data are averages ± SEM. PI stands for post-injection.

B Cell tracker green-labeled LM2-mock and LM2-RARRES3 cell lines were injected via the tail vein and allowed to lodge in the lungs. Two day post-injection, mice were inoculated with rhodamine-lectin and 30 min later were perfused with 5 ml of PBS to remove cells attached to the vasculature. Lungs were extracted, flushed with PBS, and fixed-frozen in OCT, and frozen sections were obtained. Representative confocal images of extravasated cells (green) to the lung parenchyma are shown. In red, vasculature staining using rhodamine-lectin. Right panel: Quantification of extravasated cells in each condition is plotted. n = 5 mice per group. 10 sections per mouse were scored. Data are averages ± SD.

C, D Adhesion to collagen I and fibronectin, respectively. The MBA-MB-231 shControl, shRARRES3 #1 and #2, LM2-Mock and LM2-RARRES3 cells were labeled with cell tracker green and plated (5×10^4) in triplicate in 24-well inserts coated with Collagen I (C) and Fibronectin (D). One hour post-plating, inserts were washed twice with PBS to remove non-attached cells and fixed in PFA. Images were taken, and the area covered by cells was determined using Image J. The percentage of area covered by cells in Collagen I (C) or Fibronectin (D) inserts is shown. Data are averages of three independent experiments ± SD (n = 3).

into the lung parenchyma was determined. While 39.75 (± 4.5) LM2-Mock cells were observed per lung section, the overexpression of *RARRES3* significantly reduced these levels to 24.5 (± 1.9) cells (Fig 4B), suggesting that alterations in features required for extravasation, such as migration through an endothelial barrier, invasion or adhesion, could account for this observation. It has been previously reported that the capacity of LM2 cells to migrate through an endothelial cell layer is 5-fold greater than that of MDA-MB-231 parental cells (Gupta *et al*, 2007). However, RARRES3 expression levels did not affect the migratory capacity of either of these cell populations

(Supplementary Fig S5A). Similarly, invasion properties, measured as the capacity of cells to degrade and invade through a matrigel layer, were not affected by *RARRES3* expression levels, either in the presence of growth factors or in growth factor-reduced matrigel (Supplementary Fig S5B). In contrast, when we determined the affinity of cells for lung extracellular matrix proteins by measuring the adhesion to Type I collagen and fibronectin, significant differences were detected (Fig 4C and D). LM2-Mock cells in contact with a Type I collagen or fibronectin matrix exhibited enhanced adhesion compared to parental MDA-MB-231 shControl cells (Fig 4C and D). Downregulation of RARRES3 in MDA-MB-231 parental cells caused a marked increase in the capacity of cells to adhere to these two matrices (Fig 4C and D). Correspondingly, RARRES3 overexpression in highly metastatic LM2 cells reduced adhesion to Type I collagen and fibronectin to the levels shown by parental shControl cells (Fig 4C and D), confirming that RARRES3 expression attenuates cell adhesion to the lung parenchyma.

RARRES3-PLA$_1$/2 catalytic activity stimulates differentiation

The emergence of metastasis reflects the capacity of cancer cells not only to overcome the need to adhere to the vasculature and extracellular matrix but also to initiate a new lesion. Our observation that high levels of *RARRES3* expression reduced the number of metastatic foci suggested that RARRES3 inhibits metastatic colony initiation. We hypothesized that RARRES3 blocks the initiation of metastatic lesions by promoting cellular differentiation signals through its intrinsic phospholipase $A_{1/2}$ catalytic activity. $PLA_{1/2}$ activity is pivotal for the production of the arachidonic and lysophospholipid precursors that result from the hydrolysis of the acyl chain of phospholipids (Wang & Dubois, 2010). Upon downstream modifications by cyclooxygenases, these precursors are modified to active compounds called eicosanoids (prostaglandins and leukotrienes), which may signal as lipid secondary messengers and promote differentiation (Wang & Dubois, 2010). We modeled the three-dimensional structure of RARRES3 based on the structure of the HREV107 family member and identified the key residues that form the $PLA_{1/2}$ catalytic domain of the human gene (Fig 5A and B), including His23, His35, Arg18, and Cys113. These four residues, conserved across species, comprise a well-defined catalytic core of the above-described enzymatic activity (Fig 5B and Supplementary Fig S6) (Uyama *et al*, 2009). By mutating two of the catalytic core amino acids (H23P and C113S), we confirmed that RARRES3 $PLA_{1/2}$ activity and its catalytic core residues were responsible for changes in cellular arachidonic acid content (Fig 5C).

Next, we evaluated the contribution of RARRES3 $PLA_{1/2}$ catalytic activity to cell differentiation processes through lipid signaling mediators such as arachidonic derivatives. Interestingly, Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptor proteins that function as transcription factors and whose activity is dependent on arachidonic derivatives (Sertznig *et al*, 2007). PPARs play an essential role controlling cellular metabolism, development, and differentiation (Sertznig *et al*, 2007). We investigated whether PPAR function was sensitive to RARRES3 catalytic activity, by using a PPAR-specific luciferase reporter assay based on three copies of the rat acyl-CoA oxidase peroxisome proliferator response element, Aox-3x-PPRE-Luc. We found that RARRES3 expression increased the reporter transcription and a RARRES3-DEAD mutant abrogated this effect (Fig 5D). To further evaluate the clinical relevance of the catalytic activity of RARRES3 and its association with differentiation markers in BC, we initially focused on 13 well-known PPAR target genes associated with differentiation processes (Sertznig *et al*, 2007), including lipid metabolism enzymes, fatty acid transport and uptake genes, the peroxisome maintenance gene, and gene transcription. We found that the expression of these genes significantly correlated with RARRES3 expression in ER^- BC primary tumors (Fig 5E). Interestingly, the expression levels of six of these genes (PEX11A, ACOX2, ACAD8, HMGCS2, SLC27A2, and FABP5) were individually and significantly associated with risk of lung metastasis recurrence in these tumors (Fig 5E). Next, we performed a cross-validation of our group of PPAR-dependent RARRES3-correlated genes in the combined expression dataset of 211 clinically annotated human primary ER^- breast tumors (MSK/EMC dataset). The outcome of interest was time to lung recurrence (TTR) after primary tumor surgical removal. Using gene set enrichment analysis (GSEA) (Subramanian *et al*, 2005), we found a strong negative association between the PPAR-dependent RARRES3-correlated gene set and an increased risk of lung recurrence upon therapeutic treatment (normalized enrichment score of -1.88 and a false discovery rate of 0.001) (Fig 5F). On the basis of these lines of evidence, we interrogated whether RARRES3 expression in primary ER^- tumors also correlated with well-established mammary epithelial differentiation genes. With this aim and to determine the genes significantly associated with changes in *RARRES3* expression, we performed a correlation analysis between *RARRES*3 and all the other genes in the MSK/EMC primary tumor expression dataset (Affymetrix U133Aplus2). RARRES3 expression correlated positively with the differentiation genes GATA2 and GATA3, and inversely with the EZH2 polycomb protein, a pluripotency marker gene (Fig 5G). Collectively, these observations strongly support the notion that the retention of RARRES3 expression and the production of signaling mediators and precursors through its $PLA_{1/2}$ activity are associated with BC tumors preserving some of their differentiation attributes.

RARRES3 prevents metastasis colony initiation

A potential consequence of RARRES3 expression in BC cells is the retention of certain differentiation properties, which could challenge metastasis-initiating functions. To test this hypothesis, we measured the lung metastasis-initiating capacity of limiting dilutions of Mock and RARRES3-expressing LM2 cell populations upon intrapulmonary injections into BALB/c Nude mice. The cells were injected directly into the lung parenchyma (absence of extravasation/adhesion) as opposed to subcutaneously or in the MFP, since the extracellular matrix component of the lung metastatic niche has been reported to be crucial for lung metastasis-initiating capacity (Oskarsson *et al*, 2011). Tumor emergence was used as a surrogate of the metastasis-initiating capacity of cells. Mock and RARRES3-overexpressing LM2 cells inoculated in high numbers (5,000 cells) colonized the lungs with a similar frequency and latency (Supplementary Fig S7A). At lower dosages (500 or 50 cells), Mock LM2 cells retained the capacity to colonize the lungs with high efficiency, whereas RARRES3-expressing ones displayed a reduced capacity (Fig 6A). Similarly, at low dosages (500 and 50 cells), 4T1 murine metastatic cells expressing RARRES3 showed reduced metastatic initiation capacity in the lungs (Fig 6B and Supplementary Fig

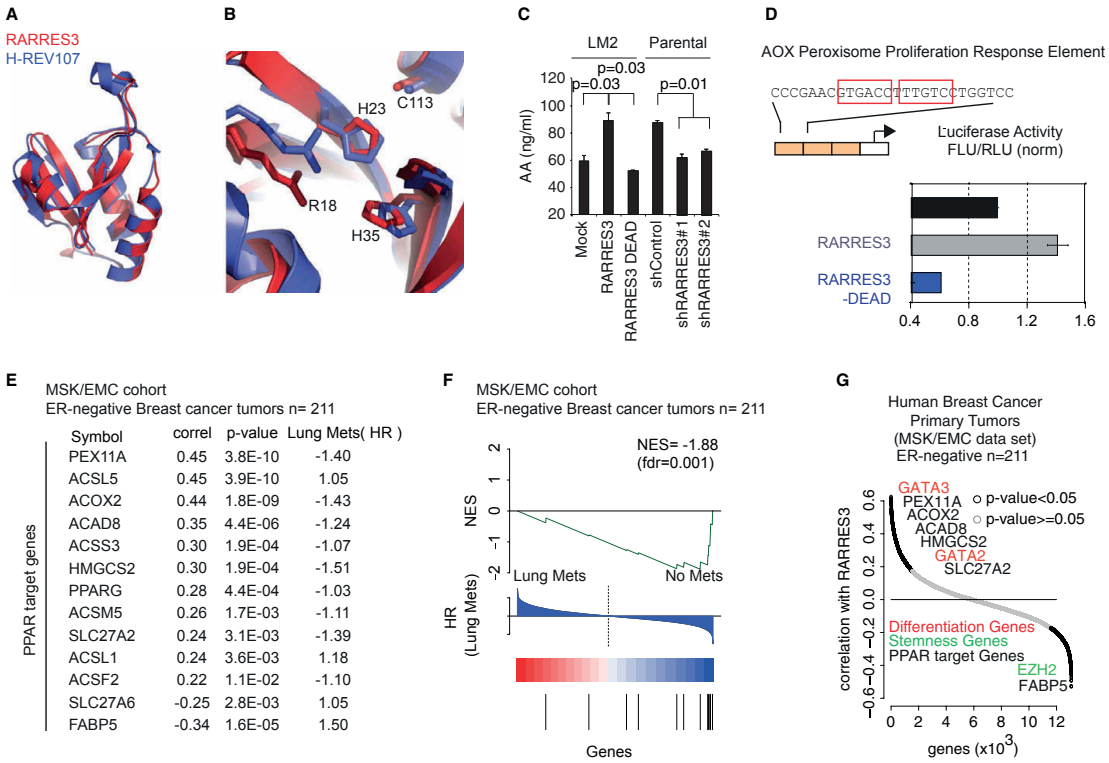

Figure 5. RARRES3 $PLA_{1/2}$ catalytic activity triggers differentiation-signaling mediators.

A Homology 3D structural model of RARRES3 (red) aligned with the structure of H-REV107 (violet) (Ren *et al*, 2010).

B The conserved catalytic residues of RARRES3 and H-REV107 showing the proposed similarity of function.

C Human Arachidonic Acid (AA) levels (ng/ml) measured in cell extracts of MDA-MB-231 Parental and LM2 cellular derivatives expressing different levels of RARRES3. Data are represented as the mean of three independent experiments ± SEM.

D Luciferase activity of 3x-AOX peroxisome proliferation response element reporter plasmid in MDA-MB-231 parental cells transiently transfected with Mock, RARRES3- and RARRES3-DEAD-expressing vectors. Activity of 3xAOX promoter was normalized to control condition and presented in arbitrary units. Data are mean of three independent experiments with ± SD.

E The correlation coefficient and significance of *RARRES3* expression levels in ER^- BC primary tumors from the MSK/EMC meta-cohort against the expression of 13 PPAR target genes represented in the U133A affymetrix array are shown, together with the p value associated with each correlation. Moreover, the risk of lung metastasis (HR) associated with the expression of each of those genes in primary tumors is also reported.

F Gene set enrichment analysis (GSEA) representing association of HR of lung metastasis with the PPAR target *RARRES3*-correlated gene set in the human breast cancer dataset (MSK/EMC expression dataset). NES-normalized enrichment score; FDR-false discovery rate; HR-hazard ratio.

G The correlation coefficient of RARRES3 expression levels in ER^- BC primary tumors from the MSK/EMC meta-cohort against the expression of all the genes represented in the U133A affymetrix array is shown. In red, differentiation GATA transcription factors. In black, some PPAR target genes described in (E). In green, stemness gene.

S7A). In contrast, increased frequency of lung colonization for RARRES3-depleted CN37 cells in low dosages (1,000 cells) was detected (Fig 6A). Therefore, the reduction in metastasis-initiating capacity paralleled the expression of RARRES3.

Next, we analyzed the contribution of *RARRES3* expression to the rate of oncosphere formation in 3D and 2D culture conditions, a readout of pluripotency (Dontu *et al*, 2003; Liao *et al*, 2007; Grimshaw *et al*, 2008) that determines the ability of a single BC cell to start a new colony. For this purpose, we grew the various RARRES3-expressing cell populations previously established (MDA-MB-231, CN37, and 4T1 cells) in matrigel. 3D culture systems recapitulate organotypic growth with respect to a polarized phenotype, specialized cell–cell contacts, and attachment to an underlying basement membrane (Schmeichel & Bissell, 2003; Debnath & Brugge, 2005). All of these features are required for the proper control of cellular proliferation, survival, and differentiation. We seeded the various RARRES3-expressing LM2, CN37, and 4T1 cells into a 3D matrix as described in the experimental section. The number of colonies formed was quantified and compared in each cellular population (Fig 6C and Supplementary Fig S7B).

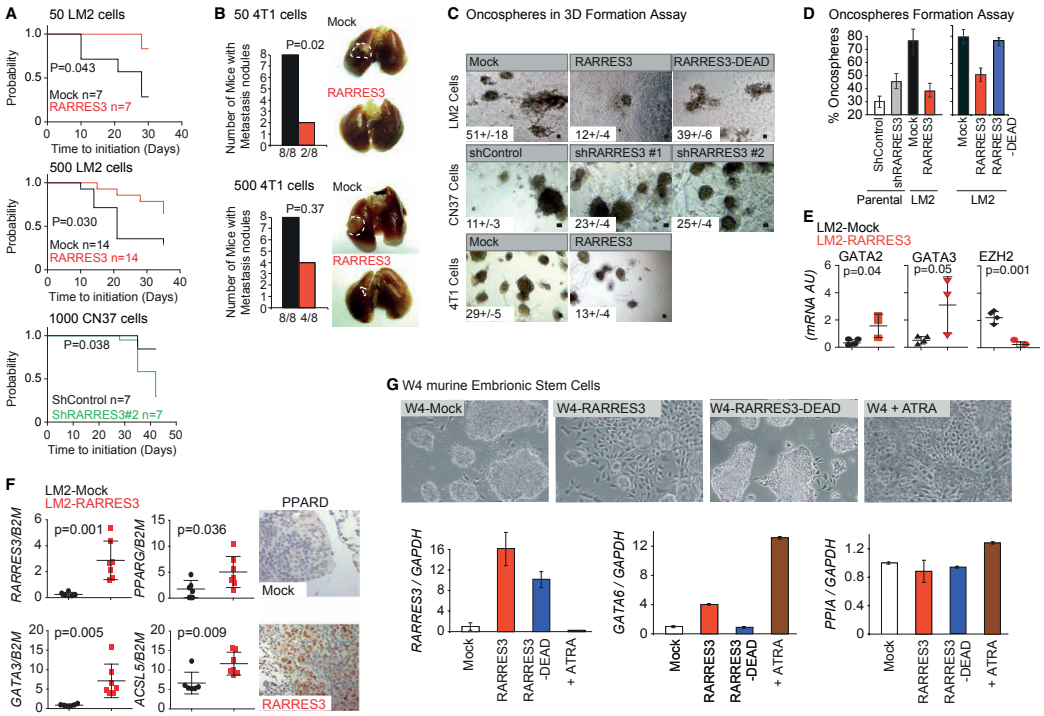

Figure 6. RARRES3 induces cellular differentiation thus impairing metastasis initiation.

A Mock- and RARRES3-expressing LM2 cells or shControl and shRARRES3 CN37 cells at the indicated dilutions were intrapulmonary injected, and grow in the lungs assessed by luciferase bioluminescence over time. Kaplan–Meier plots of the probability of lung metastasis-free survival and log-rank test were used. $n = 7$ mice per group was used unless indicated otherwise.

B Mock- and RARRES3-expressing 4T1 cells at the indicated dilutions were intrapulmonary injected, and grow in the lungs was confirmed macroscopically 20 days post-implantation. Contingency plots and Fisher exact test were used. Representative images are shown (white dashed line limits tumor area). $n = 8$ mice per group were used.

C Equal limiting dilutions of the indicated cell lines were plated in Matrigel. The number of organotypic spheres per plate was counted. Shown is the average of three independent experiments ± SD. Representative images are shown. Scale bar represents 50 μm.

D Limiting dilutions of the indicated cell lines were performed, and one cell was plated per well of 96-well plates. One plate per cell line was cultured. The percentage of wells that generated oncospheres was calculated. Shown is the average of three independent experiments ± SD.

E Human mRNA qPCR analysis of the indicated genes and cell lines in oncospheres cultures is shown. Shown is the average of at least three independent experiments ± SD.

F Human mRNA qPCR or IHC analysis of the indicated genes on lung metastasis of mice inoculated with Mock and RARRES3-expressing LM2 cells is shown ($n = 7$). Shown is the average all samples ± SD or a representative image of these tumors.

G RW.4 cells expressing a control vector (W4), a RARRES3, or a RARRES3-DEAD mutant-overexpressing vector (W4-RARRES and W4-RARRES3-DEAD) were cultured in the presence or absence of 1M all-trans retinoic acid (ATRA) for 3 days. Upper panels: representative images of the cultures are shown. Lower panels: qPCR analysis of *RARRES3*, *GATA6*, and *PPIA* (control) mRNAs of the indicated cultures was performed. Data are average of three independent experiments ± SD.

Interestingly, increased *RARRES3* expression reduced the capacity of all the cell types to undergo organotypic growth. Catalytic RARRES3-DEAD mutant overexpression in LM2 cells did not abrogate the capacity of these cells to form 3D spheroids (Fig 6C and Supplementary Fig S7A). This observation suggests that the enzymatic activity of the protein is necessary for RARRES3 to prevent the initiation of the 3D structures from a single cell. Similarly, oncosphere formation from single cells in low-attachment plates was also tested in various RARRES3-expressing MDA-MB-231 cell populations. One cell per well was seeded in a 96-well plate, and the ability to form oncospheres was determined 2 weeks later. While only 30.2% of parental shControl cells formed oncospheres, up to 76% of LM2 cells showed this potential (Fig 6D). *RARRES3* downregulation enhanced the capacity of the parental cell line to form oncospheres, while overexpression of RARRES3 dramatically abrogated this property in LM2 cells in a $PLA_{1/2}$ activity-dependent manner (Fig 6D). Moreover, the levels of *RARRES3* mRNA were decreased in oncospheres produced by MDA-MB-231 cells compared

to the respective original cells in culture (Supplementary Fig S7C). The reduction in the capacity of RARRES3-expressing LM2 cells to form spheroids was correlated with higher expression of the differentiation markers GATA3 and GATA2, associated with RARRES3 expression in primary tumors (Fig 6E). Similarly, lung metastatic tumors in mice originated from RARRES3-expressing LM2 cells also showed increased expression of some differentiation attributes, including the differentiation transcription factor GATA3, as well as some PPAR targets (PPARD, PPARG, and ACSL5) significantly correlated with RARRES3 expression in primary tumors (Fig 6F).

The maintenance of a stem phenotype is frequently associated with the expression of pluripotency genes and the absence of differentiation markers (Dontu *et al*, 2003; Sparmann & van Lohuizen, 2006; Chou *et al*, 2010). Our previous observations reinforced the notion that RARRES3 promotes differentiation. To test this hypothesis and assess the capacity of RARRES3 to engage differentiation, we used a pluripotent embryonic mouse cell line, RW-4, where differentiation can be easily monitored. Treatment of RW-4 cells with all-trans retinoic acid (ATRA) induces differentiation, a process that is controlled by GATA6, since its absence precludes differentiation (Capo-Chichi *et al*, 2005). Thus, GATA6 controls and also can be used as a marker of the differentiation status in these cells (Capo-Chichi *et al*, 2005). RW-4 cells were cultured in gelatin plates in the absence of feeders. Under these conditions, the cells grew in tight groups, and only a few isolated cells that accomplished differentiation presented a long shape and attached to the plate (Fig 6G). Overexpression of RARRES3 induced the differentiation of RW-4 cells, a phenotype that is easily detected by the presence of numerous differentiated cells attached to the plate and the size reduction of the groups of pluripotent cells (Fig 6G). This phenotype was abrogated in the absence of RARRES3 catalytic activity (Fig 6G). As a control, we treated RW-4 cells with ATRA. As expected, almost all the cells in the plate engaged in differentiation (Fig 6G). To quantify the extent of differentiation induced by RARRES3, we analyzed the mRNA levels of GATA6 by quantitative PCR. RARRES3 induced the expression of GATA6 by up to fourfold, whereas ATRA induced an increase of 13-fold (Fig 6G). These results indicate that the catalytic activity of RARRES3 induces the differentiation of pluripotent mouse embryonic cells and is associated with the retention of differentiation markers in experimental systems of BC and primary tumors. These observations support the concept that RARRES3 prevents the initiation of lung metastatic lesions by enforcing the retention of differentiation features.

Discussion

Here we provide novel evidence on the role of RARRES3 in preventing BC lung metastasis by the combined inhibition of metastatic adhesion and initiation. We have shown that RARRES3 impedes the adhesion of BC cells to the lung parenchyma while enforcing the retention of differentiation properties, thus restraining the adhesion and initiation of new lesions by the metastatic cells in the lungs (Fig 7).

RARRES3 expression did not cause any differences in primary tumor growth, angiogenesis, or proliferation. On the contrary, this metastasis suppressor modulated mainly steps required at the metastatic site, including metastatic initiation. The acquisition of low expression levels of *RARRES3* in ER$^-$ BC primary tumors that metastasize to the lung is directly associated with a reduction in *GATA* differentiation genes (Chou *et al*, 2010) and inversely correlated with expression of the *EZH2* pluripotency gene marker (Sparmann &

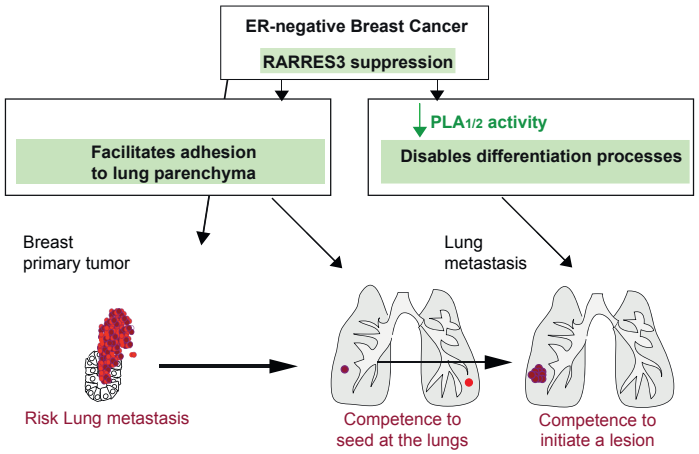

Figure 7. Schematic model.
Model showing how RARRES3 suppression contributes to ER$^-$ breast cancer primary tumors metastasis to the lung. This suppression enables adhesion to the lung parenchyma, thus facilitating seeding at the lungs. Moreover, RARRES3 suppression and loss of its $PLA_{1/2}$ catalytic activity disable differentiation signals, which, in turn, provide metastasis initiation competence to breast cancer cells to colonize the lung.

van Lohuizen, 2006). GATA3 has been shown to suppress lung metastasis from mouse and human mammary tumors by a mechanism that involves cell fate specification (Kouros-Mehr *et al*, 2008a; Dydensborg *et al*, 2009). In contrast, the maintenance of a stem phenotype, associated with self-renewal properties, is critical for cells to establish new lesions (Al-Hajj *et al*, 2003; Dontu *et al*, 2003; Li *et al*, 2007; Liao *et al*, 2007), which in ER^- BC would be favored by the loss of expression or catalytic activity of RARRES3.

Our data suggest that the $PLA_{1/2}$ catalytic activity of RARRES3 is central to its differentiation function. $PLA_{1/2}$ enzymes are catalytically responsible for the production of arachidonic acid, which is subsequently processed to produce prostanoids and leukotrienes (Wang & Dubois, 2010). Prostaglandins and leukotrienes modulate the proliferation, migration, and invasion of tumor epithelial cells through multiple signaling pathways in both an autocrine and paracrine fashion (Wang & Dubois, 2010). Moreover, these signal mediators are key molecules in the regulation of differentiation and stem cell homeostasis (Ginestier *et al*, 2009; Wang & Dubois, 2010). Here we show that the strong requirement of the $PLA_{1/2}$ catalytic activity of RARRES3 to sustain differentiation reflects, in part, an increase in PPAR activity and expression of its downstream targets. PPAR signaling provides a survival advantage to BC cells upon loss of attachment (Carracedo *et al*, 2012), and activation of PPARA with the chemical agonist compound Wy14643 reduces the development of malignant mammary tumors in a tumor-prevention setting (Pighetti *et al*, 2001). Some of the PPAR-regulated pro-differentiation activities described herein may be reduced in BC cells expressing low levels of RARRES3, thus facilitating metastatic features. This may explain why BC primary tumors that will metastasize express low levels of *RARRES3*. The induction of differentiation appears to be a common mechanism by which cells restrain their metastatic capacity in tumors of distinct origin. However, this observation does not explain why, among ER^- tumors, those expressing low levels of *RARRES3* exhibit significantly poorer lung metastasis-free survival, while metastasis to bone or brain remains largely unaffected.

