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Estudio del Efecto Angiocrino Endotelial Sobre Células Epiteliales de Cáncer Prostático

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Por

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Tabla de contenidos

Abreviaciones

CaP	Cáncer de próstata
ADT	Terapia de deprivación androgénica
HUVEC	Células endoteliales de vena de cordón umbilical humano
AR	Receptor de andrógenos
T	Testosterona
DHT	Dihidrotestosterona
ARE	Elementos de respuesta a andrógenos
CRPC	Cáncer de próstata resistente a la castración
CAF	Fibroblastos asociados al cáncer
MSC	Células madre estromales
CAM	Macrófagos asociados al cáncer
EPC	Células precursoras endoteliales
CE	Células endoteliales
VEGF	Factor de crecimiento endotelial vascular
MVD	Densidad microvascular
PSA	Antígeno prostático especifico
TRAMP	Modelo de ratón transgénico de adenocarcinoma prostático

Resumen

El cáncer de próstata (CaP) es la segunda causa de muerte por cáncer en países occidentales, incluyendo Chile. La terapia de deprivación androgénica (ADT) continúa siendo el tratamiento estándar para el cáncer localmente avanzado o metastásico, sin embargo, como resultado de esta terapia, el CaP progresa desde un fenotipo sensible a andrógenos, hacia uno resistente a la ADT, el cual es frecuentemente letal. Datos previos indican que la progresión hacia un fenotipo resistente a la ADT está asociado a un aumento en la angiogénesis. Más aún, existe evidencia que sugiere que la masa tumoral prostática se encuentra bajo un estricto control de la microvasculatura, y que la perfusión de oxígeno y nutrientes por los capilares, estaría reforzada por un potente efecto paracrino (angiocrino) por parte de las células endoteliales. Estudios previos de nuestro laboratorio sugieren que las células endoteliales modularían el crecimiento de células tumorales mediante un mecanismo de tipo angiocrino, el cual es independiente del flujo sanguíneo. Sin embargo, hasta el momento se desconoce la extensión de este efecto angiocrino sobre células tumorales prostáticas, así como también, los mecanismos moleculares que subyacen estos efectos biológicos. Por lo tanto, la hipótesis central de esta tesis es: “Las células endoteliales promueven un aumento en las características que determinan agresividad tumoral en células de CaP a través de un mecanismo de tipo angiocrino”.

Para desarrollar esta tesis se utilizaron líneas celulares de CaP que representan el fenotipo sensible a andrógenos, resistente a la castración y metastásico, así como también, cultivos primarios de células endoteliales obtenidas de vena umbilical humana HUVEC (Human Umbilical Vein Endothelial Cell). En primer lugar, se analizó la expresión y funcionalidad del receptor de andrógenos (AR) para determinar la dependencia androgénica de estas células en el contexto de la biología celular del CaP. Se analizó a la vez los efectos biológicos de los andrógenos y el papel de la AR en estos efectos, principalmente en la proliferación y supervivencia de las células HUVEC y de las líneas celulares de CaP. Posteriormente, utilizando medios condicionados obtenidos de células HUVEC, se estudió el efecto de la estimulación paracrina sobre la viabilidad, proliferación, migración e invasión en las líneas celulares de CaP. Finalmente, se estudió la contribución de las células endoteliales sobre los procesos de migración y crecimiento tumoral *in vivo* usando los modelos de pez cebra y xenoinjertos en ratones inmunodeficientes, respectivamente. Además, se estudió el perfil de secreción de citoquinas de dos líneas celulares de CaP expuestas a medio condicionado de células endoteliales HUVEC.

Nuestros resultados mostraron que las células HUVEC, expresan funcionalmente el AR y que, si bien los andrógenos no afectan la supervivencia de las células HUVEC, si estimulan la proliferación de estas células. Por otro lado, nuestros resultados indicaron que las líneas celulares de CaP seleccionadas para el trabajo experimental de esta tesis, representan los

fenotipos específicos con respecto a su dependencia androgénica. Estos resultados validan el uso de estos modelos, tanto de células HUVEC como de las líneas celulares de CaP, para el estudio de la comunicación entre ambos tipos celulares. Los estudios *in vitro* con medios condicionados sugieren un aumento en la viabilidad y proliferación de las células de CaP, sin embargo, al analizar la capacidad de migración e invasión se observó que las células resistentes a la ADT y metastásicas serían más susceptibles que las células sensibles a andrógenos al efecto angiocrino proveniente de las células endoteliales. En los experimentos *in vivo*, se observó que las células endoteliales a través de una comunicación paracrina aumentan la migración, el crecimiento tumoral, la microvasculatura tumoral y la metástasis. De manera interesante, también se observó que existe un efecto diferencial en la secreción de citoquinas cuando se compara una línea sensible a andrógenos y una resistente a la deprivación androgénica al estimularlas con medio condicionado.

La importancia de este estudio reside en mostrar que las células endoteliales, a través de un mecanismo paracrino/angiocrino, pueden promover un comportamiento más agresivo de las células de CaP y que este efecto es más extenso en células de mayor agresividad versus células de menor agresividad. Esta característica indicaría que células más agresivas podrían adaptar su biología para poder responder a más señales presentes en el microambiente, y de esa manera, promover un aumento en su agresividad. En consecuencia, este mecanismo de comunicación paracrina podría ser un factor que contribuya a la adquisición de un fenotipo más avanzado, y probablemente metastásico, en pacientes con CaP. Por lo tanto, resulta esencial definir los mecanismos moleculares que están implicados en esta comunicación paracrina, ya que podrían resultar en la identificación de nuevos blancos terapéuticos o biomarcadores para contrarrestar el CaP, especialmente el cáncer de próstata avanzado.

Introducción

Aspectos epidemiológicos y biológicos básicos del cáncer de próstata

El cáncer de próstata (CaP) es la principal neoplasia maligna en hombres adultos y la segunda causa de muerte por cáncer en la mayoría de los países desarrollados o en vías de desarrollo (Siegel et al. 2018). Aunque esto representa un problema significativo y creciente en la población, los mecanismos de inicio, progresión y metástasis del CaP siguen siendo poco conocidos. No obstante, la prevalencia de esta enfermedad depende en gran medida de la edad, historia familiar, raza y susceptibilidad genética (Sluka y Davis 2013, Arora y Barbieri 2018). La mayoría de los pacientes que se diagnostican con esta enfermedad, lo hace en una etapa temprana, en la cual el tumor se encuentra confinado a la glándula prostática, la cual es potencialmente curable con terapias locales, tales como la prostatectomía radical y/o radioterapia. Sin embargo, aproximadamente 33% de los pacientes tratados con este tipo de terapias experimentan recurrencia local o enfermedad metastásica en un periodo de 5-10 años (Yap et al. 2012, Cioni et al. 2018).

Tanto las células epiteliales de próstata normales como las células malignas dependen de los andrógenos y de la activación del receptor de andrógenos (AR, por sus siglas en inglés) para su supervivencia y crecimiento. El AR es un factor de transcripción activado por ligando que pertenece a la super familia de proteínas denominadas receptores nucleares hormonales (esteroides-tiroideos). La vía de señalización clásica de los andrógenos requiere la unión de AR a los andrógenos, dihidrotestosterona (DHT) o testosterona (T), y su posterior disociación de las proteínas de shock térmico HSP90. AR es posteriormente fosforilado y traslocado al núcleo donde se une a secuencias consenso, o elementos de reconocimientos de AR (ARE), presentes en las regiones promotoras de los genes blancos para andrógenos. La unión de AR al ADN

induce el reclutamiento de una serie de proteínas co-reguladoras, las cuales modulan la estructura cromatínica e inducen el reclutamiento de la maquinaria transcripcional básica (Arora y Barbieri 2018). Debido a la dependencia de las células tumorales a los andrógenos, la terapia de deprivación androgénica (ADT "*androgen deprivation therapy*"), a través de la castración química, es la forma estándar de tratamiento para el CaP localmente avanzado o metastásico, la cual generalmente es efectiva por 24 meses (de Brot et al. 2015). La recurrencia de esta enfermedad a la ADT se denomina CaP resistente a la castración o CRPC ("*castration-resistant prostate cancer*"). En este punto, el CaP se vuelve resistente a la terapia hormonal y las células cancerosas adquieren la capacidad de invadir y hacer metástasis a los ganglios linfáticos y órganos distantes, por lo que representa el fenotipo letal de esta enfermedad (Egan et al. 2014). El tratamiento de CRPC es complejo y ha abierto nuevos campos de investigación durante la última década, los cuales se enfocan en la mejor comprensión de la biología de la enfermedad y en desarrollo de nuevas terapias.

Microambiente tumoral: Una visión integrativa de la biología del cáncer prostático

Históricamente, la investigación sobre la biología del CaP se ha enfocado primariamente en el componente epitelial como único tipo celular de relevancia biológica y terapéutica. Sin embargo, una mayor comprensión acerca del microambiente tumoral ha dado lugar a la reorientación del estudio del cáncer hacia las células que componen el microambiente (estroma). Como en el caso de la mayoría de los tumores, las células del CaP no existen de forma aislada; en su lugar, las células epiteliales están en estrecha cercanía, y dependen de las interacciones de una amplia gama de otros componentes estromales. El tejido epitelial prostático normal está compuesto de células luminales secretoras, células basales y células neuroendocrinas. El tejido

no epitelial, referido como estroma, está compuesto por células musculares lisas, fibroblastos, células vasculares y componentes del sistema inmune. Aparte de los componentes celulares, existen factores solubles como las citoquinas y otras moléculas extracelulares que constituyen la matriz extracelular, entre las que se encuentran principalmente de colágeno, laminina, proteoglicanos y glicoproteínas (Levesque y Nelson 2018).

Cambios neoplásicos en el epitelio prostático a menudo son acompañados por cambios fenotípicos histológicos en el estroma, ampliamente llamado estroma reactivo. En la activación del estroma, una de las principales alteraciones observadas en el microambiente es la pérdida de células de músculo liso diferenciadas y un dramático incremento en la población de fibroblastos (Levesque y Nelson 2018). Una mayor caracterización del estroma reactivo muestra un incremento en la secreción y una composición modificada de la matriz extracelular, que se caracteriza por un aumento en la secreción y deposición de colágeno y de diversas proteasas, como metaloproteinasas (MMPs), una disminución de laminina, y un aumento de la densidad microvascular. (Wang et al. 2012, Chiarugi et al. 2014, Singh et al. 2014). Además, el estroma reactivo prostático exhibe la expresión de marcadores biológicos consistentes con aquellos expresados en el estroma presente en el sitio de reparación de una herida (Kruslin et al. 2015). Entre las células estromales que pueden afectar el comportamiento y la malignidad del CaP, se encuentran los fibroblastos asociados al cáncer (CAF), las células del estroma mesenquimatosas derivadas de la médula ósea (MSC), las células precursoras endoteliales (EPC), los macrófagos asociados al cáncer (CAM) y las células endoteliales (CE). (Chiarugi et al. 2014, Kruslin et al. 2015).

Las células endoteliales representan elementos claves en la determinación de la biología del cáncer

Uno de los componentes importantes en el microambiente tumoral, y que se ha demostrado que juega un papel crítico en la progresión tumoral, es el sistema vascular. El sistema vascular está compuesto por células endoteliales (CE) que recubren el interior de los vasos sanguíneos y de células musculares lisas o pericitos que soportan la estructura del vaso (Sturtzel 2017). Estudios previos han demostrado que las funciones de las células endoteliales no están únicamente limitadas a ser parte de estructuras vasculares, sino que, además, incluyen múltiples roles en procesos de organogénesis y regulación de procesos biológicos en general. Específicamente, está establecido que las CE son metabólicamente activas y juegan un rol crítico en varios procesos fisiológicos entre los cuales se encuentra el control del tono vascular, el tráfico de células entre la sangre y el tejido subyacente, la mantención de la fluidez de la sangre, permeabilidad, angiogénesis e inmunidad innata y adaptativa (Aird 2012). Bajo condiciones normales, el endotelio presenta un fenotipo vasodilatador, anticoagulante y anti-adhesivo (antinflamatorio), que se conoce como "quiescente" o "no-activado". Las CE están constantemente activas detectando y respondiendo a las señales del microambiente celular, por lo tanto, adaptando su funcionamiento a las exigencias de orden superior. La "activación" endotelial describe la inducción de ciertos genes que hacen que el endotelio cambie su fenotipo a uno pro-adhesivo (proinflamatorio), procoagulante y vasoconstrictor (Kliche et al. 2011). El endotelio responde a varios estímulos humorales, neurales y mecánicos y libera tanto señales contráctiles como relajantes que afectan la capa de musculo liso subyacente (Schwartz et al. 2010). Cuando, sin embargo, el proceso es sostenido o espacial y/o temporalmente inadecuado, la función endotelial se deteriora, estado llamado disfunción endotelial. La disfunción endotelial

se considera como un paso temprano y crucial en la progresión de las enfermedades vasculares, (Kliche et al. 2011) como por ejemplo, una amplia gama de enfermedades cardiovasculares asociadas con afecciones patológicas como la vasoconstricción, la trombosis y el estado inflamatorio. (Godo y Shimokawa 2017, Konukoglu y Uzun 2017).

Además de las funciones ya mencionadas, los vasos sanguíneos pueden inducir la formación de órganos o influenciar procesos de crecimiento y diferenciación directos al proporcionar señales de manera paracrina (angiocrina) (Butler et al. 2010). El contacto entre las CE y el tejido adyacente se produce de forma temprana tanto en el embrión, antes del reclutamiento de células que forman parte de los vasos sanguíneos, y más tarde en los lechos capilares, donde las células murales individuales (pericitos) escasamente cubren el endotelio. Por lo que, es posible que la señalización mutua entre el endotelio y tejidos circundantes durante el desarrollo constituya la base molecular para la relación física y fisiológica que dura hasta la edad adulta (Cleaver y Melton 2003). Por lo tanto, la señalización celular endotelial se cree actualmente que es fundamental para promover la especificación del destino celular, la diferenciación de órganos y la remodelación postnatal de tejidos (Crivellato et al. 2007). El sistema cardiovascular es el primer órgano funcional que penetra en el embrión de vertebrados en desarrollo. Por tanto, es fácil imaginar que muchos (si no todos) los órganos en desarrollo interactúan con los vasos preexistentes y reciben señales vasculares. Hasta la fecha se ha demostrado una potente señalización paracrina/angiocrina de parte del endotelio en el páncreas, hígado, riñón y pulmones, en donde las células endoteliales, de forma independiente a la perfusión sanguínea, son esenciales para la diferenciación y desarrollo de estos órganos (Coultas et al. 2005, Ramasamy et al. 2015, Woik y Kroll 2015). Por otra parte, en la edad adulta las CE que forman los capilares son reconocidas como un nicho especializado que, a través de la expresión

balanceada de factores angiocrinos, participan activamente en la inducción, especificación y en la guía de la regeneración de órganos, así como también en la mantención de la homeostasis y metabolismo. Después de una lesión, las células endoteliales orquestan la auto regeneración y diferenciación de las células madre residentes y células progenitoras en el órgano funcional (Woik y Kroll 2015, Rafii et al. 2016).

La noción de que las CE promueven una reparación fisiológica cuando están sanas, y procesos de enfermedad, cuando están disfuncionales, representan puntos en un espectro regulador del endotelio notablemente plástico. (Franses y Edelman 2011) Precisamente, como las células tumorales penetran el epitelio normal e interactúan con la vasculatura que las rodean, se ha establecido que las CE son capaces de regular varios aspectos de la biología tumoral, desde la entrega de oxígeno y nutrientes al tumor, hasta la modulación de la respuesta inmune, presentando un rol crucial en la iniciación, dormancia, progresión y metástasis del tumor. (Butler et al. 2010, Choi y Moon 2018)

Neo-angiogénesis

Para apoyar la alta tasa proliferativa de células cancerosas, los tumores necesitan desarrollar rápidamente una nueva red vascular, y lo hacen principalmente mediante la activación del proceso de angiogénesis (Hanahan y Coussens 2012). La angiogénesis consiste en la formación de nuevos vasos sanguíneos a partir de vasos preexistentes, y está regulada por una compleja red dinámica de mecanismos promotores y supresores, en forma de factores solubles, estrés fisiológico e interacciones célula-célula, cuyo equilibrio se presume que se altera en procesos tumorales provocando un cambio o “switch” angiogénico hacia estados hiper- o hipo-vascularizados (Sakurai y Kudo 2011). Los vasos sanguíneos asociados al tumor son importantes para proporcionar nutrientes y oxígeno al tumor en crecimiento, y a la vez que

juegan un rol clave en la diseminación del tumor/metástasis (Wang et al. 2018). Sin embargo, los vasos sanguíneos del tumor se caracterizan por presentar una morfología aberrante y cercana al fenotipo inmaduro con una falta de recubrimiento de pericitos, filtración vascular, variación del diámetro del lumen vascular y exceso de ramificaciones (Hanahan y Coussens 2012).

En 1971, Folkman propuso que el crecimiento tumoral y la metástasis son dependientes del proceso de angiogénesis, y que la inhibición de la neovascularización de los tumores sólidos podría proporcionar una nueva estrategia terapéutica contra el cáncer (Folkman 1971). La primera parte de esta hipótesis es actualmente aceptada por la comunidad científica, considerando a la angiogénesis como un sello distintivo en el desarrollo y la progresión de un cáncer (Hanahan y Weinberg 2011). Sin embargo, las terapias anti-angiogénicas como estrategias para el tratamiento del cáncer no han recibido similar aceptación (Abdollahi y Folkman 2010). El concepto de terapia anti-angiogénica ha llevado a la aprobación de diferentes agentes para el tratamiento del cáncer, la mayoría de ellos dirigidos hacia los componentes de la vía del factor de crecimiento endotelial vascular (VEGF) (Folkman 2007, Abdollahi y Folkman 2010, Taverna et al. 2013, Bilusic y Wong 2014, Jain 2014, van Beijnum et al. 2015). Desafortunadamente, estos agentes utilizados como monoterapia, o en combinación con quimioterapia, han demostrado beneficios terapéuticos restringidos, de aumento de la sobrevida de los pacientes del orden de solo semanas o meses. Más desconcertante aún, pese a que el aumento en la angiogénesis parece ser un evento generalizado en cáncer, existe un grupo considerable de tumores para los cuales las terapias anti-angiogénicas no presentan ningún tipo de efecto (De Falco 2014, Jain 2014, van Beijnum et al. 2015). En consecuencia, la inhibición farmacológica de la señalización de VEGF inhibe la angiogénesis tumoral en algunos, pero no en todos los modelos de cáncer de ratón, y no bloquea totalmente la progresión tumoral en

ratones y humanos. En contraste, la inactivación genética de VEGF afecta la angiogénesis durante el desarrollo y es letal en embriones. Estas observaciones respaldan la noción de que la regulación de la angiogénesis tumoral es un proceso multidimensional que es menos dependiente de la señalización de VEGF que la angiogénesis del desarrollo. Además, los tumores pueden adaptarse rápidamente a la neutralización de factores de crecimiento pro angiogénicos individuales, incluyendo VEGF, a través de rutas que implican la adaptación metabólica y la reprogramación, la aplicación de señales compensatorias pro angiogénicas o la adquisición de modos de crecimiento tumoral independiente de angiogénesis (De Palma et al. 2017).

Angiogénesis y Cáncer de Próstata

En CaP, al igual que en otros tipos tumorales, existe evidencia que sugiere que la angiogénesis tendría un papel importante en el desarrollo y progresión de esta patología (Sakurai y Kudo 2011, Tomic et al. 2012, Taverna et al. 2013, Bilusic y Wong 2014). Se ha observado que las células de CaP sobre expresan VEGF con respecto a su contraparte normal (Bilusic y Wong 2014), y que el aumento en la angiogénesis se correlaciona con la progresión y el potencial metastásico de este tipo tumoral (Welen et al. 2009). Existe evidencia que sugiere que la masa tumoral está bajo control estricto del endotelio microvascular y que la transición del CaP sensible a andrógenos al CaP resistente a la castración se asocia con una mayor densidad de micro vasos (MVD) (Gustavsson et al. 2008, Karagiannis et al. 2014). Varios estudios han propuesto que la extensión de la angiogénesis en CaP se asocia con características clínicas, como la puntuación de Gleason y el estadio patológico (Weidner et al. 1993, Lissbrant et al. 1997, Bono et al. 2002), y podría ser un factor pronóstico independiente de recurrencia bioquímica y supervivencia del paciente (Miyata et al. 2015, Yang et al. 2016, Kobayashi et al. 2018). Los

estudios realizados por Gustavsson y colaboradores (Gustavsson et al. 2005, Gustavsson et al. 2008) ampliaron aún más el valor pronóstico de MVD en CaP, lo que indica que la transición del fenotipo sensible a andrógenos a resistente a la castración se asoció con una mayor actividad angiogénica en tumores de xenoinjerto derivados de la sub-línea resistente a la castración LNCaP-19 (Gustavsson et al. 2008, Tomic et al. 2012), y que el nivel de expresión de varios genes asociados con la angiogénesis se alteró durante esta transición, incluidos ADAMTS1, fibronectina, neuropilina, Ang-2 y VEGF. Por lo tanto, un creciente cuerpo de literatura apoya la idea de que la angiogénesis es un evento crucial en el desarrollo y la progresión de la CaP y que la angiogénesis dirigida podría ser un tratamiento efectivo para contrarrestar el fenotipo resistente a la castración. Sin embargo, y al igual que lo ocurre en otros tipos de cáncer, se observa un fracaso con las terapias anti-angiogénicas utilizadas como monoterapias o cuando se administran en combinación con otras terapias en ensayos controlados aleatorios (Adesunloye et al. 2014). Como monoterapias, la mayoría de los agentes anti-angiogénicos no logran una respuesta tumoral objetiva o una disminución del PSA, y además no muestran efectos considerables en la mejora de la sobrevivencia de pacientes con CaP (Bilusic y Wong 2014)

Esto sugiere que la interacción entre los tumores y las CE es más compleja de lo que se creía en un principio (van Beijnum et al. 2015), y a su vez, sugieren que las CE podrían estar cumpliendo otro rol, independiente del suministro de nutrientes y oxígeno al tumor (Butler et al. 2010). Las CE podrían, al igual como se ha observado en la organogénesis y reparación de tejidos (Rafii et al. 2016), liberar distintos factores de crecimiento u otras moléculas que afecten de forma paracrina a las células tumorales. Es así, como se ha observado que distintas moléculas liberadas por las CE impactan de forma positiva en la progresión del cáncer y, además, se ha propuesto

que estas células forman un nicho que promueve la sobrevivencia y proliferación de células tumorales y les otorgaría una función quimio resistente (Butler et al. 2010).

Paradigma “angiocrino” en cáncer.

La investigación sobre la angiogénesis tumoral se ha concentrado principalmente en tratar de entender cómo los vasos son reclutados y estructuralmente distorsionados para promover el crecimiento del tumor (Franses y Edelman 2011, Kim et al. 2011). Sin embargo, debido al relativo fracaso de las terapias anti-angiogénicas (Bilusic y Wong 2014), el interés de la comunidad científica se ha centrado en identificar y caracterizar funciones alternativas de las CE que permitan un efecto más directo de las CE en la regulación de la biología del cáncer. En esta línea investigativa, Butler y colaboradores postularon la hipótesis de que las CE promueven el crecimiento del tejido tumoral no solo por la entrega de oxígeno y nutrientes, sino también a través de un mecanismo “angiocrino”, que involucra la producción y liberación al medio extracelular de factores de crecimiento que podrían promover la proliferación de las células tumorales (Butler et al. 2010). Esta hipótesis ha sido fuertemente reforzada por un conjunto considerable de evidencia experimental previa que indicaría la participación de "factores angiocrinos" durante procesos como la organogénesis (Lammert et al. 2001, Matsumoto et al. 2001, Woik y Kroll 2015) y la regeneración tisular (Butler et al. 2010, Nolan et al. 2013, Rafii et al. 2016).

Varios estudios han demostrado que las CE secretan factores solubles y citoquinas de una forma paracrina que tienen un impacto positivo en la progresión tumoral (Butler et al. 2010, Levesque y Nelson 2018), sin embargo, los mecanismos moleculares por los cuales las CE otorgarían ventajas fenotípicas y funcionales a las células tumorales aún no están del todo claros y requieren mayor investigación. La mayoría de los estudios *in vitro*, se han realizado utilizando

ensayos de co-cultivo entre CE y células de cáncer para estudiar la interacción y comunicación directa entre ambos tipos celulares en conjunto con el efecto paracrino. Estos estudios, sugieren que las CE proveen de un nicho fértil que incrementaría la proliferación, sobrevida, invasión y migración de las células de cáncer. Entre estos, se observó que las CE permiten la proliferación y propagación de clones leucémicos agresivos (Poulos et al. 2014), la proliferación, supervivencia y propiedades pro-metastáticas de células de cáncer de mama (Ghiabi et al. 2014, Ghiabi et al. 2015), aumentan la invasión de células de CaP (Wang et al. 2013), y promueven la capacidad de formación de células madre en meduloblastoma (Wang et al. 2017). Este nicho, además se ha visto que confiere en modelos de células de leucemia, linfoma y cáncer colorrectal, resistencia a la quimioterapia (Cao et al. 2014, Poulos et al. 2014, Wang et al. 2017). Sin embargo, también se han proporcionado líneas de evidencia que demuestran que las CE, sin tener contacto directo con las células de cáncer, podrían secretar factores solubles que promueven la proliferación, migración, invasión e incrementan la metástasis en modelos de carcinoma hepatocelular, cáncer colorrectal y en glioblastoma (Galan-Moya et al. 2011, Feng et al. 2017, Wang et al. 2017). Además, se ha observado que las CE, a través de un mecanismo paracrino, promueven el fenotipo de células madre en células de cáncer colorrectal, células de glioblastoma y células de cáncer de mama (Lu et al. 2013, Ghiabi et al. 2014, Wang et al. 2017). Esta comunicación ha sido estudiada con más detalle, por el grupo de Zhao, utilizando modelos de ratones y peces cebra, el cual mostró que cordones de CE penetran los tumores y permanecen sin circulación durante varios días antes de someterse a perfusión sanguínea vascular y que durante este tiempo factores secretados por CE son responsables de la estimulación de la proliferación de células tumorales (Zhao et al. 2016). Todas estas evidencias indican que las CE

desempeñarían un papel mucho más temprano y más amplio en la promoción del crecimiento tumoral, independiente de la circulación vascular.

En CaP, Existen líneas de evidencia "indirecta" que sugieren que existiría una comunicación entre el epitelio y endotelio prostático (Levesque y Nelson 2018). Se ha observado que la co–inoculación en la capsula renal de CE con células epiteliales luminales o basales resultan en el crecimiento de tejido prostático (Bates et al. 2008). Sin embargo, hasta el momento solo existen dos reportes que han abordado este concepto a nivel molecular en CaP. En el primer trabajo, realizado por Wang y colaboradores (Wang et al. 2013), se determinó que las CE fueron capaces de estimular el proceso de metástasis. Este fenómeno se correlacionó con un aumento en la secreción de interleucina-6 por parte de las CE, lo cual resultaría en una disminución de la señalización del receptor de andrógenos en células tumorales y la activación de la vía TGF-b/MMP-9, gatillando un aumento en las capacidades invasivas de las células tumorales (Wang et al. 2013). En el segundo trabajo, realizado por Pedrosa y colaboradores (2015), utilizando una cruza de un ratón mutante *Jag1* endotelial con el modelo de ratón de adenocarcinoma de próstata (TRAMP), el cual desarrolla CaP espontáneamente, demostraron que la pérdida de *Jag1* en el endotelio no solo tiene un efecto inhibitorio en la neo-angiogénesis y maduración de los vasos sanguíneos, sino también en el efecto angiocrino, a través de la inhibición de Notch3/Hey en células tumorales, restringiendo la proliferación, aumentando la apoptosis y previniendo la adquisición de un fenotipo invasivo (Pedrosa et al. 2015).