In addition to supporting a reduction in metastasis initiation capacity, we show that RARRES3 modulates the ability of metastatic tumor cells to specifically attach to the lung parenchyma, which may explain the specific differences observed in lung extravasation capacity. *RARRES3* loss of expression favored the adhesion of ER^- BC cells to extracellular matrix proteins of the lung. While the lung parenchyma is composed mainly of Types I and III collagen, elastin, fibronectin, proteoglycans, and glycosaminoglycans (Suki *et al*, 2005; Pelosi & Rocco, 2008), the brain extracellular matrix has a unique composition, and matrix proteins common in other tissues are virtually absent in the brain (Dityatev *et al*, 2010). The lack of association of *RARRES3* expression levels with brain metastasis may be explained by the fact that *RARRES3* specifically modulates adhesion to the lung parenchyma and not to that of the brain. In addition to extravasation and homing through an endothelial cellular layer and to a specific matrix, the blood–brain barrier (BBB) may be a limiting step for cell colonization of the brain (Cardoso *et al*, 2010). Thus, extravasation to the brain may require the concerted acquisition and loss of expression of multiple genes, while a single gene would not have sufficient strength to drive this phenotype (Bos *et al*, 2009). On the other hand, the absence of a vasculature barrier or collagen/fibronectin-rich matrix to overcome in bone metastasis might be the molecular rationale for the lack of *RARRES3* downregulation in highly metastatic populations to the bone (Kang *et al*, 2003). In summary, the decrease in *RARRES3* expression may confer metastatic cells an advantage to adhere to the lung parenchyma, thus facilitating subsequent lung colonization over other potential metastatic tissues.

Our data indicate that *RARRES3* is a clinically relevant gene that restrains the lung metastatic capacity of BC cells and whose levels in the primary tumor may also predict risk of specific relapse. The contribution of RARRES3 to differentiation over self-renewal suggests that reduced *RARRES3* expression would also be predictive for cancer patients that exhibit therapy-resistant tumors. In fact, the characteristics that stem cells exhibit underlie their capacity to survive conventional therapies (Schott *et al*, 2013). Therefore, tumors expressing low levels of *RARRES3* may require new therapies designed to target BC-initiating cells. In addition, our results support the notion that RARRES3 activation leads to the differentiation of BC tumor cells and contributes to limiting metastasis progression. Thus, screening for compounds that activate RARRES3 may contribute to the development of new differentiation-inducing strategies to target therapy-resistant tumors. Alternatively, depending on the latter strategies effectiveness to enforce differentiation, RARRES3 activation could offer a useful pretreatment to improve the effect of conventional therapies. On the basis of the mechanistic and clinical data presented above, it is suggestive to use retinoic acid in the adjuvant setting to induce RARRES3 metastasis suppressor function, given its current use to treat certain hematological diseases. The chemopreventive use of retinoids has been described to reduce the appearance of secondary neoplasias in patients with lung, head and neck, liver, and breast cancer (Fields *et al*, 2007). Nevertheless, some contradictory results have strongly curtailed its potential in the treatment of solid tumors (Lotan *et al*, 1995) and may support the development of alternative strategies to increase *RARRES3* expression.

Materials and Methods

Cell culture

LM2 cell derivative is a lung metastatic subline derived from the MDA-MB-231 breast cancer cell line in Prof. Massagué's laboratory (Minn *et al*, 2005). CN37 is a pleural effusion patient-derived cellular population (Gomis *et al*, 2006). 4T1 cells were originated from a spontaneous BALB/c mouse breast cancer tumor (Aslakson & Miller, 1992). Stable cell lines expressing the shRNA RARRES3 or a non-silencing shRNA were generated as described (Tarragona *et al*, 2012). For RARRES3 overexpression in cells, the RARRES3 sequence was cloned into the retroviral vector pBabePuro/hygro. Stable cell lines expressing the various vectors described were generated under puromycin selection for 48 h or hygromycin selection for 14 days. All cell lines were stably transfected with TK-GFP-Luciferase construct and sorted for GFP.

Animal studies and xenografts/syngeneic models

All animal work was approved by the institutional animal care and use committee of IRB Barcelona. Female BALB/c Nude (MDA-MB-231 cells), NOD/SCID (CN37 cells), or BALB/c wild-type mice (4T1 cells) were used.

For tail vein injections, cells were resuspended in 1× PBS and injected into tail vein of mice using a 26G needle, as previously described (Tarragona *et al*, 2012). Prior to the injection of tumor cells, mice were anesthetized with ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight), and immediately after injection they were imaged for luciferase activity. Mice were monitored weekly using IVIS imaging, unless otherwise indicated. Lung tumor development was followed once a week by bioluminescence imaging, taking a photo of upper dorsal region that corresponds to lung position. Bioluminescent images were quantified with Living Image 2.60.1 software. All obtained values were normalized to those obtained at day 0. 4T1 lung colonization capacity was scored 20 days post-inoculation by H&E. Three sections per mouse lungs separated 25 μm were counted. The average of the total area of the metastasis normalized to total lung area was measured. Then, the average of all mice total lung metastasis area was plotted.

For the injection of tumor cells at the orthotopic site, mice were anesthetized as described above, and tumor cells mixed with growth factor-reduced matrigel (BD Bioscience) before inoculation (1:1). Once palpable, tumors were measured with a digital caliper, and the tumor volume was calculated. For metastatic experiments in Fig 2A, tumors were resected when reaching 300 mm^3.

For the injection of cells directly into the lungs, mice were anesthetized as described above, and the indicated number of cells was counted and then suspended in 25 μl of 1× PBS and mixed 1:1 with growth factor-reduced matrigel (BD Bioscience). To avoid injecting the heart of BALB/c Nude, NOD/SCID, or BALB/c wild-type mice, a total of 50 μl of this solution was injected directly between the 3rd and the 4th costal bone. Dilutions including 50, 500, 1,000, and 5,000 cells were used. On the day of injection (day 0), luciferase activity was assessed with IVIS. Subsequently, this activity was measured to score tumor initiation of colonization. In case of 4T1 cells, 20 days post–injection, mice were killed and lungs analyzed for macroscopic lesion detection. Lesions were confirmed by H&E staining.

For *in vivo* lung extravasation assays, CellTracker™ Green (Invitrogen)-marked cells (5×10^5) were suspended in 200 μl of cold 1× PBS and injected into the tail vein. After 48 h, we then injected 50 μg (100 μl) of rhodamine-lectin into the same vein to label the vasculature. Mice were perfused via heart with 5 ml of 1× PBS and sacrificed 30 min later. Lungs were removed, the trachea was perfused, and lungs frozen in OCT. OCT sections were then analyzed.

For *in vivo* tumor permeability assays, mice were injected intravenously with rhodamine-dextran (70 kDa, Invitrogen) at 2 mg per 20 g of body weight, and 3 h later they were perfused via heart with 5 ml of PBS and sacrificed. Tumors were extracted and fixed in formalin. Paraffin-embedded tumors were sectioned and analyzed.

Oligonucleotide array assays

RNA sample collection and generation of biotinylated complementary RNA (cRNA) probe were carried out essentially as described in the standard Affymetrix (Santa Clara, CA) GeneChip protocol. Ten micrograms of total RNA was used to prepare a cRNA probe using a Custom Superscript kit (Invitrogen). For expression profiling, 25 ng of RNA per sample was processed using isothermal amplification SPIA Biotin System (NuGEN technologies). Each sample was hybridized with an Affymetrix Human Genome U133APlus2.0 microarray at the IRB Barcelona Functional Genomics Facility. All microarray statistical analyses were performed using Bioconductor (Gentleman *et al*, 2004). Background correction, quantile normalization, and RMA summarization were performed as implemented in Bioconductor's affy package (Irizarry *et al*, 2009).

Patient gene expression datasets

The patients' information is publically available and was downloaded from GEO Barrett *et al* (2007). The following cohorts were used: (i) MSKCC set. GSE2603, including 82 breast cancer samples (Minn *et al*, 2005); (ii) MSK/EMC. Pooled GSE2603, GSE2034, GSE5327, and GSE12276. This pooled cohort has 560 patients' samples. In order to remove systematic biases, the expression measurements were converted to *z*-scores for all genes prior to merging. ER^+ patients were selected based on the bimodality of gene ESR1. More information is provided in Supportive materials and methods.

Lentiviral and retroviral production

293T cells were used for lentiviral production. Lentiviral vectors expressing shRNAs against human *RARRES3* from the Mission shRNA Library were purchased from Sigma-Aldrich. Cells were transfected with lentiviral vectors following standard procedures, and viral supernatant was used to infect MDA-MB-231 and CN37 cells. Selection was done using Puromycin (2 μg/ml) for 48 h. As a negative control in all the infections, a lentivirus with control shRNA was used. 293T cells transfection with retroviral vectors was done using standard procedures, and viral supernatant was used for infection. An empty vector was used as a Mock control.

RARRES3 short hairpins sequence:
sh#1: CCGGCCCGCTGTAAACAGGTGGAAACTCGAGTTTCCACCTGTTTACAGCGGGTTTTTG
sh#2: CCGGGCGCTTGGAATCCTGGTTGTTCTCGAGAACAACCAGGATTCCAAGCGCTTTTTG
Control short hairpin:
ShControl: CCGGCATCGACAAGACTGCTAACCACTCGAGTGGTTAGCAGTCTTGTCGATGTTTTTG

Statistical analysis

Metastasis-free survival curves of mice were plotted using Kaplan–Meier estimates and compared using the log-rank test. Categorical variables were compared with the Fisher exact test. Continuous variables were compared nonparametrically with the Wilcoxon test or with a Student *t*-test depending on normality of the distribution. Irrespectively of whether the direction of the differences was biologically expected to follow a certain direction (i.e. gene silencing), two-sided tests were used, unless indicated otherwise. We considered $P < 0.05$ to be statistically significant.

Kaplan–Meier survival and correlation analysis in patient samples: Publicly available and clinically annotated breast cancer cohorts with gene expression profiles (GSE2603, GSE2034, GSE5327, and GSE12276) were pooled as described above. Various probes of the same gene were summarized via mean. Patients were divided into groups on the basis of levels of expression using natural

divisions (i.e. tertiles, median), and the Kaplan–Meier survival function was plotted. The hazard ratio (HR) and *P* value for each gene of interest (RARRES3 or ESR1) were calculated using a Cox proportional hazards model and performing likelihood ratio tests. The HR was checked for constancy over time, fulfilling Cox model assumptions. All significance measurements were done using the parameter of interest, RARRES3 or ESR1, expression as a continuous variable.

GSEA analysis

GSEA analysis was done as implemented in the phenoTest package of Bioconductor.

RARRES3 gene expression correlation

Gene expression data of ER^- patients ($n = 211$) of GSE2603, GSE2034, GSE5327, and GSE12276 pooled breast cancer sample cohort were used. A Spearman correlation test was performed for each gene against RARRES3. We corrected for multiple testing using the Benjamini and Hochberg method.

Protein extraction and Western blot

Cells were lysed with a buffer containing 1% Triton in 50 mM Tris/HCl (pH 7.4) for protein extracts and processed as in Tarragona *et al*, 2012. The antibodies used were anti-RARRES3 (Abyntek SA) and α-Tubulin (Sigma). RARRES3 rabbit polyclonal antibody was generated using RARRES3 (23-117Aa) produced in *E. coli*.

Quantitative real-time PCR

Total RNA was isolated and processed as described (Tarragona *et al*, 2012). Human *RARRES3,* the other genes described (i.e. *PPARG, ACSL5,* and *ID1*), human *B2M* and mouse *B2M or GAPDH* as endogenous controls were amplified with commercially designed TaqMan gene expression assays (Applied Biosystems).

RNA isolation from metastasis tumors

Lungs positive for luciferase observed *ex vivo* were collected, and RNA was obtained by adding 600 μl of lysis buffer (from Ambion kit) plus 1% β-Mercaptoethanol directly to the lungs. Lung tissue was homogenized using a Pre-cellys 24 machine (20 s, two cycles) (Bertin Tech). The homogenized extract was then passed through a QIAshredder column (Qiagen, cat no. 19656), and RNA was purified using a PureLink RNA mini kit (Ambion, cat no. 12183918A), following the manufacturer's instructions.

Histopathology and immunohistochemistry

Tissues were dissected, fixed in 10% buffered formalin (Sigma), and embedded in paraffin or fixed-frozen OCT. Sections (2- to 3-μm thick) were stained with hematoxylin and eosin (H&E). For Ki67 and Vimentin IHC staining, paraffin sections were deparaffinized and rehydrated through a series of alcohols. Next, sections were treated with peroxidase-blocking solution for 15 min and washed two times with distilled water. In particular, for Ki67 IHC antigen retrieval, sections were boiled for 20 min in citrate buffer pH6. They were then washed three times with 1× PBS and blocked with 0.05% BSA in 1× PBS for 30 min at room temperature. Then, sections were incubated with primary antibody against human Ki67 (Novocastra NCL-ki67p; dilution 1:500 in 0.05% BSA, 1× PBS) for 1 h at room temperature. They were then washed three times with 1xPBS and incubated with HRP-conjugated secondary antibody raised against rabbit IgGs (BrightVision poly HRP-Anti_Rabbit IgG ready to use; ImmunoLogic) for 45 min at room temperature. Slides were washed three times with 1× PBS and incubated with DAB for 3 min. Hematoxylin counterstaining was then performed.

For Vimentin IHC antigen retrieval, sections were autoclaved for 10 min in citrate buffer (pH 6.0). Next, sections were washed three times with 1× PBS and blocked with 1× PBS for 30 min at room temperature. They were then incubated with primary antibody against human Vimentin (Novocastra NCL-L-VIM-V9; dilution 1:100 in 1× PBS) for 2 h at room temperature. Further, sections were washed three times with 1× PBS and incubated with HRP-conjugated secondary antibody raised against mouse IgGs (Bright Vision poly HRP-Anti Mouse IgG ready to use; ImmunoLogic) for 30 min at room temperature. Slides were then washed three times with 1× PBS and incubated with DAB for 3 min. Hematoxylin counterstaining was then performed.

For PPARD IHC antigen retrieval, sections were incubated in citric buffer (pH 6.0) at 95°C 30 min. Mouse monoclonal PPARD F-7 antibody (Santa Cruz Biotechnology SC-74440) was used in 1:15 dilution. IHC detection was performed with the ABC kit, from Vector Laboratories. Slides were counterstained in Harris hematoxylin, dehydrated, cleared, and cover-slipped.

For quantification of the number of foci per field, images from Vimentin-immunostained lung sections were taken at 2× magnification (three sections per lung and five animals per group). For each section, the average number of foci per field was plotted. To analyze the metastatic area, images were taken at 4× magnification, and the area of each metastatic lesion was quantified with the Image J software. Five images per section/animal were evaluated, and the average area plotted.

For quantification of Ki67, images from Ki67-immunostained lung sections were taken at 40× magnifications (five fields per section and five sections per lung lesion). Percentage of Ki67-positive cells relative to total number of cells was quantified. Total of five mice per each group were analyzed.

Reporter assays

Renilla and luciferase reporter assays were performed as previously described (Tarragona *et al*, 2012). The plasmid 3xAOX PPRE-TK-LUC containing three copies of the peroxisome proliferator-response element (PPRE) from the rat acyl-CoA oxidase was used. A Renilla plasmid (Promega) was included to control for transfection efficiency.

Migration assay

Cells were marked with 5 μM CellTracker™ Green (Invitrogen) following the manufacturer's instructions and kept overnight in medium with 0.1% FBS. Next day, 5×10^4 cells were seeded on human fibronectin-coated Biocoat Cell Culture Inserts (Becton Dickinson Labware) in medium with 0.1% FBS, while the wells were

loaded with complete medium. Eight hours after seeding, cells were washed and fixed with 4% paraformaldehyde. Cells on the apical side of each insert were scraped off, and migration to the basolateral side was visualized with Nikon Eclipse TE2000-U fluorescent microscope. Each sample was seeded in triplicate, and five fields from each well were counted.

Invasion assay

Cells were marked with 5 μM CellTracker™ Green (Invitrogen) following the manufacturer's instructions and were kept overnight in medium with 0.1% FBS. Next day, 5×10^4 cells were seeded on chambers coated with growth factor-reduced or completed matrigel (BD Bioscience) in medium with 0.1% FBS, while the wells were loaded with complete medium. Eight hours after the seeding, cells were washed and fixed with 4% paraformaldehyde. Cells on the apical side of each insert were scraped off, and the migration to the basolateral side was visualized with Nikon Eclipse TE2000-U fluorescent microscope. Each sample was seeded in triplicate, and five fields from each well were counted.

Adhesion assay

Cells were marked with 5 μM CellTracker™ Green (Invitrogen) following the manufacturer's instructions and kept overnight in medium with 0.1% FBS. Next day, 5×10^4 cells were seeded in triplicates on collagen- or fibronectin-coated 24-well inserts. One hour after the seeding, cells were washed and fixed with 4% paraformaldehyde. They were then visualized with a Nikon Eclipse TE2000-U fluorescent microscope. Each sample was seeded in triplicate, and five fields from each well were counted.

Flow cytometry analysis

Cells were stained using the Annexin V Apoptosis Detection kit (BD Pharmingen), following manufacturer's instructions. Data were obtained using a BD FACSAria cell sorter and analyzed using FlowJo software.

Oncospheres formation assay

To assess tumor initiation capacity *in vitro*, cells were counted and plated into low-attachment 96-well plates at dilution of 1 cell per well. They were then cultured in mammary epithelial basal medium (MEBM, Lonza, cat no. CC-3151), supplemented with MEGM SingleQuots (which contain Insulin, EGF, Hydrocortisone and GA-1000, LONZA cat no. 4136), 1X B27 without retinoic acid (GIBCO, cat no. 12787-010), and 20 ng/ml of recombinant fibroblast growth factor (GIBCO, cat no. PHG0026), and incubated in 5% CO2, 37°C in order to obtain a first generation of oncospheres (anoikis and pluripotency selection) after 15 days. The process was repeated to ensure second-generation oncospheres (pluripotency selection). After 2 weeks of culture, the oncospheres were counted under the microscope.

Organotypic 3D formation assay

Cells from first-generation oncospheres were spun down at 100 ×*g* for 5 min. The pellet was then disaggregated using 0.5% trypsin (Sigma, cat no. T-3924) for 5 min at 37°C. Trypsin was blocked using DMEM/F12 medium (GIBCO) supplemented with 10% FBS, and subsequently cells were spun down at 600 ×*g*. Cells were counted and then resuspended in growth-reduced factor matrigel (BD Bioscience, cat no. 354230) in order to obtain 1,000 cells per 50 μl. Each drop was placed in the center of one well of an adherent 24-well plate and incubated for 15 min. After gel solidification, each well was replenished with 400 μl of MEBM medium supplemented with the same factors described in the oncosphere formation assay. Media was replaced every 2 days, and organotypic 3D structures were grown for 15 days. Total spheroids were counted in each drop and considered positive when exceeding 50 cells and a diameter of 50 μm.

Arachidonic acid levels determination

To determine arachidonic acid in cell extracts, a total of 10 million cells were collected in 1 ml of 1× PBS and stored at −20°C. In order to break the membranes, two cycles of freeze-thawing were performed. Cells were then spun down at 2,000 ×*g* for 5 min at 4°C, and supernatant was collected. Fifty microliters of each condition was dispensed into the human arachidonic acid (AA) ELISA kit (cat. no CSB-E09040 h, CUSABIO). Each condition was assessed in triplicate, and standard curve and concentrations were assessed using the professional soft "Curve Expert 1.3" provided by CUSABIO. The data plotted are the average of three independent experiments.

Circulating tumor cells

Blood from mice was collected in tubes containing EDTA/heparin. The fluid was transferred to 2-ml plastic tubes and centrifuged for 10 min at 86 ×*g* at 4°C. The supernatant was discarded. If the pellet was bloody, 1 ml of ACK lysis buffer (Cambrex: 10-548) was added for 5 min at room temperature and after that, the collected sample was mixed with PBS to a total volume of 10 ml, centrifuged again and decanted. RNA of the remaining cells was extracted. Human B2M and mouse GAPDH taqman probes were used to assess the amount of human versus mouse RNA in mouse blood.

Supplementary information for this article is available online: http://embomolmed.embopress.org

Acknowledgements

We would like to thank the Functional Genomics, Microscopy, and Cytometry core facilities of IRB Barcelona, and the UB. We thank C. Caelles for the 3AOX-luc construct. We thank Angel Nebreda for his scientific suggestions. EJA is supported by "La Caixa" PhD fellowship programme, and JU is a Juan de la Cierva Researcher (MICINN). JM is a Howard Hughes investigator. The work of A.C. and S.F-R is supported by the Ramón y Cajal award to AC (Spanish Ministry of Education) and the ERC (336343). JM was supported by HHMI. RRG and XS are ICREA Research Professors (Institució Catalana de Recerca i Estudis Avançats). Support and structural funds were provided by the *Associación Española Contra el Cáncer* (AECC), Fundación BBVA, *Generalitat de Catalunya* (2009 SGR 1429), and Spanish *Ministerio de Ciencia e Innovación* (MICINN) (SAF2010-21171) to RRG.

Author contributions

MM and EJA designed and performed experiments, analyzed data, and wrote the paper. JU performed and analyzed experiments and wrote the paper. EF

The paper explained

Problem
Breast cancer is the most frequently diagnosed cancer in women in Europe and the United States. Despite a recent decrease in the incidence of this disease, it continues to be the second leading cause of death by cancer. Most of these deaths are caused by the metastatic spread of the tumor. The lung is a common site of metastatic relapse in ER-negative breast cancer patients, and metastasis is responsible for the fatal outcome of the disease. Thus, a better understanding of the biology of the metastatic process is needed if we are to tackle this problem.

Results
In this study, we show that *RARRES3* is a metastatic suppressor gene in breast cancer. Using the MDA-MB-231 breast cancer cell line model and derivatives, which have a strong metastatic capacity to lung, we functionally validated that RARRES3 loss of expression in ER-negative breast cancer cells confers a selective advantage for the colonization of the lung. Tumor cells sometimes cannot grow or survive in the absence of a supportive microenvironment. We show that loss of *RARRES3* expression facilitates the ability of the tumor cells to extravasate and adhere to the lung extracellular matrix and facilitates the initiation of proliferation to colonize the lung. Collectively, our results show that genes selected for metastasis contribute to the different steps of this process and represent the random accumulation of traits that provide the necessary advantage for adaptation to the microenvironment of a different organ.

Impact
This study shows that *RARRES3* restrains the lung metastatic capacity of breast cancer cells and that *RARRES3* levels in the primary tumor are clinically relevant as may predict risk of relapse. The contribution of RARRES3 to differentiation over self-renewal suggests that reduced *RARRES3* expression could be also predictive of therapy-resistant tumors, identifying patients possibly requiring new therapies designed to target breast cancer-initiating cells. Thus, screening for compounds that activate RARRES3 may contribute to the development of new differentiation-inducing strategies to target therapy-resistant breast tumors.

and MG performed experiments with cells, IHC, and all experiments with mice, together with MM. SF-R performed IHC experiments. EP performed all the bioinformatics experiments. RBF and XS generated and analyzed the 3D structural model of RARRES3. DR produced RARRES3 fragment in *E. coli*. AC analyzed data and design experiments. JM designed experiments and analyzed data. RRG supervised the project, designed experiments, analyzed data, and wrote the paper.

Conflict of interest
The authors declare that they have no conflict of interest.

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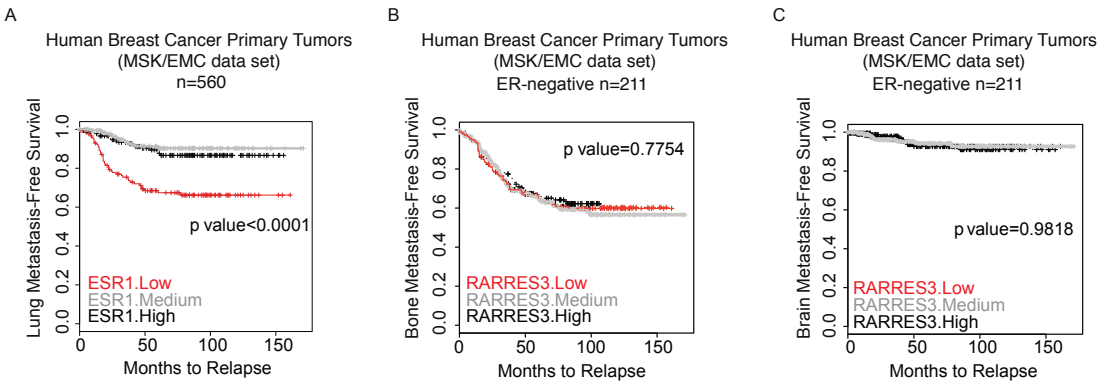


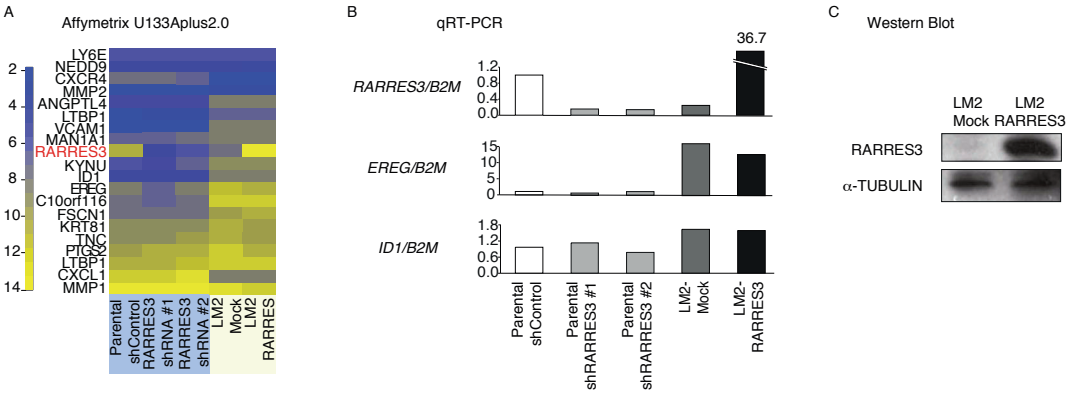
Supplementary Figure S1

Supplementary Figure S1

(A) Kaplan-Meier representation of the probability of lung metastasis-free survival in the MSK/EMC breast cancer tumor data set (n=560) according to *ESR1* levels of expression. Low, Med and High represent *ESR1* expression levels in the following way: low (<mean - SD), medium (≥ mean - SD and ≤ mean + SD) and high (> mean + SD).

(B) Kaplan-Meier representation of the probability of bone metastasis-free survival in the MSK/EMC breast cancer tumor data set (focused on ER-negative tumors n=211) according to *RARRES3* levels of expression. Low, Med and High represent *RARRES3* expression levels in the following way: low (<mean - SD), medium (≥ mean - SD and ≤ mean + SD) and high (> mean + SD).

(C) Kaplan-Meier representation of the probability of brain metastasis-free survival in the MSK/EMC breast cancer tumor data set (focused on ER-negative tumors n=211) according to *RARRES3* levels of expression. Low, Med and High represent *RARRES3* expression levels in the following way: low (<mean - SD), medium (≥ mean - SD and ≤ mean + SD) and high (> mean + SD).



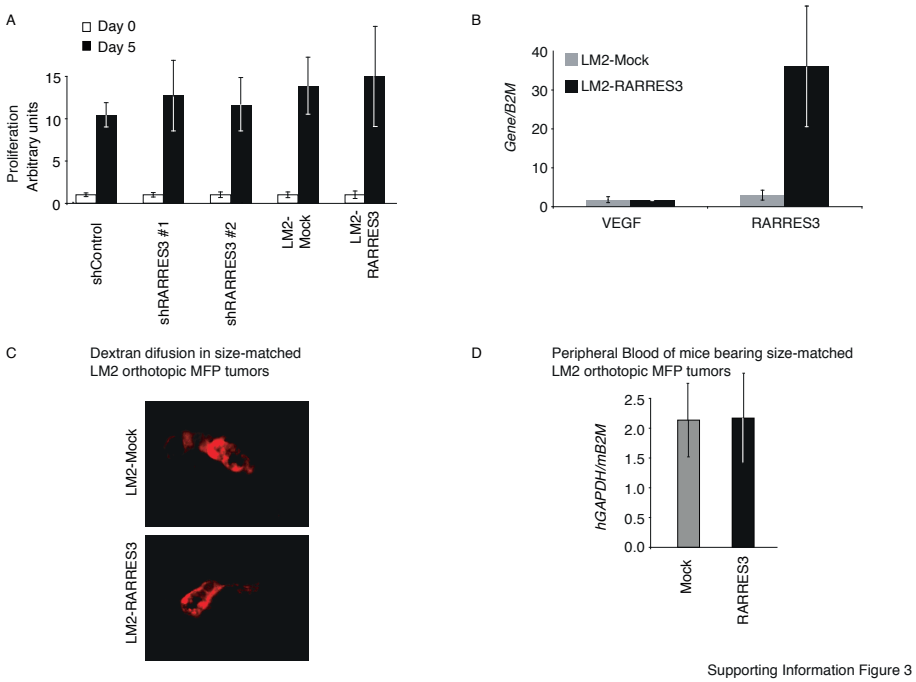
Supporting Information Figure 2

Supplementary Figure S2

(A) Knockdown of RARRES3 in Parental MDA-MB-231 cells and RARRES3 overexpression in LM2 metastatic derivatives as confirmed by gene expression profiling (Affymetrix U133APlus2.0). None of the other previously defined Lung Metastasis Signature Genes is consistently affected under the previous conditions.

(B) *RARRES3, EREG* and *ID1* mRNA expression levels measured by qRT-PCR and normalized to *B2M* levels. Data are presented as mean of three independent experiments.

(C) Western Blot analysis of RARRES3 levels in the indicated cell populations. α-TUBULIN was used as a loading control.



Supporting Information Figure 3

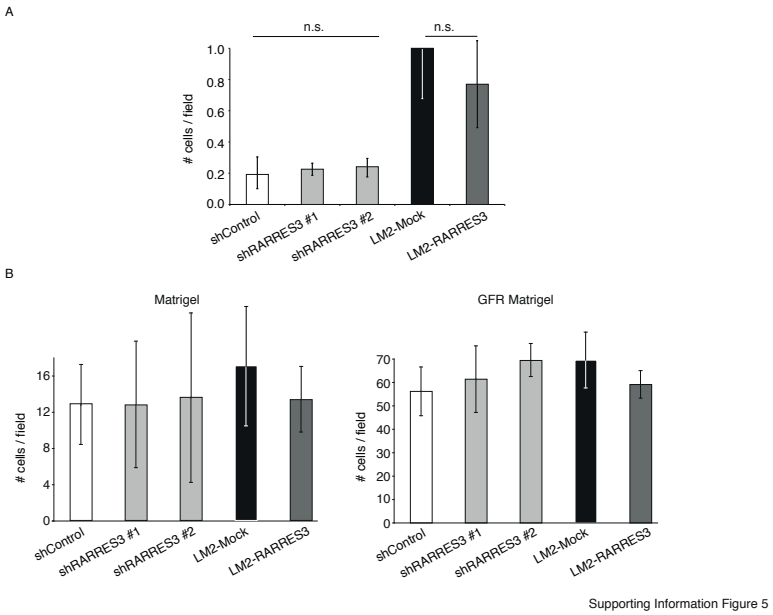
Supplementary Figure S3

(A) Proliferation assay. $5x10^4$ cells were seeded on day 0 and grown in regular media. At 5 days post-plating, cells were counted and normalized to day 0. Data are presented as mean of three independent experiments with SD.

(B) *VEGF* and *RARRES3* mRNA expression levels measured by qRT-PCR normalized to *B2M* levels. Data is presented as mean of three independent experiments with SD.

(C) Rhodamine conjugated dextran (70 KDa) was injected into mice bearing size-matched LM2 and LM2-RARRES3 mammary tumors. At 3 hours post-injection, mice were perfused to remove dextran from the vasculature, tumors were extracted, and sections were microscopically analyzed to detect dextran extravasation (five sections and n=5 per group). Representative images of LM2 and LM2-RARRES3 tumors are shown.

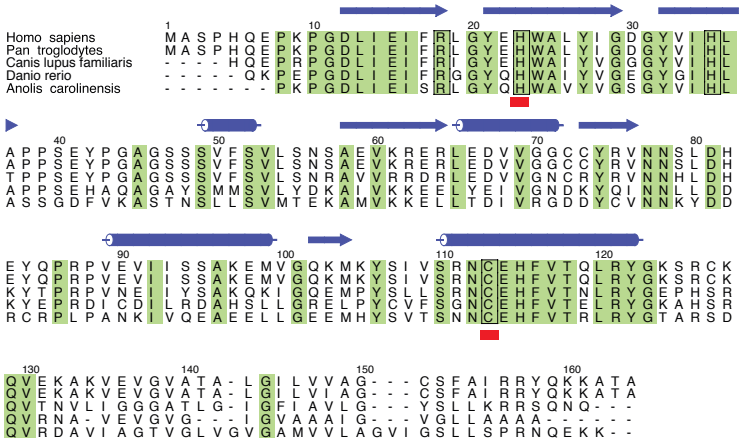
(D) Circulating human metastatic cells were measured by qPCR using a human B2M and mouse GAPDH mRNA probe in blood samples obtained from mice bearing mammary fat pad size-matched tumors of the indicated origin (n=5 per group). Data are averages ± SD.



Supplementary Figure S5

(A) Migration of parental and LM2 cells expressing different levels of RARRES3 were scored in Boyden chambers covered with fibronectin. Each cell line was seeded in triplicate and 5 fields per chamber were counted. Results represent the average of three independent experiments ± SD

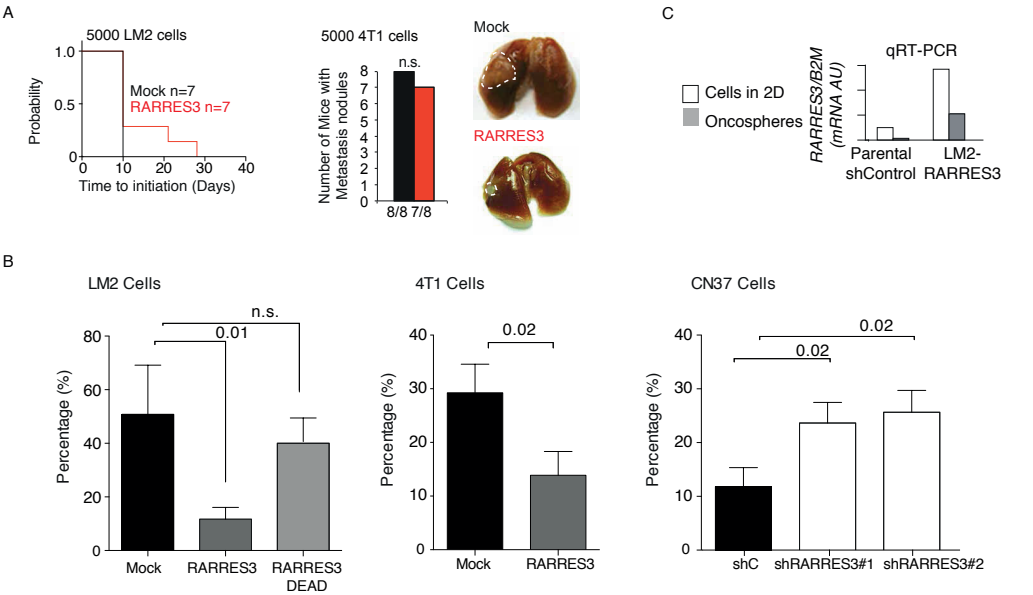
(B) Invasion of parental and LM2 cells expressing different levels of RARRES3 were scored in Boyden chambers covered with Matrigel (left panel) or growth factor-reduced Matrigel (right panel). Each cell line was seeded in triplicate, and 5 fields per chamber were counted. Results represent the average of three independent experiments ± SD



Supporting Information Figure 6

Supplementary Figure S6

Sequence alignment of various RARRES3 homologous sequences showing conserved residues (green) and proposed active site residues (black boxes). The secondary structures of H-REV107 are shown above the alignment (purple).



Supporting Information Figure 7

Supplementary Figure S7

(A) Mock and RARRES3-expressing LM2 and 4T1 cells were injected directly into mice lungs, and ability to grow in the lungs was assessed by luciferase bioluminescence over time or macroscopically 20 days post inoculation. Kaplan-Meier plot of the probability of lung metastasis-free survival was used for LM2 cells (log rank test) and a contingency plot was used for 4T1 cells (Fisher exact test) and representative images shown (white dashed line limits tumor area). n=7 mice per group was used in LM2 cells experiment. n=8 mice per group was used in 4T1 cells experiment.

(B) Equal dilutions of the indicated cell lines were performed, and cells were plated in Matrigel. The % of 3D oncospheres was calculated. The average of 3 experiments ± SD is shown.

(C) *RARRES3* mRNA qPCR analysis of the indicated cell lines either in attached culture (white bars) or in oncospheres culture (grey bars) is shown.

Supplementary Table S 1. Clinical validation of potential metastasis suppressors described in the literature (Horack et al. 2008). In bold, those whose expression in the primary tumor predicts risk of site-specific relapse.

Gene symbol	Hazard Ratio of overall Mets	Hazard Ratio of Lung Mets*	Hazard Ratio of Brain Mets*	Hazard Ratio of Bone Mets
PEBP1	-1,10	**-1,44****	**-1,43**	-1,01
RARRES3	-1,03	**-1,31**	-1,01	1,02
SMAD7	-1,08	-1,20	-1,37	-1,05
ARHGDIB	-1,02	-1,16	1,21	1,04
BRMS1	-1,06	-1,15	-1,12	-1,03
CASP8	1,02	-1,15	1,02	1,01
CDH1	-1,06	-1,12	1,07	-1,03
MAP2K7	-1,06	-1,12	-1,24	-1,04
AKAP12	1,01	-1,09	1,11	1,04
CLDN4	-1,03	-1,09	1,07	1,04
SOCS3	-1,03	-1,06	-1,16	1,05
GPR68	1,01	-1,03	-1,19	1,06
MAP2K6	1,01	-1,02	-1,21	1,04
MAP2K4	-1,03	-1,01	1,18	-1,04
MATN2	-1.01	1.01	1.29	-1.11
MED23	1.04	1.02	-1.11	1.08
GSN	-1.03	1.06	1.16	-1.00
RECK	1.05	1.07	-1.06	1.01
CTGF	1.04	1.11	1.03	1.05
CD44	1.01	1.11	1.13	-1.01
DCC	1.06	1.13	1.25	1.07
NME1	-1.00	1.16	1.24*	-1.13
KISS1	1.08	1.18	**1.67**	1.06
DLC1	1.07	1.21*	-1.04	1.11
DRG1	1.04	**1.33***	1.01	-1.10
CD82	1.04	**1.39**	1.26	-1.03

*p<0.01
**p=0.019

Supplementary Materials and Methods

Description of the MSK/EMC data set

We used the EMC-344 and MSK-82 data sets, which are based on HG-U133A and were combined, and also the EMC-189 data set, which is based on HG-U133plus2 and was processed separately (GSE2603, GSE12276, GSE5327, and GSE2034 available at the Gene Expression Omnibus (GEO) public database). In order to remove systematic biases, prior to merging the sets, the expression measurements were converted to z-scores for all genes. Patient clinical records of the 615 primary tumor samples has been extracted from the supplemental material described in Zhang, X.H., et al "Latent bone metastasis in breast cancer tied to Src-dependent survival signals" Cancer Cell. 2009, 6: 67-78. Following the indications of the Cancer Cell manuscript (Table S1, page 33 of supplemental material), we retrieved the metastasis site annotation from Table 8 of Bos, P., et al. "Genes that mediate breast cancer metastasis to the brain" Nature. 2009, 459: 1005-9. The metastasis site annotation was reported for 560 of the 615 samples. The median duration of follow-up was 7.667 years (range, 0 to 14.25) for the 268 patients without metastasis and 1.917 years (range, 0 to 9.583) for the 292 patients with metastasis. The median follow-up for all 560 patients was 4 years (range, 0 to 14.25). Those 55 patients lacking of time to metastasis annotation were not included in any ulterior time to metastasis analysis.

To examine the prognostic value of RARRES3 in different subsets of breast cancers, we divided the breast cancer samples on the basis of their ER status. For ER status, we used the intensity of ESR1 on the Affymetrix chip, as the pathological status was not available (for GSE12276). The distribution of ESR1 gene showed strong bimodality. We defined the ER+ and ER- tumors based on this bimodality. We defined the ER+ and ER−tumors based on this bimodality (ER+ BC n=349).

Missing Values Report

Five hundred and sixty patients are represented in the cohort. Of which, the variables "ER, "HER2mod", "tumor size" have 15(2%), 20 (3%), and 63 (11%) missing values, respectively.

RARRES3 three-dimensional structural analysis

The homology model of RARRES3 was constructed using the I-TASSER (Roy et al., 2010). This model was then minimized using the charm22 GBSW implicit solvent model (Chen et al., 2006) with distance restraints on the heavy side chain atoms for the catalytic residues to ensure that their orientation from the structure of H-REV107 (PDB ID 2KYT) was conserved (Ren et al, 2010). Finally, the structure was minimized without restraints using the KOBA knowledge-based potential (Chopra et al., 2010). The quality of the model was verified using MolProbity (Chen et al., 2010). Structures were visualized with Pymol, and the sequence alignment was generated with Alscript (Barton, 1993).

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CHAPTER 3.2
SC-like transcriptional reprogramming mediates metastatic resistance to mTOR inhibition BC

OPEN

Oncogene (2016), 1–13

www.nature.com/onc

ORIGINAL ARTICLE

Stem cell-like transcriptional reprogramming mediates metastatic resistance to mTOR inhibition

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Inhibitors of the mechanistic target of rapamycin (mTOR) are currently used to treat advanced metastatic breast cancer. However, whether an aggressive phenotype is sustained through adaptation or resistance to mTOR inhibition remains unknown. Here, complementary studies in human tumors, cancer models and cell lines reveal transcriptional reprogramming that supports metastasis in response to mTOR inhibition. This cancer feature is driven by EVI1 and SOX9. EVI1 functionally cooperates with and positively regulates SOX9, and promotes the transcriptional upregulation of key mTOR pathway components (REHB and RAPTOR) and of lung metastasis mediators (FSCN1 and SPARC). The expression of *EVI1* and *SOX9* is associated with stem cell-like and metastasis signatures, and their depletion impairs the metastatic potential of breast cancer cells. These results establish the mechanistic link between resistance to mTOR inhibition and cancer metastatic potential, thus enhancing our understanding of mTOR targeting failure.

Oncogene advance online publication, 19 December 2016; doi:10.1038/onc.2016.427

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Received 10 March 2016; revised 31 August 2016; accepted 10 October 2016

INTRODUCTION

The mechanistic target of rapamycin (mTOR) kinase integrates cues from nutrients and growth factors and is thus a master regulator of cell growth and metabolism.[1] As such, mTOR is activated in most cancer types and is frequently associated with poor prognosis.[2] Moreover, oncogenic mTOR signaling has a direct role in promoting cancer progression by inducing a pro-invasion translational program.[3] This program includes the downregulation of the tuberous sclerosis complex 2 (*TSC2*) gene, whose product, in a heterodimer with the *TSC1* product, serves as a negative regulator of mTOR complex 1 (mTORC1).[4] Consequently, loss of *Tsc2* in mice promotes breast cancer progression and metastasis.[5] Collectively, current knowledge supports the notion that mTOR signaling has a key role in cancer initiation, progression and metastasis.

As mTOR is a key factor in cancer biology, therapies based on its inhibition have been widely studied[6] and are central to the treatment of advanced metastatic breast cancer.[7] However, the success of monotherapy assays has been limited. Critically, within a relatively short term, allosteric mTOR inhibition concomitantly induces upstream receptor kinase signaling, which mediates therapeutic resistance.[8] Thus, therapies that combine allosteric inhibitors (rapamycin (sirolimus) and rapalogs) with inhibitors of growth factor signaling have been extensively evaluated.[9] Intriguingly, recent studies have further linked mTOR activity to a stem cell-like cancer phenotype that mediates breast cancer metastasis[10,11] and, using triple-negative (TN) breast cancer cell lines, have described that mTORC1/2 inhibition spares a cell population with stem cell-like properties and enhanced NOTCH activity.[12] These results are consistent with previous observations concerning the required activation of mTOR signaling in breast cancer stem-like viability and maintenance,[13] the enhancement of NOTCH signaling in poorly differentiated breast tumors[14] and the increase of tumor-initiating capacities with mTOR inhibition in liver cancer.[15] In this scenario, a fundamental question emerges as to whether relative long-term adaptation or resistance to mTOR inhibition is functionally linked to tumor-initiating properties and, eventually, metastasis.

Here, we explored the hypothesis that mTOR signaling supports metastasis and remains active in therapeutic resistance in metastatic breast cancer. We found that abnormal mTOR signaling enhances tumor-initiating properties and metastatic potential. This activity is dependent on EVI1, which in cooperation with SOX9 sustains a transcriptional reprogramming response.

RESULTS

Active mTORC1 signaling associates with distant metastasis

mTORC1 is the target of one of the latest drugs approved for the treatment of breast cancer in the advanced metastatic setting,[7] which suggests that this protein complex has a potential role in supporting metastasis and aggressive features. To study this relationship, a tissue microarray of primary breast tumors was assessed for mTORC1 activity by means of immunohistochemical determination of phospho-Ser235/236-ribosomal protein S6 (pS6), a well-established downstream target of mTORC1.[1] An association between pS6 positivity and the basal-like tumor phenotype or CK5 positivity was observed (Figure 1a; Mann–Whitney test $P < 0.01$). Most importantly, an association was also detected between medium-high pS6 positivity and the development of distant metastases (Fisher's exact test $P = 0.02$; odds ratio (OR) = 2.64, 95% confidence interval (CI) 0.95–7.35). Intriguingly, whereas the analyses by tumor subtypes were underpowered, both estrogen receptor (ER)-positive and ER-negative cases suggested a trend toward increased metastatic risk (ORs = 4.44 and 1.96, respectively). Thus, enhanced mTOR activity and breast cancer metastatic potential appear to be linked.

Metastasis dependence on mTORC1 signaling

To test the contribution of mTOR signaling to metastasis, we used the well-defined MDA-MB-231 breast cancer cell line, including its parental poorly metastatic population and the lung metastatic derivatives LM1 and LM2.[16] Western blot analyses showed increased levels in LM2 cells of several components of the mTORC1 signaling pathway, and particularly of RAPTOR and RHEB across the sub-populations (Figure 1b). The enhanced signaling in LM2 cells compared with the poorly metastatic parental population was confirmed by quantification of immunohistochemical staining of pS6 in the lung metastases that developed the cells upon tail vein injection (Figure 1c). Expanding on these observations, analysis of TCGA data showed negative correlations between *TSC1/2* and an upregulated gene set whose expression was clinically and experimentally associated with breast cancer metastasis to lung (lung metastasis signature (LMS)-up; Pearson's correlation coefficients (PCCs) < -0.25; *P*-values $< 10^{-8}$). Notably, this set was derived from the study of LM2 cells.[16]

Next, we tested the causal role of mTOR activity in the experimental model of lung metastasis. The capacity of LM2 cells to colonize the lung was assessed in the presence or absence of an allosteric mTOR inhibitor. LM2 cells stably expressing green fluorescent protein (GFP) and luciferase were injected into the lateral tail vein of immunocompromised mice, which were then randomly allocated to a group treated with dimethyl sulfoxide (DMSO) or a group treated with everolimus, both for 38 days. A significant reduction of lung colonization (and, as expected, of pS6 intensity) was observed in the latter group, both by measurements of *in vivo* photon flux and the relative lung metastasis area *ex vivo* by histology (Figure 1d). Collectively, these data suggest that mTORC1 signaling is associated with breast cancer metastatic potential and that inhibition of mTOR prevents lung metastasis. However, it is unclear whether this association persists in settings of resistance to mTOR inhibitors.

Metastatic resistance to mTOR inhibition

To evaluate the mechanisms responsible for resistance to mTOR inhibitors, we used two independent metastatic tumor models, namely a human TN *BRCA1*-mutated breast tumor orthotopically engrafted in nude mice (hereafter ortho-xenograft; Supplementary Figure 1) and the TN 4T1 murine breast carcinoma cell line engrafted in syngeneic background mice. Cells from both tumor models showed substantial mTORC1 signaling activity, particularly at the tumor invasive front (Supplementary Figure 1).[17] Unexpectedly, although systemic treatment with sirolimus or everolimus blunted primary tumor growth in each model, it did not reduce the number or size of lung metastases (Figure 2a). In addition, and contrary to expectations, the intensity of pS6 staining at the invasive tumor fronts of the primary lesion and in the lung metastases of the sirolimus-treated ortho-xenografts was significantly higher than in the control animals (Figure 2b). Similarly, a key factor in cancer metastasis initially identified in LM2 cells and human data analyses (thus included in LMS-up), FSCN1,[16,18] was found to be significantly overexpressed in both experimental models exposed to mTOR inhibitors (Figure 2c). Subsequent gene expression analysis of the treated tumors revealed coordinated changes concurrent with mTOR inhibition that were associated with LMS activation (Supplementary Figure 2). These changes included overexpression of LMS-up in the sirolimus-treated ortho-xenografts and underexpression of LMS-down in the everolimus-treated 4T1 tumors (as measured by the gene set expression analysis (GSEA), *P*-values < 0.05; Supplementary Figure 2).