Si bien estos estudios son alentadores, en términos de validar un rol angiocrino de las CE sobre la progresión del CaP (capacidad metastásica), es insuficiente en términos de entender: 1) La extensión de los procesos biológicos afectados de manera angiocrina por las CE en células

tumorales de CaP y 2) Los mecanismos moleculares que subyacen estos efectos biológicos y que pudiesen tener un rol preponderante en el contexto del desarrollo y progresión del CaP.

Hipótesis

Proponemos que: "**Las células endoteliales promueven un aumento en la proliferación y en las capacidades migratorias e invasivas de células de cáncer prostático a través de un mecanismo celular de tipo angiocrino"**.

Objetivos Específicos

1. Caracterizar los modelos biológicos *in vitro* de células endoteliales humanas y líneas celulares de cáncer prostático humano, en términos de su dependencia androgénica.

2. Caracterizar el efecto angiocrino del endotelio humano sobre los parámetros biológicos que determinan agresividad tumoral (proliferación, sobrevida, migración y capacidad invasiva) en modelos *in vitro* de cáncer de próstata sensible y resistente a la deprivación androgénica.

3. Analizar los efectos biológicos asociados a la interacción entre células endoteliales y células de cáncer de próstata sensible y resistente a la deprivación androgénica en modelos biológicos *in vivo* de pez cebra y ratones severamente inmunodeficientes.

Resultados

Objetivo 1

Caracterizar los modelos biológicos *in vitro* de células endoteliales humanas y líneas celulares de cáncer prostático humano, en términos de su dependencia androgénica.

El cáncer de próstata tiene la particularidad que al igual que las células epiteliales normales de próstata dependen de los andrógenos para su proliferación y sobrevida, lo cual es la base para las terapias contra esta enfermedad. A esto se suma la evidencia que sostiene que las células endoteliales de cáncer de próstata también presentan una dependencia a los andrógenos. Por lo que es de vital importancia la validación de los modelos celulares a utilizar para los estudios de comunicación paracrina entre células de cáncer y de endotelio.

Los resultados tanto de los antecedentes y la validación de las células endoteliales HUVEC como modelo para el estudio en cáncer de próstata están descritos en las siguientes publicaciones:

Torres-Estay, V., Carreño, D., San Francisco, I., Sotomayor, P., Godoy, AS, Smith, G. Androgen Receptor in Human Endothelial Cells. **J Endocrinol, 224: R131-R137, 2015**.

Torres-Estay, V., Carreño, D., Fuenzalida, P., Watts, A., San Francisco, I., Montecinos, V., Sotomayor, P., Ebos, J., Smith, G., Godoy, AS. Androgens Modulate Male-Derived Endothelial Cell Homeostasis Using Androgen Receptor-Dependent and -Independent Mechanisms. **Angiogenesis, 20(1):25-38.2017.**

Publicación 1

Review | V TORRES-ESTAY, A S GODOY, G J SMITH and others | *Androgen receptor in human endothelial cells* | 224:3 | R131–R137

Androgen receptor in human endothelial cells

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Abstract

Androgen receptor (AR) is a ligand-inducible transcription factor, and a member of the steroid-thyroid-retinoid receptor superfamily, that mediates the biological effects of androgens in a wide range of physiological and pathological processes. AR expression was identified in vascular cells nearly 20 years ago, and recent research has shown that AR mediates a variety of actions of androgens in endothelial and vascular smooth muscle cells. In this mini-review, we review evidence indicating the importance of AR in human endothelial cell (HUVEC) homeostatic and pathogenic processes. Although a role for AR in the modulation of HUVEC biology is evident, the molecular mechanisms by which AR regulates HUVEC homeostasis and disease processes are not fully understood. Understanding these mechanisms could provide critical insights into the processes of pathogenesis of diseases ranging from cardiovascular disease to cancer that are major causes of human morbidity and mortality.

Key Words
- endothelium
- androgen receptor
- angiogenesis
- cancer

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Journal of Endocrinology

Androgen receptor and vascular cells

Androgens are male sex hormones that are critical for the development and maintenance of the male reproductive system. Given the extensive role of androgens in normal physiology, abnormal androgen activity has been implicated in a wide variety of pathological conditions, including androgen-dependent prostate cancer (CaP; Matsumoto *et al.* 2013).

The incidence of cardiovascular and vascular disease is greater in men compared with age-matched premenopausal women. However, during menopause the incidence in women increases dramatically supporting a long-standing hypothesis that estrogens might provide vascular protection. However, the results of clinical trials raise an important controversy about the risks and benefits of hormone replacement therapy (Campelo *et al.* 2012). Epidemiological and clinical data have indicated that the male hormones, androgens, are independent factors that contribute to the higher male susceptibility to atherosclerosis through adverse effects on lipids, blood pressure, and glucose metabolism (Liu *et al.* 2003, Nheu *et al.* 2011). There is evidence that androgen use has been associated with premature coronary disease in athletes and impaired vascular reactivity in female-to-male trans-sexuals (Death *et al.* 2004). On the other hand, it has been shown that a decrease in androgens, particularly testosterone, as a result of aging in men or bilateral ovariectomy in women, is associated with hypertension, diabetes, and atherosclerosis and that testosterone replacement therapy may benefit these people (Nheu *et al.* 2011). Restoration of physiological concentrations of testosterone may have a beneficial

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influence on the hemostatic system through enhancement of anti-coagulant activity (Jin *et al.* 2007), and may exert anti-thrombotic effects (Jin *et al.* 2009, 2010). Furthermore, short-term administration of testosterone to men with coronary artery disease reduced myocardial ischemia and improved endothelial vasomotor function (Yu *et al.* 2010). However, despite the growing evidence of the protective effect of androgens on atherosclerosis, the picture is far from clear (Liao *et al.* 2012).

Vascular endothelial cells (EC) and vascular smooth muscle cells (VSMC) are key cellular components of blood vessels that play important roles in vascular health and disease. During the development and progression of atherosclerosis, changes occur both in the structure and function of these cells resulting in a wide range of abnormalities that affect growth, death, and physiological function. These cells contain functional androgen receptor (AR) and are targets for hormone action (Liu *et al.* 2003). Results have indicated that exposure to testosterone (mediated through AR) enhanced tumor necrosis factor α (TNFα)-induced apoptosis after serum deprivation in cultured human endothelial cells (HUVECs; Ling *et al.* 2002). In ECs dihidrotestosterone (DHT) induced VCAM-1 expression that resulted in increased monocyte binding to the endothelium. The pathway leading to VCAM-1 expression was dependent of the interaction of functional AR with the NF-κB signaling pathway (Death *et al.* 2004, Nheu *et al.* 2011). In addition, testosterone rapidly induced nitric oxide (NO) production in human aortic endothelial cells (HAEC) that was associated with phosphorylation/activation of eNOS that was inhibited by incubation with nilutamide, or an AR siRNA (Yu *et al.* 2010, 2012). The biological action of testosterone in ECs is mediated predominantly by AR; however, some consequences may be mediated by ER after conversion of testosterone to estradiol (Mukherjee *et al.* 2002). Indeed, in ECs, estrogens alone rapidly activated eNOS and stimulated NO production in an ER-dependent manner (Yu *et al.* 2010). However, the nonaromatizable DHT and DHT analog, R1881, elicited significant eNOS phosphorylation and NO production (Goglia *et al.* 2010, Yu *et al.* 2010). Therefore, the critical effects of testosterone on eNOS phosphorylation and NO production appear to be AR-dependent (Koizumi *et al.* 2010, Yu *et al.* 2010, Campelo *et al.* 2012). DHT at physiological concentrations stimulated AR-mediated proliferation of HAEC, probably through upregulation of the expression of VEGF-A, cyclin A, and cyclin D1. The stimulation of EC proliferation in the cardiovascular system by activation of AR could contribute to the repair of EC injury/damage, and prevent EC dysfunction, minimizing a primary risk factor for vascular stiffness, hypertension, and atherosclerosis (Cai *et al.* 2011).

AR and angiogenesis

Angiogenesis, the formation of new blood vessels from pre-existing endothelium, is subject to a complex control system regulated by endogenous pro-angiogenic and anti-angiogenic factors. Physiologically, the formation of new vessels is crucial for wound healing, organ regeneration, enabling ovulation in the female reproductive system, implantation, and placenta formation. Pathological angiogenesis is an important factor in multiple disease processes, such as tumor growth, diabetic retinopathy, macular degeneration, and psoriasis (Hoeben *et al.* 2004). However, the role of angiogenesis in the repair of cardiovascular damage and the role of androgens in the causation versus the repair of cardiovascular disease (CVD) remain controversial (Sieveking *et al.* 2010*a*). Furthermore, anti-diabetic drugs increase serum testosterone levels in hypo-androgenemic obese men (Kapoor *et al.* 2008), whereas these drugs reduce androgen levels in hyper-androgenemic obese women (Sahin *et al.* 2007), indicating that men and women may respond to androgens differently or that insulin resistance has different effects on androgens in men and women. These results are indicative of a significant deficiency in our understanding of the differential role of androgens in men and women: is the expression of androgens and AR (and more importantly, androgen regulation) the same in males and females (Moulana *et al.* 2011)? Sieveking and colleagues showed that DHT, through activation of AR, increased EC migration, proliferation, tubulogenesis, and the production of vascular endothelial growth factor, a pivotal molecule in angiogenesis, in cells from male donors. However, in striking contrast, DHT treatment did not induce similar changes in ECs derived from female donors. These results indicate that androgens, through AR, might regulate vascular regeneration in a sex-dependent manner; these results could explain, in part, the observed sex differences in the outcomes of CVD (Sieveking *et al.* 2010*b*). In contrast, Yoshida *et al.* (2013), showed that AR is essential for robust re-vascularization in response to ischemia in both male and female mice. This work documented a sex-independent physiological role of AR in angiogenic potency and provided evidence of a novel cross-talk between androgen/AR signaling and the VEGF/kinase insert domain protein receptor signaling pathways. The differences between the studies of Yoshida and

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colleagues and Sieveking and colleagues may reflect the differences in model systems: Sieveking and colleagues used cultured HUVECs, while Yoshida and colleagues utilized an *in vivo* model of ischemic rodent tissues, which included skeletal muscle, vasculature, bone, lymphatic tissue, and nerves. Results of a recent study (Annibalini *et al*. 2014) have indicated that under identical culture conditions, endothelial cells (HUVECs), regardless of sex, predominantly use pathways responsive to the action of androgens rather than those responsive to the action of estrogens. Also these results indicated that male and female HUVECs expressed high levels of AR and 5α-reductase-1, but very low levels of estrogen receptors and aromatase.

AR and endothelial progenitor cells

In the last decade, our knowledge of vascular homoeostasis and repair has evolved significantly with the discovery of circulating endothelial progenitor cells (EPCs) in adult human blood. EPCs that originally reside in the bone marrow and other putative niches are mobilized to the peripheral circulation in response to many stimuli, and once in the bloodstream take an active part in EC replacement and formation of new blood vessels (Fadini *et al*. 2009). Because of their role in maintenance of functional endothelium, EPCs are currently considered to be an integrated component of the cardiovascular system. Subjects with risk factors for or with established CVD have a depletion of circulating EPCs. Interestingly, patients with lower levels of EPCs in the bloodstream have a higher risk of cardiovascular events. These results indicate that depletion of EPCs is a pathogenic event that contributes to an inability to maintain an intact endothelium and to promote angiogenesis, risk factors that can translate into the development and progression of CVD (Di Mambro *et al*. 2010).

Human EPCs express AR, and androgens influence EPC mobilization from bone marrow (Foresta *et al*. 2006, 2008). Furthermore, a direct effect of testosterone on EPC function was indicated by the evidence that hypo-gonadal men had lower numbers of circulating EPCs compared with normal men, and that the numbers of EPCs increased significantly after testosterone replacement therapy (Foresta *et al*. 2006). Moreover, subjects with Klinefelter syndrome (KS), a condition characterized by hypogonadism and associated with a significant morbidity related to vascular diseases, have a marked reduction in the number of EPCs in circulation (Di Mambro *et al*. 2010). These *in vivo* observations were supported by the results of *in vitro* studies that demonstrated that testosterone, acting through AR-mediated pathways, increased EPC proliferation, colony formation, and migration (Foresta *et al*. 2008). Recently, it has been suggested that DHT modulated EPC proliferation and adhesion, and the PI3-K/AKT pathway played an important role in this process. The positive effects of androgen (DHT) on EPCs may explain the finding that low levels of circulating androgens are associated with increased male CVD morbidity and mortality (Liu *et al*. 2014).

AR and cancer-associated vasculature

Although the importance of tumor angiogenesis was initially met with skepticism, it is now accepted as a hallmark of cancer progression and has been explored as a therapeutic target in almost every type of neoplastic disease, including CaP (Galsky & Oh 2013). CaP is a common malignancy in humans, representing the second leading cause of cancer-related deaths in American men (Siegel *et al*. 2012). Increasing evidence has indicated that the tumor microenvironment has a role equally important as the cancer cells in the progression of a tumor (Dayyani *et al*. 2011). In the tumor microenvironment, one of the key components thought to have a critical role in tumor progression is the vasculature (Godoy *et al*. 2013).

Our group determined that human prostate endothelial cells (HPEC) isolated from fresh human clinical specimens of benign prostate and CaP expressed functional AR *in vivo* and *in vitro*, and that androgen modulated *in vitro* EC proliferation and gene expression in a cell-type-specific manner. Also dihydrotestosterone (DHT), through AR, directly increased proliferation of primary cultures of HPECs in a dose-dependent manner without affecting the formation of endothelial tube structures in the matrigel, indicating that the differentiation and migration processes involved in endothelial tube formation are independent of proliferation in prostate ECs. These studies provide evidence of a potential role for AR in regulation of human prostate vascular EC homoeostasis (Godoy *et al*. 2008). Our group also demonstrated that androgen withdrawal induced acute involution of the human prostate vasculature in primary xenografts of human benign prostate or CaP tissues that had been transplanted into humanized SCID mice that had received implants of testosterone pellets to maintain a human level of circulating testosterone (Godoy *et al*. 2011). In this preclinical model, vascular involution was correlated temporally with the induction of apoptosis in the human prostate ECs, indicating that testicular androgen signaling had an important role in the maintenance of prostate EC homeostasis in intact men. This observation also indicated

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that androgen ablation negatively affected EC viability in human prostate tissue independently of epithelial cell apoptotic death (Godoy *et al*. 2011). Supporting this hypothesis, withdrawal of androgenic signaling using AR antagonists (e.g., flutamide and bicalutamide) or inhibitors of steroid metabolism (e.g., finasteride and dutasteride) reduced hematuria during prostate surgery or in patients with benign prostatic hyperplasia (BPH) and in combination therapy with bicalutamide-goserelin (a gonadotropin-releasing hormone agonist) and dutasteride (an inhibitor of 5α-reductase isoenzymes types 1 and 2) induced profound vascular collapse and reduced prostatic tissue vascularity in human CaP patients (Godoy *et al*. 2013). Results from other studies also indicated that prostate tumor cells regulated EC growth through a paracrine mechanism, which was mainly mediated by VEGF, and demonstrated that DHT was able to modulate EC growth via tumor cells (Wen *et al*. 2013) and that the induction of VEGF was mediated by binding of the transcription factor AR and SP1 to the core promoter region of VEGF (Eisermann *et al*. 2013).

Androgen deprivation therapy (ADT), the standard treatment for advanced CaP, is rarely curative and, in virtually all cases, the initial response to ADT is followed by relapse of the disease as castration-resistant CaP, the lethal phenotype of the disease. The evidence of expression of functional AR in human prostate ECs in CaP tissue and of the acute effect of ADT on human prostate vascular integrity indicates that human prostate vasculature has a unique potential as a first-line target for ADT (Godoy *et al*. 2008, 2013). ADT-induced transient destabilization of the human prostate EC compartment may present a 'therapeutic window' for the delivery of chemotherapeutic agents. Therefore, the study of the regulatory role of androgens in the prostate microvasculature may provide the molecular basis for the development of new therapeutic modalities. This paradigm-shifting approach would change the monolithic paradigm of ADT as a first-line therapy, which is focused on induction of apoptotic death in CaP cells, to a dynamic paradigm where ADT is employed in a neo-adjuvant setting to improve therapeutic efficacy of conventional and new treatment modalities (Godoy *et al*. 2013). This new approach would capitalize on the 'therapeutic window' opened by the acute apoptotic death of androgen-responsive prostate ECs to allow access to the

Table 1 Summary of research studying the effects of androgens on endothelial cells

Model	Type of androgen	AR mediated[a]	Effect	Author
Human aortic endothelial cell (HAEC)	Testosterone	Not demonstrated	Increase in the production of nitric oxide	Goglia *et al*. (2010) and Yu *et al*. (2010, 2012)
	DHT	Yes	Increase in proliferation	Cai *et al*. (2011)
Human umbilical vein endothelial cell (HUVEC)	DHT	Yes	Increase in monocyte binding	Death *et al*. (2004) and Nheu *et al*. (2011)
	DHT	Yes	Increase in proliferation and tubulogenesis	Sieveking *et al*. (2010*b*)
	DHT	Not demonstrated	Induction of a pro-inflammatory state	Annibalini *et al*. (2014)
	Testosterone			
Human prostate endothelial cell (HPEC)	DHT	Yes	Increase in proliferation	Godoy *et al*. (2008)
Human endothelial progenitor cell (EPC)	Testosterone	Not demonstrated	Increase in proliferation, colony formation, and migration	Foresta *et al*. (2006, 2008)
	DHT	Not demonstrated	Increase in proliferation and adhesion	Liu *et al*. (2014)
Prostate vasculature (primary xenograft model)	Withdrawal	Not directly demonstrated	Increase in apoptosis[b]	Godoy *et al*. (2011)
Vascular endothelial cells (mouse hind limb ischemia model)	AR knockout	Not directly demonstrated	Reduced angiogenic capability	Yoshida *et al*. (2013)
Murine endothelial cell line (MEC)	Conditioned media from Prostate cancer cell line (DHT)	Not demonstrated	Increase in proliferation	Wen *et al*. (2013)

[a]Effect mediated by the androgen receptor (AR).
[b]Effect observed between 1 and 4 days after androgen withdrawal.

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interstitial tissue space to chemotherapeutic agents that are usually blocked by the intact endothelial barrier (Godoy *et al*. 2013). Furthermore, the potential generality of this therapeutic approach is indicated by the observation that ECs of all human hormonally responsive tissues, including breast, endometrium and ovary, as well as prostate, express AR.

Summary and conclusions

The role of androgen signaling and AR-mediated transregulation of the expression of genes associated with normal EC processes associated with viability, proliferation, and angiogenesis/repair, as well as with pathogenic processes, such as atherosclerosis and neoplasia, is poorly understood. To further cloud the issue, the literature is rife with reports espousing diametrically opposed conclusions (Table 1). AR is expressed in ECs in a largely sex- and species-independent manner in bone and bone marrow, skin, pancreas, brain, skeletal muscle, cornea, and endothelial progenitor cells (Liang *et al*. 1993, Abu *et al*. 1997, Mantalaris *et al*. 2001, Suzuki *et al*. 2001, Sinha-Hikim *et al*. 2004, Liu *et al*. 2005, Ohtsuki *et al*. 2005, Foresta *et al*. 2008, Morales *et al*. 2008, Fadini *et al*. 2009). Furthermore, AR is expressed in ECs in all steroid-responsive tissues in humans. However, a species difference was observed for prostate, in which ECs of both benign and malignant human prostate were demonstrated to express functional AR whereas ECs of rodent prostate and CaP were reported to lack expression of AR (Prins *et al*. 1991, Ralph *et al*. 2003, Europe & Tyni-Lenne 2004). Regarding sex differences, Sieveking and colleagues and Death and colleagues reported that, in both *in vitro* and *in vivo* models, androgens stimulated the proliferation and angiogenesis/vascular repair by ECs of male origin, or in male organisms, but not by ECs of female origin, even if the female ECs were supplemented with exogenous androgen or AR (Death *et al*. 2004, Sieveking *et al*. 2010*b*). However, Yoshida *et al*. (2013) have recently reported a novel sex-independent protective mechanism against ischemic injury mediated by AR.

The mechanisms by which AR mediates its biological effects in ECs are equally unclear. Demonstration that the nonaromatizable androgens, DHT and R1881, induced biological endpoints similar to those obtained with testosterone, and that the biological consequences were abrogated by flutamide, bicalutamide (casodex), nilutamide, or AR siRNA, clearly implicated AR in the modulation of EC function, proliferation, and gene expression via conventional nuclear-receptor-mediated transcriptional transactivation (Goglia *et al*. 2010, Yu *et al*. 2010, Cai *et al*. 2011, Nheu *et al*. 2011). However, AR localized to the cell membrane in caveolae has been implicated in nongenomic regulation of EC function/gene expression via activation of the c-Src/PI3-K/AKT cascade that ultimately results in the activation of eNOS and NO production (Somjen *et al*. 2004, Goglia *et al*. 2010, Yu *et al*. 2010, 2012). The role of AR in the modulation of all responses of ECs to circulating androgens is complicated further by reports that the adrenal androgen DHEA(S), in addition to its role as the precursor of T/DHT and estrone/estradiol synthesis, also binds to a cognate receptor on the EC membrane and induces NO synthesis due to enhanced expression and stabilization of eNOS, and that this induction is not blocked by antagonists of ER, AR, PR, or GR (Simoncini *et al*. 2003, Williams *et al*. 2004, Zapata *et al*. 2005, Liu *et al*. 2008).

In summary, while it is clear that circulating androgens and their metabolites have significant effects on the normal biology and pathological processes that affect ECs, the processes through which AR-mediated mechanisms regulate EC homeostasis and angiogenic responses to injury or disease require significant clarification. Identification of these processes will be the key for the development of better models for investigating the biology of hormonally responsive tumors, tumor angiogenesis, and atherosclerosis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Publicación 2

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ORIGINAL PAPER

Androgens modulate male-derived endothelial cell homeostasis using androgen receptor-dependent and receptor-independent mechanisms

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Abstract
Background Sex-related differences in the role of androgen have been reported in cardiovascular diseases and angiogenesis. Moreover, androgen receptor (AR) has been causally involved in the homeostasis of human prostate endothelial cells. However, levels of expression, functionality and biological role of AR in male- and female-derived human endothelial cells (ECs) remain poorly characterized. The objectives of this work were (1) to characterize the functional expression of AR in male- and female-derived human umbilical vein endothelial cell (HUVEC), and (2) to specifically analyze the biological effects of DHT, and the role of AR on these effects, in male-derived HUVECs (mHUVECs).
Results Immunohistochemical analyses of tissue microarrays from benign human tissues confirmed expression of AR in ECs from several androgen-regulated and non-androgen-regulated human organs. Functional expression of AR was validated in vitro in male- and female-derived HUVECs using quantitative RT-PCR, immunoblotting and AR-mediated transcriptional activity assays. Our results indicated that functional expression of AR in male- and female-derived HUVECs was heterogeneous, but not sex dependent. In parallel, we analyzed in depth the biological effects of DHT, and the role of AR on these effects, on proliferation, survival and tube formation capacity in mHUVECs. Our results indicated that DHT did not affect mHUVEC survival; however, DHT stimulated mHUVEC proliferation and suppressed mHUVEC tube formation capacity. While the effect of DHT on proliferation was mediated through AR, the effect of DHT on tube formation did not depend on the presence of a functional AR, but rather depended on the ability of mHUVECs to further metabolize DHT.
Conclusions (1) Heterogeneous expression of AR in male- and female-derived HUVEC could define the presence of functionally different subpopulations of ECs that may be affected differentially by androgens, which could explain, at least in part, the pleiotropic effects of androgen on vascular biology, and (2) DHT, and metabolites of DHT, generally thought to represent progressively more hydrophilic products along the path to elimination, may have differential roles in modulating the biology of human ECs through AR-dependent and AR-independent mechanisms, respectively.

Gary J. Smith and Alejandro S. Godoy have contributed equally to this work.

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Introduction

Androgen receptor (AR) is a ligand-inducible transcription factor, and a member of the steroid–thyroid–retinoid receptor superfamily, that mediates the biological effects of androgens in a wide range of physiological and pathological processes [1]. An increasing body of the literature that demonstrates expression of AR in endothelial cells (ECs) from several human tissues [2–8] suggests a potential role for androgens, acting through AR-mediated processes, in modulation of human EC homoeostasis [9–11]. However, up until now, the biological effects, and the molecular signals/mechanisms, driven by AR in an EC context, still remain poorly characterized.

Androgen signaling plays a key role in several high-incidence and high-prevalence human diseases, such as cardiovascular diseases (CVDs), benign prostate hyperplasia (BPH) and prostate cancer (CaP) [9–11]. Interestingly, in these pathological processes, androgens have been hypothesized to exert their role, at least partially, by modulating EC homeostatic function, presumably acting through AR [8, 12–15]. In the case of CVDs, interaction between androgens and the endothelial cells of the blood vessel wall has been hypothesized based on the following observations: (1) testosterone (T) supplementation inhibits the formation of atheroma in animal models [16], (2) androgen withdrawal is associated with decreased central arterial compliance in humans [17], (3) T is a protective factor against atherosclerosis through immunomodulation of plaque development and stability [18], (4) long-term oral administration of T induces endothelium-dependent and endothelium-independent vaso-relaxation [19], and (5) conversely, men have a higher incidence of cardiovascular disease than women during their reproductive years, with gender differences diminishing after female menopause [20], suggesting that androgens are associated causally with an increased risk of cardiovascular disease in both men [20] and women [21]. As supported by these pieces of evidences, the role of androgens in cardiovascular physiology and physiopathology remains controversial, and therefore, a better understanding of the molecular links between androgen and endothelial cell biology are required in order to unravel the pathogenesis of CVDs.

In androgen-responsive human prostate tissue, withdrawal of androgenic signaling by AR antagonists (e.g., flutamide or bicalutamide) and/or inhibitors of steroid metabolism (e.g., finasteride or dutasteride) inhibited hematuria due to BPH or after prostate surgery [22]. Moreover, two clinical studies [23, 24] in CaP patients have shown that a combination therapy with bicalutamide/goserelin (a gonadotropin-releasing hormone agonist) and dutasteride induced a profound vascular collapse and reduced prostate tissue vascularity. Our group [25] confirmed prostate vascular involution was induced by androgen withdrawal in benign and malignant human prostate tissue transplanted to SCID mice and that this effect was correlated temporally with induction of EC apoptosis. However, the androgen signaling in human prostate ECs requires more in-depth characterization at the mechanisms of action of AR.

Even though an increasing body of the literature documenting the effects of androgens on human vasculature has been developed over the last 10 years, the paradigm of the mechanism of action of androgens on the homeostatic function of human ECs generally is attributed to the effects of androgens being mediated through modulation of other (non-endothelial) cell types in the tissue microenvironment, specially in prostate tissue microenvironment. In this study, the androgen-responsive HUVECs were utilized as model to characterize mechanistically the biological role of androgens, acting through endogenous AR-mediated signaling, in human ECs. Our analysis indicated that expression of AR in HUVECs could define the presence of functionally different subpopulations of EC that may be affected differentially by androgens, and that DHT, and metabolites of DHT, may have differential roles in modulating the biology of human ECs through AR-dependent, and AR-independent, mechanisms, respectively.

Materials and methods

Cell cultures

Primary cultures of HUVECs were isolated from fresh umbilical cords obtained from male and female fetuses according to previously published reports [26, 27]. Human umbilical cords were collected with the approval of the Ethics and Biosafety Committee at Pontifical Catholic University. HUVECs also were obtained commercially from PromoCell (PromoCell, Heidelberg, Germany). All HUVECs were cultured in EC growth media (PromoCell) supplemented with 5 % FBS. LNCaP cells were obtained commercially from ATCC (ATCC, Manassas, VA) and cultured in RPMI-1640 media supplemented with 10 % FBS.

Reverse transcription, PCR and quantitative real-time PCR

Total RNA from primary cultures of HUVEC and LNCaP cell was prepared using the RNAeasy mini-kit (Qiagen,

Valencia, CA). Reverse transcription from mRNA was performed using the SuperScript™ III First-Strand kit (Invitrogen, Carlsbad, CA) [25]. Quantitative real-time PCR (QRT-PCR) was performed using power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) in the ABI PRISM 7300 system [25]. The cycle conditions were 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Primer sequences for RT-PCR and QRT-PCR are detailed in Supplementary Table 1.

Immunostaining

Immunostaining was performed according to standard procedures [8]. HUVECs were previously stimulated with or without DHT for 24 h. AR (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) and CD31 (Dako, Carpinteria, CA) co-localization analyses were performed using the mouse/rabbit EnVision™ G/2 Double Stain System (Dako) [28]. AR expression was detected using an HRP-conjugated secondary antibody and 3,3-diaminobenzidine tetrahydrochloride as substrate (brown precipitate). CD31 expression was visualized using an alkaline phosphatase-conjugated secondary antibody and Permanent Red as substrate (red precipitate). Immunostaining in the absence of primary antibody provided negative controls.

AR ligand-binding assay

HUVECs were pre-incubated for 24 h in EC growth media supplemented with 5 % charcoal-stripped FBS before binding assays. Total R1881 binding to AR was determined by incubation of cells for 4 h at 37 °C in increasing concentrations of [17∝-methyl-^{3}H]-R1881 (Perkin-Elmer, Waltham, MA) that ranged from 0.01 to 6 nM. Non-specific binding was determined by analysis of total binding in the presence of a 500-fold excess of non-radiolabeled R1881 (Perkin-Elmer) under the same experimental conditions. Specific binding of [^{3}H]-R1881 was calculated by subtraction of the non-specifically bound radioactivity from the total bound radioactivity. The K_d value for R1881 was determined using Scatchard analysis [8] (reciprocal of the slope [$-1/K_d$]) and represents the average of three independent experiments, each performed in triplicate.

AR-mediated luciferase assay

HUVEC and LNCaP cell were incubated for 24 h in EC growth media (HUVEC) or RPMI media (LNCaP), supplemented with 5 % charcoal-stripped FBS. Cells were infected for 3 h with an adenoviral expression vector that encoded either an MMTV promoter- or PSA promoter-driven luciferase reporter (10–20 infectious units/cell) and then stimulated with or without (vehicle, ethanol) DHT for 36 h [8, 29]. Demonstration of inhibition of MMTV-driven luciferase reporter activity by the anti-androgen bicalutamide was achieved by the maintenance of HUVEC cultures in the presence of designated concentrations of bicalutamide throughout the entire duration of the experiment (3-h infection + 36-h incubation).

Flow microfluorimetry

Cell permeabilization was performed using the Caltag Fix and Perm Cell permeabilization kit (Caltag Laboratories, San Francisco, CA). HUVEC were stained immunofluorescently with mouse antihuman CD31 (1:20; Dako) and rabbit antihuman AR (1:100; Santa Cruz Biotechnology) primary antibodies and subsequently with fluorescently labeled species-specific secondary antibodies. Staining was assessed using the FACSCalibur (BD Bioscience with CellQuest software for Macintosh) and analyzed using FCS Express (DeNovo Software, Los Angeles, CA). Briefly, cohorts of cells were deprived of androgen for 24 h (−24 h) or 48 h (−48 h), or were deprived of androgen for 48 h after which androgen (1.0 nM DHT) was re-introduced into the culture media for an additional 24-h period (−48 + 24). As a positive control, cells were treated with serum-free medium for the same intervals of time. Both floating and attached cells were collected from cultures and subjected to CD31 staining, as well as to staining with annexin-V-FITC and propidium iodide (PI) using protocols provided by the manufacturer (BD Biosciences, San Jose, CA).

Immunoblotting

Proteins from cytosol and nuclear cell extracts were isolated using the NE-PER nuclear and cytoplasmic extraction reagents (ThermoFisher Scientific). Cytosol, nuclear and total cell extracts (50 μg of protein) were separated electrophoretically using SDS–polyacrylamide gel electrophoresis (10 % w/v; Bio-Rad Laboratories, Hercules, CA) and the separated proteins transferred to nitrocellulose membranes [8]. Nitrocellulose membranes were incubated with the primary antibodies: anti-AR (1:1000, Santa Cruz Biotechnology), anti-cleaved PARP (1:1000, Cell Signaling, Beverly, MA), anti-caspase-3 (1:500, Cell Signaling), anti-Bcl2 (1:1000, Dako), anti-FXR (1:500, R&D systems, Minneapolis, MN), anti-RXRα (1:500, Santa Cruz Biotechnology), anti-β-actin (1:1000, Santa Cruz Biotechnology), anti-β-tubulin (1:3000, AbCam), anti-Histone H3 (1:1000, Cell Signaling). After this, nitrocellulose membranes were incubated with the corresponding HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibody. Antibody localization was visualized using enhanced chemiluminescence (Pierce Biotechnology).

Microtiter tetrazolium (MTT) and cell counting assays

The effect of DHT on population growth of HUVECs was assessed over a 10-day period using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] method. Cells (5×10^2 cells/well) were inoculated into 96-well plates in 100 µl of medium. The plated cells were allowed to attach overnight, after which the media was replaced with fresh media containing either 1.0 nM DHT, or vehicle (ethanol). Cell growth was determined by the MTT assay on days 0, 1, 3, 5, 7 and 10 after DHT stimulation. For the MTT assay, medium was removed from the wells, and 200 µl of Hepes buffer was added to each well, followed by addition of 50 µl of MTT (2.5 mg/ml in PBS, Sigma-Aldrich, St. Louis, MO). The wells were incubated for 4 h at 37 °C, after which the liquid in the well was aspirated and replaced by 200 µl of dimethyl sulfoxide (Sigma-Aldrich) and 25 µl of Sorensen's buffer. The optical density was measured at 570 nm using an ELISA reader (EL800, BioTek Instruments, Winooski, VT). For cell counting assays, wells were treated with either 1.0 nM DHT or vehicle, and cell number counted on days 0, 1, 3, 5, 7 or 10 after DHT stimulation. Attached cells were collected by trypsinization and counted using a hemocytometer. Trypan blue dye exclusion was used to determine viable cells.

EC tube formation assay

Matrigel (176 µl, BD Biosciences) was dispensed into wells of a 24-well plate and incubated for 30 min at 37 °C in 5 % CO_2 for the Matrigel to solidify [8]. HUVECs were suspended at a density of 100×10^3 cells/ml in 500 µl of EC growth media supplemented with 5 % charcoal-stripped FBS and designated concentrations of DHT (0.01, 0.1, 1, 10 nM). Aliquots of HUVECs (50×10^3 cells) were seeded into wells that contained solidified Matrigel and were incubated for 24 h at 37 °C in 5 % CO_2. The effect of DHT on EC tube formation was analyzed by collection of four random digital images per well (4× magnification), and total length of tubular structures was quantified per image using Optimas 6.2 (Media Cybernetics, Bethesda, MD).

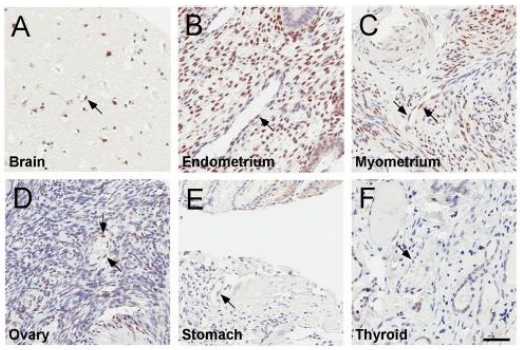
Statistical analysis

Statistical evaluation of data was performed using SuperANOVA software (Abacus Concepts, Berkeley, CA). All data differences were considered statistically significant when the p value was <0.05.

Results

AR is expressed in human ECs of multiple organs

Expression of AR in human ECs was validated by immunostaining analyses of microarrays of tissue sections of a diversity of benign human tissues (Fig. 1). AR protein was observed in ECs in brain, endometrium, myometrium, ovary and prostate tissue (Fig. 1a–d, g). However, several benign human tissues, such as thyroid, colon, liver, lung, pancreas, spleen, stomach and kidney, showed no AR immunostaining in ECs (Fig. 1e–g). Within the immunopositive tissues, AR immunostaining was present in ECs of both micro- and macro-vasculatures; however, AR expression at the level of the individual EC was heterogeneous; some ECs showed AR immunostaining, others did not.



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Benign Human Tissue	AR Expression in EC
Brain	+
Colon	-
Endometrium	+
Kidney	-
Liver	-
Lung	-
Myometrium	+
Ovary	+
Pancreas	-
Prostate	+
Spleen	-
Stomach	-
Thyroid	-

Fig. 1 AR expression in ECs from benign human tissues. AR expression was analyzed in a tissue microarray containing benign human specimens of brain, breast, colon, endometrium, kidney, liver, lung, myometrium, ovary, pancreas, prostate, skeletal muscle, skin, spleen, stomach, testes, thyroid and tonsil. Expression of AR at the EC level was observed in brain (**a**), endometrium (**b**), myometrium (**c**), ovary (**d**), prostate and skin (**g**) (*black arrows*). However, ECs from stomach (**e**), thyroid (**f**), lung, skeletal muscle and kidney (**g**) showed no expression of AR in ECs. *Black bar* 50 µm

Functional expression of endogenous AR in female- and male-derived HUVECs does not depend on the sex

Primary cultures of HUVECs isolated from umbilical cords obtained from female and male fetuses were utilized to characterize in vitro the expression and functionality of AR. First, we analyzed the expression of AR protein in female- and male-derived HUVEC cultures that were cultured through 10 consecutive passages after isolation (Fig. 2a). Our results indicated that AR expression was maintained during all these passages in female- and male-derived HUVEC culture (Fig. 2a). Therefore, all subsequent studies in HUVECs were performed using cultures within the first 5 passages. Interestingly, both female and male HUVECs expressed both the 110-kDa full-length AR and a shorter band, around 85 kDa, which might indicate the presence of a short AR variant [30]. Notably, this band was more robust in female than in male HUVEC cultures (Fig. 2a). In parallel, we analyzed expression of AR at the mRNA and protein level in 10 different cultures of female- and male-derived HUVECs (data not shown). In Fig. 2b, c, we show expression of AR mRNA and protein in 4 different male- and 4 different female-derived HUVEC cultures. Our results indicated that expression of AR was variable in both female-derived and male-derived HUVEC cultures. Interestingly, this variability was not related to the gender of the fetus from which HUVECs were obtained (Fig. 2b, c). Functional activity of the endogenous AR in female and male HUVEC was confirmed by measurement of AR-mediated transcriptional activity using an adenoviral MMTV promoter-driven luciferase gene reporter after stimulation with or without DHT for 36 h (Fig. 2d). Our results confirmed that functionality of endogenous AR in female-derived and male-derived HUVECs did not depend on the sex of the HUVEC cultures and it was not correlated with the level of expression of the AR protein. Together, these data indicate that the levels of expression and functionality of AR in HUVECs associated more to a still unexplained inter-donor variability rather than a sex-related variability. In order to pursue with our analysis, and to correlate our results with what we previously observed using human prostate-derived ECs, we decided to focus our study on the expression and functionality of AR in male-derived HUVEC cultures (here after mHUVECs) (Fig. 3). Expression of AR mRNA and protein, and AR protein translocation to the nucleus after 24 h of DHT stimulation was demonstrated in mHUVECs using RT-PCR (Fig. 3a), immunocytochemistry (Fig. 3b) and immunoblotting (Supplementary Figure 1). Analysis of co-localization of AR and CD31 confirmed expression of AR in ECs (Fig. 3c). Ligand-binding affinity of AR in mHUVECs was analyzed using the radiolabeled synthetic AR agonist/ligand, R1881. Specific binding of R1881 to AR in mHUVECs increased in a dose-dependent manner, and binding saturated above a ligand concentration of 2.0 nM (Fig. 3d). Scatchard transformation of the ligand-binding data resulted in a straight line, indicative of a single ligand-binding site for R1881, with a K_d 0.1 nM (Fig. 3e). Functional activity of the endogenous AR in mHUVECs was confirmed by measurement of AR-mediated transcriptional activity using an adenoviral MMTV promoter- or PSA promoter-driven luciferase gene reporter. DHT increased the reporter activity driven by the MMTV promoter fivefold in mHUVECs (Fig. 3f, MMTV). However, DHT did not stimulate expression of the luciferase reporter driven by the epithelial cell-specific PSA promoter (Fig. 3f, PSA). Functionality of the PSA-driven reporter construct was validated using LNCaP cells (Fig. 3f, PSA). The MMTV promoter is promiscuous, and MMTV-driven transcription can be stimulated by nuclear steroid receptors others than AR (i.e., GR and PR) [31]. Consequently, expression of GR and PR nuclear receptors was analyzed in mHUVECs using immunocytochemistry (data not shown). Both nuclear receptors (GR and PR) were expressed in mHUVECs ; however, neither of them translocated to the nucleus after DHT treatment, which suggested that they were not involved in MMTV-driven transcriptional activity. Furthermore, causal participation of AR in the MMTV-driven transcriptional activity in mHUVECs was validated using the anti-androgen bicalutamide. DHT-stimulated, AR-mediated, MMTV-driven luciferase reporter activity was inhibited in a dose-dependent manner by bicalutamide (Fig. 3g).

As was observed for ECs in multiple benign human tissues in situ (Fig. 1), expression of AR in mHUVECs in vitro was heterogeneous; only a fraction of these cells expressed AR in culture (Fig. 3h). The proportion of AR-expressing male-derived HUVEC was quantitated using flow cytometry to identify cells that co-expressed AR and CD31 (Fig. 3h). The percentage of AR-positive male-derived HUVEC varied according to the cell culture population analyzed, ranging from less than 5 %, to as high as 60 % (Fig. 3h).

Androgens do not affect survival in vitro in male-derived HUVECs

Androgen deprivation was associated with a reduction in microvessel density (MVD), and appearance of apoptotic ECs, in primary xenografts of human benign and malignant prostate tissue [25], which suggested androgen modulated directly EC survival. The role of androgen in EC survival was investigated in vitro in mHUVECs by exposure of cells to 24, or 48 h, of androgen deprivation. As a control of loss/maintenance of AR protein and functionality in the

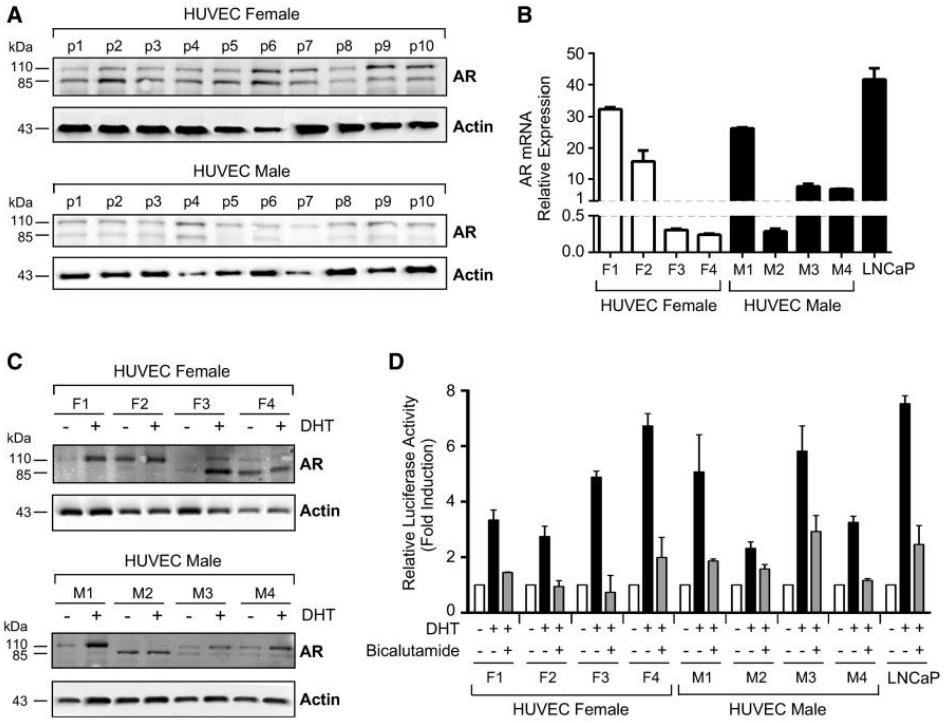

Fig. 2 AR expression and functionality in female- and male-derived HUVECs. **a** AR protein expression was analyzed using immunoblotting in a female- and a male-derived HUVEC culture, which was cultured through 10 (p1–p10) consecutive passages. β-Actin (actin) was used as loading control, **b** analysis of AR mRNA expression in 4 different female-derived HUVEC cultures and 4 different male-derived HUVEC cultures using QRT-PCR. LNCaP cells were used as positive control, **c** the same sets of HUVEC cultures were analyzed for AR protein expression using immunoblotting, **d** AR-mediated transcriptional activity was analyzed in the same set of female- and male-derived HUVECs infected with an adenoviral MMTV-driven luciferase gene reporter in the absence (vehicle, *white bars*) and presence (*black bars*) of 1 nM DHT. AR-mediated transcriptional activity was inhibited by the anti-androgen bicalutamide (*gray bars*). LNCaP cells were used as positive control

absence of androgen, mHUVECs also were exposed to 48 h of androgen deprivation followed by 24 h of replenishment with exogenous androgen. Quantitative RT-PCR analyses demonstrated no significant variation in the levels of AR mRNA in mHUVECs exposed to androgen deprivation (Fig. 4a). However, AR protein level was diminished consistently in mHUVECs by androgen deprivation, as demonstrated using immunoblotting (Fig. 4b) and immunostaining (Fig. 4c) analyses. Automated image analysis of AR immunostaining was used to quantitate the number of nuclei immunopositive for AR as a percentage of the total nuclei. Statistical evaluation of the data demonstrated the acute removal of androgen resulted in a significant ($p < 0.001$) decrease (up to ~ 60 %) in the number of AR immunopositive nuclei (Fig. 4d). Under these conditions, immunoblotting analyses revealed that the level of expression of the pro-apoptotic marker, cleaved PARP, was unaffected by the removal of androgen from mHUVECs (Fig. 4e). The protein level of the pro-apoptotic marker cleaved caspase-3 was slightly decreased, and of the anti-apoptotic marker bcl-2 slightly increased, by androgen deprivation. However, replenishment of androgen (1.0 nM DHT) did not return bcl-2 nor the cleaved-caspase-3 levels to baseline. In parallel, the induction of apoptosis in mHUVECs by androgen deprivation was evaluated using the Annexin V-FITC Apoptosis Detection Kit with flow cytometric analysis (Fig. 4f). Apoptotic cells were defined as Annexin V-FITC positive, PI negative cells (bottom right quadrant of multi-parameter data plot). Only a slight increase in the number of apoptotic mHUVECs was observed after either 24 h (from 1.16 to 1.98 %) or 48 h (from 1.16 to 2.41 %) of androgen deprivation, and this increase was reversed when androgen was replenished (Fig. 4f). As a positive control for induction of apoptosis as detected by immunoblotting and Annexin V-FITC analyses, mHUVECs were exposed to serum starvation for 24 and 48 h. Serum starvation resulted in a robust increase in the number of apoptotic cells, as well as in the expression

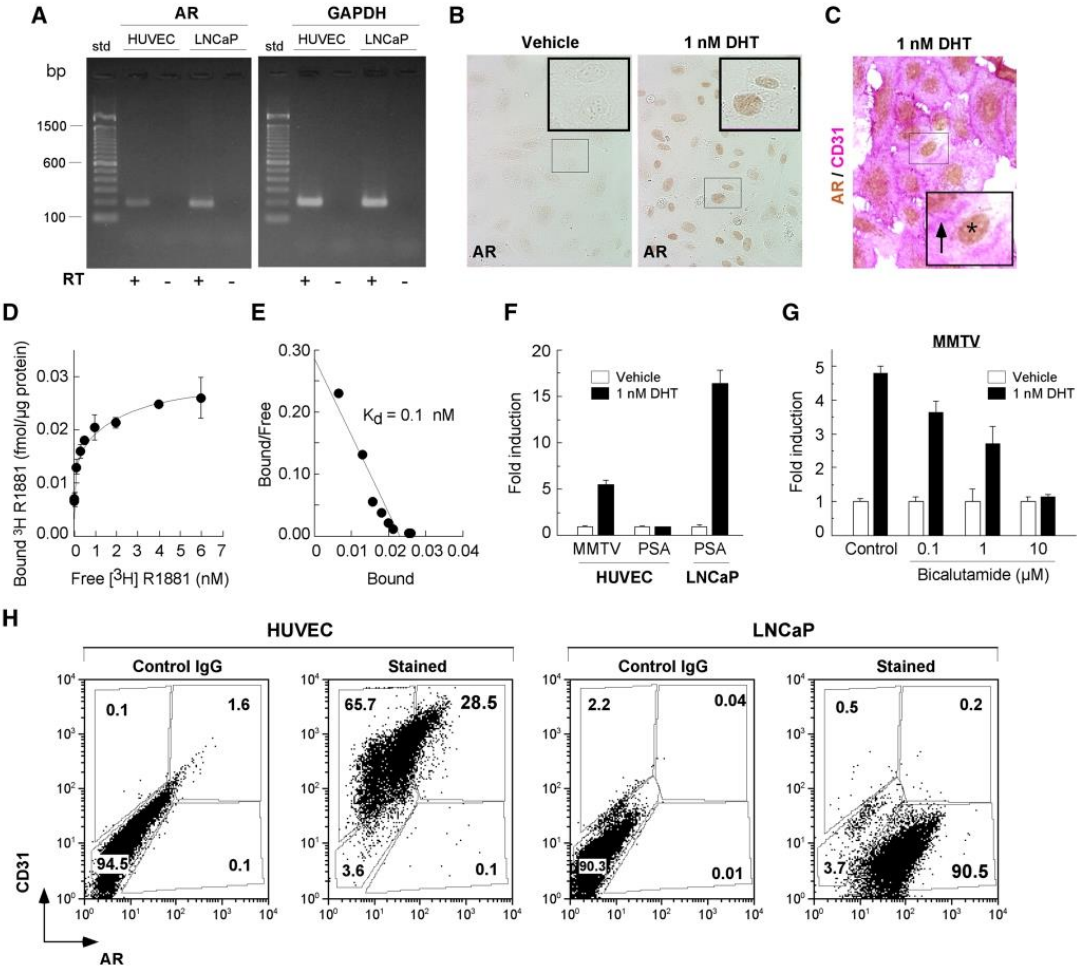

Fig. 3 Functional expression of AR in mHUVECs. **a** AR mRNA expression was analyzed using RT-PCR. LNCaP cells were used as positive control. Std: marker ladder. *bp* base pair, *RT* reverse transcription, **b** AR immunostaining in HUVEC in the absence (vehicle) or presence of 1 nM DHT, **c** AR and CD31 double immunostaining in HUVEC exposed to 1 nM DHT, **d** specific [^{3}H]-R1881 binding activity in HUVEC, **e** scatchard plot of the binding data from graph D, **f** AR-mediated transcriptional activity in HUVEC infected with adenoviral MMTV- or PSA-driven luciferase gene reporter, **g** AR-mediated transcriptional activity was inhibited in a dose-dependent manner by the anti-androgen bicalutamide, and **h** co-expression of AR and CD31 was analyzed in HUVEC using flow cytometry. LNCaP cells were used as positive control for AR expression

of cleaved PARP and a decrease in bcl-2 expression at 24 and 48 h (data not shown).

Androgen modulates proliferation and tube formation capacity in male-derived HUVECs using independent mechanisms

In vitro studies in primary cultures of human prostate ECs indicated that activation of AR by exogenous DHT increased EC proliferation [8]. The effect of androgen on cell growth in mHUVECs was analyzed using the MTT cell proliferation assay of cell number (Fig. 5a) and trypan blue exclusion assay of cell viability (Fig. 5b). mHUVECs were cultured for 1–10 days in the absence (vehicle) or presence of 1.0 nM DHT. Both methodologies independently demonstrated a significant ($p < 0.05$) increase in mHUVEC growth in the presence of 1.0 nM DHT compared to vehicle (ethanol). The causal role of AR in the induction of EC growth was demonstrated by the capacity of the anti-androgen bicalutamide to block the DHT-activated, AR-mediated growth (Fig. 5c).

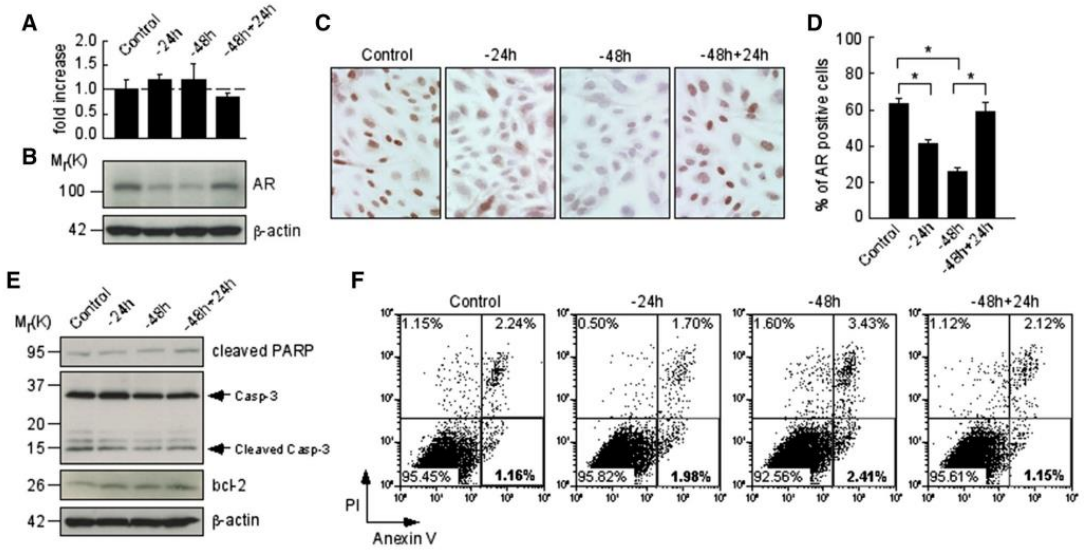

Fig. 4 DHT does not affect EC survival in mHUVECs. HUVEC were exposed to 24 or 48 h of androgen deprivation. HUVEC also were exposed to 48 h of androgen deprivation followed by 24 h of replenishment of androgen (1 nM DHT). Control cells were maintained in regular media supplemented with 1 nM DHT. Expression of AR was analyzed using QRT-PCR (**a**), immunoblotting (**b**) and immunostaining (**c**) analyses. **d** Automated image analysis of the AR immunostaining in HUVECs ($^*p < 0.001$); 10 (×40) independent images were quantified per condition. **e** Expression of pro-apoptotic (cleaved PARP, cleaved caspase 3) and anti-apoptotic (bcl-2) markers were analyzed using immunoblotting. **f** Apoptotic levels were confirmed using Annexin-V/PI kit

The effect of androgen on tube formation in Matrigel by mHUVECs was analyzed in the presence of increasing concentrations of androgen (Fig. 5d). DHT significantly decreased, in a dose-dependent manner, the ability of mHUVECs to form tubes in Matrigel. This effect was less pronounced than the effect of DHT on mHUVEC proliferation and was more evident at higher concentrations of DHT (10 nM). In order to confirm/discard the role of AR in the inhibitory action of DHT on tube formation, the effect of bicalutamide on the ability of DHT to inhibit tube formation by mHUVECs was tested (Fig. 5e). Bicalutamide did not revert the inhibitory effect of DHT on tube formation by mHUVECs, which suggested that AR was not involved in mediating this effect. Because higher concentrations of DHT were needed to affect tube formation capacity than to inhibit proliferation by mHUVECs which occurred at concentrations consistent with the K_d of DHT for AR (0.1 nM), and considering that AR appeared not to be involved in this effect because bicalumatide did not reverse the inhibition of tube formation, we hypothesized that the inhibition of tube formation was dependent on the ability of mHUVECs to metabolize DHT to other bioactive moieties. To test this hypothesis, R1881, a synthetic, non-metabolizable (Fig. 5f) AR agonist, was utilized to explore whether the inhibition of tube formation was dependent on further bioconversion of DHT into one or more metabolites. R1881 did not reproduce the inhibitory effect of DHT on the ability of mHUVECs to form tubes in Matrigel (Fig. 5g), in contrast to the ability of R1881 to substitute DHT for stimulation of proliferation (data not shown), supporting the hypothesis that further metabolization of DHT is required to produce the molecule(s) that inhibit(s) tube formation in vitro.