To further study resistance to allosteric mTOR inhibition, we subjected MCF7 ER-positive and HCC1937 TN cells to long-term exposure to 50 and 150 nM of everolimus, respectively. After a period of sensitivity defined by undetectable or very low levels of

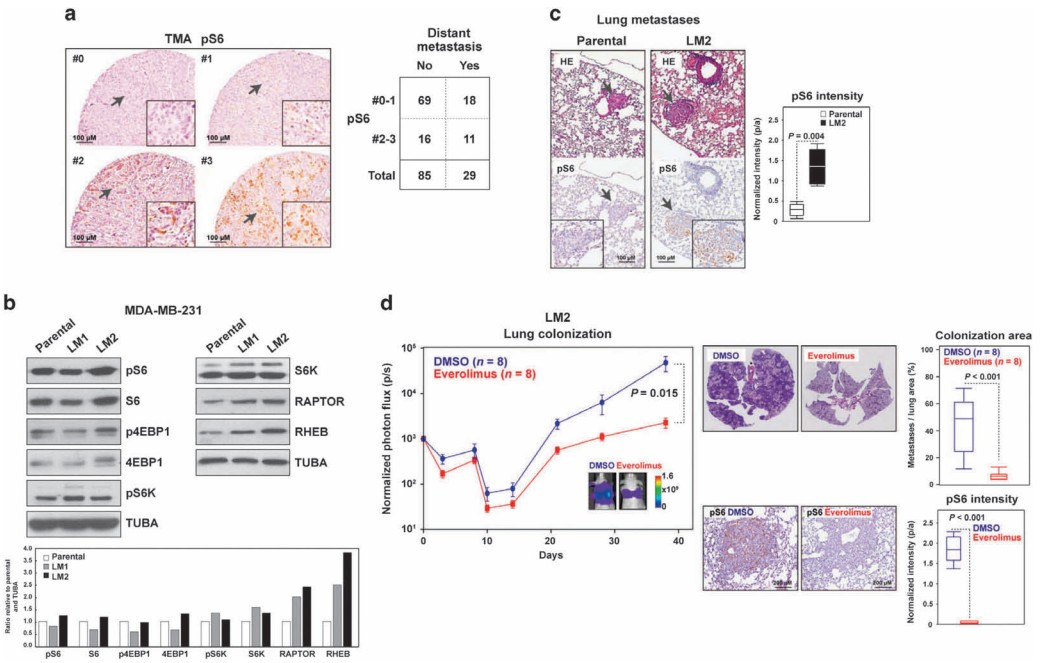

Figure 1. mTORC1 activity concurrent with enhanced metastatic potential. (**a**) Left panels, representative immunohistochemical scores (0, negative, to 3, highest expression) of pS6 staining in the tissue microarray (TMA) of primary breast tumors. Right panel, results for the association between pS6 staining and distant metastasis. (**b**) Increased expression of mTORC1 pathway components with enhanced metastatic potential of MDA-MB-231 cells. The loading control (α-tubulin, TUBA) is shown. Bottom panel, graph showing quantifications of protein levels relative to parental and TUBA (per sample). (**c**) Increased pS6 expression in lung metastases developed by LM2 cells. The arrows mark magnified fields. Right panel, box-and-whisker plots for the quantification (pixels/area, p/a) of pS6 intensity; three mice and three similar lung metastases were analyzed in each setting. The *P*-value of the two-tailed Mann–Whitney test is shown. (**d**) Left panel, graph showing the *in vivo* photon flux quantification in mice injected with LM2 and treated with DMSO or everolimus. Representative images from bioluminescence in lungs from DMSO- or everolimus-treated mice are shown. The scale bar depicts the range of photon flux values as a pseudo-color display, with red and blue representing high and low values, respectively. Right top panels, quantification of lung colonization (total metastasis area normalized per total lung area, based on HE). Right bottom panels, representative immunohistochemical results for pS6 and quantification of normalized intensities.

pS6, both cell lines recovered canonical mTORC1 signaling in 90–120 days (Figure 2d, top panels). Similarly to the *in vivo* observations, FSCN1 increased concurrently with adaptation to everolimus in both cell settings (Figure 2d, bottom panels). Subsequently, transcriptome analyses showed a significant change of the LMS in both cell lines, and particularly of the LMS-up in HCC1937 cells (Supplementary Figure 3).

Interestingly, both everolimus-adapted cell models showed significantly higher colony-forming capacity, with the higher relative difference found in HCC1937 cells (Figure 2e). Accordingly, fluorescence-activated cell sorting revealed an increase of CD49f+ and of CD44+/CD24 − cells in everolimus-adapted MCF7 and HCC1937 cultures, respectively (Figure 2f). Although MCF7 did not show an increase in CD44+/CD24 − , CD49f positivity has been linked to cancer stem cell-like properties.[19] In addition, quantitative gene expression analysis revealed a significant increase of *SOX2* in everolimus-adapted MCF7 cells and, in turn, an increase of *NANOG* and *OCT4* (but not *SOX2*) in everolimus-adapted HCC1937 cells (Supplementary Figure 4). Notably, an increase in *SOX2*, but not the two additional stem cell-like markers, has also been described in MCF7 cells resistant to tamoxifen.[20] Therefore, by combining *in vivo* and *in vitro* models of breast cancer, we reveal that exposure to allosteric mTOR inhibitors consistently promotes metastatic and tumor initiation properties. However, the precise regulators of this aggressive reprogramming remain to be determined.

TSC1/2 expression correlates negatively with tumor-initiating features

Given that differences in colony formation assays and tumor initiation properties were observed in mTOR inhibitor-resistant cell populations, we then explored the association between mTOR signaling and cancer cell initiation features in gene expression profiles from patient samples. To this end, we computed the expression correlations between *TSC1* or *TSC2* (*TSC1/2*) and 20 previously defined gene expression signatures using breast cancer data from The Cancer Genome Atlas (TCGA).[21] The signatures (full annotation is provided in Supplementary Table 1) include a consensus set derived from the study of embryonic stem cell-like cells (sESCs),[22] a consensus set of correlated master regulators of breast cancer stemness-like functions (hereafter sMRS),[23] and a MYC-centered regulatory network (sMYC);[24] importantly, these signatures were originally associated with poor prognosis and/or metastatic potential of ER-negative breast cancer and other types of cancer.[22–24] The expression profiles of

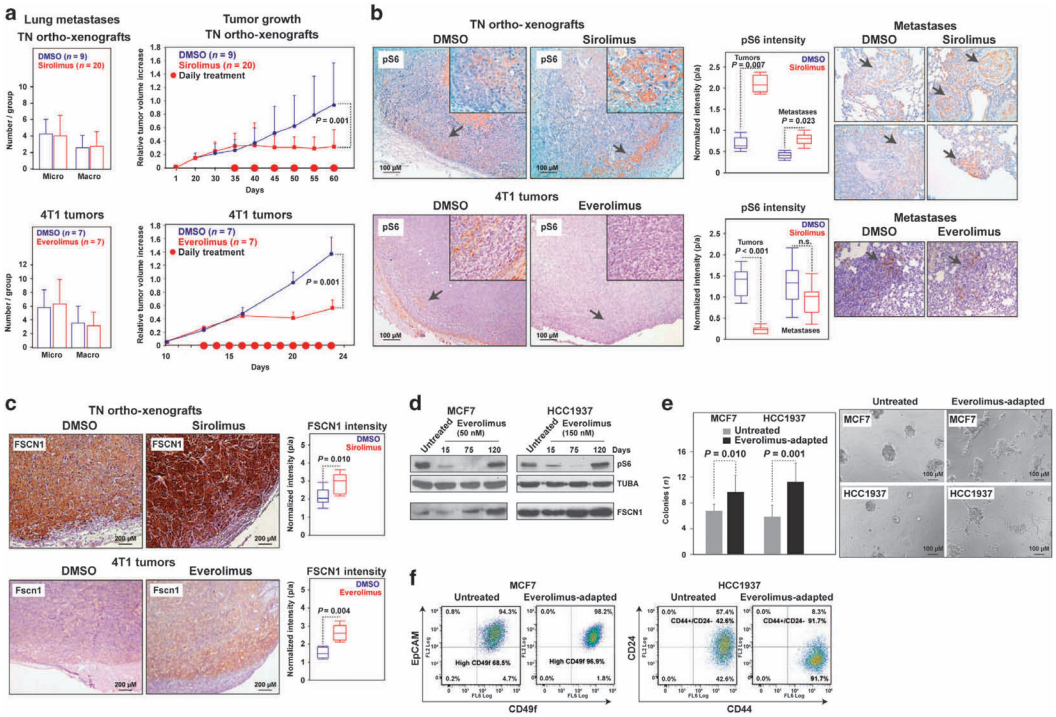

Figure 2. Metastatic resistance to mTOR inhibition. (**a**) Left panels, graphs showing the average and standard deviation of micro- and macro-metastases observed in the lungs of the DMSO- and sirolimus- or everolimus-treated orthoxenografts and 4T1 tumors, respectively. The results correspond to the last day of treatment, and micro- versus macro-metastases were defined using a 2 mm width threshold, and by examining at least three tissue levels separated by $> 20\,\mu m$. Right panels, growth rates of the DMSO- and sirolimus- or everolimus-treated tumors. (**b**) Representative immunohistochemical results for pS6 at the invasive tumor fronts (magnifications; top right panels) and the lung metastases (right panels) of DMSO- or sirolimus/everolimus-treated mice. The middle panels show quantifications, which correspond to three tumors, three equal front areas, and three metastases in each case. (**c**) Representative immunohistochemical tumor results for FSCN1/Fscn1 in DMSO- or sirolimus/everolimus-treated mice; quantifications are shown in right panels. (**d**) Recovered pS6 signal with concurrent FSCN1 overexpression through adaptation to everolimus in MCF7 and HCC1937 cells. Days of treatment are shown. (**e**) Left panel, graph showing the quantification of colonies from untreated and everolimus-adapted cells (12 culture fields were analyzed). The one-tailed *t*-test *P*-values are shown. Representative images of cell cultures are shown in right panels. (**f**) Flow cytometry results showing the cell counts for CD49f/EpCAM and of CD44/CD24 positivity in untreated or everolimus-adapted MCF7 and HCC1937 cells, respectively.

TSC1/2 were found to be negatively correlated (PCCs < -0.10; *P*-values < 0.05) with most of the signatures (Figure 3a). In turn, positive correlations were observed with the downregulated genes that characterize mammary epithelial basal and luminal progenitor cells (Figure 3a).[25] Moreover, the expression of *TSC1* was positively correlated with a downregulated gene expression signature associated with oncogenic PI3KCA activity in breast cancer.[26,27] Collectively, these results confirmed that mTOR activity is associated with stem cell-like gene expression profiles in breast cancer.

Next, we observed that the sESC, sMRS and sMYC largely distinguished the expression profiles of mTOR inhibitor-treated tumors from those treated with DMSO (Figure 3b). The regulators of the sMRS (originally defined as Core-9)[23] were commonly overexpressed upon mTOR inhibition (Figure 3c). Furthermore, a strong overexpression of the three signatures was detected when the ortho-xenografts were allowed to re-grow following treatment with sirolimus (relative to the DMSO-treated re-growth, GSEA *P*-values < 0.001; Figure 3d and Supplementary Table 2). Analysis of the three signatures defined above did not reveal significant changes in the cell line models, but most of the regulators of sMRS were found to be overexpressed in HCC1937 cells (Figure 3e). These results were confirmed by western blot analysis of HMGA1 (Figure 3f), which has been associated with poor prognosis and metastatic breast cancer.[28] The observed differences between the *in vitro* and *in vivo* expression changes may be due to the molecular specificity and/or biological conditions involved in each setting. Globally, however, inhibition of mTOR appears to be coupled to the transcriptional reprogramming that sustains metastatic and tumor initiation features.

EVI1 couples mTOR signaling to metastasis

Feedback activation of known mediators of resistance to rapalogs was not observed in the sirolimus-treated ortho-xenografts, but an increase in phospho-Thr202/Tyr204 ERK (pERK) was detected in 4T1 tumors treated with everolimus (Supplementary Figure 5). Exome sequence comparison between one DMSO- and one sirolimus-treated ortho-xenograft did not identify acquired mutations affecting components of the canonical TSC/mTOR pathway (Supplementary Table 3). *In vitro*, only modest time-dependent

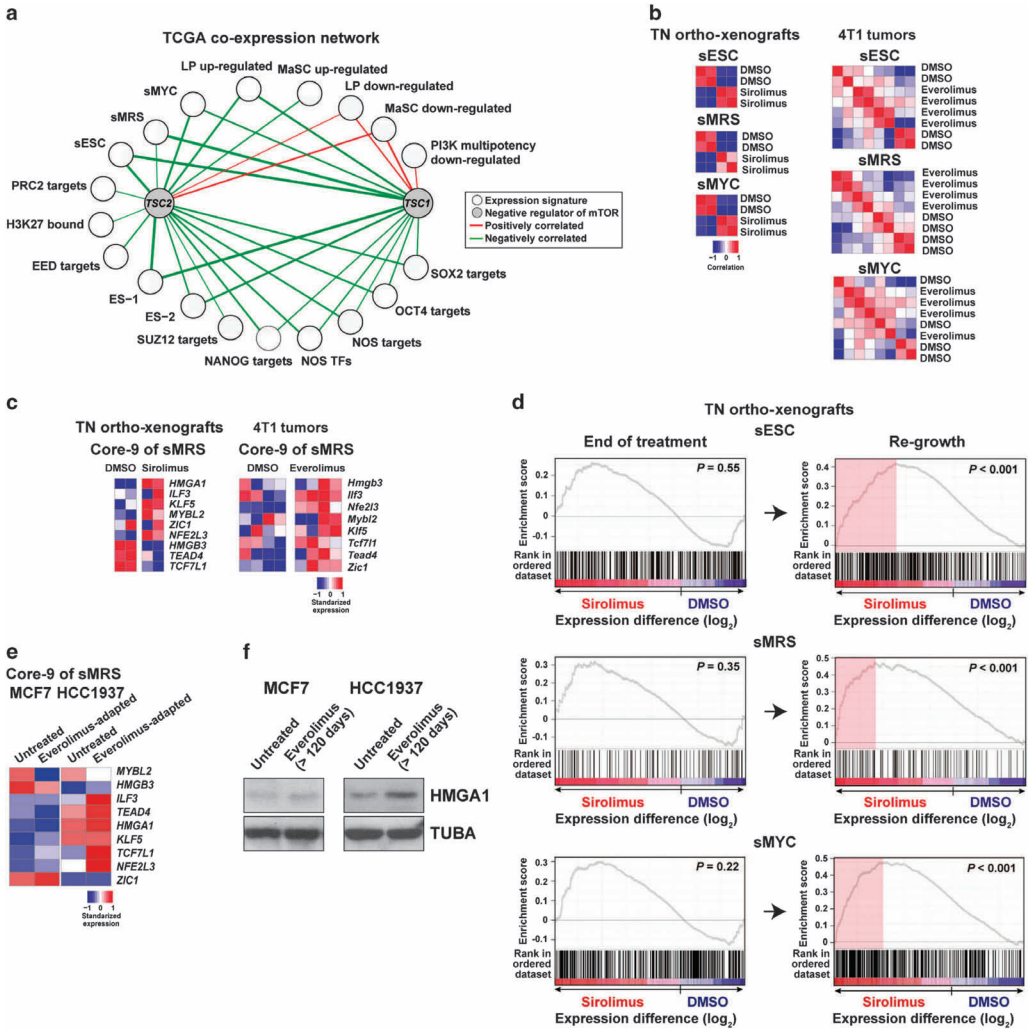

Figure 3. Co-expression analysis and stem cell-like signatures. (**a**) TCGA network of significant co-expression levels (PCC *P*-values < 0.05) between *TSC1* or *TSC2* and signatures derived from stem cell-like cell studies (Supplementary Table 1). The nodes represent *TSC1/2* and the signatures, and the edges positive (red) or negative (green) correlations. Edge width is proportional to the corresponding PCC value. (**b**) Clustering correlation of sESC, sMRS and sMYC. The ortho-xenografts are differentially clustered relative to the treatment, and a similar trend is observed for 4T1 tumors. (**c**) The master regulators of the sMRS (that is, Core-9) are found to be relatively overexpressed upon mTOR inhibition. (**d**) Significant overexpression of sESC, sMRS and sMYC in regrown ortho-xenografts after sirolimus treatment. The GSEA ESs and the nominal *P*-values are shown. (**e**) Most of the regulators of sMRS are relatively overexpressed in everolimus-adapted HCC1937 cells. (**f**) HMGA1, which is encoded in Core-9, is upregulated upon adaptation to mTOR inhibition, particularly in HCC1937 cells.

changes of phospho-S473 AKT (pAKT) and phospho-Y703 STAT3 (pSTAT3) were observed (Supplementary Figure 6). Although the lack of detection of known factors of resistance in our *in vitro* and *in vivo* analyses may be due to long-term treatments, we next sought to analyze a different mechanism that could be common to all models.

Given the transcriptomic changes observed across the *in vivo* and *in vitro* models, the data were analyzed to identify alternative regulators. A significant association (false discovery rate $< 5\%$) was observed in the gene expression profiles from ortho-xenograft samples and a target gene set of the ecotropic viral integration site-1 (EVI1) proto-oncogene (Transfac V$EVI1_02; Supplementary Figure 7). Similar associations for predicted EVI1 target sets were observed using data from the 4T1 tumors and MCF7 cells (Supplementary Figure 7). Importantly, expression analysis using TCGA data revealed positive correlations between *EVI1* and stem cell-like signatures (sMYC and from mammary stem and progenitor cells),[25] in addition to metastatic signatures,

including LMS-up,[16] a signature from low-burden breast cancer metastatic cells,[10] and of breast cancer multipotency promoted by oncogenic PI3KCA[26,27] (Figure 4a). Moreover, a set of 79 commonly overexpressed genes (>0.25 $\log_2$; Supplementary Table 4) across the *in vivo* and *in vitro* models of mTOR inhibitor resistance showed significant positive co-expression with *EVI1* (Figure 4b). Of note, this set included *LEF1*, which regulates stem cell maintenance in different contexts and is functionally connected to SOXs.[29] In addition, this set showed over-representation of gene products involved in actin-cytoskeleton remodeling (Supplementary Table 4).

EVI1 is essential for hematopoietic stem cell self-renewal[30] and its overexpression has been associated with worse recurrence-free, overall and distant metastasis-free survival of ER-negative breast cancer.[31] *In vitro* depletion of EVI1 reduced the levels of pS6, particularly in everolimus-adapted HCC1937 cells (60% reduction, and MCF7 showed a reduction of 10% in any condition; Figure 4c), whereas GFP-EVI1 overexpression conferred higher cellular viability in response to everolimus (Figure 4d). Of note, the levels of pS6 in the GFP-EVI1 overexpression assays were relatively low (Figure 4d), which could be due to the lack of full adaptation and/or the need for EVI1 co-factors.

To further validate the direct transcriptional role of EVI1, chromatin immunoprecipitation (ChIP) assays of predicted transcriptional targets were performed. This analysis revealed increased (one-tailed *P*-values < 0.01) EVI1 binding at the following *loci* with adaptation to everolimus in at least one cell model: *FSCN1* and *SPARC* (from the LMS-up); *SCUBE3* and *TCF4* (from V $EVI1_02); and *RHEB*, *RPS6KA1* and *RAPTOR* (from the mTOR pathway) (Figure 4e). In addition, RAPTOR and RHEB were found to be overexpressed as a function of everolimus adaptation in MCF7 and HCC1937 cells, respectively (Figure 4f; RAPTOR showed transitory underexpression in HCC1937), and EVI1 depletion reduced the expression of both proteins in the everolimus-

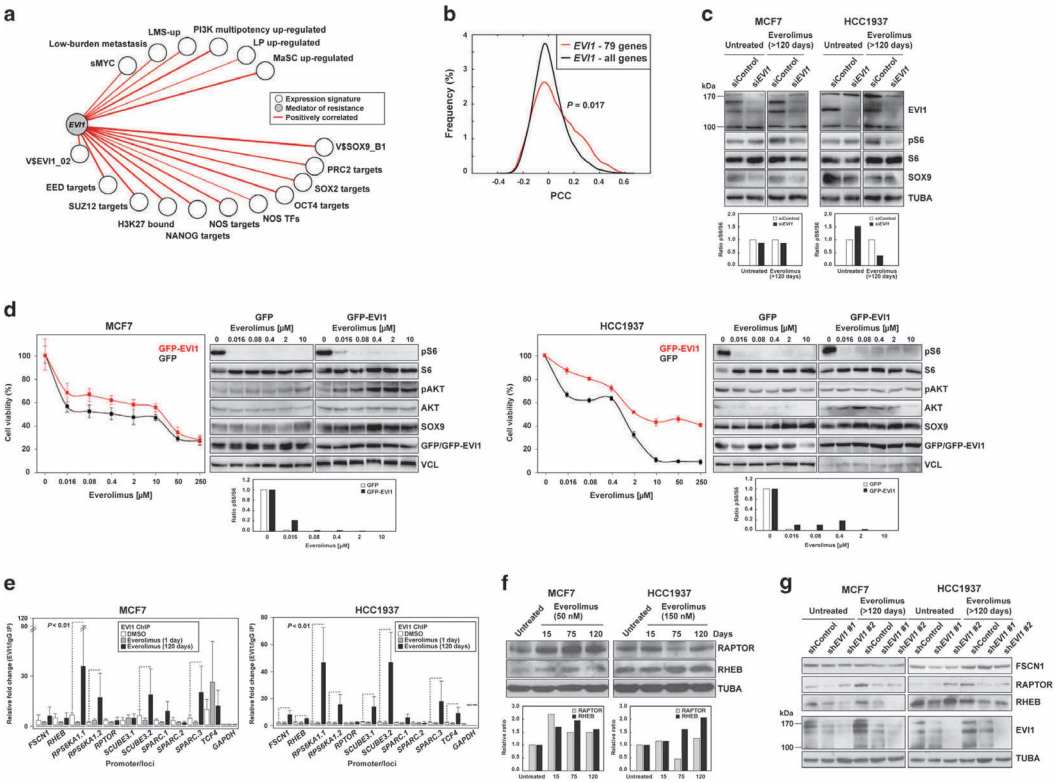

Figure 4. EVI1 couples stemness, metastatic potential and resistance to mTOR inhibition. (**a**) TCGA network of significant co-expression (PCC *P*-values < 0.05) between *EVI1* and signatures derived from stem cell-like cells and/or metastatic settings (Supplementary Table 1). (**b**) Distributions of PCCs between *EVI1* and the commonly overexpressed 79 genes across the studied models or the complete microarray gene list as background control. The *P*-value of the Mann–Whitney test for the comparison of the distributions is shown. (**c**) Reduced pS6 levels with *EVI1* depletion in cell models. The quantification of pS6/S6 signal ratios is show at the bottom (relative to siControl). (**d**) Ectopic overexpression of GFP-EVI1 in MCF7 (left panels) and HCC1937 (right panels) cells provides higher viability upon exposure to everolimus, relative to GFP-only overexpression. Also shown are the western blot results for defined markers across the drug-exposed cell cultures. The quantification of pS6/S6 signal ratios is show at the bottom (relative to TUBA per sample). (**e**) Increased EVI1 binding at predicted target promoters/gene loci with adaptation to everolimus. The fold changes are relative to the immunoglobulin control and the promoter gene targets are shown in the *X* axis. (**f**) Relative overexpression of RAPTOR and/or RHEB with adaptation to everolimus in MCF7 and HCC1937 cells. The quantification is show at the bottom (relative to untreated and TUBA per sample). (**g**) Relative reduction of RAPTOR and RHEB expression following EVI1 depletion, in particular in the everolimus-adapted setting.

adapted settings (Figure 4g). However, the expression of FSCN1 was not significantly reduced with EVI1 depletion (Figure 4g) and, in turn, EVI1 was found to be overexpressed with FSCN1 depletion (Supplementary Figure 8). Therefore, these results may reflect the initial response towards mTOR inhibition; in fact, a similar effect was observed for RAPTOR and RHEB when EVI1 was depleted in parental HCC1937 cells (Figure 4g, right panels).

EVI1 cooperates with SOX9

Based on the strong association between EVI1 and stem cell-like/tumor initiation gene expression signatures, we searched for potential EVI1-transcriptional target genes mediating such functions. The V$EVI1_02 gene set and several other stem cell-like cells and/or metastasis-associated gene signatures were positively co-expressed with a key regulator of these functions, *SOX9* (Figure 5a).[32,33] Subsequently, whole-genome EVI1 ChIP data corroborated the positive correlation between EVI1 and SOX9 binding sites in HCC1937 cells (Supplementary Figure 9). Interestingly, EVI1 ChIP data also showed a positive correlation with SLUG targets[34] in both cell models, and with SNAIL targets[34] in HCC1937 (Supplementary Figure 9). Notably, MCF7 differentiation to a basal-like phenotype requires SLUG activity.[35]

Expanding on the above results, sirolimus-treated ortho-xenografts and everolimus-adapted cells showed increased SOX9 expression compared with their corresponding controls

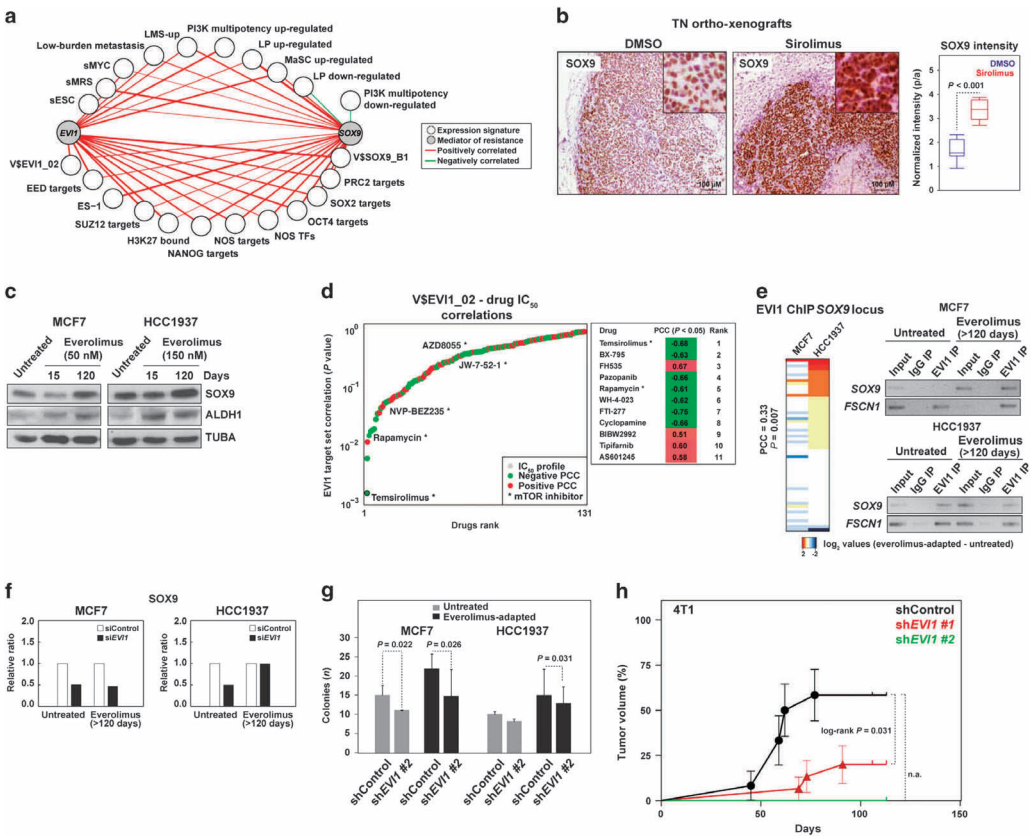

Figure 5. EVI1 cooperates with SOX9 and regulates its expression. (**a**) TCGA network of significant co-expression (PCC *P*-values < 0.05) between *EVI1* or *SOX9* and signatures derived from stem cell-like cells and/or metastatic settings (Supplementary Table 1). (**b**) Increased SOX9 expression in ortho-xenograft tumor fronts of mice treated with sirolimus; the results correspond to at least three ortho-xenografts of each group. (**c**) Increased SOX9 and ALDH1 expression in everolimus-adapted cells. (**d**) Graph showing the results from the analysis of the complete drug panel for the correlation between IC_{50} profiles and the expression of the V$EVI1_02 gene set; drugs are ranked according to PCC log *P*-values. Negative and positive PCCs are indicated with different colors, and the mTOR inhibitors in the panel are denoted. (**e**) Left panel, unsupervised clustering and correlation analysis of the difference in EVI1 ChIP results at the *SOX9* locus between everolimus-adapted and untreated cells. Right panels, results of ChIP assays targeting a predicted EVI1-binding site in the *SOX9* promoter (Supplementary Table 5); the input, control immunoglobulin immunoprecipitation (IP), and EVI1-IP results are shown. The control results for the binding site in *FSCN1* are also shown. (**f**) Depletion of EVI1 leads to a reduction of SOX9 expression in three cell conditions (the results correspond to Figure 4c; the ratios are relative to siControl and TUBA per sample). (**g**) Depletion of EVI1 leads to a reduction of colony-forming capacity. The results of the one-tailed *t*-test are shown. (**h**) Depletion of Evi1 impairs the tumorigenic potential of 4T1 cells. The log-rank *P*-value is shown for the comparison between the shControl and short hairpin RNA (shRNA)-*EVI1* #1; note that transduction with shRNA-*EVI1* #2 completely impaired tumor formation so a *P*-value could not be computed (n.a.).

(Figures 5b and c, respectively; the same antibody did not recognize mouse Sox9). The expression of ALDH1—canonical stem/progenitor marker in normal breast tissue and tumors, and also associated with poor prognosis[36]—was also detected to be increased with adaptation to everolimus in both MCF7 and HCC1937 cells (Figure 5c). The higher expression at the tumor invasive front is consistent with previous observations of invasive leader cells showing positivity for basal/stem cell-like markers.[10,37]

In addition to the results from the models, the analysis of data from hundreds of cell lines[38] revealed significant positive correlations between *EVI1* and *SOX9* expression, and with *EVI1* locus copy number (PCCs >0.20, *P*-values $<10^{-4}$). Moreover, in this data set, both *EVI1* and *SOX9* expression correlated positively (that is, linked to resistance) with the half maximal inhibitory concentration (IC_{50}) of temsirolimus (PCCs = 0.22 and 0.24, respectively, *P*-values $<10^{-4}$). In fact, a ranking-based analysis using the V$EVI1_02 gene set as a surrogate of EVI1 activity showed equivalent results, and the second and fourth most correlated drugs, respectively, were BX-795 (inhibitor of PDK1 activity) and rapamycin (Figure 5d). To further assess these findings, an ER-positive and HER2-positive breast cancer cell model, BT-474, was exposed to 150 nM of everolimus for approximately 100 days and subsequently profiled for gene expression changes; the results showed significant associations with EVI1 and SOX9 targets, and with the commonly over-expressed genes detected across the above *in vitro* and *in vivo* models (Supplementary Figure 10).

Further analysis of the predicted functional relationship between *EVI1* and *SOX9* revealed a positive correlation in the differential (between control and everolimus-adapted) binding of EVI1 at the *SOX9* locus (Figure 5e, left panel). Next, targeted ChIP assays confirmed that EVI1 binds at the *SOX9* locus in untreated HCC1937 and both everolimus-adapted cell models (Figure 5e, right panel). Thus, depletion of EVI1 reduced the expression of SOX9 in three out of four conditions; however, it remains to be determined which co-factor(s) may maintain SOX9 expression at normal levels in everolimus-adapted HCC1939 cells (Figure 5f). In parallel, depletion of EVI1/Evi1 reduced the colony-forming capacity of both models of everolimus adaptation (Figure 5g) and impaired *in vivo* tumorigenic potential of 4T1 cells (Figure 5h). Collectively, ChIP, gene/protein expression analyses and *in vivo* functional assays depict a link between EVI1 and SOX9 in the regulation of stem cell-like and tumor initiation features.

In vivo evaluation of the EVI1, SOX9 and mTOR relationship

The functional cooperation between EVI1 and SOX9 was evaluated *in vivo* using LM2 and 4T1 cells transduced with a short-hairpin RNA scrambled control or directed against *EVI1*, and with or without concomitant overexpression of Sox9 (mouse protein). Thus, EVI1 depletion significantly reduced the capacity to colonize the lungs, and concurrent *Sox9* overexpression partially rescued metastatic potential (Figures 6a and b). As shown in everolimus-adapted cell lines, EVI1/Evi1 depletion caused a significant decrease of both SOX9 and pS6 expression in the corresponding metastasis (Supplementary Figure 11). Concurrent Sox9 over-expression recovered pS6 signal in 4T1 but not in LM2 cells (Supplementary Figure 11), which suggest differences in the precise regulation of mTOR activity between the models.

In addition to EVI1, depletion of SOX9 in LM2 cells led to a significant decrease in lung colonization capacity and, conversely, Sox9 overexpression increased this capacity (Figure 6c and Supplementary Figure 12). Depletion of Sox9 in 4T1 cells and everolimus treatment of both cell models also reduced lung colonization (Figures 6c and d). In addition, depletion of FSCN1 in both models also led to a substantial impairment of lung colonization (Figures 6e and f). Moreover, a greater effect was observed when the animals were simultaneously treated with everolimus (Figures 6e and f), which fully suppressed pS6 signal (Supplementary Figure 11). Collectively, these results indicate that transcriptional reprogramming mediated by EVI1-SOX9 is one of the key factors in metastatic resistance to mTOR inhibition.

DISCUSSION

We provide evidence of the association between EVI1-SOX9 function, mTOR inhibition resistance and metastasis in breast cancer. We also show that EVI1-SOX9-mediated transcriptional reprogramming drives the molecular processes that support breast cancer tumor initiation features and metastatic potential in therapeutic resistance (Figure 7). These data are coherent and expand on the concept that cancer stem cell-like cell populations have high tumor-initiating capacity and are frequently the source of therapy resistance and metastasis.[39] Data from hundreds of cell lines[38] suggest that the proposed mechanism is relevant in settings beyond breast cancer. Importantly, *EVI1* maps in a genomic region (including *PI3KCA* and *SOX2*) whose amplification is an independent predictor of breast cancer recurrence.[40] This region is frequently found to be amplified in basal-like and *BRCA1*-mutated breast cancer,[21] as well as in non-small cell lung and ovarian cancers.[41] In addition, *EVI1* amplification is independent of *PIK3CA* mutations,[41] which further reinforces the link with basal-like breast cancer and is consistent with a role in resistance to allosteric mTOR inhibition. It remains to be determined whether *EVI1* expression is upregulated through genomic amplification and/or whether its function is enhanced by biochemical modifications by casein kinase II[42] and/or ERK signaling as seen to be activated in our 4T1 assays.

Our preclinical findings and mechanistic model are consistent with and expand on recent observations across different neoplastic settings. EVI1 contributes to epithelial-to-mesenchymal transition (EMT) and invasion of acute myeloid leukemia,[43] and influences EMT in ovarian cancer cells,[44] whereas EMT mediates resistance to rapamycin.[45] In the latter study, MCF7 cells transfected with constitute active SNAIL showed increased ERK signaling and decreased sensitivity to rapamycin. Thus, our study provides a mechanistic explanation for these observations. In addition, it has recently been shown that SOX2 and SOX9 mediated the maintenance of latent metastatic stem cell-like cells,[46] and it was previously demonstrated that SLUG and SOX9 cooperatively determine mammary stem cell state,[32] and that SOX9 function links tumor initiation and invasion.[33] Moreover, a stem cell-like cancer phenotype that mediates breast cancer metastasis is predicted to exhibit abnormal mTORC1 signaling[10] and, in turn, mTORC1/2 inhibition promotes stem cell-like properties and enhanced NOTCH1 activity in TN breast cancer cell lines.[12] Thus, enhanced mTOR signaling impairs cell differentiation by potentiating NOTCH1 activity, and this signaling is found to be increased in poorly differentiated breast tumors.[14] Intriguingly, NOTCH may also regulate *SOX9* expression,[47] which, in turn, is a master regulator of stem and progenitor cells.[48] In parallel, allosteric mTOR inhibition increases the number of tumor-initiating cells in a model of liver cancer[15] and the metastatic potential in a model of pancreatic neuroendocrine cancer.[49] In this scenario, our study proposes that EVI1 and SOX9 functionally cooperate to sustain mTORC1 activity, EMT and metastatic potential, thereby providing new insights into therapeutic resistance.

Our findings—particularly those from MCF7 cell assays—may have clinical implications for the established use of mTOR inhibitors in endocrine-resistant ER-positive advanced metastatic breast cancer,[7] in which resistance to treatment is eventually reported. Our results indicate that exposure to allosteric mTOR inhibition selects a stem cell-like cancer cell population with metastatic capacity, which may therefore promote disease progression. Although *in vivo* assays may be warranted to further

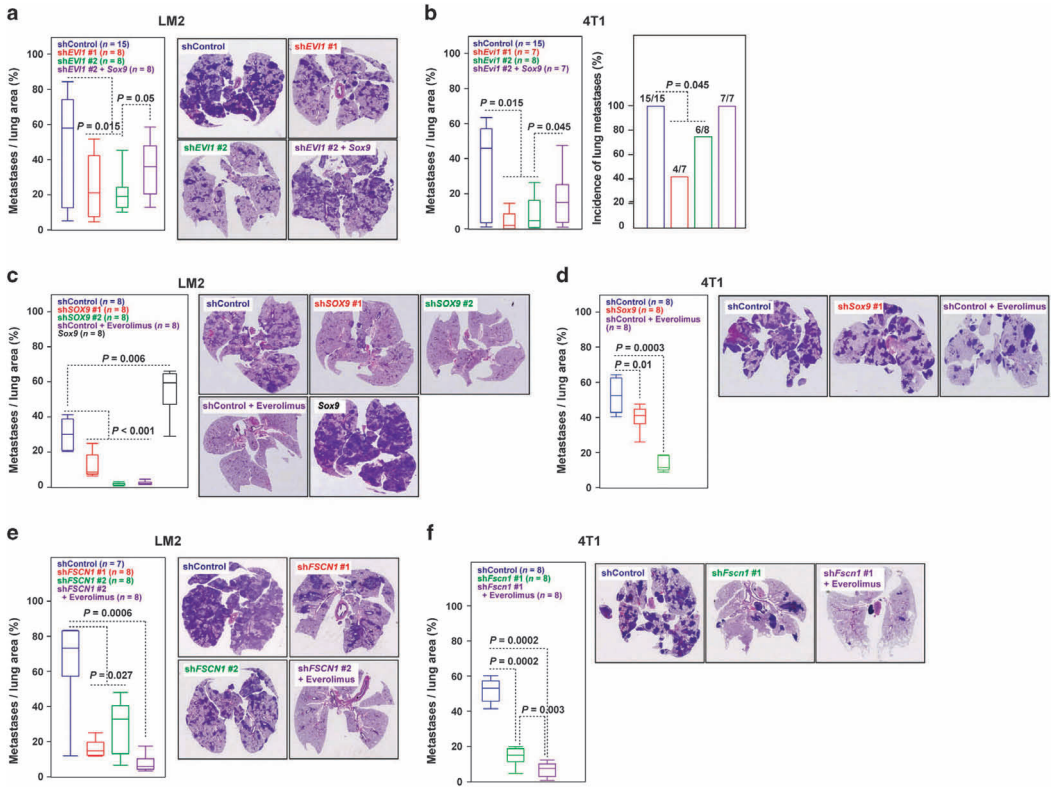

Figure 6. *In vivo* assessment of the role of EVI1 and SOX9. (**a**, **b**) Depletion of *EVI1*/*Evi11* expression (using two different short hairpin RNAs (shRNAs)) in LM2 and 4T1 cells reduced lung colonization, and *Sox9* overexpression partially recovered this potential, left panels. (**a**) Right panels show representative images of lungs and their respective HE staining. (**c**) Depletion of *SOX9* and overexpression of *Sox9* reduced and increased, respectively, lung colonization of LM2 cells. Treatment with everolimus of shControl LM2 cells also reduced lung colonization. (**d**) Depletion of *Sox9* or treatment with everolimus of 4T1 cells reduced lung colonization. (**e**, **f**) Depletion of *FSCN1*/*Fscn1* expression in LM2 and 4T1 cells reduced lung colonization, and concurrent treatment with everolimus further impaired this potential.

assess this observation, and while we cannot rule out that the specific population may arise through the acquisition of new mutations, it is noteworthy that the Breast Cancer Trials of Oral Everolimus-2 (BOLERO-2) study for the efficacy of everolimus plus exemestane in endocrine resistance showed similar benefits for patients with or without visceral metastases.[50] Nevertheless, full and durable pathway inhibition—such as obtained that by the next generation of targeted drugs[51]—may fully impair metastatic resistance.

MATERIALS AND METHODS

Tissue microarray

The tissue microarray included 138 infiltrating ductal breast carcinoma tumors collected at the Department of Pathology of the MD Anderson Cancer Center, Madrid (Spain). The patients underwent surgery between 2003 and 2004, and all tumors were classified as grade 3. According to the TNM system, 45 tumors belonged to stage I, 48 to stage II and 45 to stage III-IV. The linked data included ER ($n = 104$), progesterone receptor ($n = 127$) and epidermal growth factor receptor 2 (HER2; $n = 125$) status, and CK5 expression ($n = 128$), absence/presence of lymph node metastasis ($n = 124$), and absence/presence of distant metastasis ($n = 127$). The tissue microarray contained duplicated cases and normal tissue, and the immunohistochemical results were scored independently and blindly (to molecular and clinical status). Selection of the highest value for a given case, blindly to its status, solved discordant scores. The study was approved by the ethics committee of the MD Anderson Cancer Center and written informed consent was obtained from all patients.

Gene expression analyses

Pre-processed and normalized data of human breast cancer were taken from the corresponding publication[16] and from the TCGA repository (http://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp).[52] RNA samples were extracted using TRIzol Reagent (Invitrogen, Karlsruhe, Germany) and RNeasy Kit (Qiagen, Venlo, Netherlands), and quality was evaluated in an Agilent Bioanalyzer (Foster City, CA, USA) 2100. The RNAs were amplified using the Ribo-SPIA system (NuGEN Technologies Inc., San Carlos, CA, USA) and subsequently hybridized on the Human Genome U219 microarray platform (Affymetrix, Santa Clara, CA, USA; IRB Core Facility, Barcelona, Spain). Gene expression data from the ortho-xenograft, and MCF7 and HCC1937 cell lines have been deposited under the GEO reference GSE39694. Gene expression data from the 4T1 tumors and BT-474 cells have been deposited under the GEO references GSE50712 and GSE85801, respectively. The GSEA and DAVID (for functional term analyses) tools were used with standard parameters.[53,54] The signature correlations were computed by selecting genes with s.d. > 1.0 and using the average *Z*-score value per gene set. The quantification of *NANOG*, *OCT4* and *SOX2* gene expression was performed as previously described.[55]

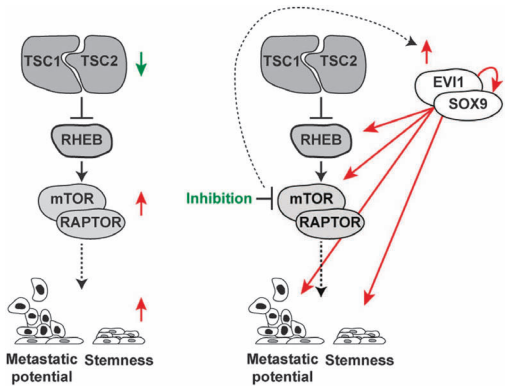

Figure 7. Proposed mechanistic model. In untreated cancer, low *TSC1/2* expression is associated with enhanced mTORC1 activity and, therefore, with a primary metastatic and stemness phenotype. In cancer treated for mTOR inhibition, EVI1-SOX9 become activated (in part by overexpression) and positively sustain the following features: mTOR signaling (through upregulation of RHEB and RAPTOR), metastatic potential (through LMS-up and other signatures) and stemness (through at least SOX9).

Antibodies

Anti-total and pAKT (#9272 and #9271, respectively, Cell Signaling Technology, Danvers, MA, USA; #4060 for immunohistochemistry assays), anti-ALDH1 (#611194, BD Biosciences, Oxford, UK), anti-4EBP1 (#9452, Cell Signaling Technology), anti-p4EBP1 (#2855 and #9451, Cell Signaling Technology), anti-ER (#IR151, Dako, Glostrup, Denmark), anti-total and phospho-Thr202/Tyr204 ERK (#4695 and #4376, respectively, Cell Signaling Technology), anti-EVI1 (#2265 and #2593, Cell Signaling Technology; and #A301-691A, Bethyl Laboratories, Montgomery, TX, USA), anti-FSCN1 (#SC-56531, Santa Cruz Biotechnology, Dallas, TX, USA), anti-GFP ChIP grade (#ab290, Abcam, Cambridge, UK), anti-GLUT1 (#652, Abcam), anti-HER2 (#790-100, Ventana, Tucson, AZ, USA), anti-HMGA1 (#129153, Abcam), anti-pIGF1R (#39398, Abcam), anti-IRS1 (#2382 and #9451, Cell Signaling Technology), anti-KI67 (#IR626, Dako), anti-CK19 (#IR615, Dako), anti-PR (#IR168, Dako), anti-RAPTOR (#SC-81537, Santa Cruz Biotechnology), anti-RHEB (#SC-6341, Santa Cruz Biotechnology), anti-S6 (#SC-74459, Santa Cruz Biotechnology), anti-pS6 (#4858, Cell Signaling Technology), anti-S6K (#9202, Cell Signaling Technology), anti-pS6K (#9205, Cell Signaling Technology), anti-SOX9 (#5535, Abcam), anti-total and pSTAT3 (#9132 and #9145, respectively, Cell Signaling Technology), anti-TUBA (#44928, Abcam) and anti-VCL (V9131, Sigma-Aldrich, St Louis, MO, USA). The antibodies used for fluorescence-activated cell sorting were anti-CD24-PE, anti-CD44-APC, anti-CD49f-Alexa-647, and anti-EPCAM-FITC (#555428, 559942, 562473, and 347197, respectively; BD Biosciences).

Immunohistochemistry

The assays were performed on serial paraffin sections (3–4 μm thick) using the EnVision (Dako) or Ultraview (Ventana) systems. Antigen retrieval was performed using citrate- or EDTA-based buffers. Endogenous peroxidase was blocked by pre-incubation in a solution of 3% H_2O_2 and blocking was performed in 1X phosphate-buffered saline with 5% goat serum or 1% bovine serum albumin and 0.1% Tween 20 (Sigma-Aldrich). In all experiments, equivalent sections were processed without incubation with the primary antibody, which did not reveal immunostaining in any case. Sections were hematoxylin and eosin (HE)-counterstained and examined with an Olympus BX51 (Tokyo, Japan) microscope. The immunohistochemistry microscopic images were color deconvoluted and quantitated using the regions of interest methodology in ImageJ (http://rsb.info.nih.gov/ij). Quantification of tumor fronts was based on rectangular areas of 25 μM×50–300 μM. When quantifying the results from lung metastases, the complete metastatic area was considered because the fronts were often difficult to outline histologically.

Cell culture

The LM2 cell derivative is a lung metastatic sub-line originated from MDA-MB-231 breast cancer cells from the laboratory of Professor Massagué.[16] The 4T1 cells derived from a spontaneous BALB/c mouse breast cancer tumor[56] and were obtained from the ATCC (Rockville, MD, USA). The LM2 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (GIBCO), 1x L-glutamine (Biowest, Nuaillé, France) and 1% penicillin/streptomycin (Biowest). The 4T1 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 1x L-glutamine and 1% penicillin/streptomycin. All MDA-MB-231 cells/sub-lines were stably transfected with a thymidine kinase and GFP luciferase construct and sorted for GFP expression. The MCF7 and HCC1937 cell lines were obtained from ATCC and cultured in supplemented Dulbecco's modified Eagle's medium and RPMI-1640 medium, respectively. The Matrigel (BD Biosciences) colony formation assays were performed using standard protocols with 5% fetal bovine serum. Everolimus was purchased from Selleck Chemicals (Houston, TX, USA) and LC Laboratories (Woburn, MA, USA). Fluorescence-activated cell sorting was performed using FACS Canto (Becton Dickinson, Franklin Lakes, NJ, USA) and Diva software (Becton Dickinson) package, and antibody-based cell labeling was performed as previously described.[55]

Western blotting

To analyze extracts, cells were lysed in standard 150 mM NaCl buffer supplemented with protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany) and, in some instances, a phosphatase inhibitor was added (1 mM NaF, Sigma-Aldrich). Lysates were clarified twice by centrifugation at 13 000× *g* and protein concentration was measured using the Bradford method (Bio-Rad, Solna, Sweden). Lysates were resolved in sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred to Immobilon-P (Merck Millipore, Billerica, MA, USA) or PVDF membranes (Roche Molecular Biochemicals). Target proteins were identified by detection of horseradish peroxidase-labeled antibody complexes with chemiluminescence using the ECL Western Blotting Detection Kit (GE Healthcare, Amersham, UK).

Lung colonization assays

The Animal Care and Use Committee of IRB Barcelona approved the following animal studies. Female BALB/c nude (MDA-MB- 231 cells) or BALB/c wild-type mice (4T1 cells) were used. For tail vein injections, cells were suspended in 1x phosphate-buffered saline (GIBCO; 200 μl per mouse) and injected into the lateral tail vein of mice using a 26G needle, as previously described.[57] Before the injection of cells, mice were anesthetized with ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight), and immediately after injection they were imaged for luciferase activity by injecting 50 μl of beetle luciferin potassium salt (Promega, Madison, WI, USA) at 15 mg/ml. To induce the expression of short hairpin RNA *in vivo,* doxycycline (1 mg/ml, Sigma-Aldrich) was administered *ad libitum* in drinking water containing 25 mg/ml sucrose (Sigma-Aldrich). When indicated, DMSO solution (at the same concentration as for the compound test, 5%) or everolimus (5 mg/kg; SC-218452, Santa Cruz Biotechnology) was administered daily by intraperitoneal injection. Mice were monitored weekly using IVIS imaging, unless otherwise indicated. Lung tumor development was followed up once a week by bioluminescence imaging of the upper dorsal region that corresponds to lung position. Bioluminescent images were quantified with Living Image 2.60.1 software (Perkin-Elmer, Waltham, MA, USA). All values were normalized to those obtained at day 0. The HE staining of lung sections scored the lung colonization capacity of 4T1 cells 3 weeks post-inoculation. Five sections, separated by 50 μm, per mouse lung were counted. The average of the total metastatic area normalized to total lung area was measured. The average total lung metastasis area for all mice was then plotted. The tissue was dissected, fixed in 10% buffered formalin (Sigma-Aldrich), and embedded in paraffin. Sections (3 μm thick) were stained with HE. To analyze the metastatic area, images were taken with a scanner, and the area of each metastatic lesion was quantified with the ImageJ software. Five images per section/animal were evaluated, and the average area was plotted. The Fiji Trainable Weka segmentation, an ImageJ plugin based on the Weka[58] Java machine learning library, was used to classify images on the basis of local colorimetric, textural and structural features in the neighborhood of each pixel. Images were processed with a custom macro created at the Microscopy Core Facility of IRB Barcelona.

The two-tailed non-parametric Mann–Whitney test was used to assess significance of the immunohistochemical staining results.

Ortho-xenograft

The patient was a 33-year-old woman with a pathological germline *BRCA1* mutation and diagnosed with breast cancer shortly after pregnancy. At diagnosis she presented a locally advanced TN ductal infiltrating carcinoma of the breast (T4) with involvement of ipsilateral nodes (N2) and lung metastasis. Primary systemic chemotherapy was initiated with TAC regimen for four cycles, followed by mastectomy to prevent local complications because of extensive breast involvement. Following surgery, the patient received further chemotherapy with the same regimen. The patient was diagnosed with brain metastases shortly after and died 8 months post-diagnosis as a result of disease progression. Mutational analysis of *BRCA1* was carried out by the Molecular Diagnostics Unit (Catalan Institute of Oncology, Barcelona, Spain) following standards for genetic testing and pathological determination. The patient provided written informed consent, and the study was approved by the IDIBELL Ethics Committee. Female athymic (*nu/nu*) mice (Harlan, Harlan Laboratories, Barcelona, Spain) between 4 and 6 weeks of age were used for engraftment. The orthotopic model developed histologically detectable lung metastases in a period of approximately 50 days after engraftment. The protocol was reviewed and approved by the IDIBELL Animal Care and Use Committee. A daily oral treatment with sirolimus (Rapamune) or control solution (DMSO, Sigma-Aldrich) was applied.

Exome analysis

The National Centre for Genomic Analysis (CNAG) carried out exome sequencing. Sequence capture and amplification was performed using Agilent (Agilent Technologies, Palo Alto, CA, USA) SureSelect Human All Exon kit (Agilent) according to the manufacturer's instructions. Paired-end sequencing was performed on a HiSeq2000 instrument (Illumina, San Diego, CA, USA) using 76-base reads. Reads were aligned to the reference genome (GRCh37) and BAM files were generated using SAMtools. Duplicates were removed using SAMtools and custom scripts, and single-nucleotide variant calling was performed using a combination of SAMtools and Sidrón algorithms as described previously.[59] The reads were first aligned to mouse genome (mm9), and those read-pairs that did not align to mouse were then aligned to the human genome following the same pipeline as above. Only mismatch variants were taken into account and small insertions and deletions were not counted. Common variants, defined as those present in dbSNP135 with a minor allele frequency $> 1\%$, were filtered out.