DHT could potentially be converted to 3α-androstanediol/androsterone metabolites in male-derived HUVECs

DHT can be metabolized via two different pathways [32] (Fig. 6a). The family of enzymes called 3β-hydroxysteroid dehydrogenase (3β-HSD), of which two members (HSD3β1 and HSD3β2) have been described in human cells, metabolize DHT to 3β-androstanediol (3β-diol). Alternatively, DHT can be metabolized to 3α-androstanediol (3α-diol) and androsterone by the sequential action of two distinct families of enzymes, the 3α-hydroxysteroid dehydrogenase (3α-HSD) and the 17β-hydroxysteroid dehydrogenase (17β-HSD) or to androstanedione and androsterone by the sequential action of 17β-HSD and 3α-HSD (Fig. 6a). Four members of the 3α-HSD family and fourteen members of the 17β-HSD family have been described in human cells [32]. In this study, RT-PCR was

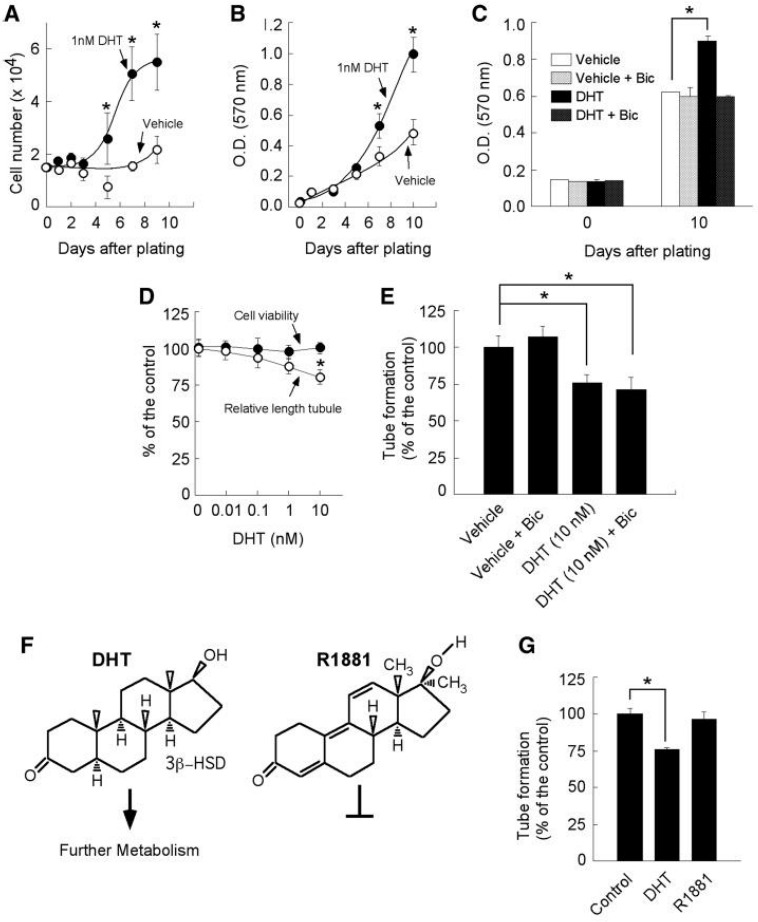

Fig. 5 Androgen modulates mHUVEC proliferation and tube formation using independent mechanisms. **a**, **b** The effect of androgen on HUVEC growth was analyzed by counting viable cells using the trypan blue exclusion method (**a**) and the MTT cell proliferation assay (**b**). HUVEC were cultured for 1–10 days in the absence (*white circles*, vehicle) or presence (*black circles*, 1 nM DHT) of DHT. **c** Treatment with bicalutamide confirmed AR-activated EC proliferation. The effect of DHT on endothelial tube formation in Matrigel™ was analyzed in the presence of increasing concentrations of DHT (0.01, 0.1, 1, 10 nM). **d** Relative tube length was expressed as percentage of the control (*white circles*, 0 nM), and cell viability was evaluated in parallel using the trypan blue exclusion method (*black circles*). Effect of DHT on endothelial tube formation was not affected by bicalutamide (**e**) and not observed when R1881 was used (**f**, **g**). $*p < 0.05$

utilized to analyze mRNA expression of the two members of the family of enzymes 3β-HSD and select members of the families of 3α-HSD (3α-HSD2, 3α-HSD3, 3α-(20α) HSD) and 17β-HSD (17β-HSD2 and 17β-HSD3) enzymes (Fig. 6b, c). mHUVECs lacked expression of mRNA for both members of the 3β-HSD enzyme family (Fig. 6b). However, mHUVECs expressed mRNA of three members (3α-HSD2, 3α-HSD3, 3α-(20α) HSD) of the 3α-HSD enzyme family (Fig. 6c) and at least one member (17β-HSD2) of the 17β-HSD enzyme family (Fig. 6c). The pattern of expression of enzymes involved in metabolic deactivation of DHT suggests 3α-androstanediol and/or androsterone as candidates for mediation of the effect of androgen on EC tube formation.

Androsterone has been reported to be a ligand capable of activation of the farnesoid X receptor (FXR), a signaling pathway with the potential to modulate EC homeostasis [33–35]. This hypothesis was tested by analysis of the expression of FXR at the mRNA and protein levels in mHUVECs. Neither mRNA (Fig. 6d) nor protein (Fig. 6e) for FXR was expressed in mHUVECs. mRNA isolated from five different donors of mHUVECs (Supp. Figure 2A) was analyzed, demonstrating that inter-patient variability was not responsible for the lack of expression of FXR. Furthermore, the possibility that lack of FXR mRNA in mHUVECs reflected the presence of splicing variants of FXR was excluded through the design of primer sets that covered multiple regions of the FXR coding sequence of the two known human FXR splice variants [36]. None of the variants were expressed in mHUVECs (Supp. Figure 1B). In contrast, expression of the retinoid X receptor alpha (RXRα), a known partner for FXR for production of heterodimers, was detected in mHUVECs (Fig. 6e). Together, these data support the hypothesis that 3α-androstanediol/androstanedione/androsterone might modulate mHUVECs tube formation through a mechanism that does not involve FXR.

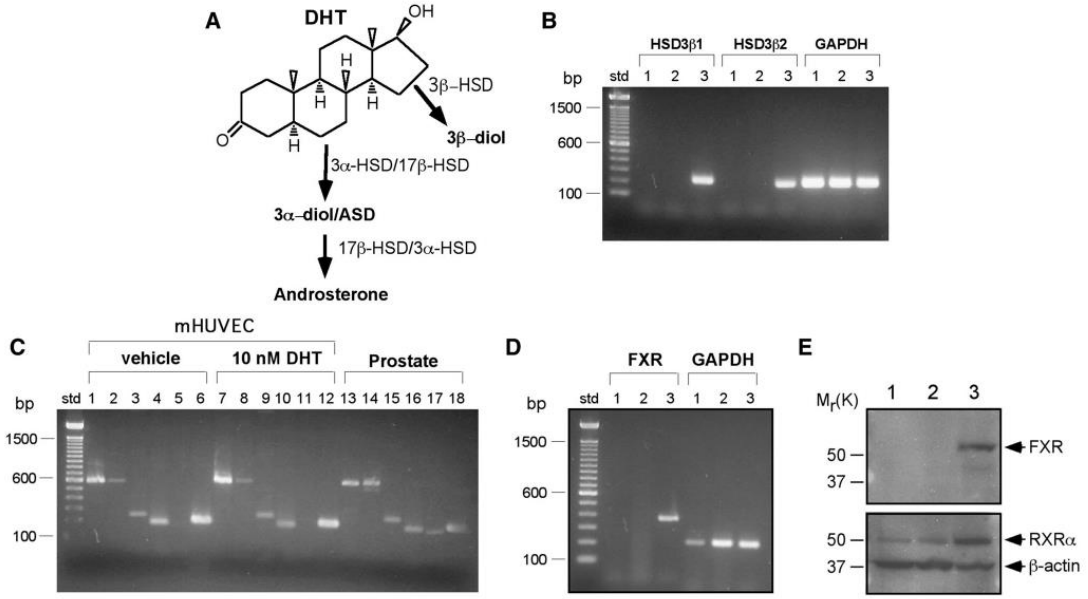

Fig. 6 Expression of the enzymes involve in the metabolism of DHT in mHUVECs. **a**, **b** Expression of the mRNA for the enzymes HSD3β1 and HSD3β2 was analyzed in HUVEC exposed to vehicle (*lanes 1*) and 1 nM DHT (*lanes 2*). LNCaP cells were used as positive control (*lanes 3*). GAPDH was used as a loading control. **a**, **c** Expression of the mRNA for the enzymes 3α-HSD2 (*lanes 1*, *7*, *13*), 3α-HSD3 (*lanes 2*, *8*, *14*), 3α-(20α) HSD (*lanes 3*, *9*, *15*), 17β-HSD2 (*lanes 4*, *10*, *16*) and 17β-HSD3 (*lanes 5*, *11*, *17*) in HUVEC. GAPDH was used as a loading control (*lanes 6*, *12*, *18*). Prostate tissue was used as positive control (*lanes 13–18*). Expression of the FXR receptor was analyzed using RT-PCR (**d**) and immunoblotting (**e**) analyses in HUVEC exposed to vehicle (*lanes 1*) and 1 nM DHT (*lanes 2*). HepG2 cells were used as positive control (*lanes 3*). Expression of RXRα was analyzed using immunoblotting (**e**). GAPDH and β-actin were used as loading control for RT-PCR and immunoblotting, respectively

Discussion

Over the last two decades, a considerable number of studies have focused on elucidation of the role of androgen on different aspects of vascular biology and atherogenesis. However, the role of androgen as a causal or a protective factor and the importance of the interplay of androgens with estrogens in the etiology and pathogenesis of cardiovascular disease remain controversial [15–21, 37]. Furthermore, while the role of androgen in hormonally sensitive tissues has been explored extensively, the role of circulating androgens in vascular biology in hormonally sensitive tissues, such as prostate tissue, is not understood [12, 13, 23, 25].

Typically, circulating T is the source of androgen that is metabolized in the target tissue to DHT, by the action of the 5α-reductases, or to 17β-estradiol by the action of aromatase (CYP19). Both enzymatic processes occur in ECs [38, 39]; therefore, the net biological effect of T on EC biology may depend on the tissue-specific relative contribution of the activities of both families of enzymes (reductases and aromatase). Based on the fact that DHT is the most bioactive androgen in human cells, having fivefold higher affinity for AR than T [40], and that AR expression in ECs is documented in several human tissues [2–8], in this study we utilized DHT as the source of androgen rather than T, because DHT cannot be aromatized, avoiding the potential for confounding effects mediated through the estrogen receptor in response to the conversion of T to 17β-estradiol.

Previous reports [2–8] of expression of AR in ECs from several human tissues were confirmed in this study. Interestingly, AR-positive ECs were observed both in "classical" and in "non-classical" hormonal target tissues, which suggest an EC-specific role for AR in these tissues. In support of this hypothesis, the present study showed that prostate epithelial cell-specific promoters that are trans-activated by AR, specifically the PSA (Fig. 3f) and probasin (not shown) promoters, were not transactivated by endogenous AR in endothelial cells, suggesting EC-specific roles for AR/co-regulators. Furthermore, the functional characterization of AR in HUVEC in this study demonstrated a dissociation constants (K_d) of AR for DHT around 0.1 nM, consistent with the reported K_d for AR in prostate epithelial cells [41], suggesting also an important role for cell-type specific co-regulators of AR-mediated transcription. On this regard, it is

important to highlight that the experimentally determined K_d predicts a "theoretical" maximum capacity for activation of AR in ECs at concentrations around 1–2 nM DHT. However, some studies [42] that investigated the biological effects of androgen in HUVECs used concentrations of DHT that were far above the predicted range for maximal activation of AR, reaching levels of DHT as high as 400 nM. Using such high concentrations of DHT and claiming that the biological effects are produced by activation of AR, therefore, appear of unknown predictive value.

An interesting observation of this work was the detection of a shorter band (around 85 kDa) for AR. The molecular size of this band was concordant with expression of a shorter AR variant [43], which up until now have been described to be expressed only in prostate cancer cells, in which they have been proposed as a possible explanation for the transition from androgen-sensitive to castration-resistant phenotype [44]. Even though these observations are preliminary, it would be interesting to investigate whether these AR variants may have a physiological role in normal/benign cells, especially endothelial cells, and whether or not these AR variants may show differential expression between male- and female-derived ECs [43–45].

A previous study by Sieveking et al. [42] reported that androgen modulated angiogenesis capacity in HUVECs in a gender-related manner, indicating that AR was expressed at a higher level in male fetus-derived HUVECs compared to female fetus-derived HUVECs. Our study, however, challenged these results indicating that differences in the level of expression of AR in HUVECs were more associated with an inter-donor rather than a gender-related variability. In our study, AR expression and functionality were variable in male- and in female-derived HUVECs, showing male and female HUVEC cultures with high, medium or low levels of expression of AR (Fig. 2b, c). In accordance with our results, a study reported by Yoshida et al. [11, 45] indicated that both male and female AR knockout mice showed impaired revascularization after ischemia. Yoshida et al. [11, 45] attributed their different results, compared to the Sieveking's study, to the different animal models utilized in both studies [11]. Although our results support Yoshida's conclusions, they cannot rule out the possibility that this variability might be more associated with difference in the number of AR immunopositive ECs observed in either male or female ECs nor the type of organ involve in each analysis. Our results in mHUVEC (Fig. 3h) indicated that the AR immunopositive cells varied from 0 to up to 60 % between donor and donor, which might explain, at least in part, this controversy.

Even though AR expression and functionality have been studied in great detail in prostate luminal epithelial/epithelial cancer cells, few studies have focused on analysis of the expression and functionality of AR in non-epithelial cell compartments. Our group has a long-standing interest in unraveling the molecular mechanisms associated with the AR function in non-epithelial (endothelial and stromal) prostate cells [8–10, 14, 46–48]. Interestingly, mHUVECs have demonstrated to reproduce most of the biological effects of androgens observed in prostate endothelial cells [8]. Therefore, in this study we concentrated our efforts to unravel the biological effects of androgen, and the role of AR on these effects, in mHUVECs. Our study indicated that DHT had differential effects on multiple on mHUVEC processes: DHT did not affect EC survival; stimulated EC proliferation; and suppressed EC tube formation on Matrigel. From these biological processes, only EC proliferation was modulated directly by activation of AR. Endothelial cell survival has been demonstrated to be affected negatively by androgen deprivation in animal models [12, 23, 24]. Our group demonstrated that apoptosis of human prostate ECs was induced acutely by androgen deprivation in human prostate tissue transplanted to SCID mice [25]. However, whether endothelial cell apoptois was a direct response to androgen deprivation, and the role of perturbation of AR-mediated gene transcription in this process, are questions that remain unanswered. In the current study, androgen deprivation demonstrated no effect on survival of mHUVEC in vitro, in contrast to marked effect on ECs in human prostate xenografts in situ [25]. Considering that both types of ECs express AR, the lack of induction of apoptosis by androgen deprivation in the in vitro studies suggests that androgen deprivation also might activate/deactivate paracrine signaling in the prostate microenvironment that could induce/contribute to EC death in vivo.

In androgen-responsive human prostate tissue, the primary role of AR has been hypothesized to be to drive and maintain differentiation of the secretory luminal epithelial cells. However, in CaP, AR activity has been associated with regulation of cancer epithelial cell growth and/or survival. Mechanistic investigations revealed that AR acts as a master regulator of the G1-S phase transition in CaP epithelial cells. AR-mediated signaling promotes G1 cyclin-dependent kinase (CDK) activity and induces phosphorylation/inactivation of the retinoblastoma tumor suppressor (RB) protein, thereby governing androgen-stimulated proliferation [49]. The current study and our previous studies with primary cultures of human prostate ECs [8] suggest that AR is involved causally in the regulation of human EC proliferation (Fig. 6). Studies by Cai et al. [50] suggested that androgens acting on AR stimulated human aortic EC proliferation through upregulation of VEGF-A, cyclin A and cyclin D. However, the detailed molecular mechanism(s) by which AR regulates proliferation in human ECs is still poorly understood. Interestingly, in vitro, EC proliferation was "activated" by, but not

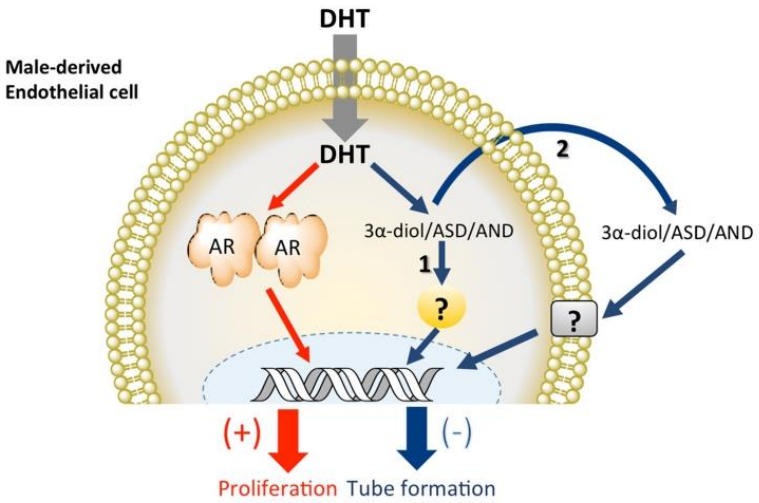

Fig. 7 Schematic representation of the effects of androgen in human ECs. Androgen affects EC homeostasis at multiple levels. DHT stimulates (+) EC proliferation through activation of androgen receptor (AR, *red arrows*). On the other hand, conversion of DHT to 3α-androstanediol/androsterone (3α-diol/AND) seems to be necessary for androgen to negatively regulate (−) tube formation (3α-diol/AND, *blue arrows*). Two options for the mechanism of action of 3α-diol/AND are hypothesized in this schema: *1* 3α-diol/AND modulates target genes through binding/activation of a putative intracellular receptor and *2* 3α-diol/AND needs to be exported outside the cell in order to activate membrane receptors which in turn activate signaling cascades that result in modulation of target gene expression/angiogenic capacity. (Color figure online)

"dependent" on, the presence of an active AR, since EC proliferation was observed, although at a reduced level, in the absence of androgen, as well as in the presence of the anti-androgen bicalutamide. This response could be related to the presence of both AR-expressing and AR-negative compartments in the HUVEC population.

Angiogenesis is an integral part of both normal and pathological processes. The role of androgens in angiogenesis has been considered in some detail in animal and human models, but mostly at the tissue level. In humans, the effect of androgen on tumor angiogenesis has been studied in prostate tissue, with changes in microvessel density correlated with tumor progression both in an androgenic environment and after ADT [13]. Consequently, androgenic effects on tumor angiogenesis have been proposed to be driven largely by indirect mechanisms principally associated with androgen-mediated regulation of VEGF expression by prostate epithelial cells [13], and the subsequent effect on EC survival. Surprisingly, no studies have explored the direct effect of androgen on the angiogenic capacity of human ECs. In order to explore the direct effect of androgen on human EC function, we utilized the tube formation assay, a well-established assay that models the in vitro formation of tubular structures that resembles a capillary network in a three-dimensional architecture. Unexpectedly, DHT decreased the ability of mHUVEC to form tubes on Matrigel, and the effect was not dependent on the presence of an active AR in mHUVECs, but rather depended on the ability of mHUVECs to metabolize further DHT.

DHT can be converted to 3β-androstanediol by the action of the enzyme 3β-hydroxysteroid dehydrogenase [32]. Alternatively, DHT can be metabolized directly to 3α-androstanediol and subsequently androsterone by sequential activity of two families of enzymes: the 3α-hydroxysteroid dehydrogenases and the 17β-hydroxysteroid dehydrogenases, respectively. A role for 3β-androstanediol in regulation of EC homeostasis was discarded based on the lack of expression of the mRNA for any members of the family of 3β-hydroxysteroid dehydrogenases in mHUVEC. Therefore, we initially hypothesized that the DHT metabolite androsterone was a plausible candidate for the DHT metabolite likely to affect tube formation capacity of mHUVEC through activation of the FXR [33, 34]. Consistent with this hypothesis, this study demonstrated that several members of the families of enzymes that participate in the conversion of DHT to androsterone were expressed in mHUVEC. Furthermore, FXR expression was reported in rat pulmonary ECs, where activated FXR leads to down-regulation of endothelin-1 expression [34]. In addition, activated FXR was reported to promote MMP-9-dependent EC motility through regulation of focal adhesion kinase (FAK) activity [51] in human ECs. However, expression of mRNA or protein for FXR was

absent in mHUVEC, contrasting with the reports of Bishop-Bailey et al. [33] and He et al. [34] that FXR was expressed broadly in human ECs.

The current study appears to exclude the possibility of androsterone-mediated activation of FXR as a key modulator of endothelial tube formation by mHUVEC. However, more analyses are necessary to clarify whether conversion of DHT to androsterone is responsible for the inhibitory effect of androgen on EC tube formation in vitro. An alternative explanation for our results would posit that 3α-androstanediol is the/a key modulator of angiogenic capacity in mHUVEC. However, further studies are necessary to clarify whether 3α-androstanediol, androstanedione, and/or the further metabolite androsterone are modulators of tube formation capacity and the putative mechanism involved in this effect (Fig. 7).

In summary, our analysis supports the following conclusions: (1) expression of AR in human ECs could define presence of functionally different subpopulations of ECs that may be affected differentially by androgens, which could explain, at least in part, the paradigm of the contradictory effects of androgen on vascular biology, and (2) DHT, and metabolites of DHT, generally thought to represent progressively more hydrophilic products along the path to deactivation and elimination, may have differential roles in modulating the biology of human ECs using AR-dependent and AR-independent mechanisms, respectively. This unique observation provides a new paradigm for regulation of endothelial cell homeostasis and opens new avenues of exploration to better understand the role of androgen in human ECs.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest.

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Resultados Objetivo 1

En orden de poder realizar los estudios de comunicación paracrina de células de cáncer y células endoteliales, se realizó una caracterización de los modelos de líneas celulares de CaP a utilizar. Se tomaron cuatro líneas celulares que representaban los fenotipos del CaP: RPWE-1, línea celular de epitelio benigno, LNCaP, línea celular que representa fenotipo sensible a andrógenos, LNCAP-C4-2 fenotipo resistente a la castración y la línea celular PC-3 representa un fenotipo agresivo y metastásico. Las células LNCaP y LNCaP-C4-2 expresan el AR a nivel de proteína y mensajero (Fig. 1A-B). La diferencia entre ambas es su dependencia a los andrógenos. Si bien en ambas líneas aumenta la actividad transcripcional de AR en presencia de su ligando (Fig. 1C) la línea celular LNCaP, presenta mayores niveles de AR, en presencia de andrógeno (DHT), y solo en presencia de este andrógeno se expresa el PSA, ya que el gen que expresa el PSA se encuentra regulado por los andrógenos, no así la línea celular LNCaP-C4-2 que expresa el AR de forma similar, en presencia o ausencia de andrógenos al igual que PSA (Fig 2B). También se analizó la modulación de la proliferación de los cultivos celulares en presencia y ausencia de andrógenos. Solo la línea celular LNCaP, presento una dependencia de su crecimiento a los andrógenos (Fig. 2).

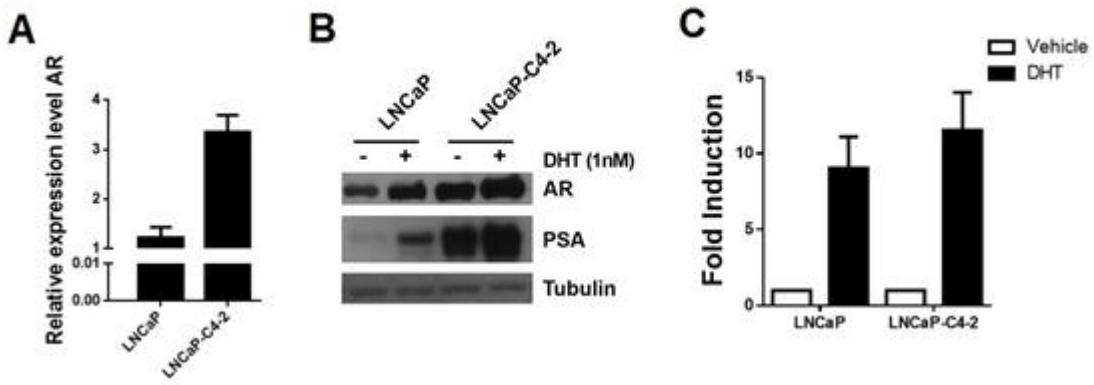

Figura 1 . Expresión funcional de AR en celulas de cáncer de próstata. A) Medición de los niveles de expresión del ARNm de AR mediante RT-PCR cuantitativo. HPRT1 se usó como gen reportero. B) Niveles de proteína de AR y PSA (Prostate Specific Antigen) en células LNCaP y LNCaP-C4-2 en ausencia y presencia de DHT (1nM). La expresión de PSA es dependiente de AR. B-tubulina se utilizó como control de carga. C) actividad transcripcional de AR en células de cáncer de próstata infectados con un vector MMTV adenoviral con un gen reportero de luciferasa.

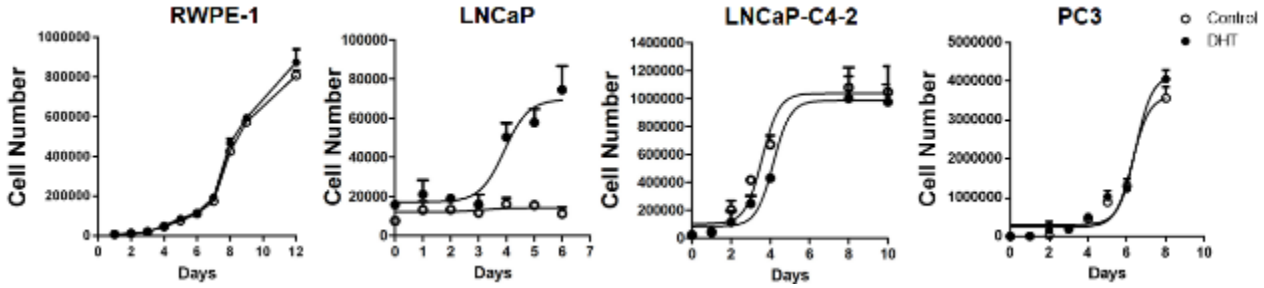

Figura 2. Modulación de la proliferación de células de próstata por andrógenos. Las células RWPE-1, LNCaP, LNCaP-C4-2 y PC3 fueron cultivadas en presencia (circulos negros) y ausencia (circulos blancos) de DHT 1nM. El numero total de células fue contado utilizando el contador automatico LUNA^{TM} utilizando el metodo de exclusión por azul de tripán.

Objetivos 2 y 3

2. Caracterizar el efecto angiocrino del endotelio humano sobre los parámetros biológicos que determinan agresividad tumoral (proliferación, sobrevida, migración y capacidad invasiva) en modelos *in vitro* de cáncer de próstata sensible y resistente a la deprivación androgénica.