4T1 tumors

The animal studies were conducted using protocols that had undergone appropriate review and approval at the New York University School of Medicine. Balb/C mice were injected subcutaneously with 5×10^4 4T1 cells, measured for tumor size at day 10, and randomly organized in two equivalent groups that were treated with DMSO solution (the same concentration as for the compound test) or everolimus (5 mg/kg; SC-218452, Santa Cruz Biotechnology) daily by intraperitoneal injection. Tumors were excised at day 23 and processed. Half of each tissue sample was used for immunohistochemistry and half for gene expression microarray analysis. For the tumorigenicity assays, 250 000 4T1 cells were injected at the orthotopic site, mixed with growth factor-reduced Matrigel (BD Biosciences) before inoculation (1:1). Once palpable, tumors were measured with a digital caliper, and the tumor volume was calculated. The ethics committee of the CIC bioGUNE approved these assays.

ChIP assays

Assays were prepared using 10^7 cells of each cell line per condition. Chromatin was fragmented by sonication (Bioruptor, Diagenode, Denville, NJ, USA) for 30 min (30-s pulses, 30-s pauses) and assays were carried out following the manufacturer's protocol (kch-mahigh-A16, HighCell# ChIP Kit, Diagenode), using anti-EVI1 (#2593, Cell Signaling Technology) or an equal amount of IgG isotype as negative control (#2729, Cell Signaling Technology). The amount of DNA was analyzed by real-time polymerase chain reactions using SYBR Green-based assays (Applied Biosystems, Life Technologies, Foster City, CA, USA). The results were calculated using the ΔCt method. A genomic region of the *GAPDH* gene was used as negative control (Diagenode). The corresponding human genome coordinates, EVI1-binding sites and primers designed for the assays are detailed in Supplementary Table 5. Whole-genome ChIP data were obtained by hybridization to SurePrint G3 Human Promoter 1x1M microarrays (IRB Core Facility) and analyzed by MACS (version 2.0.9).[60] The data have been deposited under the GEO reference GSE50905. The complete ranking of differential EVI1 binding between adapted and sensitive MCF7 or HCC1937 cells was used as input for the GSEA of transcription factor targets.

Gene expression alterations

Stable LM2 and 4T1 cell lines expressing short hairpin RNAs were generated as described previously.[57] The sh*FSCN1/Fscn1* (that is, targeting both human and mouse gene expression) #1 and sh*EVI1/Evi1* #2 were encoded in lentiviral vectors (inducible pTRIPZ lentiviral short hairpin RNAs, GE Dharmacon, Lafayette, CO, USA). The short hairpins were induced by 1 μg/ml doxycycline for 72 h. The shControl and sh*EVI1/Evi1* #1 were encoded in a retrovirus pGFP-V-RS (OriGene, Rockville, MD, USA). The sh*FSCN1/Fscn1* #1 was encoded in a pSUPER (Addgene, Cambridge, MA, USA) vector. The sh*Sox9* (against mouse gene sequence) and sh*SOX9* (human) were obtained from the MISSION library (SHCLND-NM_011448 and SHCLND-NM_000346, respectively; Sigma-Aldrich). An additional sh*SOX9* was obtained from Addgene, catalog #40644. For *Sox9* overexpression, the corresponding coding sequence was cloned into a lentiviral pWXL vector. Stable cell lines expressing the various constructs described above were generated under puromycin selection for 48 h. The siRNA against *EVI1* expression was an ON-TARGETplus SMARTpool (L-006530-02-0010, Dharmacon). The following primer sequences were used to assess gene expression changes in real-time (using SYBR Green, Applied Biosystems) polymerase chain reaction assays: *EVI1*, 5′-CATT GGGAACAGCAACCAT-3′ and 5′-GGTCACCAAAGCCTTTTCAT-3′; *Evi1*, 5′-CAC AGAAAGTCCAAATCACAGG-3′ and 5′-GCCACACGTTGGAGGAAC-3′; *Sox9*, 5′-GTACCCGCATCTGCACAAC-3′ and 5′-CTCCTCCACGAAGGGTCTCT-3′; *ACTB*, 5′-GGAGTGGGTGGAGGCAG-3′ and 5′-AACTAAGGTGTGCACTTTTGTTC-3′; and *mL32*, 5′-GAAACTGGCGGAAACCCA-3′ and 5′-GGATCTGGCCCTTGAACCTT-3′.

Genomics of drug sensitivity data analyses

For the correlation analysis between the basal expression of *EVI1*, its predicted target genes and the drug responses across cancer cell lines, data were downloaded from the GDS project (web-release April 2012).[38] This data set included IC_{50} values for 131 drugs that were assessed in a panel of 638 human cancer cell lines. The basal gene expression data were downloaded from ArrayExpress reference E-MTAB-783. Non-annotated probes were removed and expression values were averaged when multiple probes mapped to the same gene. Correlation scores and *P*-values were computed using the PCC. The EVI1 target set included 20 genes that were represented by at least one microarray probe (Supplementary Table 6). The extent of the basal expression of the EVI1 targets was quantified using an enrichment score (ES) computed with a Matlab implementation of the GSEA algorithm. To estimate ES significance, a null model was created by generating 10 000 random gene sets (of the same size as the EVI1 target set) and used to query the data set through GSEA. Next, two inverse Gaussian distributions (for positive and negative ES values) were fitted on the resulting empirical distribution and used to compute *P*-values. The correlations between IC_{50} profiles and ESs were computed by considering only cell lines whose basal expression profile yielded a significant ES ($P < 0.05$), according to the null model. The enrichment *P*-values of mTOR inhibitors among drugs whose IC_{50} profile was anti-correlated with the EVI1 target ES were computed using Fisher's exact test and considering the total set of 131 drugs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Ander Urruticoechea, Gabriel Capellá and George Thomas for helpful discussion; Ana Isabel Extremera, Antoni Xaubet and Julio Ancochea for administrative support; and Sebastien Tosi from the Advanced Digital Microscopy Facility at IRB Barcelona for image analysis. We also thank Xiyun Deng, Yanna Cao, Tien C Ko, Yi Zhang (The University of Texas Health Science Centre at Houston) and Adrian W Moore (RIKEN Brain Science Institute) for their support in evaluating antibodies against EVI1. This study was supported by the following bodies and grants:

the Scientific Foundation 'Asociación Española Contra el Cáncer' (AECC, Stable Coordinated Group, Hereditary Cancer); the BBVA Foundation; the Eugenio Rodríguez Pascual Foundation grant 2012; Generalitat de Catalunya AGAUR SGR 2012 grants 283, 290 and 312, and SGR 2014 grants 364, 530, and 535; Spanish Ministry of Health ISCIII FIS grants PI10/00057, PI10/00222, PI10/01422, PI12/01528, PI13/00132, and PI14/00336. ISCIII RTICC grants RD06/0020/1051, RD12/0036/0007, RD12/0036/0008 and RD12/0036/0063; Spanish Ministry of Science and Innovation, 'Fondo Europeo de Desarrollo Regional (FEDER), una manera de hacer Europa', MINECO grants SAF2010-20203 and SAF2013-46196; and the Telemaraton 2014 'Todos Somos Raros, Todos Somos Únicos' grant P35. EJA was supported by 'la Caixa' PhD fellowship program, F Iorio was supported by a fellowship from the EMBL-EBI and the Wellcome Trust Sanger Institute Postdoctoral (ESPOD) program, and NL-B, ME and RRG were supported by ICREA. No funding bodies had any role in study design, data collection and analysis, decision to publish, or preparation of the manuscript, either in the submission form or the text of the manuscript.

AUTHOR CONTRIBUTIONS

RRG and MAP: performed the design and interpretation of experiments, and wrote the manuscript; FM, EJA, HA, GRG, J Boni, XP, CH, AL, HS, MS, LG-B, XS, J Cerón and JG-M: molecular and cell biology experiments; FM, EJA, HA, SD, MHB-H and A Villanueva: *in vivo* studies; FM, HA, MM and MDO: chromatin immunoprecipitation assays; JS-M, FI, AI, DC, NB, LP, AG, NL-B, LG-A and JS-R: bioinformatic analyses; FM, HA, J Boni, S Puertas, NG, VH, MM-I and AF: immunohistochemistry experiments; AR-S, A Martínez, MPB, M Gil, CF, AF, IM, S Pernas, MJP, XA, MAS, RB, EC, SM, JV, A Velasco, XM-G, MAQ, AS and GM-B: tissue microarray studies; RV-M and XSP: exome analyses; A Petit, A Vidal, IC, TS and GV: pathological evaluations; AMS, VW, MPL, M Nellist, JVS-M, ME, MJ, LB and JWMM: genetic analyses; FM, HA, J Boni, EG-S and A Cordero: three-dimensional cell culture experiments; JH-L, SRC and J Cortés: breast cancer tumor analyses; JL, FC, IB, A Perkins, J Brunet, FV, OC, M Graupera, NM-M, A Matheu, A Carracedo, TFG, EEWC, MS-C, M Nanjundan and CL: experimental design and interpretation.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)

Figure S1

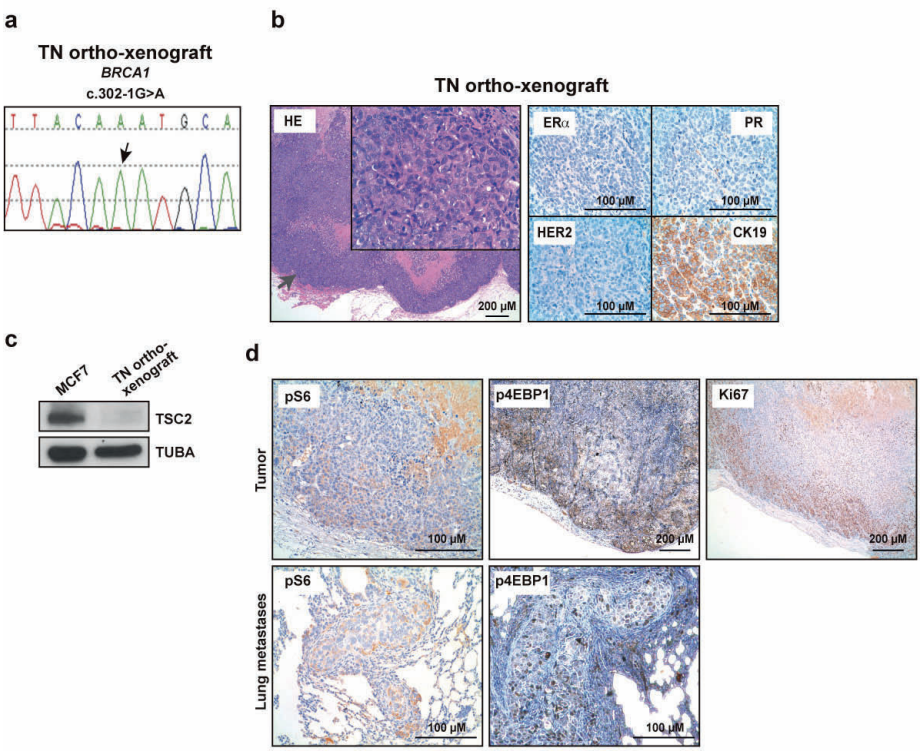

a
TN ortho-xenograft
BRCA1
c.302-1G>A
T T A C A A A T G C A
b
TN ortho-xenograft
HE
200 µM
ERα
PR
HER2
CK19
100 µM
c
MCF7
TN ortho-xenograft
TSC2
TUBA
d
Tumor
Lung metastases
pS6
p4EBP1
Ki67
100 µM
200 µM

Figure S2

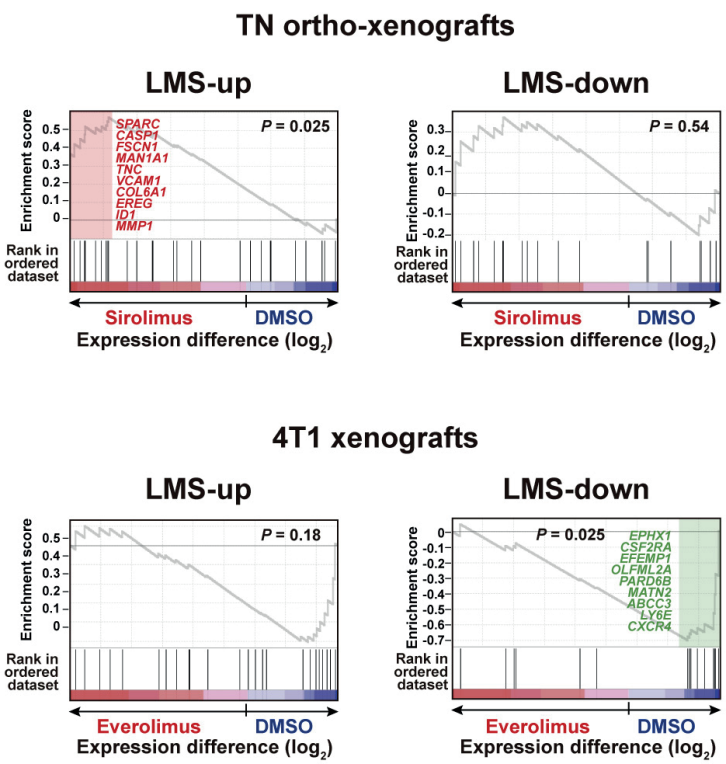

TN ortho-xenografts
LMS-up
LMS-down
Enrichment score
Rank in ordered dataset
SPARC
CASP1
FSCN1
MAN1A1
TNC
VCAM1
COL6A1
EREG
ID1
MMP1
P = 0.025
P = 0.54
Sirolimus
DMSO
Expression difference (log2)
4T1 xenografts
LMS-up
LMS-down
P = 0.18
P = 0.025
EPHX1
CSF2RA
EFEMP1
OLFML2A
PARD6B
MATN2
ABCC3
LY6E
CXCR4
Everolimus
DMSO
Expression difference (log2)

Figure S3

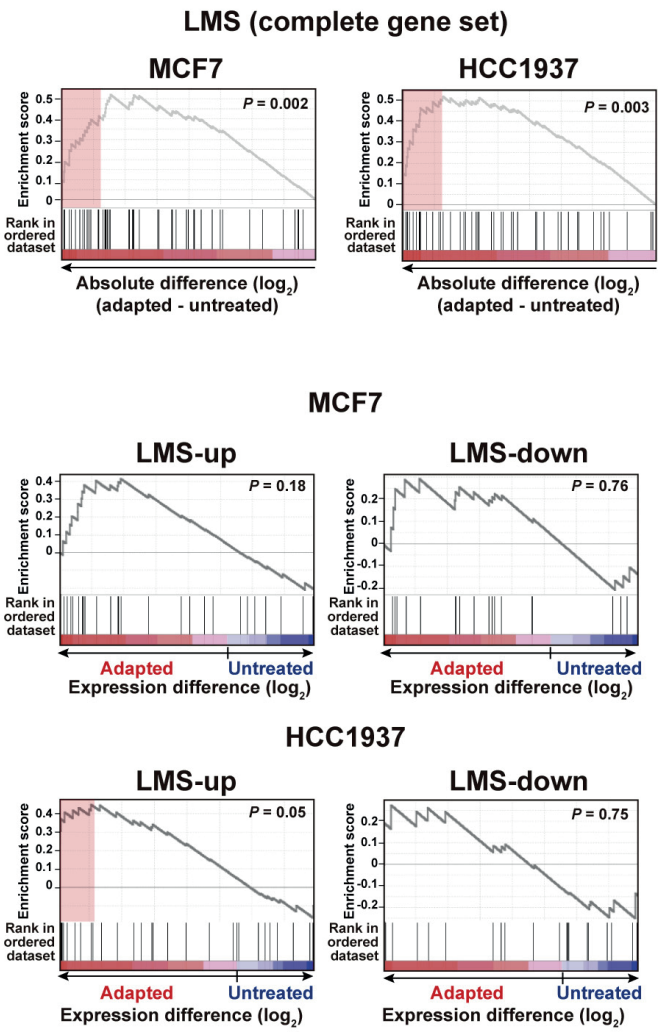

LMS (complete gene set)
MCF7
HCC1937
Enrichment score
Rank in ordered dataset
P = 0.002
P = 0.003
Absolute difference (log2) (adapted - untreated)
MCF7
LMS-up
LMS-down
P = 0.18
P = 0.76
Adapted
Untreated
Expression difference (log2)
HCC1937
LMS-up
LMS-down
P = 0.05
P = 0.75

Figure S4

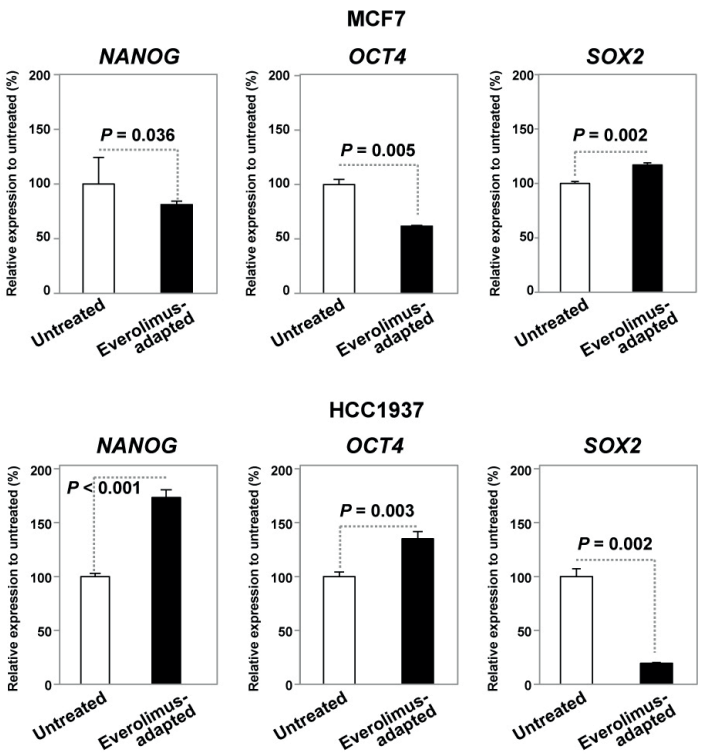

MCF7
NANOG
OCT4
SOX2
Relative expression to untreated (%)
200
150
100
50
0
P = 0.036
P = 0.005
P = 0.002
Untreated
Everolimus-adapted
HCC1937
NANOG
OCT4
SOX2
P < 0.001
P = 0.003
P = 0.002
Untreated
Everolimus-adapted

Figure S5

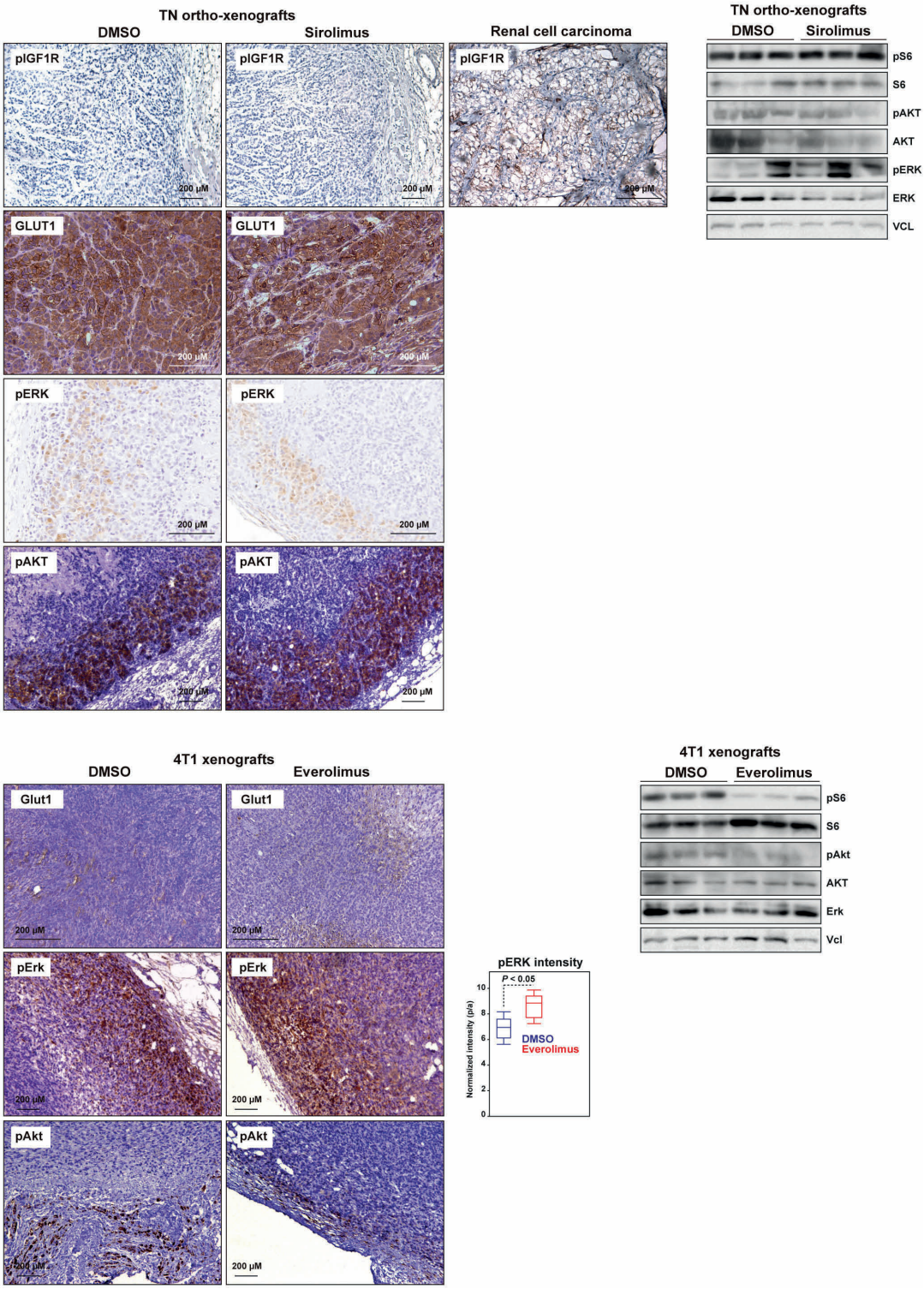

TN ortho-xenografts
DMSO
Sirolimus
Renal cell carcinoma
pIGF1R
GLUT1
pERK
pAKT
200 µM
TN ortho-xenografts
DMSO
Sirolimus
pS6
S6
pAKT
AKT
pERK
ERK
VCL
4T1 xenografts
DMSO
Everolimus
Glut1
pErk
pAkt
pERK intensity
P < 0.05
Normalized intensity (pix)
DMSO
Everolimus
4T1 xenografts
DMSO
Everolimus
pS6
S6
pAkt
AKT
Erk
Vcl

Figure S6

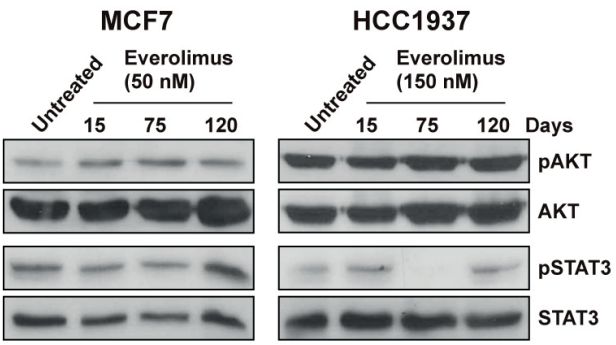

MCF7
HCC1937
Untreated
Everolimus (50 nM)
Everolimus (150 nM)
15
75
120
Days
pAKT
AKT
pSTAT3
STAT3

Figure S7

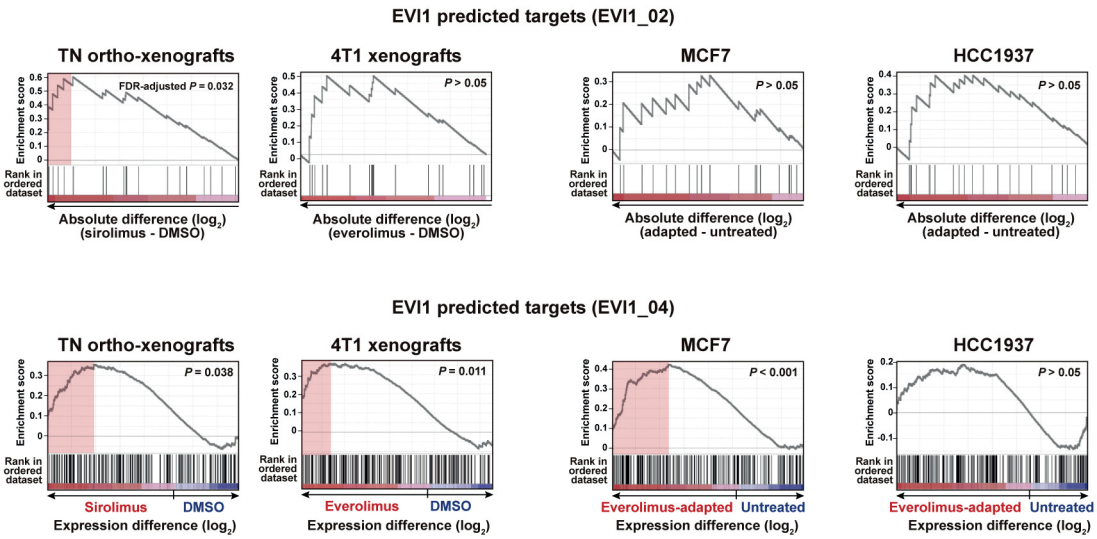

EVI1 predicted targets (EVI1_02)
TN ortho-xenografts
FDR-adjusted P = 0.032
Enrichment score
Rank in ordered dataset
Absolute difference (log2) (sirolimus - DMSO)
4T1 xenografts
P > 0.05
Absolute difference (log2) (everolimus - DMSO)
MCF7
P > 0.05
Absolute difference (log2) (adapted - untreated)
HCC1937
P > 0.05
Absolute difference (log2) (adapted - untreated)
EVI1 predicted targets (EVI1_04)
TN ortho-xenografts
P = 0.038
Sirolimus
DMSO
Expression difference (log2)
4T1 xenografts
P = 0.011
Everolimus
DMSO
Expression difference (log2)
MCF7
P < 0.001
Everolimus-adapted
Untreated
Expression difference (log2)
HCC1937
P > 0.05
Everolimus-adapted
Untreated
Expression difference (log2)