3. Analizar los efectos biológicos asociados a la interacción entre células endoteliales y células de cáncer de próstata sensible y resistente a la deprivación androgénica en modelos biológicos *in vivo* de pez cebra y ratones severamente inmunodeficientes.

La evidencia sugiere que las células endoteliales no solo proveen de oxígeno y nutrientes al tumor por un incremento en la angiogénesis, si no que tienen un rol independiente de la circulación, y que estas células a través de la secreción de factores paracrinos (angiocrino) pueden incrementar el desarrollo y progresión de las células de cáncer. El efecto de las células endoteliales sobre las células de cáncer se ha estudiado en distintos modelos de cáncer, en los cuales se ha determinado que en algunos modelos las células necesitan tener contacto directo, en cambio en otros modelos solo es necesario la secreción de factores al medio extracelular. El objetivo de esta tesis es entender la comunicación paracrina entre ambos tipos celulares, y el efecto sobre la progresión tumoral de las células endoteliales sobre las células de CaP , en ausencia de contacto directo entre ellas, tanto en modelos in vitro como en modelos in vivo.

Los resultados de la interacción paracrina entre células endoteliales y líneas celulares de cáncer de próstata están desarrollados en la siguiente publicación:

Publicación 3

Differential Angiocrine Role of the Endothelium on Prostate Cancer Cells

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Short title: Angiocrine Role of Endothelial Cells on Prostate Cancer

Abstract

A growing body of literature supports the notion that angiogenesis is a crucial event in the development and progression of prostate cancer (PCa) and that therapeutic targeting of angiogenesis could be an effective treatment to counteract this disease. However, the outcome of human clinical trials, in which anti-angiogenic drugs were delivered in conjunction with chemotherapy, has been limited to a transient increase in survival of patients with advanced solid tumors. These data suggest that the mechanism by which endothelial cells regulate tumor growth is complex and is not driven merely by the establishment of normalized passive and permissive conduits for delivery of oxygen and nutrients to the tumor tissue. Here, we investigated the biological impact of endothelial cells, through endothelium-derived paracrine/angiocrine factors, on prostate cancer epithelial cells using cell conditioned media collected from human umbilical vein endothelial cells (HUVEC). Our results indicate that conditioned media stimulates proliferation of PCa cell lines and selectively promotes migration and invasion of more aggressive PCa cell lines in vitro and in vivo. Using a cell line-derived xenograft model, we observe that endothelial cells, through paracrine communication, enhance tumor growth, increase tumor microvasculature and promote metastasis. In accordance with our in vitro and in vivo data, gene enrichment analyses obtained from RNA seq data show that conditioned medium induces a differential effect on gene expression profile when comparing low versus highly aggressive prostate cancer cell lines. Moreover, the highly aggressive cell lines enrich for epigenetic changes and known migratory mechanisms. Together, these results indicate that endothelial cells, through the secretion of paracrine/angiocrine factors a paracrine mechanism, could promote more aggressive behavior of PCa cells in vitro and in vivo and that these biological effects might be broader in PCa cells of greater tumorigenicity. Therefore, blood

flow-independent effects of endothelial cells could have potential roles in boosting the aggressiveness of PCa cells, especially in patients with advanced PCa.

Introduction

Prostate cancer (PCa) is the most common non-cutaneous cancer of men and the second leading cause of cancer-related death in men worldwide [1]. Huggins and Hodges reported in 1941 [2] that growth of prostate cancer depended on androgens, and this conceptual break-through led to the development of androgen deprivation therapy (ADT), which has represented the standard treatment for locally advanced and metastatic disease for almost 80 years. Even though ADT-based therapeutic modalities reduce the level of circulating testicular androgens, and initially provide palliation for patients, these therapies do not provide durable/permanent remission/cure for PCa. Further, because of these therapies, PCa progresses from an androgen-sensitive (AS-PCa) disease to a more aggressive, and eventually lethal, castration-resistant phenotype (CR-PCa) [3].

Cancer is a disease that alters/results from complex interactions between the cancer epithelial cells and their surrounding stromal compartment in which the cancer cells live, which is called the tumor microenvironment (TME) [4, 5]. The TME of PCa includes multiple types of non-malignant cells, such as activated fibroblast, infiltrating macrophages and other immune cells, as well as the tumor microvasculature composed of endothelial cells and pericytes [6]. The prostate cancer TME exhibits an activated phenotype composed of a myofibroblast/fibroblast mixture, with a significant decrease of fully differentiated smooth muscle cells, increased extracellular matrix remodeling, increased protease activity, the influx of inflammatory cells,

and increased angiogenesis. All these cancer-associated stromal alterations resemble the tissue changes that accompany normal wound healing [7].

Like other solid tumors, growth and dissemination of PCa is dependent on tumor angiogenesis. Cancer epithelial cells release soluble pro-angiogenic factors that induce neovascularization, a process referred to as the "angiogenic switch" [8]. Several studies have proposed that the extent of angiogenesis in PCa (expressed as increases in micro vessel density [MVD]) was associated with clinical features, such as Gleason score and pathological stage, [9, 10] and could be an independent prognostic factor of biochemical recurrence and patient survival [11-13]. Studies by Gustavsson and colleagues [14, 15] further expanded the prognostic value of MVD in PCa, indicating that the transition from AS-PCa to a CR-PCa phenotype is associated with increased angiogenic activity in xenograft tumors derived from the castration-resistant subline LNCaP-19 [15, 16]. They also found that the level of expression of several genes associated with angiogenesis were altered during this transition, including ADAMTS1, fibronectin, neuropilin, Ang-2, and VEGF. Thus, a growing body of literature supports the notion that angiogenesis is an important event in the development and progression of PCa and targeting angiogenesis could be an effective treatment to counteract CR-PCa. Thus far, the primary focus of therapeutic modalities designed to disrupt angiogenesis was to inhibit proliferation of tumor-associated endothelial cells [17-19]. Despite the demonstrated efficacy of these drugs in improving survival of tumor-bearing mice, the outcome of human clinical trials in which these drugs were delivered in conjunction with chemotherapy, has been limited to a "transient" increase in survival of patients with advanced solid tumors, with most patients ultimately succumbing to tumor progression [18]. Paradoxically, in some mouse tumor models, the inhibition of specific angiogenic pathways enhanced tumor invasiveness and metastasis [20]. In PCa, several large

trials [21, 22] using antiangiogenic agents have been so far inconclusive or produced disappointing results. These failures have been seen with antiangiogenic agents used as monotherapies and when they are given in combination with other agents in randomized controlled trials [21, 22]. Together, this evidence suggested that the mechanism(s) by which endothelial cells regulate tumor growth is complex and is not driven merely by the establishment of normalized passive and permissive conduits for delivery of oxygen and nutrients to tumor tissue [23].

One possible explanation for the failure of anti-angiogenic therapies to promote long-term survival of cancer patients could be related to the ability of endothelial cells to release specific growth factors that directly regulate tumor growth in a proliferation state- and perfusion-independent manner [23-25]. In support of this concept, during developmental processes, the invasion of endothelial cells into incipient organs conferred inductive signals that promoted organogenesis, even in the absence of blood flow [26, 27]. Also, a growing body of literature [28-34] supports the hypothesis that endothelial cells could have an important paracrine (angiocrine) role in tumor development and progression. In vitro studies [35-38], in which cancer cells were co-cultured with endothelial cells, indicated that endothelial cells provide a fertile niche that can increase proliferation, survival, invasion, and migration of cancer cells. In these studies, it was observed that endothelial cells induced proliferation and propagation of aggressive leukemic clones [36], promoted proliferation, survival and pro-metastatic potential of breast cancer cells [35], and increased the capacity of stem cell formation in medulloblastoma [38]. In PCa, previous studies [37, 39, 40] have documented the paracrine effect of endothelial cells on PCa cells. Again, in these reports, the “paracrine effect” was analyzed through an experimental setting in which cancer cells were co-cultured with endothelial cells. From these

reports, Wang et al.,[37] suggested that an increase in the secretion of interleukin-6 by endothelial cells resulted in a decreased in androgen receptor signaling in tumor cells and the activation of the TGF-b/MMP-9 pathway which promoted invasion of PCa cells. Despite these interesting observations, the full extent of the biological effects of endothelial cells on cancer cells, including PCa cells, and the cellular and molecular mechanisms by which endothelial cells modulate cancer cells, and more specifically PCa cell homeostasis, are far from being understood. Consequently, in this work, we aimed to 1) determine the extent of the biological effect of endothelial cells on PCa cells, 2) characterize in vivo the contribution of paracrine (angiocrine) effect versus the cell-to-cell contact effect of endothelial cells on PCa cells, and 3) elucidate the potential molecular mechanism(s) that underlie the in vitro biological effects of endothelial cell con PCa cells.

Materials and Methods

Cell cultures: Primary cultures of HUVEC cells were isolated from fresh umbilical cords obtained from male fetuses according to previously published reports [41-43]. Human umbilical cords were collected with the approval of the Ethics and Biosafety Committee at Pontifical Catholic University of Chile (PUC). All HUVEC cells were cultured in SFM-endothelial medium (GIBCO) supplemented with 10% fetal bovine serum (FBS) and 10 µg/ml of endothelial growth factor (Merck). The human prostate cell lines RWPE-1, LNCaP, and PC3 were obtained commercially from ATCC (ATCC, Manassas, VA). The cancer cell line LNCaP-C4-2 was kindly donated by Dr. Sergio Oñate, University of Concepción, Concepción, Chile. Cell culture media for each cell line were used as follows: RPMI-1640 media (GIBCO) for LNCaP-C4-2 and PC3 cell lines, RPMI-1640 media supplemented with 1 nM of

dihydrotestosterone (DHT) for LNCaP cell line and Keratinocyte Serum Free Medium (K-SFM, GIBCO) for RWPE-1 cell line. All media were supplemented with heat-inactivated fetal bovine serum (10%), 100 μg/ml streptomycin and 100 U/ml penicillin. Cell cultures were maintained at 37°C with 5% CO2 in a humified incubator.

In vivo models: Immunocompromised male NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice were acquired from The Jackson Laboratory, USA, and housed under specific pathogen-free conditions and 12 h day/night cycles at the animal facility of PUC. Zebrafish (Danio rerio) model was acquired from Zebrafish International Resource Center (ZIRC, USA) and kept at 28°C with a 14/10 h day/night cycle at the animal facility from PUC. Embryos raised beyond 24 h post-fertilization (hpf) were treated with phenylthiourea (PTU; 0.003%, w/v; Sigma) to make them translucent. All animal protocols were conducted under the approval of the ethical committee of PUC.

Conditioned media: HUVEC cells at 90% confluence were washed three times with PBS? and then cultured in growth medium supplemented with 1% FBS for 36 h. Conditioned media (CM) was harvested and centrifuged at 3,000 g for 5 min to remove cell debris. The resulting supernatant was collected and stored in aliquots at -80°C.

Cell proliferation assays: Cell proliferation was measured using both, CellTiter 96 aqueous non-Radioactive Cell Proliferation kit assay and immunofluorescence staining of the proliferation marker, Ki-67. Briefly, PCa cell lines were seeded at a density of 1x103 cells/well in 96 well plates and incubated overnight. After that, culture media was replaced with CM from HUVEC in different proportions mixed with culture media supplemented with 5% FBS for 96 h. The media was replaced every 48 h. The number of viable cells was determined using

CellTiter 96 aqueous non-Radioactive Cell Proliferation kit (Promega) assay according to the manufacturer's instructions. The optical density was measured at 570 nm using an ELISA reader (EL800, BioTek Instruments, Winooski, VT). Cell survival rate was expressed as A/B × 100, where A was the absorbance value from the cell treated with CM and B was the absorbance value from control (untreated) cells. For Ki-67 immunofluorescence staining, PCa cell lines were cultured on coverslips to the appropriated density. After that, culture media was replaced with CM from HUVEC in a proportion of 50/50 with regular culture media for 96 h replacing this media every 48 h. Cells were fixed in 4% paraformaldehyde and then permeabilized with 0.5% Triton X-100 for 10 min. Fixed and permeabilized cells were blocked with 5% w/v BSA for 30 min and incubated with rabbit anti-Ki-67 (1:500, Abcam) antibodies overnight at 4°C. Immunostaining was then visualized by incubating the cells in a secondary antibody Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen) for 1 h at room temperature. Nuclei were counterstained using 4,6-diamidino-2-phenylindole (DAPI) (1:20000, Sigma-Aldrich). Lastly, cells were photographed using a fluorescence microscope (DM IL LED, Leica).

Transwell migration and invasion assays: Migration and invasion assays were performed using the CytoSelect™ 96- Well Cell Migration and Invasion Assay (Cell Biolabs, INC), which consists of a 96-well plate with cell culture inserts containing a filter with 8 μm diameter pores. The transwell for the invasion assay was coated with a uniform layer of basement membrane matrix solution. Prostate cell lines were put into starvation overnight and then trypsinized and suspended in RPMI medium or CM without FBS at 5×104 cells/ml for the migration assay and at 2×105 cells/ml for the invasion assay. Cell suspension (100 μl) was added to the upper well, and 150 μl of RPMI media containing 10% FBS was added to the lower well. Cells in the wells were incubated for 24 h for migration assay and 48 h for invasion assay. Lastly,

migrated/invaded cells were dissociated from the membrane and subsequently detected with CyQuant® GR Dye. Fluorescence was quantified using a fluorescence plate reader at 480 nm/520 nm (Synergy™ 2 Multi-Detection Microplate Reader, BioTek Instruments, Inc).

Zebrafish xenograft model: LNCaP and PC3 cells were fluorescently labeled using CellTracker™ Red CMTPX (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Labeled cells were washed in PBS twice and re-suspended in PBS/EDTA at a concentration of 1×106 cells/ml. For the microinjection of labeled cells into the embryos, zebrafish embryos of 2 days post-fertilization (dpf) were dechorionated and anesthetized with tricaine (Sigma, USA). Embryos were injected into the middle of the embryonic yolk sac region with approximately 250 PCa cells and then incubated at 28°C. Embryos were imaged individually at 3 days post-implantation under an inverted wide-field fluorescence microscope (DM IL LED, LEICA). Tricaine (0.04 mg/ml) was added to their water, and then the embryos were mounted on a slide with 0.5% agarose to prevent their movement during the live imaging process. Cell fluorescence (red pixels) was measured in the tail of the fishes and quantified using ImageJ software.

Cell line-derived xenograft model: NSG mice were subcutaneously injected at the upper left flank region with 0.1 ml of PC3 cell suspension (in phosphate-buffered saline containing 50% Matrigel) containing 1x106 PC3 cells for control conditions, 1x106 PC3 cells preincubated with CM from HUVEC cells for 48 h, or a mixture of 1x106 PC3 cells and 2x105 HUVECs cells (5:1). Tumor growth was evaluated by measuring the length and width of tumor mass at the inoculation site with a caliper. The tumor volumes were determined using the following formula V= w2l/2, where "w" is the width, and "l" is large of tumors. After 7 weeks, the tumor-bearing

mice were sacrificed. The tumors, kidneys, lungs, and liver were removed and fixed in 10% phosphate-buffered formalin and were embedded in paraffin for pathological analysis.

Immunohistochemistry: Immunostaining analyses were performed as previously described.[44, 45] Briefly, after rehydration and antigen retrieval, tissue slides were incubated with primary antibodies against Ki-67 (1:100, Abcam), CD31 (1:100; Santa Cruz Biotechnology), cleaved caspase-3 (1:100, Cell signaling) or VEGF (1:300; Santa Cruz Biotechnology), followed by an HRP-conjugated anti-rabbit or anti-mouse secondary antibody (1:200, Dako). Immunostaining in the absence of primary antibody provided negative controls. Immunostaining was revealed using an Impact NovaRED substrate kit (Vector Lab) according to the manufacturer's protocol.

Human cytokine array: Presence of cytokines and chemokines in the CM from HUVEC and in the cell culture media of LNCaP and PC3 cell lines exposed to conditioned medium from HUVEC cells for 48 hrs, were determined using the Proteome profiler human XL cytokine array kit (R&D systems) according to the manufacturer's instruction. Cell culture media from LNCaP and PC3 was collected 48 h after the removal of CM from HUVEC. Average signal (pixel density) of three independent CM(s) from HUVEC and two independent cell culture media were determined using ImageJ. Expression level changes more than a 1.5-fold change was considered as a significant perturbation. Protein-protein interaction of secreted proteins of conditioned media from HUVEC cells was generated using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database.[46]

Expression analysis: Expression profiling was performed by the Genomic shared resource at Roswell Park Comprehensive Cancer Center, Buffalo NY. LNCaP and PC3 cell lines were treated with a mix of CM obtained from three independent HUVEC cultures. PCa cell RNA was isolated using a RNeasy kit (Qiagen). 500 ng of total RNA was converted to cDNA, followed by in vitro transcription to generate biotin-labeled cRNA using the Ambion Illumina Total Prep RNA Amplification (Ambion, Inc) as described in the manufacturer's instructions. Differential gene expression analysis of RNA sequencing was conducted using log-transformed fragments per kilobase per million mapped fragments (FPKM+1) with the Bioconductor Limma package in the statistical analysis software "R". A Bayesian empirical model was then constructed based upon the transformed FPKM data and compared to generate Differentially Expressed Gene (DEG) lists. Genes with an adjusted p-values of less than 0.05 and greater than a 1.5-fold change/less than a -1.5-fold change in expression were considered differentially expressed. DEGs were then used to construct a pre-ranked list for Gene Set Enrichment Analysis (GSEA). Ranks were assigned by multiplying the –log (adjusted p value) and the fold change, together. GSEApre-ranked was then performed on these lists of enriched pathways. The results of the GSEA analysis were then used to generate networks in Cytoscape, with the implementation of EnrichmentMap and AutoAnnotate plug-ins.

Statistical analysis: Statistical evaluation of data was performed using GraphPad Prism software (version 6). Mean and SEM values as well are indicated in each figure. Statistical significance of the differences between the means was evaluated using the one-way analysis of variance test (ANOVA). Student t test was performed when two conditions were compared. All data differences were considered statistically significant when the p-value was less than 0.05.

Results

Conditioned media from human endothelial cells increases cell viability and proliferation of prostate cancer cell lines.

The effect of CM from endothelial cells on viability and proliferation of prostate epithelial cell lines was examined using both, MTT assay and Ki-67 immunostaining (Fig. 1). Since an established human prostate endothelial cell line is not commercially available at this moment, HUVEC cells were utilized as a model of human endothelial cells due to their particular characteristic to express androgen receptor and similarly respond to androgens as human prostate endothelial cells do [43, 47]. To assess cell viability, we tested increasing concentrations (0, 10, 20, 50, 80, 100%) of CM (mixed with standard media of PCa cell lines supplemented with 5% FBS) from HUVEC cells on the benign RWPE-1 human prostate epithelial cell line, and the malignant, LNCaP, LNCaP-C4-2, and PC3, human PCa cell lines. Our result showed that cell viability of PCa cell lines was uniformly and significantly stimulated by CM from HUVEC cells (Fig. 1B). A dose-dependent increase in cell viability was observed between 0 and 80% CM, however, at a higher concentration of CM (100%), a decrease in cell viability was detected, which indicates a biphasic effect of the CM from HUVEC on cell viability of PCa cells (Fig. 1B). Interestingly, the viability of the benign RWPE-1 cell line was not stimulated by CM from HUVEC cells at any of the concentrations of CM tested (Fig. 1B). Moreover, a decrease in RWPE-1 viability was observed at concentrations of CM higher than 50% (Fig. 1B). Since the concentration of CM that produced the most significant effect on cell viability of PCa cell lines was 50%, this condition (50% of CM plus 50% of RPMI media supplemented with 5% FBS) was utilized for all further in vitro assays performed. In order to

confirm our results obtained with the MTT analyses, the percentage of proliferative prostate epithelial cells was determined using immunofluorescence for the proliferation marker, Ki-67 (Fig. 1C). As observed with the MTT analyses, Ki-67 expression levels were significantly increased in PCa cell lines when stimulated with CM from HUVEC (Fig. 1D). In agreement with our previous results, Ki-67 expression level was not affected by CM in the benign RWPE-1 cell line (Fig. 1D). Taken together, these results indicated that viability/proliferation of PCa epithelial cells was consistently stimulated by CM from HUVEC, with no effect of CM on benign prostate epithelial cells.

Conditioned media from human endothelial cells increases migration and invasion of more aggressive prostate cancer cell lines.

Migratory and invasion capacities of PCa cell lines were analyzed under the stimulation of CM from HUVEC cells using wound healing (Supp. Fig. 1) and transwell migration/invasion (Fig. 2) assays. Our results indicated that there was a differential effect of CM on its ability to potentiate migration (Supp. Fig. 1 and Fig. 2A-B) and invasion (Fig. 2B) between PCa cell lines. Using transwell assays, we analyzed the effect of CM on migration and invasion capacities in PCa cell lines under three specific conditions: 1) Cells in RPMI media in the upper chamber and FBS at the lower chamber as chemoattractant (control condition), 2) Cells plus CM in the upper chamber and FBS at the lower chamber as chemoattractant, and 3) Cells in RPMI media previously treated with CM for 48 hours in the upper chamber and FBS at the lower chamber as chemoattractant (Fig. 2A). We observed that CM from HUVEC cells significantly increased both, migration and invasion capacities, in LNCaP-C4-2 and PC-3 PCa cells. However, CM from HUVEC cells showed no significant effect on migration and invasion capacities in LNCaP

and RWPE-1 cell lines (Fig. 2B-C). Importantly, CM treated cells retained their increased migratory and invasive phenotypes even when seeded without CM (Fig 2 B, C - grey bars), indicating the durability of the phenotype induced by CM. The differential effect of CM on the migratory capacity of malignant human prostate cell lines was replicated using wound healing assay (Supp. Fig. 1). Our results indicated that CM from HUVEC cells increased migration of LNCaP-C4-2 and PC-3 cell lines, and that the effect is durable even in absence of continued exposure to CM, but not affected the migratory capacity of LNCaP cells nor the benign prostate epithelial cells, RWPE-1 (Supp. Fig. 1B-C). Together, these results indicated that CM from endothelium might selectively promote cell migration and invasion only on more aggressive PCa cell lines, which may reflect the increased ability of these cells to respond to a broader range of signals/factors present in the CM.

Human endothelial cells enhance in vivo migration of the more aggressive PC-3 cell line through a paracrine mechanism.

In order to confirm our in vitro data, we next evaluated the effect of CM from HUVEC cells on in vivo migration capacity of LNCaP and PC-3 PCa cell lines using the highly characterized zebrafish embryo model (Fig. 3) [48, 49]. LNCaP and PC-3 cell lines were previously fluorescently-labeled with CellTracker™ Red CMTPX (Invitrogen) and then engrafted at the yolk sac of zebrafish embryos using an experimental setting that included three specific conditions: 1) PCa cells engrafted alone (control condition), 2) PCa cells pre-incubated with CM from endothelial cells for 48 hours before engraftment, and 3) PCa cells co-engrafted with HUVEC cells in a 5:1 ratio (Fig. 3A). In accordance with our previous in vitro data, at day 3 post-implantation the highly aggressive PC-3 cell line showed progressive and extensive

dissemination throughout the developing embryo and migrated toward trunk and tail (Fig. 3B). PC-3 cells also could be found in the caudal hematopoietic tissue (CHT), which represents a "hot spot" region for tumor cell to extravasate vessels and invade adjacent tissues [50]. Interestingly, PC-3 cells pre-treated with CM or co-engrafted with HUVEC cells exhibited a significant increase in their migratory capacity in vivo when compared to the control condition (Fig. 3C). Conversely, and confirming what was observed in our in vitro assays, LNCaP cells did not migrate from the injection site of the zebrafish under any condition analyzed (Fig. 3D). These results confirmed that CM from HUVEC cells selectively enhanced migration or the metastatic ability of more aggressive PCa cells.

Human endothelial cells increase the tumor growth of xenografted PC-3 cells in immunodeficient mice by an increase in their proliferative capacity in vivo.

To confirm our in vitro data showing that CM from HUVEC cells enhanced the proliferative capacity of PCa cell lines, we injected subcutaneously PC-3 cells into the flank of immunodeficient NSG mice using a similar experimental design as in figure 3, which included: 1) PC-3 injected alone (control condition), 2) PC-3 pre-incubated with CM from HUVEC cells for 48 hours, and 3) PC-3 cells co-injected with HUVEC cells in a 5:1 ratio (Fig. 4A). Tumor volume was measured every week, with calipers, for a total period of 8 weeks as shown in Figure 4C. Our results indicated that tumors formed by PC-3 cells pre-incubated with CM from HUVEC or PC-3 cells co-injected with HUVEC cells were higher in size (Fig. 4B) and significantly higher in volume (Fig. 4C) when compared to tumors obtained from mice injected with PC-3 cells alone. After 8 weeks of growth, PC-3 tumors were surgically resected, weighed and processed for histological analyses. Our results indicated that tumor formed by PC-3 cells

pre-incubated with CM from HUVEC or PC-3 cells co-injected with HUVEC cells were significantly heavier than those tumors obtained from mice injected with PC-3 cells alone (Fig. 4D). Interestingly, no significant differences were observed between the conditions PC-3 cells pre-incubated with CM from HUVEC and PC-3 co-injected with HUVEC cells, which suggests that most of the effect of endothelial cells on PC-3 tumor growth could be explained by an angiocrine/paracrine mechanism, with no additional effect mediated by cell-to-cell contact between HUVEC and PC-3 cells.

The potential biological mechanisms underlying the paracrine effect of HUVEC cells on PC-3 tumor growth in vivo were assessed using immunohistochemical analyses of the cell proliferation and cell death (apoptosis) markers, Ki-67, and cleaved caspase-3, respectively (Fig. 4E-F). Our results indicated that the number of cells expressing Ki-67 marker was significantly increased in both, PC-3 pre-incubated with CM from HUVEC cells or PC-3 co-injected with HUVEC cells when compared to control condition (Fig. 4E-F). Apoptosis was determined using cleaved caspase-3 immunostaining (Fig. 4E-F). Although the levels of apoptosis in the tumor tissue specimens were generally low, we observed a significant decrease in cleaved caspase-3 immunostaining only when PC3 were co-injected with HUVEC cells when compared to control condition. No significant differences were observed between PC-3 pre-incubated with CM from HUVEC cells and control conditions (Fig. 4E-F). Taken together, our results indicated that endothelial cells increase in vivo tumor growth through a paracrine communication that enhanced mostly PCa cell proliferation without affecting PCa cell apoptosis.

Human endothelial cells increase microvascular density and VEGF-A expression in xenografted PC-3 cells-derived tumors.

We studied whether endothelial cells can modulate the proangiogenic capacity of PC-3 cells in vivo by measuring micro vessel density (MVD) and VEGF-A expression in PC-3 cell-derived xenograft tumors. Micro vessel density was determined by CD31 immunostaining (Fig. 5A-B). Both, MVD (Fig. 5A-B) and VEGF-A (Fig. 5C-D) were significantly increased in tumors derived from both, PC-3 cells co-injected with HUVEC cells or PC-3 cells pre-incubated with CM from HUVEC cells when compared to the control condition. Since there were no significant differences between the PC-3 cells co-injected with HUVEC cells or PC-3 cells pre-incubated with CM from HUVEC cells, we hypothesize that human endothelial cells increase in vivo tumor growth mostly through a paracrine communication that enhanced both proliferation and pro-angiogenic potential of prostate cancer epithelial cells, simultaneously.