Figure S8

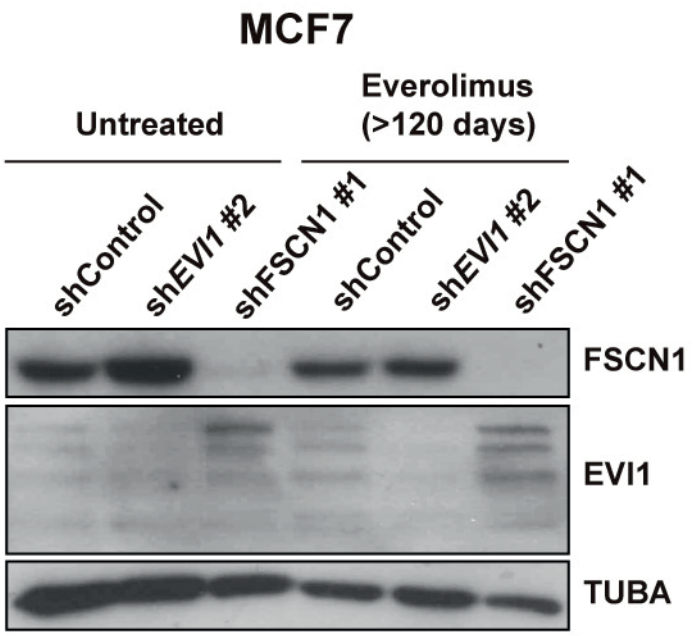

MCF7
Untreated
Everolimus
(>120 days)
shControl
shEVI1 #2
shFSCN1 #1
shControl
shEVI1 #2
shFSCN1 #1
FSCN1
EVI1
TUBA

Figure S9

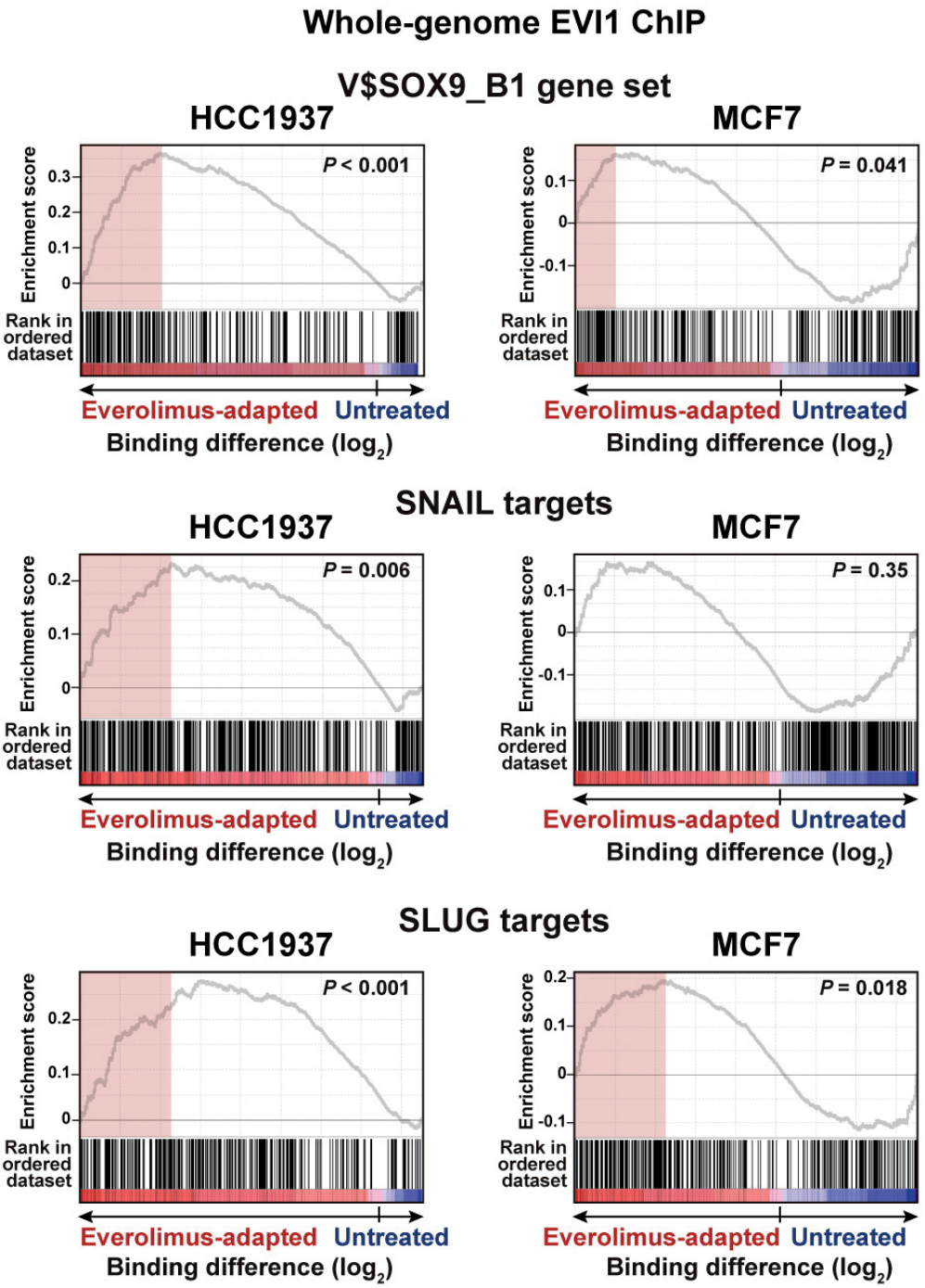

Whole-genome EVI1 ChIP
V$SOX9_B1 gene set
HCC1937
MCF7
P < 0.001
P = 0.041
Enrichment score
Rank in ordered dataset
Everolimus-adapted
Untreated
Binding difference (log2)
SNAIL targets
HCC1937
MCF7
P = 0.006
P = 0.35
SLUG targets
HCC1937
MCF7
P < 0.001
P = 0.018

Figure S10

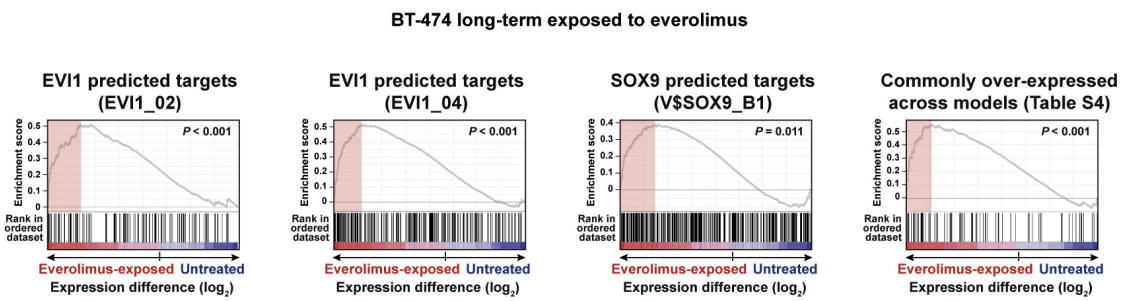

BT-474 long-term exposed to everolimus
EVI1 predicted targets (EVI1_02)
EVI1 predicted targets (EVI1_04)
SOX9 predicted targets (V$SOX9_B1)
Commonly over-expressed across models (Table S4)
Enrichment score
P < 0.001
P = 0.011
Rank in ordered dataset
Everolimus-exposed
Untreated
Expression difference (log2)

Figure S11

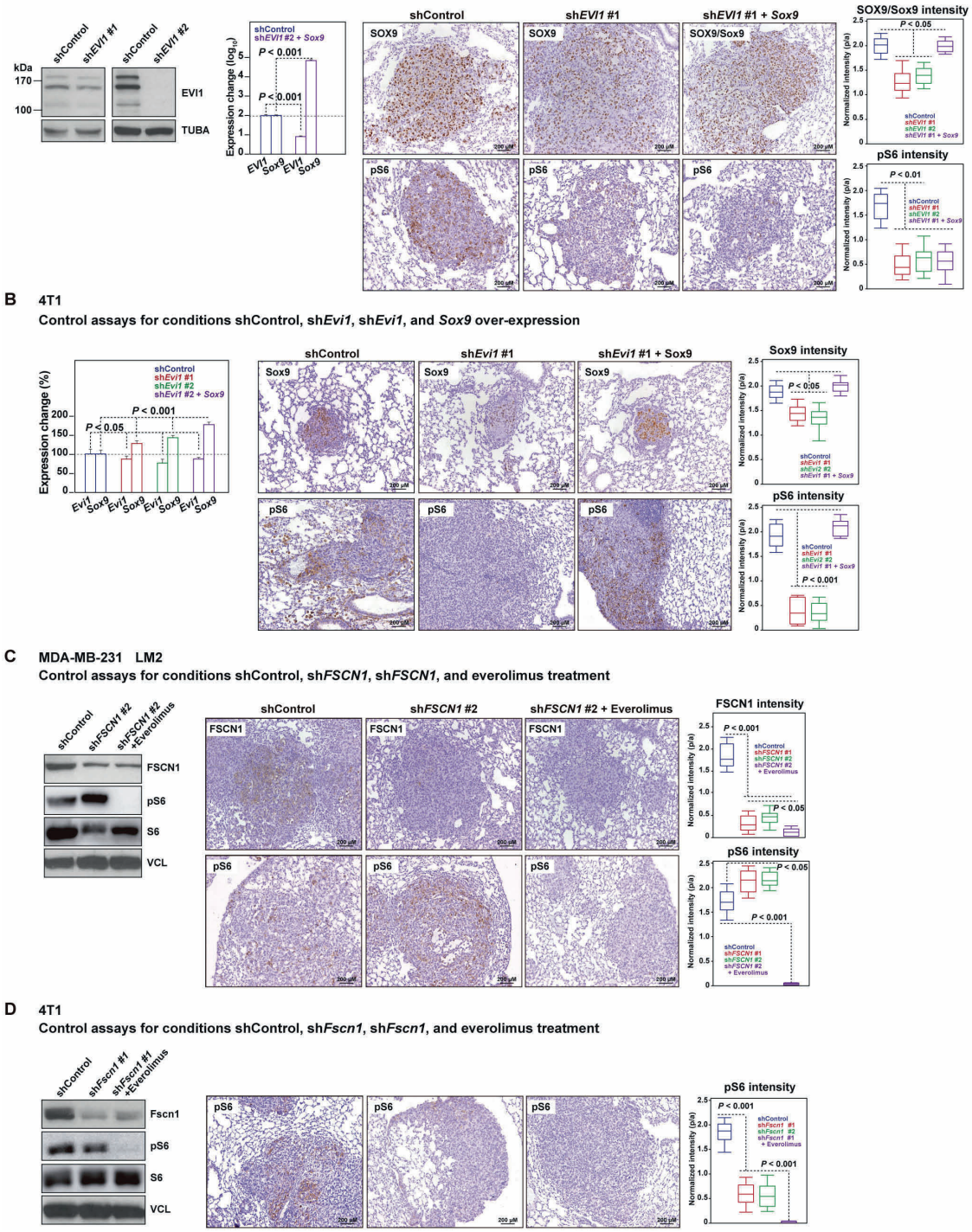
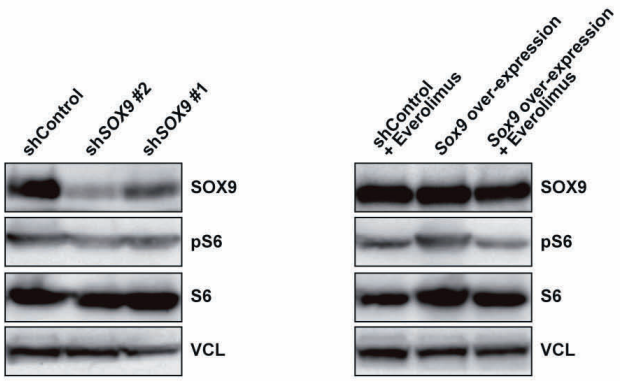

A
MDA-MB-231 LM2
Control assays for conditions shControl, shEVI1, and Sox9 over-expression
shControl
shEVI1 #1
shEVI1 #2
kDa
170
100
EVI1
TUBA
shControl
shEVI1 #2 + Sox9
P < 0.001
P < 0.001
Expression change (log2)
EVI1
Sox9
EVI1
Sox9
shControl
shEVI1 #1
shEVI1 #1 + Sox9
SOX9
SOX9
SOX9/Sox9
pS6
pS6
pS6
SOX9/Sox9 intensity
P < 0.05
Normalized intensity (p/a)
shControl
shEVI1 #1
shEVI1 #2
shEVI1 #1 + Sox9
pS6 intensity
P < 0.01
B
4T1
Control assays for conditions shControl, shEvi1, shEvi1, and Sox9 over-expression
shControl
shEvi1 #1
shEvi1 #2
shEvi1 #2 + Sox9
P < 0.001
P < 0.05
Expression change (%)
Evi1
Sox9
shControl
shEvi1 #1
shEvi1 #1 + Sox9
Sox9
Sox9
Sox9
pS6
pS6
pS6
Sox9 intensity
P < 0.05
shControl
shEvi1 #1
shEvi2 #2
shEvi1 #1 + Sox9
pS6 intensity
P < 0.001
C
MDA-MB-231 LM2
Control assays for conditions shControl, shFSCN1, shFSCN1, and everolimus treatment
shControl
shFSCN1 #2
shFSCN1 #2 +Everolimus
FSCN1
pS6
S6
VCL
shControl
shFSCN1 #2
shFSCN1 #2 + Everolimus
FSCN1
FSCN1
FSCN1
pS6
pS6
pS6
FSCN1 intensity
P < 0.001
P < 0.05
shControl
shFSCN1 #1
shFSCN1 #2
shFSCN1 #2
+ Everolimus
pS6 intensity
P < 0.05
P < 0.001
D
4T1
Control assays for conditions shControl, shFscn1, shFscn1, and everolimus treatment
shControl
shFscn1 #1
shFscn1 #1 +Everolimus
Fscn1
pS6
S6
VCL
pS6
pS6
pS6
pS6 intensity
P < 0.001
P < 0.001
shControl
shFscn1 #1
shFscn1 #2
shFscn1 #1
+ Everolimus

Figure S12

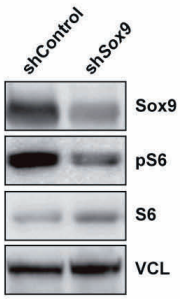
A **LM2**

Control assays for conditions shControl, sh*Sox9* + Everolimus



B **4T1**

Control assays for conditions shControl and sh*Sox9*



SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Ortho-xenograft characterization. (**a**) Model derived from a patient with a germline *BRCA1* mutation; the tumor ortho-xenograft is therefore homozygous for the mutation (arrow). (**b**) Phenotypic characterization of the ortho-xenograft showing a triple-negative receptor status and positivity for cytokeratin 19 (CK19). (**c**) Low expression of tuberin in tissue extracts from the ortho-xenograft compared to those from MCF7 cells. (**d**) Top panels, positivity for pS6, p4EBP1 and KI67 at the invasive front (arrows) of the ortho-xenograft tumor. Bottom panels, positivity for pS6 and p4EBP1 in the lung metastases generated by the ortho-xenograft.

Figure S2. Gene expression changes of the LMS sub-sets with *in vivo* exposure to mTOR inhibitors. GSEA graphical outputs for the association between the LMS-up or LMD-down (up-regulated and down-regulated sub-sets, respectively, as originally described) and the real gene expression differences between sirolimus/everolimus- and DMSO-untreated xenografts. The GSEA enrichment score and the nominal *P* values are shown. The genes contributing to the significant associations are listed.

Figure S3. Gene expression changes of the LMS set and sub-sets with *in vitro* exposure to mTOR inhibitors. GSEA graphical outputs for the association between the complete LMS (based on absolute gene expression differences), or the LMS-up and LMD-down sub-sets (based on the real gene expression differences) analysis between everolimus-adapted and untreated MCF7 and HCC1937 cells. The GSEA enrichment score and the nominal *P* values are shown.

Figure S4. Quantitative gene expression analysis of *NANOG*, *OCT4* and *SOX2* in cell models. The results are shown for MCF7 (top panels) and HCC1937 (bottom panels) untreated and everolimus-adapted cells and *P* values correspond to two-tailed *t*-tests.

Figure S5. Immunohistochemical and Western blot analyses of markers involved in mechanisms of resistance. The results are shown for ortho-xenografts (top panels) and 4T1 xenografts (bottom panels). Among the assessed markers, a significant over-expression of pERK was detected in everolimus-treated 4T1 xenografts (the corresponding antibody was not useful in Western blot analyses as it showed unspecific bands). Despite decreased tumor growth, pS6 is not reduced in the ortho-xenografts treated with sirolimus; however, it is reduced in the 4T1 xenografts treated with everolimus.

Figure S6. Western blot analysis of markers involved in mechanisms of resistance. Western blot results for total and pAKT, and total and pSTAT3, in MCF7 and HCC1937 cell extracts corresponding to untreated or everolimus-treated cultures.

Figure S7. Expression changes of predicted EVI1 target gene sets upon resistance/adaptation to allosteric mTOR inhibition in breast cancer models. Top panels, GSEA graphical outputs for the association between the EVI1_02 Transfac predicted targets and the absolute expression differences in each model. Bottom panels, GSEA graphical outputs for the association between the EVI1_04 Transfac predicted targets and the real expression differences in each model. The GSEA enrichment score and the nominal *P* value are shown.

Figure S8. Effect of FSCN1 depletion on EVI1 expression. Depletion of FSCN1 leads to an increase of EVI1 in both the parental and everolimus-adapted settings.

Figure S9. Correlation between EVI1 and SOX9 binding sites, and between EVI1 and SNAIL or SLUG targets. Top panels, GSEA graphical outputs for the association between predicted SOX9 targets (based on Transfac SOX9_B1) and the EVI1 binding differences (ChIP data) between everolimus-adapted and untreated HCC1937 (left panel) or MCF7 (right panel) cells. Middle and bottom panels show results for SNAIL and SLUG targets, respectively. The GSEA enrichment score and the nominal *P* values are shown.

Figure S10. Expression changes of BT-474 cells long-term exposed to everolimus. GSEA graphical outputs for the association between the EVI1_02, EVI1_02, and SOX9_B1 Transfac predicted targets, and the commonly over-expressed genes across the *in vivo* and *in vitro* models analyzed in this study. The GSEA enrichment score and the nominal *P* value are shown.

Figure S11. Control molecular results from the *in vivo* assays, first part. (**a**) Results for assays of LM2 cells transduced with shControl, sh*EVI1*, and *Sox9* over-expression vector. Left to right, results for EVI1 depletion (Western blot assays), *EVI1* depletion and *Sox9* over-expression (quantitative gene expression assays), SOX9/Sox9 and pS6 expression changes (immunohistochemical assays), and SOX9/Sox9 and pS6 immunohistochemical quantifications. Thus, EVI1 depletion is shown to lead to a significant reduction of both SOX9 and pS6 expression. (**b**) *Evi1* expression depletion in 4T1 cells causes reduced expression of both Sox9 and pS6, and concurrent Sox9 rescues pS6 to control levels. (**c**)

FSCN1 depletion in LM2 cells causes an increase of pS6 (but a decrease of total S6) that is prevented by exposure to everolimus. (**d**) Fscn1 depletion in 4T1 cells causes a decrease of pS6 (but a slight increase of total S6) and exposure to everolimus further reduces this signaling marker.

Figure S12. Control molecular results from the *in vivo* assays, second part. (**a**) Western blot results for assays of LM2 cells transduced with shControl or sh*SOX9*, over-expressing *Sox9* and/or treated with everolimus. (**b**) Western blot results for assays of 4T1 cells transduced with shControl or sh*Sox9*.

CHAPTER 4. DISCUSSION

4.DISCUSSION

The work presented in this thesis provides novel and clinically relevant insights into the mechanisms of BC metastasis and chemoresistance. Herein, we describe that tumor initiation features are coupled to BC metastasis and chemoresistance. First, we described RARRES3 as a tissue-specific BC lung metastatic suppressor. We show that RARRES3 prevents adhesion to the lung parenchyma and the initiation of metastatic lesions by enforcing the retention of differentiation features. Second, we identified that SC-like gene mediators EVI1 and SOX9 are critical players in mTOR chemoresistance and that they lead to an aggressive phenotype. Therefore, tumor initiation features are central drivers in cancer progression and therapeutic resistance.

4.1 Tumor initiation properties drive human lung BC metastasis

In this thesis we provide strong evidence that tumor initiation features are coupled to BC metastasis. Through experimental models *in vivo*, we demonstrate that the RA responder gene *RARRES3* impairs metastatic initiation by regulating differentiation. Therefore, differentiation attributes block metastatis in BC. This evidence is indirectly supported in the literature, as tumor initiation features have been extensively associated with metastatic phenotypes (Oskarsson *et al.*, 2014) in several types of cancer, such as those affecting the colon (Hermann *et al.*, 2007; Pang *et al.*, 2010), breast (Oskarsson *et al.*, 2011), pancreas (Dieter *et al.*, 2011) , blood (Visvader and Lindeman, 2008), brain (Guo *et al.*, 2011) and skin (Rappa *et al.*, 2008). In addition, a hierarchical model for BC metastasis has been described, in which metastases are initiated by stem-like cells that proliferate and differentiate to produce advanced metastatic disease (Lawson *et al.*, 2015). However, although a growing body of data points to the critical role of metastasis initiation in BC, few suppressor genes have been reported, as more attention has been devoted to oncogenes. For instance, there is no comprehensive illustration of diverse cellular processes regulated by metastasis suppressors during the metastasis cascade. Although an increasing number of TSGs involved in metastasis have been described using functional genomic techniques (Cancer Genome Atlas, 2012; Cancer Genome Atlas Research, 2008; Network, 2013), the relationship between TSGs and cancer risk is unclear. We illustrate the importance of metastasis suppressor genes for our understanding of cancer metastasis (Zhao *et al.*, 2015). Here we report on *RARRES3* as a key lung metastasis suppressor gene in BC.

4.2 RARRES3 blocks metastatic initiation

Of the genes identified in the LMS (Minn *et al.*, 2005a), we validated the RA responder gene *RARRES3* in BC experimental models as a lung metastasis suppressor gene. Using the ER- MDA-MB-231 breast cancer cell line model and lung metastatic derivatives, we functionally validated that *RARRES3* loss of expression confers a selective advantage for the colonization of the lung *in vivo* using xenografts. In contrast, RARRES3 gain of function in the lung metastaticderivative, LM2, suppressed lung colonization *in vivo*. Additionally, we extended our results to an immunocompetent syngeneic model, as well as using the CN37 patient-derived tumor xenograft model (**Figure 3, chapter 3.1**). Interestingly, *RARRES3* silencing engages metastasis-initiating capabilities by facilitating extravasation and adhesion of the tumor cells to the lung (**Figure 4B, C and D**). Furthermore, RARRES3 phospholipase A1/A2 activity contributes to tumor cell differentiation, thereby blocking lung metastasis, as demonstrated in 3D organotypic cultures and by a re-initiation assay *in vivo* (**Figure 6**). Hence, differentiation attributes are pivotal to prevent metastasis.

RARRES3 behaves *in vivo* as a classical metastasis suppressor gene, as it inhibits the metastasis process without preventing primary tumor formation (**Figure 2D**). In this scenario, *RARRES3* overexpression did not prevent primary growth when cells were injected into the mammary fat pat. Our data provide novel insights into the critical role of retinoid signaling in metastasis, as *RARRES3* is a RA responder gene. BC cells with low levels of *RARRES3* are selected for colonization of the lungs and then they metastasize. Consistent with our data, retinoid signaling is a critical cancer modulator that regulates genes involved in differentiation, apoptosis, cell cycle arrest and proliferation (Tang and Gudas, 2011). For instance, retinoid signaling is a major regulator of various pathways in BC. A key enzyme in RA metabolism is ALDH, which oxidizes intracellular aldehydes and plays a role in early differentiation of SCs though conversion of retinol to RA (Sophos and Vasiliou, 2003). Therefore, RA inducers are potential candidates to treat cancer. In fact, ATRA is considered the first example of targeted cancer therapy since 1997 , specifically for the treatment of AML (Tallman *et al.*, 1997), and it has been further validated (de The and Chen, 2010; Huang *et al.*, 1988; Sanz and Lo-Coco, 2011).

Importantly, ATRA treatment impairs BC tumor initiation features by inducing differentiation (Ginestier *et al.*, 2009) and blocking expansion/proliferation and invasiveness (Mangiarotti *et al.*, 1998; Van heusden *et al.*, 1998). ATRA can modulate SC gene expression programs by regulating self-renewal remodeling linked to the PRC2 EZH2 network (Ginestier *et al.*, 2009). Additionally, ATRA alters AKT/ β-catenin activity (Ginestier *et al.*, 2009), arrests BC cells by regulating the EGF pathway (Tighe and Talmage, 2004), impairs HER2 phosphorylation (Fontana *et al.*, 1992; Paroni *et al.*, 2012; Valette and Botanch, 1990), and IGF1/2 growth activity (Fontana *et al.*, 1991; Oh *et al.*, 2010), and modulates Wnt function (Mulholland *et al.*, 2005) to the same extent as estradiol (Easwaran *et al.*, 1999). In addition, ATRA reduces the invasiveness of TBNC cell line MDA-MB-231

through notch and PI3K inhibition (Farias *et al*., 2005) and imposes long-term inhibition of AKT (Paroni *et al*., 2012). Furthermore, ATRA effector function can be enhanced by TGFβ1 activity (Fontana *et al*., 1992; Valette and Botanch, 1990). Interestingly, the ATRA and HER2 signaling pathways are interconnected. HER2 overexpression causes ATRA resistance via AKT (Tari *et al*., 2002), and trastuzumab increases RAR/RXR binding via AKT stimulation (Siwak *et al*., 2003). Recently, a synergistic effect of ATRA and lapatinib (HER2 antibody) was observed (Fisher *et al*., 2015). ATRA resistance is also associated with PTEN suppression (Stefanska *et al*., 2012).

Furthermore, ATRA inhibits aggressiveness of TBNC cell lines through the p38 MAPK pathway (Wang *et al*., 2013a). In fact, RARα and RARγ are downstream targets of p38-MAPK (Alsayed *et al*., 2001; Gianni *et al*., 2012), and ATRA regulates p38 functions (Alsayed *et al*., 2001). Importantly, dual application of p38 inhibitors and ATRA have synergistic activity in AML models (Garattini *et al*., 2014), and given the importance of p38 in BC dormancy (Sosa *et al*., 2011), it would be relevant to extend this observation in BC. Dual therapy using p38 inhibitors (Garattini *et al*., 2014), AKT inhibitors or HER2 antibodies (Fisher *et al*., 2015) could be a useful strategy to treat ATRA-resistant BC. Importantly, ATRA has a beneficial impact in advanced BC patients participating in clinical trials (Budd *et al*., 1998; Connolly *et al*., 2013), thereby suggesting that differentiation therapy is useful to tackle cancer. We report evidence that by inducing RA-signaling molecules, such as RARRES3, metastasis could be impaired. In addition, differentiation therapies might be useful to overcome chemoresistance, as they stop different cancer-driving molecules.

We show that the strong requirement of the $PLA_{1/2}$ catalytic activity of RARRES3 to sustain differentiation reflects, in part, an increase in PPAR activity, as seen in our luciferase assay using the 3x-AOX peroxisome proliferation response element reporter (**Figure 5D**), and in lung metastatic lesions quantified by IHC (**Figure 2A and B**). PPAR signaling provides a survival advantage to BC cells upon loss of attachment (Carracedo *et al*, 2012). In fact, retinoids can trigger proliferation by activating PPARβ/γ (Schug *et al*., 2007; Schug *et al*., 2008). Our data suggest no differences in apoptosis or proliferation upon RARRES3 modulation. ATRA has been linked to the inhibition of proliferation in experimental models of BCs (Wei *et al*., 2015). In agreement, other reports using our ER^- MDA-MB-231 model observed that RARRES3 perturbs cell proliferation, cell cycle (facilitating the transition from G2/M phase to S phase), and apoptosis *in vitro* (Hsu *et al*., 2015). Collectively, these data are not consistent, yet these reports suggest an antiproliferative effect of RARRES3 sustained by *in vitro* models. For instance, in our hands apoptosis was quantified *in vivo* by injecting the caspase-3/7 substrate (Z-DEVD-aminoluciferin sodium salt) (**Figure 4B**), and proliferation was quantified by Ki67 IHC in lung sections (**Figure 3A and B**). In addition, we did not see enhanced proliferation after injection of low or high *RARRES3*-expressing cells into the primary site (MFP) (**Figure 1D**). These observations would suggest that, in fact, in our experimental model of metastasis, proliferation is not affected upon *RARRES3* modulation. Therefore, our findings support the notion that RARRES3 prevents the initial steps of lung colonization and

behaves as a metastatic suppressor gene, without affecting proliferation or apoptosis.

We showed that *RARRES3* expression regulates differentiation and adhesion in experimental models of ER$^-$ BC. We demonstrated that RARRES3 phospholipase catalytic activity engages differentiation in several models, an observation confirmed by others in later studies (Hsu *et al.*, 2015). This study reported that RARRES3 suppresses tumor initiation features and EMT in ER$^-$ MDA MB-231 BC cells and, contrary to our expectations, in the ER$^+$ MCF7 BC cell line. *RARRES3* overexpression was shown to decrease oncosphere formation, as we reported (**Figure 6C and D**), and partially deplete the TIC CD44$^+$/CD24^{low} subpopulation in both ER$^+$ and ER- BC cell lines. These observations are consistent with the fact that RARRES3 suppresses EMT and that SC-like features have been associated with EMT in normal and neoplastic human breast. EMT generates cells with SC-like properties (Mani *et al.*, 2008), and CD44$^+$/CD24- BC cells exhibit an EMT signature (Mani *et al.*, 2008). In addition, a novel metastasis-associated protein was also found to promote differentiation, and its downregulation is specifically associated with early occurring bone metastasis in ER$^+$ BC. Interestingly, like *RARRES3*, this gene, called *RAI2*, is related to retinoid signaling, and both maintain the integrity of breast epithelium. In fact, downregulation of *RAI2* causes *GATA3* repression (Werner *et al.*, 2015). *RAI2* downregulation leads to loss of epithelial differentiation, a process that is a key event for early steps of the metastatic cascade as it induces cellular plasticity, dissemination and dedifferentiation. RAI2 regulates the gene expression of the bona fide luminal genes *FoxA1*, *ER*α and *GATA3*. Their phenotypes recapitulate that observed upon *RARRES3* modulation (**Figure 5G, 6E, and 6F**), where an increase in *GATA3* expression was observed upon *RARRES3* overexpression in LM2 cells. In addition, we detected an increase in *GATA3* expression in lung tumors from mice injected with *RARRES3*-overexpressing cells, and a positive correlation between *RARRES3* and *GATA3* expression levels in ER- BC primary tumors, which is associated with good prognosis in BC (Albergaria *et al.*, 2009).

Retinoid signaling is essential for the maintenance of SC biology and cancer development. Interestingly, ATRA inhibits proliferation of ER$^+$, HER2$^+$ and TNBC human cells through the ablation of Pin1, a gene/protein that regulates the expression of various critical oncoproteins, such as ERα, HER2, cycling D1 and AKT (see supplementary Figure 11 from (Wei *et al.*, 2015). Since Pin1 regulates BCSC expansion (Luo *et al.*, 2014, Rustighi *et al.*, 2014), and ATRA inhibits Pin1, the effect of ATRA on BC growth could be explained by the abolishment of tumor-initiating features. Pin1 also activates ERK phosphorylation, leading to BCSC expansion and poor prognosis in BC patients (Luo *et al.*, 2015). In addition, *RAR* coincides with luminal differentiation genes such as *FOXA1* and *GATA3* (Hua *et al.*, 2009). Therefore, we propose that *RARRES3*, as a RA responder gene, modulates differentiation by regulating key luminal differentiation genes (*i.e. GATA3*), a notion that was confirmed in our experimental model. ATRA also modulates the expression of the EZH2 chromatin modulator and pluripotency gene marker (Sparmann and van Lohuizen,

2006). Indeed, we confirmed an inverse correlation between *RARRES3* and *EZH2* in primary tumor samples from humans (Figure 5G). We also corroborated that *RARRES3* negatively regulates *EZH2* expression in metastatic lesions and *in vitro* (**Figure 6D**). Therefore, RARRES3 is crucial for cell fate specification, as it regulates *GATA3* (Kouros-Mehr *et al.*, 2008{Dydensborg, 2009 #8110). In addition, *RARRES3* is critical for the maintenance of a SC phenotype—which is associated with self-renewal and metastatic re-initiation—as it regulates *EZH2* expression (Al-Hajj *et al.*, 2003; Dontu *et al.*, 2003; Li *et al.*, 2007; Liao *et al.*, 2007). Collectively, these lines of evidence confirm and complement our observations regarding the phenotype upon *RARRES3* modulation.

Next, we explored the role of RARRES3 only in ER^- BC, as it was first identified in the LMS (Minn *et al.*, 2005a). We demonstrated that low expression of *RARRES3* in primary tumors was significantly associated with the risk of lung metastasis in BC patients (**Figure 1B**), and this risk was specific for the ER^- tumors. In contrast, *RARRES3* expression was not associated with risk of bone or brain metastasis (**Supplementary Figure S1B and C**). Herein, we propose that *RARRES3* expression is important only in the ER^- BC and for lung metastasis. However, given the recent discovery of RAI2, which induces differentiation and blocks bone metastasis in a ER^+ BC model (Werner *et al.*, 2015), and RARRES3 suppression of $CD44^+/CD24^{Low}$ TIC subpopulation in MCF7 BC cells (Werner *et al.*, 2015), further work to explore the role of RARRES3 in ER^+ BC and in bone metastasis should be pursued.

We functionally validated *RARRES3* as a lung metastatic suppressor gene in BC, showing that its $PLA_{1/2}$ catalytic activity engages differentiation. Therefore, our data are based on a functional approach, without giving insight into the biochemical regulation upon RARRES3 modulation. Thus, a key issue is how RARRES3 interplays with various signaling pathways to regulate diverse biological processes. RARRES3 is regulated by RA, as well as by other signals, such as p53 (Hsu *et al.*, 2012; Tsai *et al.*, 2007), thereby pointing to another layer of regulation. In addition, it has been suggested that RARRES3 regulates several survival and self-renewal pathways, such as JNK, ERK and PI3K/AKT, in order to trigger apoptosis and cell suppression (Ou *et al.*, 2008; Tsai *et al.*, 2007). RARRES3 is a phospholipase $A_{1/2}$ and it has recently been proposed to exert acyltransferase activity in BC. RARRES3, through its activity mediates the protein deacylation of canonical Wnt/β-catenin, H-RAS, AKT and ERK signaling molecules, which ultimately leads to an impairment of tumor growth, metastatic capacity, and, more importantly, to the abolishment of tumor initiation features (Hsu *et al.*, 2015). Decreased acylation of Wnt molecules disrupts their protein expression level and subcellular localization, which results in Wnt/β-catenin blockade. We found that RARRES3 $PLA_{1/2}$ catalytic activity is central to its differentiation function, and we believe that RARRES3 may trigger deacetylation in key developmental pathways described to be pivotal in mammary tumorogenesis and SC biology (Moreno, 2010; Ramaswamy *et al.*, 2012; Wickremasinghe *et al.*, 2011).

Importantly, RARRES3 regulates the acylation status of AKT, a kinase that is essential for survival (Hsu *et al.*, 2015). Recently, activation of the AKT/mTOR pathway and RA signaling have been associated (Werner *et al.*, 2015). In this report, RAI2-depleted cells exhibited increased pAKT activation and resistance to mTOR inhibitors such as RAD001 (everolimus). Interestingly, in combination with exemestane, everolimus has already been approved to treat postmenopausal ER^+ advanced BC patients (Baselga *et al.*, 2012). In addition, an AKT inhibitor (MK-2206) is under phase II clinical trials to treat advanced BC (*see https://clinicaltrials.gov: AKT inhibitor MK2006 in treating patients with advanced BC*) (Ma *et al.*, 2016). Therefore, RA signaling genes such as *RARRES3* emerge as potential therapeutic targets. For instance, RA genes may be used to predict the response to AKT-mTOR-targeted therapeutic strategies. Accordingly, *RARRES3* expression could find use in stratifying patients for clinical benefit, and further studies to evaluate this clinical application are required.

The modulation of AKT has more implications. AKT is essential for the survival of dormant DTCs and CTCs (Bidwell *et al.*, 2012; Douma *et al.*, 2004; Kang and Pantel, 2013) and regulates the proliferation of these cell types (Grabinski *et al.*, 2011). Given that RARRES3 regulates AKT activity, the bone marrow is a RA-enriched environment, and RA regulates differentiation of HSCs in this niche (Ghiaur *et al.*, 2013; Purton *et al.*, 2006; Shiozawa *et al.*, 2011), we hypothesized that RARRES3 represents a key event at the onset of dissemination, regulating DTC survival in the BM. Therefore, understanding the molecular biology underlying metastasis-suppressing proteins, such as RARRES3, offers a potential mechanistic insight that could be translated into novel therapeutic approaches (Smith and Theodorescu, 2009).

In summary, the catalytic activity of RARRES3 $PLA_{1/2}$ restrains the lung metastatic capacity of BC cells, blocking colonization and metastatic initiation in ER^- BC experimental models.

4.3 RARRES3 is a tissue-specific suppressor of lung metastasis

We demonstrated that RARRES3 contributes to tumor differentiation by suppressing metastatic initiation in the lung, as seen by an *in vivo* re-initiation assay. In addition, we showed that differentiation was driven by the catalytic activity of phospholipase A1/A2, as seen in our oncosphere formation assays and in experiments inducing differentiation in murine embryonic SCs (**Figure 6**). However, the loss of RARRES3 phospholipase A1/2 catalytic activity cannot explain why low *RARRES3* expressing ER^- cells have a preference for the lung parenchyma. For instance, *RARRES3* overexpression in cells blocks adhesion to fibronectin and collagen-I coated plates *in vitro*. In addition, RARRES3 blocks extravasation to the lung parenchyma *in vivo* (**Figure 3B, C, and D**). Hence, RARRES3 blocks metastasis in a tissue-specific manner. Interestingly, a recent study shows that RARRES3 suppresses metastasis by interacting with MTDH. This interaction blocks EMT in a

model of CRC (Wang *et al*., 2015c). Because MTDH can promote metastasis through the induction of EMT (Tang *et al*., 2014; Zhu *et al*., 2011) and RARRES3 can suppress MTDH expression, it was proposed that RARRES3 suppresses EMT by modulating β-catenin translocation. However, given the observations that RARRES3 regulates adhesion to the lung matrix, that RARRES3 interacts with MTDH (Wang *et al*., 2015c), and that MTDH is a well-known contributor to BC lung metastasis, facilitating lung adhesion (Brown and Ruoslahti, 2004; Hu *et al*., 2009a; Hu *et al*., 2009b), we proposed that the interaction between RARRES3 and MTDH contributes to tissue-specific metastasis to the lung by modulating adhesion. Interestingly, MTDH has a lung-homing domain which mediates cell adhesion to the lung vasculature (Brown and Ruoslahti, 2004) and which has been further validated in ER$^-$ BC MDA-MB-231 xenograft models (Hu *et al*., 2009a). This observation is clinically relevant, because MTDH is overexpressed in up to 40% of BC patients (Brown and Ruoslahti, 2004; Hu *et al*., 2009a; Kang *et al*., 2005; Li *et al*., 2008a), and its overexpression activates several important pathways in BC progression, such as PI3K/AKT, nuclear factor kB (NFkB), and Wnt/β-catenin to promote aggressiveness, survival and chemoresistance (Li *et al*., 2008a). In fact, MTDH blockade leads to the impairment of BC metastasis (Qian *et al*., 2011; Zhang *et al*., 2011a). The mechanism by which the interaction between MTDH and RARRES3 is regulated and the identification of the downstream signals will be important issues to be addressed in future studies.

On the basis of our results, we can infer that the transformation of a tumor cell into a metastatic cell is not just about acquiring capacity, but equally important is the loss of certain genes, such as *RARRES3*. Genes selected for metastasis contribute to the different steps of this process and represent the random accumulation of traits that provide the advantage necessary for adaptation to the microenvironment of a different organ. In summary, cancer cells need to acquire other functions besides tumor initiation to acquire tissue specificity. In this context, loss of RARRES3 activity provides two advantages by which the cells overcome two steps of the metastasis cascade (metastatic initiation and progression functions). First, BC cells expressing low levels of RARRES3 cells can extravasate and adhere specifically to the lung parenchyma. Second, cells can reinitiate a metastatic tumor by blocking differentiation attributes.

Our data demonstrate that *RARRES3* is a tissue-specific metastatic suppressor gene whose activation blocks lung metastasis in various *in vivo* models. More importantly, we validated our findings in 580 samples from primary breast tumors, demonstrating that *RARRES3* is suppressed in ER$^-$ tumors. Therefore, *RARRES3* levels in the primary tumor are clinically relevant and predict risk of relapse in the lung in ER$^-$ BC patients. *RARRES3* loss of expression may provide a marker to identify patients with greater susceptibility to lung metastasis. Identifying patients with a greater risk of metastasis could open up the avenue for novel therapeutic opportunities. Hence, these patients might benefit from a differentiation treatment (*i.e.* ATRA) in the adjuvant setting. In fact, the synergistic action of lapatinib and ATRA through RARRES3 has been observed *in vitro* (Paroni

et al., 2012). As mentioned above, RARRES3 contributes to differentiation over self-renewal, which suggests that low expression levels of this protein might predict therapy-resistant tumors, thus identifying patients that may require novel therapies targeting TICs. This observation is clinically important, as cells with SC-like features are crucial in the metastatic cascade and in relapse. Therefore, screening for compounds that activate RARRES3 may contribute to the development of novel differentiation-inducing strategies to tackle resistance in aggressive tumors. ATRA is used for the treatment of AML (Tallman *et al.*, 1997) and has beneficial effects in advanced BC patients in clinical trials (Budd *et al.*, 1998; Connolly *et al.*, 2013), and depletion of RA signaling molecules (*i.e.* RAI2) has been recently associated with risk of bone metastasis in ER^+ BC by regulating differentiation. These observations suggest that differentiation therapy may be useful to tackle BC. In summary, we provide novel evidence supporting the use of differentiation therapy to tackle BC, as RARRES3 activation may prevent lung metastasis. After removal of the primary tumor, some patients may benefit from treatment with RA to induce *RARRES3* expression, enhance cell differentiation, and prevent metastasis.

4.4 mTOR inhibition leads to an aggressive phenotype driven by tumor initiation features

Our results highlight the importance of tumor initiation in metastasis and resistance to mTOR, demonstrating that tumor initiation properties are linked to chemoresistance to mTOR inhibitors and metastatic potential. This is supported by transcriptional reprogramming, driven by the master regulator SC-like genes *EVI1* and *SOX9* (**Figure 7, chapter 3.2**). Collectively, these results confirm previous findings reported by others, in which SC-like properties cause chemoresistance in a variety of cancers (Gong *et al.*, 2010; Li *et al.*, 2008b). In addition, this observation supports the idea that tumor initiation properties are strongly associated with metastatic potential (Oskarsson *et al.*, 2014). Herein, we show that an everolimus-adapted MCF7 cell line exhibits an increase in $CD49f^{high}$ TIC subpopulation, while an adapted HCC1937 cell line shows an increase in $CD44^+/CD24^-$ cells (**Figure 2f**). These findings are coherent with the fact that TICs have chemoresistant properties and undergo asymmetric division (Gopalan *et al.*, 2013).

mTOR is pivotal for cancer growth and resistance to various treatments, such as chemotherapy, hormone therapy, and trastuzumab (Berns *et al.*, 2007; Zhou *et al.*, 2004). For instance, a synergistic/additive effect of mTOR inhibitors and chemotherapeutic agents (*i.e.* paclitaxel, vinorelbine) was reported (Mondesire *et al.*, 2004). Importantly, the mTOR inhibitor everolimus (RAD001) is currently used to overcome resistance to hormone therapy (exemestane) in ER^+ advanced metastatic BC (Baselga *et al.*, 2012). Furthermore, mTOR drives $HER2^+$ BC progression and is responsible for resistance to trastuzumab (Margariti *et al.*, 2011), and mTOR inhibitors exhibit a synergistic effect with trastuzumab in preclinical studies (Lu *et al.*, 2007). Interestingly, in a phase III clinical

trial (BOLERO-3), everolimus in combination with trastuzumab and vinorelbine had beneficial effects in advanced metastatic BC patients (Andre *et al.*, 2014). Unfortunately, patients eventually relapsed due to drug resistance, and the link between cancer progression and resistance to allosteric inhibition of mTOR is not fully understood. To this end, we explored the mTOR resistance mechanism by means of a de novo approach. By continuous exposure to mTOR inhibitors, we generated resistant clones, and an ulterior genomic analysis pointed towards the acquisition of *EVI1* and its downstream target SOX9 tumor initiator effector. Importantly, *EVI1* overexpression confers resistance to everolimus in MCF7 and HCC1937 cell lines *in vitro* (**Figure 4d**). However, whether *EVI1* and *SOX9* overexpression are sufficient to overcome resistance to mTOR inhibitors *in vivo* needs to be clarified, especially in the everolimus-sensitive LM2 xenograft model.

Our work provides novel insights into the link between tumor initiation and resistance to mTOR inhibitors, which lead to an aggressive phenotype in BC. We explored how these two properties are coupled in the context of mTOR inhibition. We revealed that transcriptional reprogramming supports lung metastasis in response to mTOR inhibition in BC, which is driven by the EVI1-SOX9 axis. Importantly, *EVI1* and *SOX9* expression are associated with SC-like and metastasis signatures, and their depletion impairs lung metastasis *in vivo* (**Figure 6**). These signatures promote the activation of mTOR pathway components (REHB and RAPTOR) and of lung metastasis mediators (**Figure 4f and g**). The results of our study expand on the previous description of stem/progenitor regulator SOX9 (Malhotra *et al.*, 2014) as a metastasis and tumor-initiating cell factor (Guo *et al.*, 2012a; Malladi *et al.*, 2016; Ye *et al.*, 2015). SOX9 is associated with poor prognosis in several cancer types (Camaj *et al.*, 2014; Chu *et al.*, 2014; Guo *et al.*, 2012b; Lu *et al.*, 2008a; Zhou *et al.*, 2011; Zhou *et al.*, 2012). We showed that EVI1 and SOX9 are central in BC resistance to mTOR inhibitors in lung metastasis as they trigger tumor initiation features. Nevertheless, whether this mechanism is specific to mTOR inhibition or whether it can be extrapolated to other therapies requires confirmation.

Relevant to our findings is whether *EVI1* expression is upregulated through genomic amplification and/or is regulated by biochemical modifications. Some of our data reveal that biochemical modifications can modulate EVI1 expression, as seen in our 4T1 *in vivo* syngeneic model, where ERK signaling activation was observed upon everolimus treatment (**Supplementary Figure 5**). It is known that mTOR inhibition can lead to hyperactivation of the MEK pathway through a positive feedback mechanism (Zoncu *et al.*, 2011), leading to an aggressive phenotype. Given that SOX9 tumor initiation functions are linked to EMT (Guo *et al.*, 2012a), activation of the latter increases ERK activation and resistance to rapamycin (Holder *et al.*, 2015), and SOX9 is augmented through ERK in another type of cancer (Ling *et al.*, 2011). It is plausible to postulate that ERK is the upstream molecule and that it reinforces SC-like features by activating EVI1-SOX9, thereby leading to chemoresistance in lung BC metastasis. In fact, some metastatic pancreatic adenocarcinomas

are reliant on oncogenic KRAS/MEK/ERK-induced hyperactive mTOR signaling (Kong *et al.*, 2016). These data support the use of dual inhibitors of mTOR/ERK in the clinical setting. Such inhibitors could find application in the treatment of BC and and in overcoming chemoresistance.

We also report that *EVI1* ChIP data showed a positive correlation with EMT transcriptional regulators *SLUG* and *SNAIL* (**Supplementary Figure 9**). Interestingly, EMT is central in embryonic development (Nieto, 2013), when cells require mesenchymal features in order to migrate and later form tissues and organs (Acloque *et al.*, 2009). In this regard, we and others show that EVI1 contributes to EMT (Dutta *et al.*, 2013; Stavropoulou *et al.*, 2016), which suggests that EVI1 regulates MaSC plasticity under physiological conditions.

Importantly, we demonstrate that mTOR inhibition leads to an aggressive phenotype with an increase in lung metastatic mediators such as FSCN1 (Figure 2), in both immunodeficient and immunocompetent syngeneic models. Interestingly, we observed that everolimus impaired lung colonization in our LM2-immunodeficient xenograft model whereas this effect was not observed in the 4T1-immunocompetent xenografts (**Figure 6c and 6d**). Therefore, it is plausible that the immune system plays a key role in metastasis progression upon mTOR inhibition in our models. In fact, mTOR is a critical modulator of the immune system (Powell *et al.*, 2012), and mTOR inhibition has a dramatic immunosuppressive effect, including on T-cells (Kopf *et al.*, 2007), NKs (Wai *et al.*, 2008), APCs (Hackstein *et al.*, 2003; Haidinger *et al.*, 2010; Monti *et al.*, 2003), neutrophils (Gomez-Cambronero, 2003) and B-cells (Benhamron and Tirosh, 2011). These immunosuppressive effects are mediated by the activation and expansion of $Foxp3^+$ Tregs (Battaglia *et al.*, 2005; Kang *et al.*, 2008; Kim *et al.*, 2015), MDSCs (Nakamura *et al.*, 2015), and stimulation of endothelial cells (Wang *et al.*, 2013b). Whether these effects occur in our immunocompetent 4T1 model needs to be addressed.

Collectively, the appearance of a cancer cell tumor initiation phenotype in therapy resistance is consistent with the emerging role of mTOR in SC biology (Maiese, 2015; Zhou *et al.*, 2007) , and it further expands on a recent observation of a SC program in metastatic BC cells (Lawson *et al.*, 2015). We can confirm that tumor initiation features lead to an aggressive phenotype in BC and that they cause chemoresistance to mTOR inhibitors. In this scenario, we propose that EVI1 and SOX9 functionally cooperate to sustain mTORC1 activity and metastatic potential. Consequently, we provide new insights into BC resistance to mTOR inhibition. We also show that EVI1-SOX9-mediated transcriptional reprogramming drives the molecular processes that support BC tumor initiation features and metastatic potential in therapeutic resistance (**Figure 7**). Our data have important

clinical implications, as mTOR inhibitors are currently used to treat endocrine ER$^+$ advanced metastatic BC, in which resistance to treatment is reported. We depict that a central mechanism of resistance is through transcriptional reprogramming and selection of an aggressive SC-like subpopulation, driven by the SC-like genes *EVI1* and *SOX9*.

CHAPTER 5. CONCLUSIONS

5.CONCLUSIONS

1. We have functionally validated *RARRES3* as a human metastasis suppressor gene in BC.

2. We have demonstrated that RARRES3 prevents tissue-specific lung metastasis by blocking metastatic initiation and lung adhesion.

3. We have demonstrated that metastasis initiation is prevented by RARRES3 by enforcing the retention of differentiation features, driven by its $PLA_{1/2}$ catalytic activity.

5. We have identified *EVI1* and *SOX9* as SC master regulators genes in BC.

6. We have shown that SC-like properties are linked to chemoresistance, particularly to mTOR inhibitors in BC.

7. We have demonstrated that tumor initiation features promote metastatic potential in therapeutic resistance, leading to a metastatic aggressive phenotype.

CHAPTER 6. BIBLIOGRAPHY

6. BIBLIOGRAPHY

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