Human endothelial cells enhance metastasis of PC-3 cell line-derived xenograft tumors. The detection of metastatic foci in mouse tissues was performed using immunostaining analyses of Ki-67 of human origin. Seven weeks after injection of PC-3 cells into immunodeficient mice, lungs, liver, and kidneys of recipient mice were harvested to survey for metastases. Representative histological sections of the liver and kidney are shown in figure 6A. The incidence of total metastasis (sum of all organs) was slightly higher under the conditions PC-3 cells pre-incubated with CM from HUVEC cells for 48 hours and PC-3 cells co-injected with HUVEC cells when compared to control condition (Fig. 6B). However, when the incidence of metastasis was analyzed in individual organs (kidney and liver), the number of metastatic foci (Fig. 6B), as well as the extension of metastatic lesions (data not shown) were consistently

higher in both, PC-3 cells pre-incubated with CM from HUVEC or PC-3 cells co-injected with HUVEC cells, when compared to control condition (Fig. 6B). Quantitation of metastatic foci in mice lung tissues was not possible since the number of ki-67-positive PC-3 cells observed in this tissue was extremely high, and no differences were observed in all three conditions analyzed. Also, no visible metastatic lesions were observed in all three conditions analyzed (data not shown). Together, our in vitro and in vivo data support the concept that endothelial cells can promote migration, invasion, and metastasis of PCa cells through a paracrine mechanism, probably mediated by multiple signals/factors released by the endothelium, that can impact human PCa cells differentially.

Proteomic analysis of the primary signals/factors involved in intercellular communication between the endothelium and prostate cancer cells.

In order to study the potential mechanism of intercellular communication between endothelial cells and PCa epithelial cells, we performed an in vitro proteomic analysis to detect multiple cytokines, chemokines, growth factors and other soluble proteins in the conditioned medium obtained from HUVEC cells and in the supernatant of PCa cells exposed to conditioned media from HUVEC cells using the Proteome profiler human XL cytokine array kit (Fig. 7). We started by analyzing the factors secreted by the conditioned media from HUVEC cells (Fig 7A-B). Of all 105 factors detected by the array, 36 of them were significantly increased in the conditioned medium when compared to control medium (Fig 7C). To gain further insight on how the secreted factors are interconnected with each other, we performed a bioinformatic analysis for protein-protein interactions using a database called STRING with high confidence (0.700) (Search Tool for the Retrieval of Interacting Genes/Proteins) [46]. Using this program, a predicted functional

protein-protein interaction map was generated between the different secreted factors (Figure 7D). The protein-protein interaction map revealed that 29 out of the 36 secreted factors have some degree of multiple functional interactive partners. An in-depth analysis of these 29 factors for their involvement in typical biological process relevant for cancer cells, revealed that several of them are essential for processes like cell proliferation (IGFBP2, HGF, MIF, FGF2, IL27), cell migration (CCL7, CCl20, IL-6, CXCL5, CXCL11) and cell communication (CD14, GDF15, ILRL1,IL22) (Fig. 7B). Among these factors, IL-6, CCL2, CXCL1, FGF2, and HGF act like central/master regulators of the secreted factors as predicted by a protein-protein interaction (PPI) analysis (Fig. 7B). Using the same experimental approach utilized previously to analyze the presence of cytokines in the CM from HUVEC cells, we analyzed the profile of factors secreted by the PCa epithelial cells, LNCaP and PC3, which were exposed to control or CM from HUVEC cells (Fig 7E). Culture medium from LNCaP cells previously exposed to CM from HUVEC cells had 11 factors that were significantly increased compared to LNCaP culture medium obtained from cells previously exposed to control HUVEC medium (Fig. 7E). In the case of the PC-3 cell line, these factors were 18, in comparison with PC-3 culture medium used as control (Fig. 7E). When analyzing the secretion profile between the two cancer cell lines, we observed that endothelial cells increase factors related to growth, modulating immunity and increasing angiogenesis (Fig. 7F). However, there were differences between chemokines, growth factors, angiogenic factors, and inflammatory cytokines, such as ANGPT-2, PDGFA, CXCL5, G-CSF and MIP3A which were increased mainly in PC3 cells (Fig. 7G). This could explain, at least partially, the differential effect (migration and invasion) of the CM from HUVEC cells on the two PCa cell types.

Conditioned media from human endothelial cells induces differential changes in gene expression that determine aggressiveness in prostate cancer cells

To further understand the potential molecular mechanism(s) that determine the differences between the effect of endothelial cells on PCa cells, we performed transcriptomic analyses of LNCaP and PC-3 PCa cell lines treated with CM from HUVEC cells. Differential gene expression analysis of RNA sequencing was conducted DEG analysis, with a cut off of 0.05 for the adjusted p-value, and a 1.5 fold change, revealed that 2471 genes were differentially expressed in the LNCaP CM-treated vs. untreated control cells and 401 genes in the PC-3 CM treated vs. untreated control cells (Fig 8A). Both CM cell lines differentially expressed 91 of the same genes, in the same direction. (Fig 8A). DEF analysis resulted in the generation of a heatmap that reveals distinct expression patterns in the PC3 CM-treated cells and the LNCaP CM-treated cells as compared to the untreated PC3 and LNCaP untreated controls, respectively. PC3 or LNCaP cells, whether treated or untreated, still cluster with one another (Fig 8B), revealing cell line differences between more indolent and aggressive PCA cell lines result in different alterations associated with CM. To distinguish pathways differentially enriched in either the treated LNCaP or PC-3 cell lines we performed a comparative analysis of signaling pathways operating in these two cell lines. The gene set enrichment analysis (GSEA) [51] of the treated LNCaP cell line identified pathways related to proliferation were significantly enriched, as expected based on previously presented data. Conversely, the pathways related to kinase activity, metabolism, epigenetics and immune response were significantly negatively enriched in LNCaP cells. The PC-3 treated cells, were most significantly enriched for gene sets related to metastasis, epigenetics, proliferation, genetics drivers, and kinase activity pathways (Fig 8C). Together, these results were consistent with our in vitro and in vivo data obtained in LNCaP

demonstrating a hyperproliferative phenotype, and in PC-3, which acquires both a hyperproliferative phenotype as well as an invasive and metastatic phenotype that is durable even in the absence of conditioned media.

Discussion

A growing body of literature supports the notion that the TME plays a crucial role in human cancer, influencing almost every stage of its development and progression, from tumor growth to cancer cell dissemination/metastasis [52]. The TME even influences the response and resistance to anticancer therapies [6]. Endothelial cells are one of the most critical cell types that integrate the TME. It is well established that endothelial cells are central players in tumor neo-angiogenesis, a process that allows perfusion of nutrients and oxygen to the TME necessary to support cancer cell proliferation, as well as providing a way for the dissemination of metastatic clones of cancer cells [28]. For a long time, the role of blood vessels was limited to being normalized passive and permissive conduits for delivery of O2 and nutrients to the tumor tissue [23]. However, due to relative failure of anti-angiogenic therapies (which are designed to disrupt angiogenesis by inhibiting proliferation of tumor-associated endothelial cells) to promote long-term survival of cancer patients [21], it is reasonable to hypothesize that the mechanism(s) by which vasculature, and more specifically endothelial cells, regulate tumor growth is more complicated than initially thought. Tumor-associated endothelial cells could promote tumor growth through a paracrine effect that is blood flow-independent and not conducted by proliferating endothelial cells but instead could be an intrinsic property of quiescent endothelial cells [23]. A mechanism like that could explain, at least in part, why this paracrine signaling activity cannot be affected by conventional anti-angiogenic therapies, which target mostly

endothelial cells that are in a proliferative state. Although compelling, this hypothesis reveals the need for new avenues of exploration to understand better, and more broadly, the contributions that tumor-associated endothelial cells have on cancer, and more specifically, PCa cell development and progression. In the present work, we aimed to: 1) determine the extent of the biological effect of endothelial cells on PCa cells, 2) characterize in vivo the contribution of paracrine (angiocrine) effect versus the cell-to-cell contact effect of endothelial cells on PCa cells, and 3) elucidate the potential molecular mechanism(s) that underlie the in vitro biological effects of endothelial cell on PCa cells. Our results indicated that conditioned medium from endothelial cells increased proliferation, migration, and invasion of PCa cells in vitro, and promoted tumor growth, migration, and metastasis in vivo. Interestingly, these effects were selective and broader in more aggressive PCa cell lines, which suggest more aggressive PCa cells could adapt to respond to a broader spectrum of signals derived from endothelial cells, and that this endothelium/PCa cell interaction could play more significant roles in more advanced stages of this disease.

Ten years ago, our group demonstrated that ADT induced an acute vascular collapse in primary xenografts of human prostate tissue transplanted to SCID mice.[45] Moreover, our group provided a mechanistic explanation for the vascular involution after ADT by demonstrating expression and functionality of AR in human prostate endothelial cells (HPEC) both in vivo and in vitro [47]. A recent study from our group [43] and others [53, 54] have characterized AR expression and functionality in HUVEC cells. Interestingly, in this study, we showed that HUVEC cells derived from male fetuses reproduced most of the biological effects of androgens (dihydrotestosterone, DHT) observed in HPEC cells [47]. For example, in both HUVEC and HPEC cells, AR was actively expressed and causally involved in the regulation of their

proliferation activity. Based on these functional observations, and considering that HUVEC cells have been used in the past to modulate the interaction between human endothelial cells and cancer cells [31, 32, 37], we choose HUVEC cells as a representative model of HPEC for all future studies of the paracrine effect of endothelial cells on PCa cells.

Previous studies [23, 32, 35, 49, 55] indicated that endothelial cells could promote the growth of tumor cells. While studies in breast cancer and leukemia models showed that direct contact between endothelial cells and cancer cells is necessary to promote tumor growth and metastatic potential, as well as the expansion of primitive leukemia-initiating cells [35, 36], other studies in hepatocellular carcinoma and colorectal cancer have shown that conditioned media from endothelial cells represent a sufficient stimulus to produce similar effects [32, 56]. Using in vitro and in vivo models, we demonstrated that most of the effects of endothelial cells on PCa cells were mediated through paracrine mechanism/s with little to no additional contribution of the physical interaction between these two cell types. Interestingly, our in vitro studies showed that all PCa cell lines analyzed responded to the effect of endothelial cells by increasing their proliferation, and that this effect seems to be specific for malignant prostate cell lines, since no effect of the conditioned medium was observed in the proliferation of the non-malignant prostate epithelial cell line, RWPE-1. This effect was also observed in breast [57, 58], in which co-culturing of the non-malignant breast epithelial cell line MCF-10A with endothelial cells, showed no significant effect of endothelial cells on any biological characteristics of the non-malignant breast epithelial cells, such as growth of branching, EMT-like epithelial colony formation and expression of angiogenic factors. The results notably differed when performing the same experiments with malignant breast epithelial cells [57, 58]. These antecedents highlight

the difference between tumor cells and non-malignant cells concerning their ability to respond to endothelial cells-derived signals.

Within the PCa cell lines, a differential effect of the conditioned medium from HUVEC was observed in terms of its ability to affect their migration and invasion capacities. Remarkably, our results indicated that only the more aggressive PCa cells, LNCaP-C4-2, and PC-3 (a castration-resistant and metastatic human PCa cell line, respectively), showed a significant increase in their ability to migrate and invade in response to conditioned medium from HUVEC. This effect was not observed in the less aggressive androgen sensitive LNCaP cell line. In this regard, our results contrast with what was reported by Wang et al. [37] in 2013, who observed that in a co-culture experimental setting, PCa cells, including the LNCaP cell line, showed an increase in their invasion capacity when co-cultured with endothelial cells [37]. It is plausible to consider that a minimum of cell-to-cell contact is required to induce invasion of LNCaP cells. However, our in vivo data obtained using the zebrafish xenograft model, which provides a rapid and efficient method for the analysis of cancer cell migration/invasion [48, 49], independently evaluated the contribution of the paracrine signals versus physical interaction, and confirmed that co-injection of LNCaP and endothelial cells did not promote migration/invasion on LNCaP cells. This controversy might be explained, at least in part, by the different type of analyses utilized in both studies (transwell assays vs. zebrafish xenograft models). Although, it is essential to highlight that the causal effect of the cell-to-cell contact on the invasion capacity of LNCaP cells was not directly demonstrated in the experimental setting developed by Wang et al. [37]. Overall, this evidence supports the idea that the effect of endothelial cells is broader in more aggressive PCa cells, which could reflect a greater / sensitivity of these cells to either recognize signals from the conditioned medium or an increased/amplified intracellular capacity

to respond to the same signals. In any event, these observations are extremely relevant to understand the biology of this disease and might represent a source for new therapeutic target to counteract PCa, especially advanced PCa.

Because active migration of tumor cells is a prerequisite for tumor cell invasion and metastasis [59], our next step was to analyze the growth and metastatic ability of tumors using an in vivo xenograft model. A growing number of studies have shown that endothelial cells can increase the growth and aggressiveness of tumors [31, 33, 36, 38, 56]. In PCa, studies using the CWR22Rv1 cell line, which corresponds to castration resistant PCa cell line, showed that there was an increase in the incidence of metastasis to different organs when CWR22Rv1 cell line was co-injected with HUVEC cells [37]. Our results using both, zebrafish and immunocompromised mice xenograft models, replicated these results and were able to determine that a single treatment of PCa cells with conditioned medium before the injection into the animal models was sufficient to cause similar response in tumor growth as when both cell types were injected together. These observations are consistent with a model in which the paracrine signal(s) released from the endothelium produces long-term changes in the phenotype of PCa cells resulting in their ability to increase tumor growth and progression. Several studies [60-64] have determined that tumor microenvironment or non-cell-autonomous factors could promote clonal heterogeneity, allow interclonal interactions and lead to new phenotypic traits of cancer cells [64]. For example, paracrine signaling via FGF-2 and MAPK from small cell lung cancer (SCLC) subclones is a critical determinant in early steps of the metastatic process, which drives metastatic dissemination of the neuroendocrine tumor subclones [64]. Also, Neiva et al [60] demonstrated that endothelial cells could play an active role in establishing tumor cell phenotypes when observing that endothelial cell-initiated signals, CXCL8, interleukin-6 (IL-6)

and EGF, can activate three critical intracellular signaling molecules, STAT3, Akt, and ERK, in head and neck squamous cell carcinoma cells. Thus, tumor cell heterogeneity and changes in phenotypes cannot merely be ascribed to genetic diversity within a tumor, but also to the wide variation in signaling cues derived from tumor cells themselves and the many stromal cells that make up the tumor ecosystem [62].

Angiogenesis is a critical event in tumor development and metastasis [18]. Several studies [13, 15, 16, 21] have determined that PCa cells have higher expression of VEGF and as a result of that, prostate tumors show a higher microvascular density than their benign counterparts [10, 16]. Interestingly, previous studies have suggested that there would be a "two-way street" crosstalk, where endothelial cell-initiated events also have a profound impact on the behavior of tumor cells and vice-versa [33]. In this context, it has been shown that bidirectional crosstalk between endothelial and tumor cells stimulate reciprocal growth factor exchange that directly influences the angiogenic response [57]. Buchanan et al.,[57] reported that co-culture of a human endothelial cell line (HMEC-1) with a human breast carcinoma cell line (MDA-MB-231) resulted in higher expression of pro-angiogenic factors such as ANG-2 and VEGF in cancer cells [57]. Moreover, Abdel Hadi et al.,[65] observed that coculturing in vitro glioblastoma and brain endothelial cells promote expression of sphingosine kinase-2 in endothelial cells, leading to increased cellular level of sphingosine-1-phosphate (S1P), which in turn stimulated glioblastoma cell proliferation, and brain endothelial cells migration and angiogenesis[65]. In our studies, we observed that tumors from both conditions, PC-3 cells co-injected with endothelial cells and PC-3 cells previously treated with conditioned medium from endothelial cells, presented an increase in VEGF-A expression and an increase in the number of blood vessels compared to the control condition. Notably, the increase in VEGF was not as

pronounced as expected, which could reflect that, in this particular model, tumor angiogenesis could be modulated by probably more than one pro-angiogenic factor. Further proteomic analyses could help to define the profile of proangiogenic factor stimulated by endothelial cells in PCa cells.

In order to get insight into the molecular mechanisms that could mediate the biological effects of endothelial cells on PCa cells, we carried out proteomic analyses to search for extracellular signals classically involved in cell-to-cell crosstalk. Endothelial cells secreted many cytokines, chemokines and growth factors that could modulate the biology of cancer cells [23]. Among the factors secreted by HUVEC cells, we found that CCL2, IL-6, CXCL1, FGF2, and HGF were the central nodes within the entire network. FGF2 and HGF have been related to an increase in the growth of prostate cancer cells [66, 67] while, IL-6, CXCL1, and CCL2 proteins have been associated with increased migration and invasion of prostate cancer cells [37, 68, 69]. When analyzing the factors secreted by PCa cells in the presence of the conditioned media, we found remarkable differences between the cytokines, chemokines and growth factors secreted by LNCaP and PC-3 cells. Our proteomic analysis indicated that stimulated LNCaP cells mainly secrete proteins related to immune modulation, such as IL-8, IL17A, IL-11, Lipocalin-2 (NGAL), and SERPIN1 (PAI-1) protein. The Lipocalin-2, Serpin1 and IL-8 proteins are upregulated in aggressive phenotype cells such as PC-3 and in tissues of patients with advanced PCa but are downregulated in the LNCaP cell line [70-72]. However, it has been observed that these proteins can be induced by proinflammatory cytokines, both in several acute and chronic inflammatory diseases for the Lipocalin-2 protein [73], and by stimulating prostate cancer cell lines for the Serpin1 and IL-8 proteins [70, 72]. For instance, overexpression of IL-8 in LNCaP cells, either by exogenous recombinant human IL-8 or by transfection of cDNA of IL-8, resulted

in an increase in cell proliferation reducing its dependence on androgens [70]. This suggests that cytokines secreted by endothelial cells could promote the progression of the androgen-sensitive phenotype, mainly by increasing tumor growth pathways. In the case of PC-3 cells, our analysis indicated that these cells secrete factors related to inflammation, such as IL-10 and IL-22, from which there is evidence that relates them to tumorigenic roles in other types of cancer [74, 75]. PC-3 also secrete chemokines such as CCL20 (MIP3A) and CXCL5. CCL20 promotes, in an autocrine manner, survival, proliferation, and adhesion of cancer cells in vitro and enhances the growth of xenografted tumor cells vascularization, and invasion in vivo. In human prostate cancer tissues, CCL20 is overexpressed, and it has been reported to promote tumor growth in xenograft models [76]. CXCL5 has been observed to induce PCa cell malignant phenotypes in an autocrine/paracrine fashion in vitro [77]. Also, CXCL5 overexpression promoted PCa cell xenograft formation and growth [77]. Lastly, by analyzing the proteins related to angiogenesis, we were able to corroborate what was obtained in the in vivo experiment, in which the endothelial cells can increase the pro-angiogenic capacity of the cancer cells. Our proteomic analysis indicated that PC-3 cells, stimulated with CM from HUVEC cells, secreted high levels of the proangiogenic factors VEGF, ANG-2, PDGF-A, and THBS1, which could explain, at least in part, why the PC3 xenograft tumors have higher microvascular density, and higher metastatic capability.

To better understand the differences between the response of LNCaP and PC-3 cells exposed to the conditioned medium from HUVEC cells, we performed RNA-seq analysis. Our results demonstrate that when analyzing the genes that were differentially expressed in LNCaP and PC-three cells, based on the effect of the conditioned media, most of the genes were different between the cell lines. Notably, the cell lines and their treatments remain in clusters which leads

us to believe that the transcriptional differences are mostly cell line dependent. In LNCaP cells, DEGs were enriched for cellular processes related to apoptosis and RNA-binding after CM treatment, whereas in PC-3 the cellular processes related to epigenetics and cell cycle are more enriched with CM treatment. However, there are points of convergence between both cell lines that correspond to processes of cell proliferation, which correspond with the results obtained in our in vitro assays. However, the most significant differences, as well as those obtained in vitro and in vivo analyses, were increases in metastasis and kinase activity pathways. It is noteworthy that the invasive phenotype induced by CM in PC3 cells is durable even when the cells are no longer exposed to signaling induced by the CM. This suggests that epigenetic processes may have stabilized transcriptional circuits that were induced by the CM, such that changes associated with invasiveness and metastasis are maintained in the absence of continued signaling from CM. Consistent with this interpretation, we found strong enrichment of gene sets associated with epigenetics upregulated after PC3 cells were exposed to CM. On the contrary, when LNCaP cells were exposed to CM, we found downregulation of gene sets associated with epigenetics. Future studies may interrogate the hypothesis that signaling pathways induced in PC3 cells by CM lead to epigenetic reprogramming of pathways associated with cell migration and invasion and begin to explore mechanisms of how the signaling results in epigenetic reprogramming.

Conclusion

This study shows that there would be a paracrine communication between endothelial cells and prostate cancer cells, for which direct contact between both cell types is not necessary, only the secretion of different factors by the endothelial cells to the tumor microenvironment. These

findings suggest that endothelial cells have effects on the proliferation, tumor growth and metastasis of prostate cancer cells with more aggressive phenotypes, which indicates that endothelial cells could contribute to the acquisition of more aggressive and / or metastatic characteristics of this disease. Further studies of cytokines and pathways that were upregulated by the conditioned medium of endothelial cells could culminate in the identification of therapeutic targets or biomarkers to counteract PCa, especially advanced prostate cancer.

Conflict of Interest

The authors have no conflict of interest.

Acknowledgments

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Figure Legends

Figure 1. Conditioned medium from HUVEC cells increases proliferation of PCa cell lines *in vitro*. A) Schematic representation of the experimental procedures to obtain conditioned media from endothelial cells and the *in vitro* assays. B) Analysis of the effect of increasing concentration of CM (0, 10, 20, 50, 80, 100%) isolated from primary cultures of HUVEC cells mixed with standard media of PCa cell lines supplemented with 5% FBS, on RWPE-1, LNCaP, PC3 and LNCaP-C4-2 cell proliferation using MTT assay. Control condition for all experimental approaches was performed by adding fresh endothelial culture medium (without FBS or growth factors) to the standard RMPI medium in a 50/50 proportion (*$p \leq 0.05$; n=3) .C) Immunostaining analysis of the proliferation marker Ki-67 in prostate cell lines exposed to CM (50/50). DAPI was used to counterstain nuclei. D) Quantitation of the Ki-67 immunostaining data. Ki-67 positive cells were estimated as a percentage of total number of nuclei (determined by DAPI staining) (n=3; *$p \leq 0.05$, **$p \leq 0.01$ t-test)

Figure 2. Conditioned medium from HUVEC cells increases migration and invasion of more aggressive PCa cell lines *in vitro*. A) Schematic diagram of the experimental conditions for the transwell migration and invasion assays. RWPE-1, LNCaP, PC3, and LNCaP-C4-2 cells were seeded in the upper chamber (without fetal bovine serum (FBS) in the presence of RPMI medium (control, white bars), conditioned media (CM, black bars), and previously incubated with CM for 48 h before seeding (Pre CM, grey bars). 10% FBS was added to the lower chambers as a chemoattractant substance. B) 20 h later, migratory cells that passed through the polycarbonate membrane were lysed and quantified using fluorescent dye (*$p \leq 0.05$; n=3). C)

48 h later, invaded cells that passed through the layer of Matrigel and the polycarbonate membrane were lysed and quantified using fluorescent dye (n=3; *p ≤ 0.05 t-test)

Figure 3. Conditioned medium from HUVEC cells promotes cell migration of more aggressive PCa cell lines *in vivo*. A) Schematic representation of the experimental conditions for the PCa cell injection into the zebrafish model. PCa cells, marked with red cell tracker, were injected alone, co-injected with HUVEC cells (5:1 PCa:HUVEC cells), or preincubated for 48 h with CM from HUVEC cells before injection into the yolk of zebrafish embryos at 48 h post fertilization (hpf). B) Representative images of zebrafish at 72 h post-injection with red cell tracker-labeled PC-3 PCa cells. Quantitation of the intensity of the red cell tracker fluorescence associated to migratory PC-3 (C) or LNCaP (D) cells at the CV (Caudal vein) of zebrafishes using ImageJ software (n=3; *p<0.05, **p ≤ 0.01 t-test)

Figure 4. **Conditioned medium from HUVEC cells increases PCa tumor growth *in vivo***. A) Schematic representation of the experimental conditions for the subcutaneous injections of PC-3 PCa cells into NSG mice. PC-3 cells were injected subcutaneously alone ($1x10^6$), co-injected with HUVEC cells (5:1 PCa:HUVEC cells), or preincubated for 48 h with CM from HUVEC cells before injection in a mixture with Matrigel (1:1) for a total volume of injection of 100 μL. B) Representatives images of surgically resected subcutaneous tumors for the three experimental conditions. C) Average tumor volume from each experimental condition was measured weekly through the duration of the experiment. Tumor volume was determined by measuring the major (L) and minor (W) diameters with an electronic caliper and calculated according to the following formula: tumor volume= L*W*W/2. (n=11; *$p \leq 0.05$ versus PC3-

CM, #p ≤ 0.05 versus PC3-HUVEC Anova test). D) Average tumor weight (mg) was measured after surgical resection of tumors. (n=11; *$p \leq 0.05$, t-test)

Figure 5. Conditioned medium from HUVEC cells increases PCa proliferation and microvessel density *in vivo*. PC3 cells were injected subcutaneously alone ($1x10^6$), co-injected with HUVEC cells (5:1 PCa:HUVEC cells), or preincubated for 48 h with CM from HUVEC cells before injection in a mixture with Matrigel (1:1) for a total volume of injection of 100 μL. A) Representative images of the immunohistochemical analysis of Ki-67, cleaved caspased-3, CD31 and VEGF expression in tissue sections of PC-3 cell line-derived xenograft tumors. B) quantitation of the number of positive cells per field (Ki-67 and cleaved caspase-3), number of vessels per field (CD31) and immunostained area (VEGF) in tissue sections of PC-3 cell line-derived xenograft tumors. Positive controls for the expression of Ki-67 and cleaved caspase-3 were human tonsil, CD31 and VEGF, mouse adipose tissue, and human liver, respectively (n=8; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 t-test)

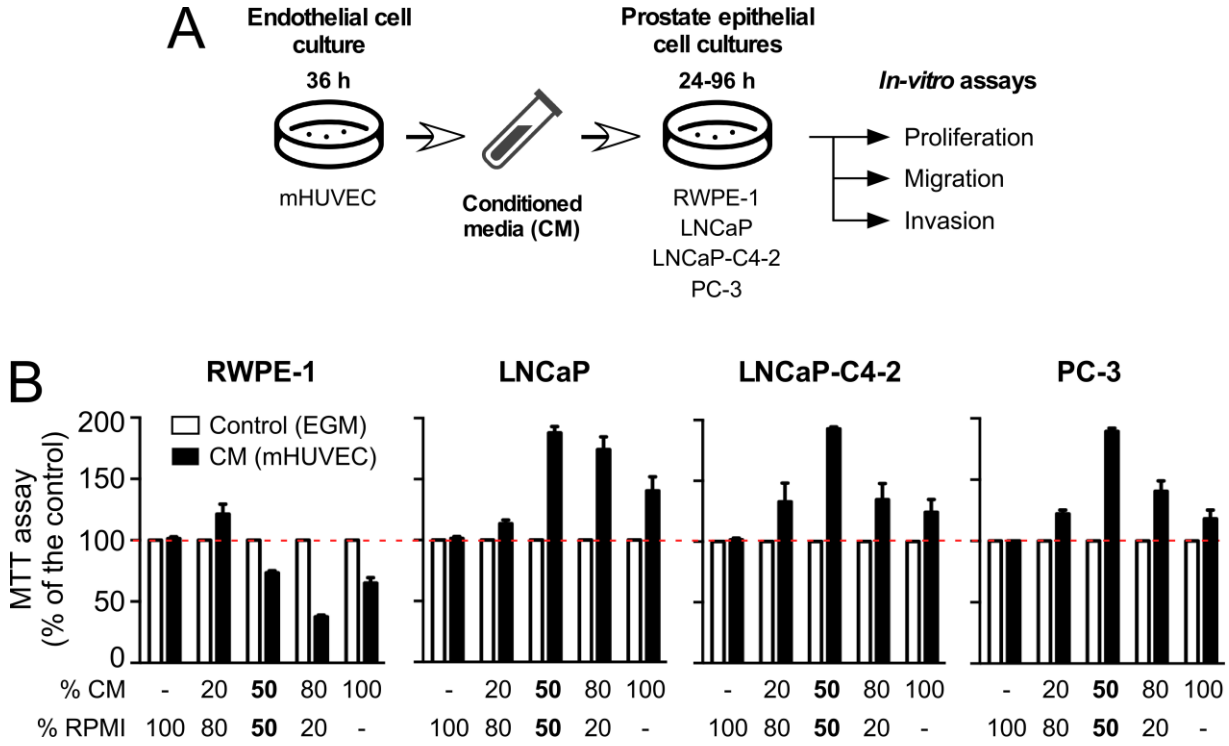
Figure 6. Conditioned medium from HUVEC cells increases metastatic foci in host tissues of xenotransplanted mice with PC-3 cells. A) Representative images of metastatic foci in host kidney and liver tissues. Metastatic PC-3 cells were immunohistochemically detected using an anti-human Ki-67 antibody. Human tonsil was used as positive control for human Ki-67 expression. B) Quantitation of the number of metastatic foci per field in host kidney and liver tissues (n=8).

Figure 7. Secreted Factors involved in crosstalk communication between endothelial and prostate cancer cell. Cytokine expression profile of the CM derived from endothelial cells and cell cultures of CaP cells exposed to CM from endothelial cells. A): Cytokine array of CM

derived from HUVEC cells (top panel) and endothelial growth media "EGM" (Bottom). B) Graphical representation of the fold change of cytokines in the CM derived from HUVEC cells. C) A protein-protein interaction network map for the 36 secreted factors was generated using the STRING program. Protein-related with cell migration (red) and cell proliferation (blue) cellular processes are colored D) Cytokine array of cell supernatant of PC-3 and LNCaP cells (Top panel) and exposed to CM of endothelial cells (bottom). E) Graphical representation of the total of accumulated fold change of cytokines in cell supernatant of PC-3 compared to LNCaP cells. F) Representation of secreted proteins that are increased by the effect of endothelial cells. The proteins are divided according to their biological function. White squares indicate that factors are not increased, and dark red squares indicate the maximal induction fold (50×). Films were scanned and analyzed using ImageJ software. Cytokines and growth factors that had 1.5-fold or more relative to the control were scored as positive.

Figure 8. **Conditioned media from HUVEC cells induces differential changes in gene expression in prostate cancer cells.** RNA-seq expression analysis of PC3 and LNCaP cells exposed to conditioned media from HUVEC cell for 48 hours. (A) Heat map of differentially expressed genes produced by PC3 CM-treated and LNCaP CM-treated cells and their untreated controls, across all cell lines, with Euclidean hierarchical clustering (B) Venn diagram of overlapping genes from the DEG lists produced by LNCaP CM-treated vs. LNCaP control untreated cells and the PC3 CM-treated vs. PC3 control untreated cells C) GSEA clusters on genes specific to LNCaP differentially expressed genes. D) GSEA clusters on genes specific to PC3 differentially expressed genes.

FIGURE 1


A
Endothelial cell culture
36 h
mHUVEC
Conditioned media (CM)
Prostate epithelial cell cultures
24-96 h
RWPE-1
LNCaP
LNCaP-C4-2
PC-3
In-vitro assays
Proliferation
Migration
Invasion
B
RWPE-1
LNCaP
LNCaP-C4-2
PC-3
Control (EGM)
CM (mHUVEC)
MTT assay (% of the control)
200
150
100
50
0
% CM - 20 50 80 100
% RPMI 100 80 50 20 -

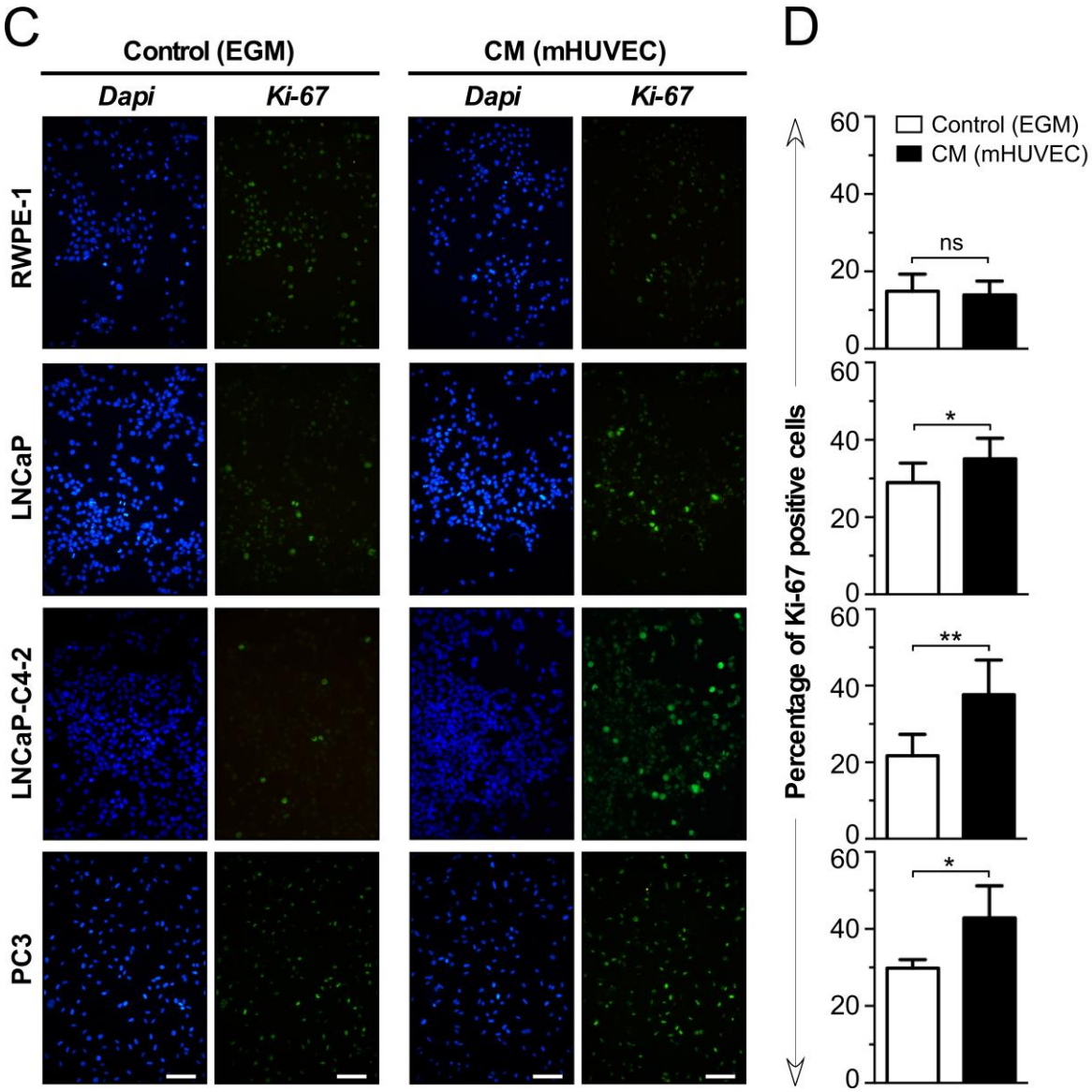

C
Control (EGM)
CM (mHUVEC)
Dapi
Ki-67
Dapi
Ki-67
RWPE-1
LNCaP
LNCaP-C4-2
PC3
D
Control (EGM)
CM (mHUVEC)
Percentage of Ki-67 positive cells
60
40
20
0
ns
*
**
*

FIGURE 2

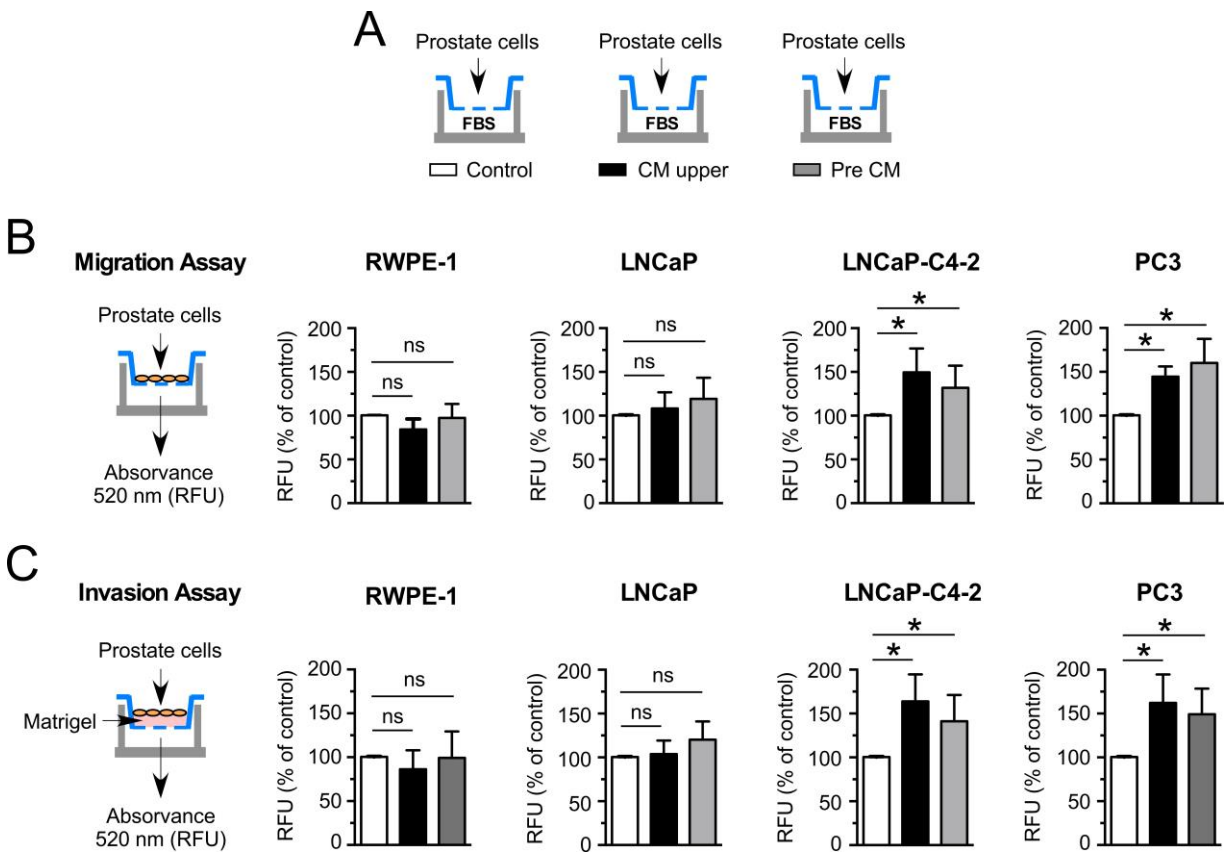

A
Prostate cells
Prostate cells
Prostate cells
FBS
FBS
FBS
Control
CM upper
Pre CM
B
Migration Assay
Prostate cells
Absorvance
520 nm (RFU)
RWPE-1
LNCaP
LNCaP-C4-2
PC3
RFU (% of control)
ns
*
C
Invasion Assay
Prostate cells
Matrigel
Absorvance
520 nm (RFU)
RWPE-1
LNCaP
LNCaP-C4-2
PC3
RFU (% of control)
ns
*

FIGURE 3

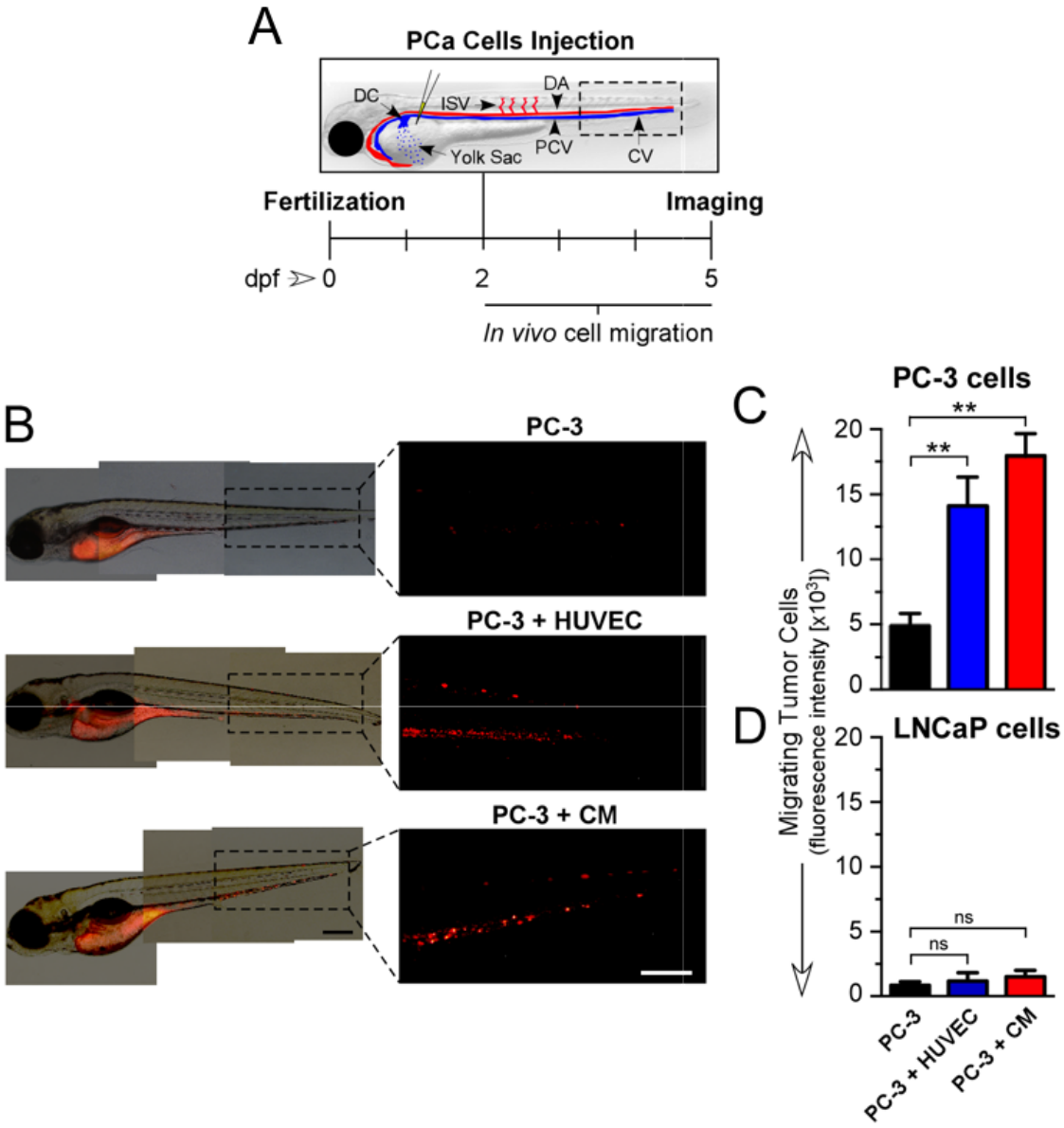

A
PCa Cells Injection
DC
ISV
DA
Yolk Sac
PCV
CV
Fertilization
Imaging
dpf
0
2
5
In vivo cell migration
B
PC-3
PC-3 + HUVEC
PC-3 + CM
C
PC-3 cells
**
**
Migrating Tumor Cells
(fluorescence intensity [x10³])
20
15
10
5
0
D
LNCaP cells
ns
ns
PC-3
PC-3 + HUVEC
PC-3 + CM

FIGURE 4

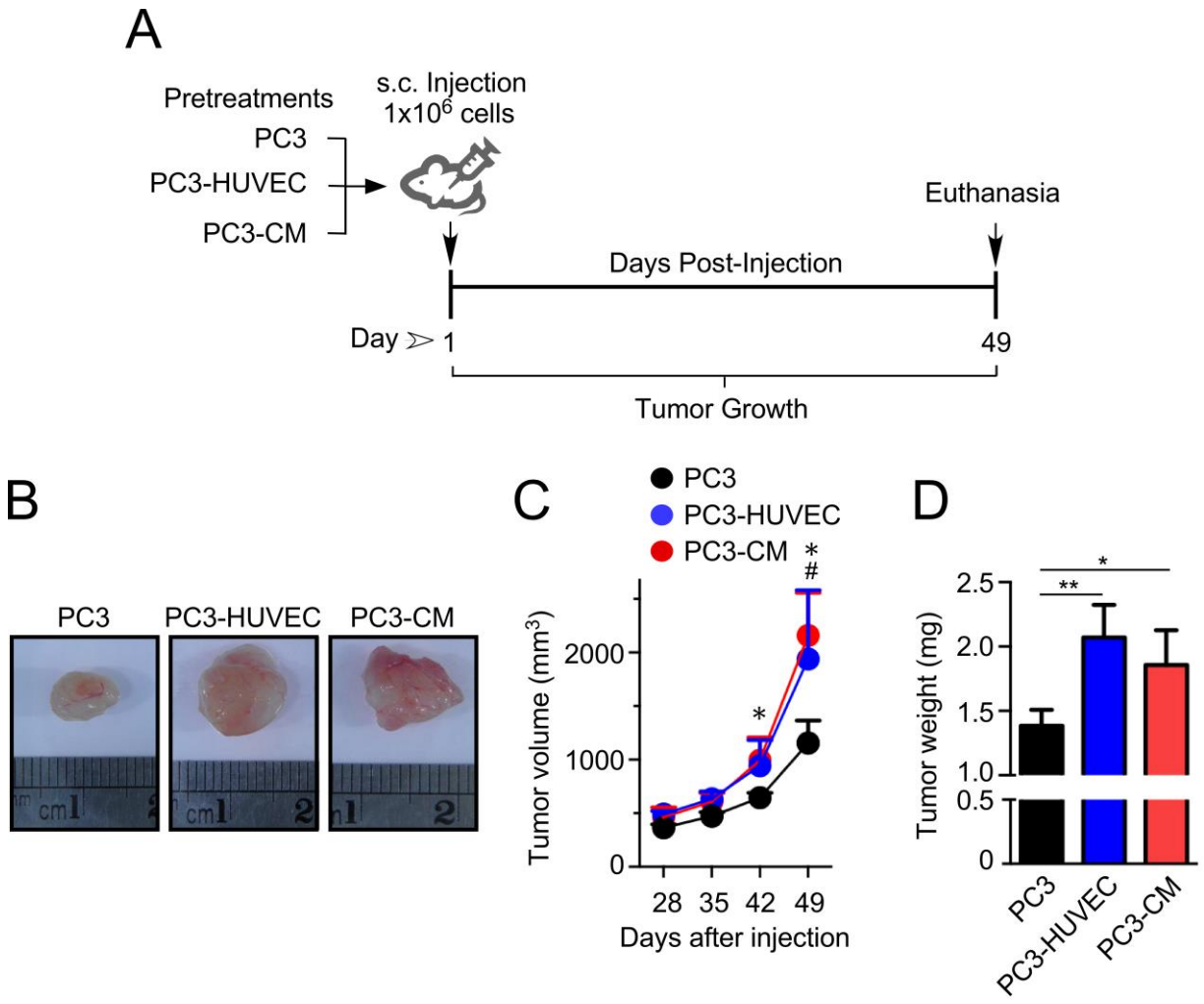

A
Pretreatments
PC3
PC3-HUVEC
PC3-CM
s.c. Injection
1x10^6 cells
Euthanasia
Days Post-Injection
Day 1
49
Tumor Growth
B
PC3
PC3-HUVEC
PC3-CM
C
PC3
PC3-HUVEC
PC3-CM
Tumor volume (mm^3)
2000
1000
0
28 35 42 49
Days after injection
D
Tumor weight (mg)
2.5
2.0
1.5
1.0
0.5
0
PC3
PC3-HUVEC
PC3-CM

FIGURE 5

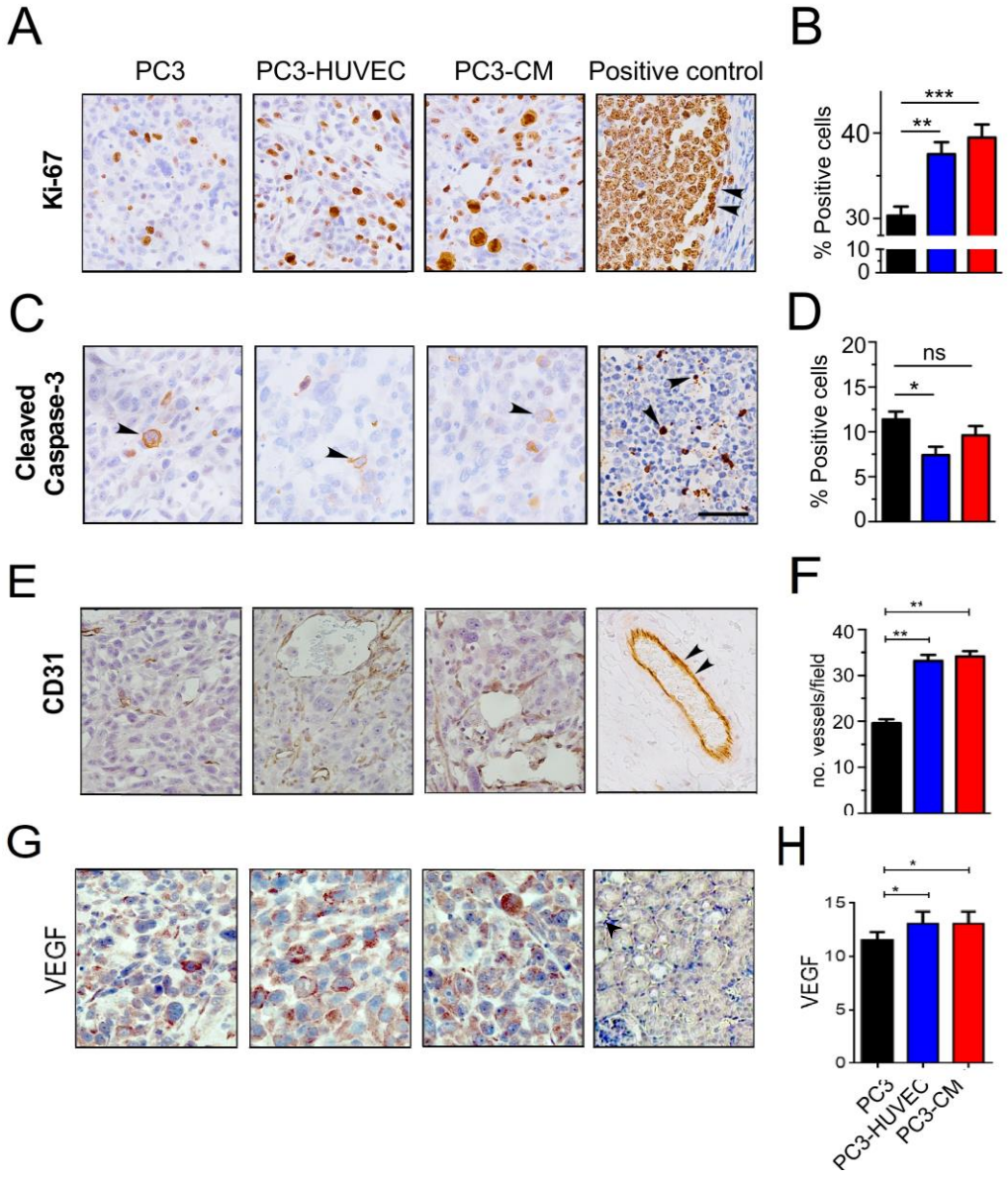
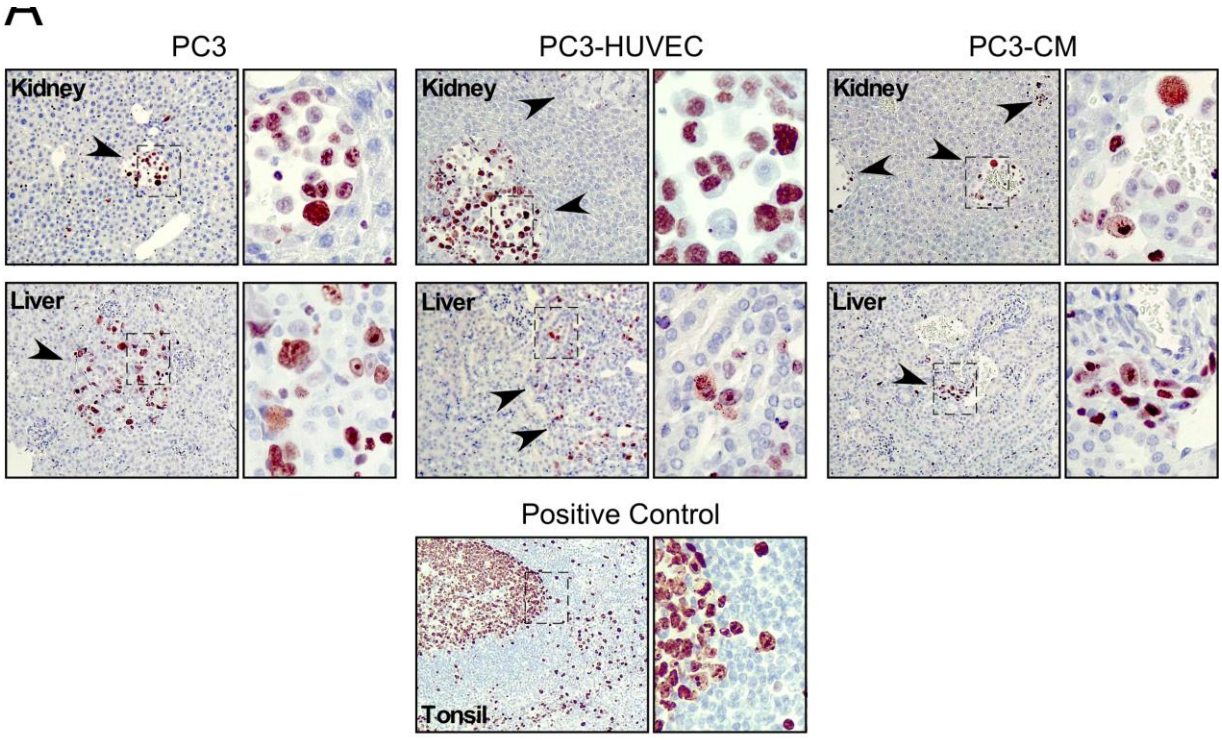

A
PC3
PC3-HUVEC
PC3-CM
Positive control
Ki-67
B
% Positive cells
***
**
40
30
10
0
C
Cleaved Caspase-3
D
% Positive cells
ns
*
20
15
10
5
0
E
CD31
F
no. vessels/field
**
**
40
30
20
10
0
G
VEGF
H
VEGF
*
*
15
10
5
0
PC3
PC3-HUVEC
PC3-CM

FIGURE 6

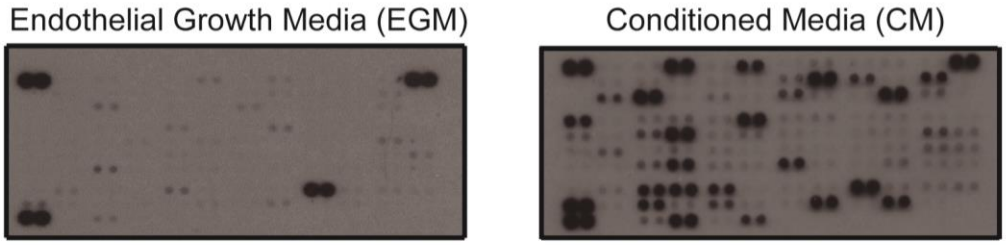


B

		Ex vivo detection of metastasis				
			Kidney		Liver	
Cell injection	Sample size	Total met. incidence (all organs)	Metastasis incidence	Average metastasic lesion (range)	Metastasis incidence	Average metastasic lesion (range)
PC3	8 mice	87.5% (7/8)	50% (4/8)	**1.6** (0.2-4)	75% (6/8)	**8** (1-18)
PC3-HUVEC	8 mice	100% (8/8)	62.5% (5/8)	**6** (2-14)	100% (8/8)	**10** (1-16)
PC3-CM	8 mice	100% (8/8)	75% (6/8)	**6** (0.5-15)	100% (8/8)	**11** (1-20)

FIGURE 7

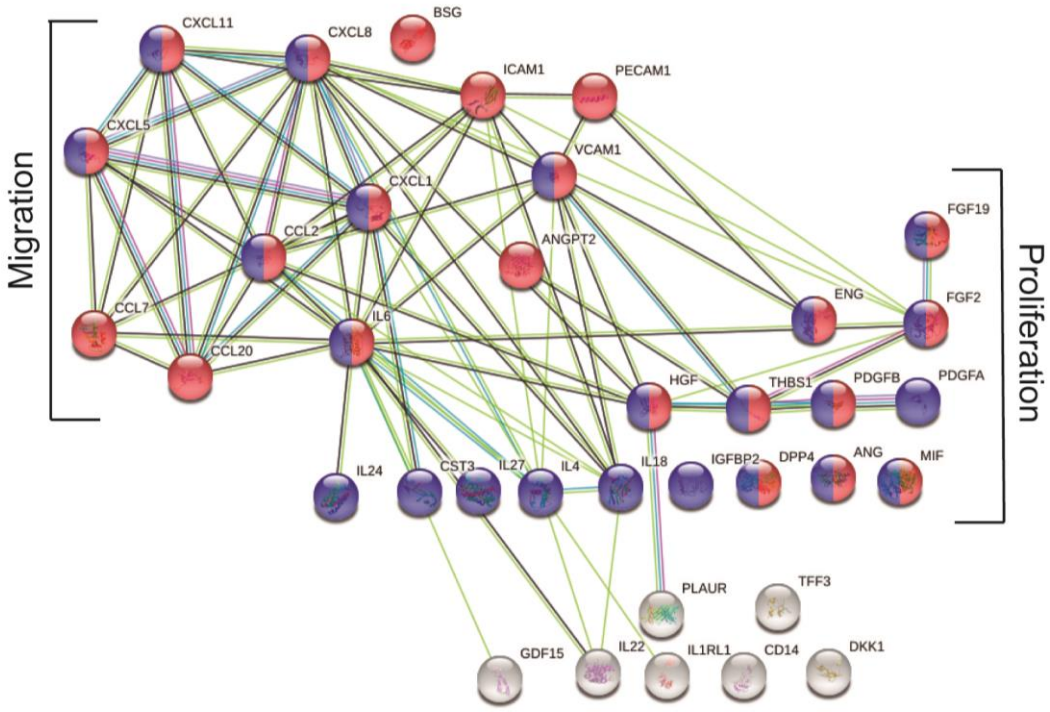
A



B

Angiogenin	ANGPT2	CD14	CST3	DKK-1	DPP4
BSG/CD147	CXCL5	ENG	FGF-2	FGF-19	GDF-15
CXCL1	HGF	ICAM-1	IGFBP-2	IL-4	IL-6
CXCL8	IL-18	IL-22	IL-24	IL-27	CXCL11
CCL2	CCL7	MIF	CCL20	PDGFA	PDGFB
IL1RL1	TFF3	THBS1	PLAUR	PCAM-1	VCAM-1

C



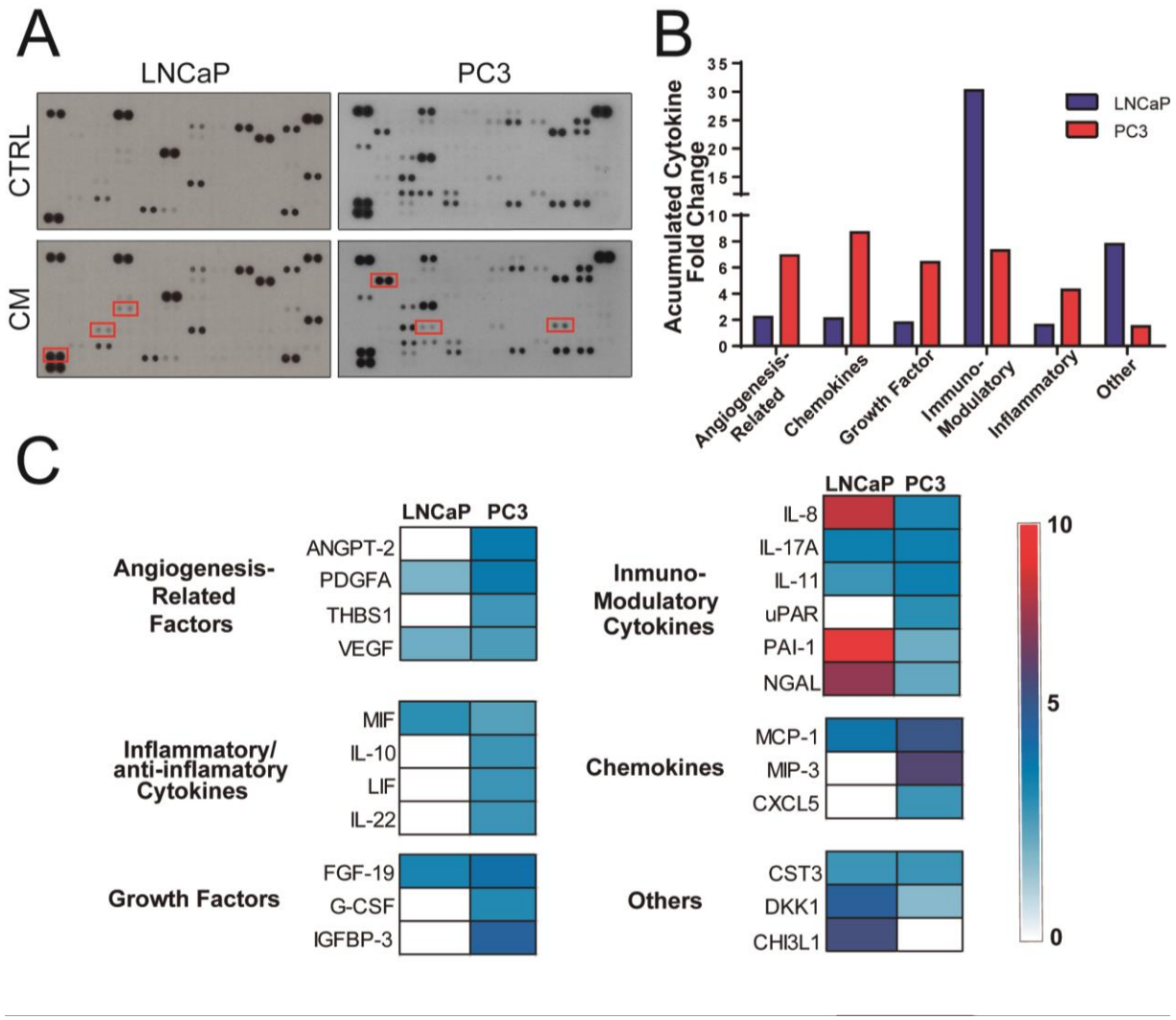
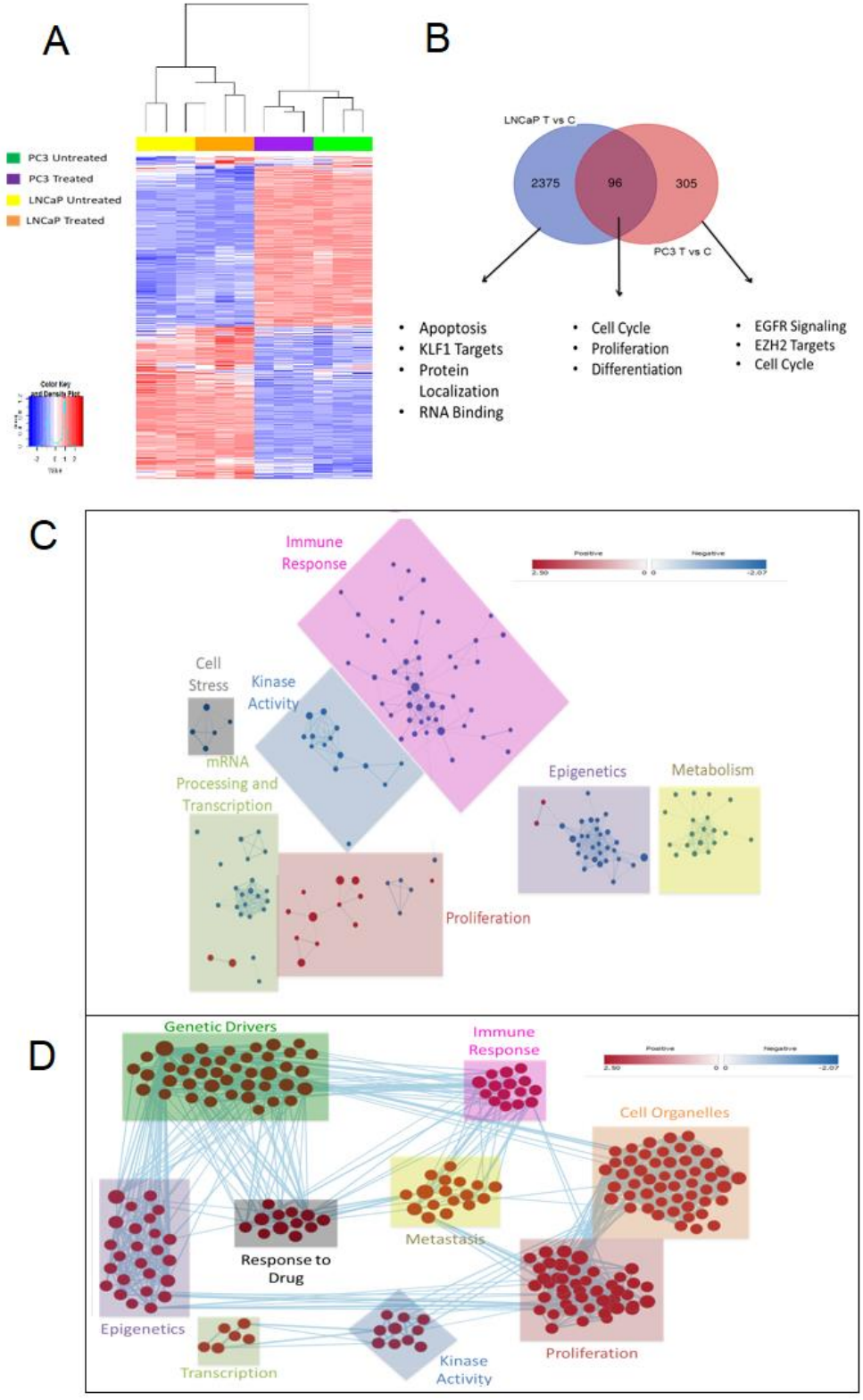
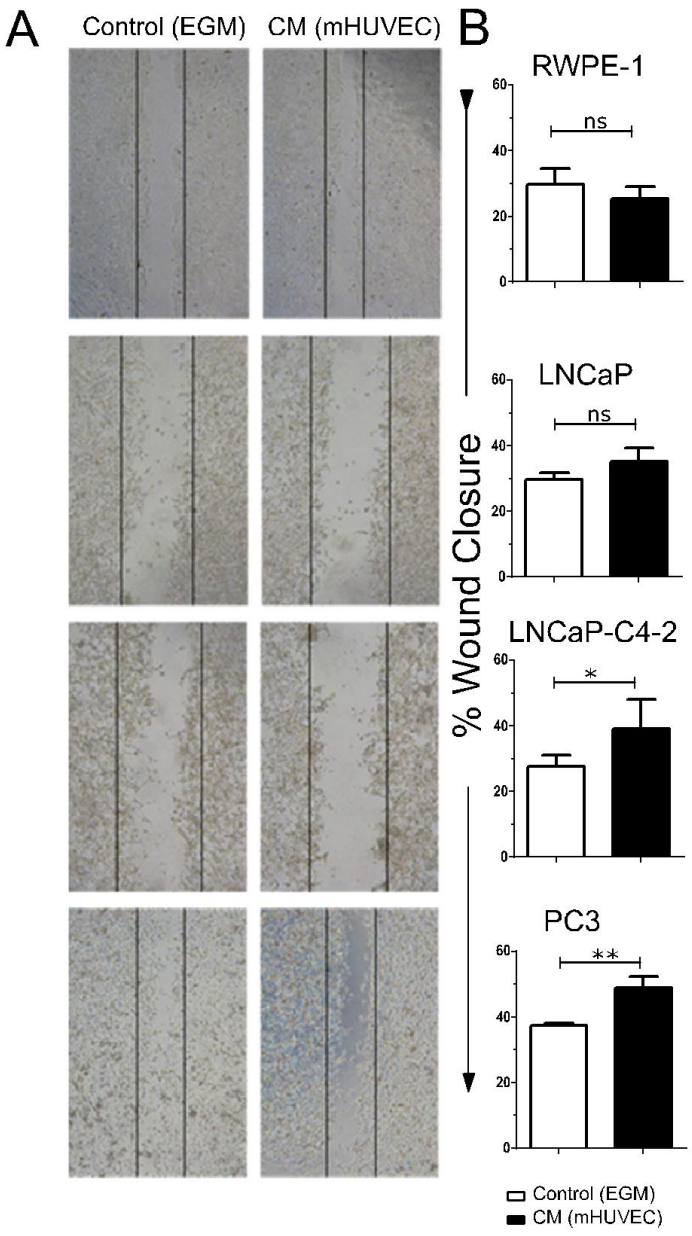

A
LNCaP
PC3
CTRL
CM
B
Acuumulated Cytokine Fold Change
LNCaP
PC3
Angiogenesis-Related
Chemokines
Growth Factor
Immuno-Modulatory
Inflammatory
Other
C
LNCaP PC3
Angiogenesis-Related Factors
ANGPT-2
PDGFA
THBS1
VEGF
Inflammatory/ anti-inflamatory Cytokines
MIF
IL-10
LIF
IL-22
Growth Factors
FGF-19
G-CSF
IGFBP-3
Inmuno-Modulatory Cytokines
IL-8
IL-17A
IL-11
uPAR
PAI-1
NGAL
Chemokines
MCP-1
MIP-3
CXCL5
Others
CST3
DKK1
CHI3L1
10
5
0

FIGURA 8


A
PC3 Untreated
PC3 Treated
LNCaP Untreated
LNCaP Treated
B
LNCaP T vs C
2375
96
305
PC3 T vs C
Apoptosis
KLF1 Targets
Protein Localization
RNA Binding
Cell Cycle
Proliferation
Differentiation
EGFR Signaling
EZH2 Targets
Cell Cycle
C
Immune Response
Cell Stress
Kinase Activity
mRNA Processing and Transcription
Epigenetics
Metabolism
Proliferation
D
Genetic Drivers
Immune Response
Cell Organelles
Metastasis
Response to Drug
Epigenetics
Transcription
Kinase Activity
Proliferation

SUPPLEMENTARY FIGURE 1



Discusión

Un creciente cuerpo de evidencia muestra que las células endoteliales no son simples conductos que se encargan de entregar O_2 y nutrientes al tumor. Por el contrario, las CE podrían ser vistas como un nicho vascular especializado que, por la secreción de diversos factores, podrían regular el crecimiento y progresión tumoral (Butler et al. 2010). De forma interesante, entender el rol de las células no cancerosas que componen el microambiente tumoral, tiene la ventaja de que estas células o sus factores podrían ser fácilmente blancos terapéuticos ya que son genéticamente estables a diferencia de las células de cáncer que están bajo frecuentes mutaciones genéticas y presentan altos índices de heterogeneidad (Lee et al. 2015). En este trabajo de tesis, estudiamos el efecto paracrino del endotelio en CaP. Por lo cual, en primer lugar, realizamos la validación de nuestros modelos celulares, los cuales corresponden a cultivos primarios de células HUVEC, y líneas celulares de CaP en base a su dependencia androgénica. Posteriormente y para estudiar el efecto paracrino utilizamos el medio condicionado obtenido a partir de células HUVEC sobre distintos parámetros biológicos que determinan agresividad en células de CaP como lo son: viabilidad, proliferación, migración e invasión, realizando ensayos *in vitro.* El crecimiento tumoral y la capacidad metastásica fue analizada *in vivo* utilizando modelos de pez cebra y xenoinjertos en ratones inmunocomprometidos. Nuestros resultados muestran que existe una comunicación paracrina entre CE y células epiteliales de CaP, en la cual no es necesaria una interacción directa entre ambos tipos celulares, si no que factores secretados por las CE ejercerían un efecto diferencial sobre las células de CaP. Asimismo, observamos que el medio condicionado de CE incrementa características biológicas que determinan agresividad y que aumentan las capacidades metastásicas de los tumores en los modelos in vivo. Finalmente, para intentar determinar los mecanismos de comunicación entre CE y células de CaP observados en

el contexto tumoral, analizamos las moléculas secretadas, principalmente citoquinas, de las CE y a la vez las moléculas secretadas desde células de CaP al ser expuestas a medio condicionado de células endoteliales. Las CE secretan una gran cantidad de citoquinas, la mayoría relacionada con procesos de proliferación, migración y comunicación celular y estos factores serían capaces de cambiar el perfil de secreción de células de cáncer. Este cambio de perfil es diferente al analizar una línea celular de baja agresividad en comparación con una de alta agresividad, lo cual concuerda con los resultados obtenidos en los experimentos in vitro.

Dependencia androgénica de células HUVEC

Para realizar los estudios de comunicación paracrina entre CE y células epiteliales de CaP, utilizamos como modelo de estudio de célula endotelial, cultivos primarios de células HUVEC, aisladas desde cordones umbilicales humanos. Si bien nuestro laboratorio tiene amplia experiencia en el aislamiento de CE de próstata humana benigna y maligna (Godoy et al. 2008), la elección del modelo experimental de células HUVEC se debe a que no fue posible obtener especímenes clínicos de CaP para poder aislar estas células, y, además, no existen líneas celulares endoteliales derivadas de próstata humana comercialmente disponibles.

No obstante, la necesidad de obtener un modelo celular de CE que se comporte lo más cercano posible, en términos de su biología, a las CE de tejido prostático, se debe a varias razones. En primer lugar, la próstata es un órgano que depende fuertemente de la presencia de andrógenos circulantes, los cuales, a través de su unión al receptor de andrógenos o AR, median los efectos biológicos sobre las células epiteliales prostáticas, lo cual permite mantener la estructura y función normal de la próstata (Chiarugi et al. 2014). Por mucho tiempo se pensó que el blanco celular principal de los andrógenos en el tejido prostático humano correspondía a las células

epiteliales. Sin embargo, estudios previos (Singh et al. 2005), han demostrado que otros tipos celulares como las células estromales (Singh et al. 2014, Wen et al. 2015) y las CE (Godoy et al. 2008, Godoy et al. 2011) de la próstata expresan el AR, y son afectadas directamente por la activación de las señales androgénicas. En el caso particular de las CE, evidencia previa sugería que los andrógenos, y/o sus metabolitos, presentaban efectos significativos sobre la biología normal y los procesos patológicos que afectan tanto a las CE de la próstata como a la de otros órganos (Cai et al. 2011, Campelo et al. 2012, Cioni et al. 2018). Entre los procesos patológicos en los cuales las CE y los andrógenos juegan un papel preponderante, se encuentran las enfermedades cardiovasculares (ECV) (Gimbrone y Garcia-Cardena 2016, Lucas-Herald et al. 2017). Esto se hace evidente cuando se observa que la incidencia de ECV es mayor en hombres adultos en comparación con mujeres premenopáusicas de la misma edad (Franconi et al. 2017). Sin embargo, el rol de los andrógenos, en especial la testosterona, aún no está clarificado, ya que si bien se ha determinado que los andrógenos son factores independientes que contribuyen a la mayor susceptibilidad masculina a la aterosclerosis (Nheu et al. 2011), también se ha determinado que las personas de sexo masculino con niveles bajos de testosterona tienen una alta prevalencia de ECV y síndrome metabólico (Lucas-Herald et al. 2017). Más aún, y a pesar de varios estudios clínicos, el efecto protector/perjudicial inducido por la terapia con testosterona sigue siendo controversial (Liao et al. 2012, Lucas-Herald et al. 2017). Se ha determinado que CE de distintos tejidos expresan funcionalmente el AR, y que son blancos de la acción hormonal (Liu et al. 2003, Ikeda et al. 2005). No obstante, los mecanismos por los cuales AR media sus efectos biológicos en las CE en el sistema cardiovascular aun no son completamente definidos. Si bien la demostración de que los andrógenos inducen efectos biológicos en las CE, como incremento en proliferación, apoptosis y producción de óxido

nítrico, y que estas consecuencias biológicas son anuladas por el uso de anti- andrógenos, claramente asocian al AR en la modulación de la función de las EC (Liu et al. 2003, Cai et al. 2011, Torres-Estay et al. 2015). En el caso específico de la próstata, nuestro grupo determinó que las CE de la próstata humana (HPEC) aisladas de muestras clínicas frescas humanas de próstata benigna y CaP expresan AR funcional. También se observó que la dihidrotestosterona (DHT), un ligando no-aromatizable a través de AR, incrementó la proliferación de cultivos primarios de HPEC de una manera dependiente de la concentración. Estos estudios proporcionan evidencia de un potencial papel para AR en la regulación de la homeostasis de las CE de la próstata humana (Godoy et al. 2008). Sin embargo, los procesos a través de los cuales los mecanismos mediados por AR regulan la homeostasis de la CE y las respuestas angiogénicas a lesiones o enfermedades requerían mayor aclaración (Torres-Estay 2015). Estudios previos de inmunohistoquímica (datos no publicados) de nuestro laboratorio sugerían que las células HUVEC expresaban el AR a nivel proteico. Los cultivos primarios de células HUVEC son probablemente las CE más populares utilizadas en la investigación porque las venas umbilicales humanas están relativamente más disponibles que otros tipos de vasos sanguíneos y pueden aislarse y mantenerse mediante un protocolo relativamente sencillo. Como modelo expresan muchos marcadores endoteliales importantes y moléculas de señalización asociadas con la fisiología vascular. Además, se ha demostrado que responden a estímulos fisiológicos y/o patológicos. Por lo tanto, aunque las venas umbilicales humanas solo se encuentran en ciertas etapas de la vida humana, las células HUVEC se han considerado como un modelo general para las CE tanto en condiciones normales como patológicas (Cao et al. 2017). Por lo que para confirmar esta observación y validar el uso de células HUVEC como modelo de CE humana similar a las CE de origen prostático, en relación con su dependencia androgénica, realizamos

una caracterización completa de la expresión de AR, su funcionalidad, y los efectos biológicos gatillados por los andrógenos en las células HUVEC. Nuestros resultados mostraron que los cultivos primarios de células HUVEC expresan el AR en niveles variables de mensajero y proteína. Mas aún, la presencia del ligando natural DHT o su ligando sintético R1881 lograron translocar el AR al núcleo de las células HUVEC, proceso que es fundamental para estimular la activación transcripcional de este receptor nuclear (Torres-Estay et al. 2017). Para confirmar estos resultados, se estudió la actividad transcripcional de AR en células HUVEC transfectadas de forma transitoria, utilizando un vector adenoviral que contiene el promotor MMTV (Mouse Mammary Tumor Virus), el cual es un modelo de expresión génico inducido por hormonas esteroidales (Hager 1988). El promotor MMTV es bastante selectivo para AR, así como para el receptor de glucocorticoides, receptor de progesterona y contiene varios sitios regulatorios que pueden ser activados por otros esteroides (Campana et al. 2016). Por lo que además de utilizar el ligando DHT, se utilizó bicalutamida, una droga que se une al receptor de andrógenos con mayor afinidad que sus ligandos, previniendo la translocación nuclear, por lo tanto, su actividad (Egan et al. 2014). La actividad transcripcional observada en las células HUVEC, se incrementó significativamente al estar en presencia del ligando DHT. A la vez, las células HUVEC presentaron una inhibición en la actividad transcripcional en presencia de bicalutamida, siendo esta respuesta dosis dependiente. En conjunto estos resultados demuestran que el AR es funcionalmente activo en estas células. Finalmente determinamos los efectos biológicos de los andrógenos y el rol del AR en células HUVEC. Nuestro estudio determinó que los andrógenos, si bien no afectan la supervivencia de las células HUVEC, ya que estas células crecen en ausencia de andrógenos y en presencia de bicalutamida, si estimulan la proliferación de estas células, siendo este efecto modulado directamente por la activación de AR (Torres-Estay et al.

2017). Con todos los antecedentes descritos, se demostró que las células HUVEC reproducen la mayoría de los efectos biológicos de los andrógenos observados en las CE de la próstata en relación con su dependencia androgénica, por lo que valida el uso de este modelo de CE para los estudios de interacción con células epiteliales de CaP.

Dependencia androgénica de líneas celulares de cáncer de próstata

Los andrógenos desempeñan funciones cruciales tanto en las células epiteliales normales de la próstata como también en la oncogénesis y la progresión del CaP (Chiarugi et al. 2014). En la glándula prostática normal, el AR se expresa en las células luminales y basales del epitelio donde su principal rol es promover la expresión de genes relacionados con la diferenciación terminal, secreción y supresión de la proliferación para la mantención de la homeostasis (Yadav y Heemers 2012). En CaP en cambio se ha observado que la señalización de AR modula la expresión de crítica de genes implicados en la proliferación y migración (Crumbaker et al. 2017). Por lo que la inhibición de la acción de AR es el pilar del tratamiento de la enfermedad localmente avanzada o metastásica. Sin embargo, a pesar de la alta tasa de respuesta a las intervenciones dirigidas a AR en pacientes metastásicos, la progresión hacia el CaP resistente a la castración es inevitable, caracterizándose por una alta morbilidad y mortalidad (Egan et al. 2014).

Debido a la progresión de esta enfermedad, las líneas celulares de CaP elegidas para este estudio, fueron escogidas para que representaran el fenotipo de cada una de las etapas de la enfermedad; epitelio no- neoplásico, CaP sensible a andrógenos, CaP resistente a la castración y CaP metastásico. Sin embargo, pese a que estas líneas están descritas en la literatura, pueden existir problemas de identificación errónea o contaminación cruzada de líneas (Corral-Vazquez et al.

2017), que cambien el fenotipo de estas líneas y lleven a una incorrecta utilización de estas, por lo que es fundamental realizar una caracterización de las líneas celulares a utilizar. La caracterización que realizamos (Datos no publicados; Anexo 1) se basó principalmente en la dependencia androgénica que poseen estas líneas. Por lo cual analizamos nuestros modelos principalmente en términos de expresión del AR y su funcionalidad, y el rol de los andrógenos y el AR en la proliferación celular. Los resultados mostraron que la línea celular RWPE-1, presenta un fenotipo de epitelio prostático no -neoplásico benigno. Esta línea celular en presencia de andrógenos no presenta cambios en su proliferación, lo que concuerda con lo esperado de una célula epitelial no neoplásica de próstata (Bello et al. 1997).

Por otra parte, las líneas celulares LNCaP, LNCaP-C4-2 y PC3 han sido los modelos más utilizados para los estudios de CaP, pero poseen características significativamente diferentes, siendo las más importantes las células LNCaP que expresan los marcadores de diferenciación luminal AR y PSA. Las células LNCaP-C4-2, si bien expresan estos marcadores, a diferencia de LNCaP su expresión no es regulada por los andrógenos, mientras que las células PC3 no expresan ninguno de estos marcadores (Tai et al. 2011). En consecuencia, las células LNCaP son dependientes de andrógenos, y la eliminación de andrógenos inhibe su crecimiento, mientras que las células LNCaP-C4-2 y PC3 no dependen de andrógenos y proliferan normalmente en medios privados de andrógenos (Liu et al. 2004, Sobel y Sadar 2005). La principal diferencia entre las líneas celulares LNCaP-C4-2 y PC-3, radica en la expresión del AR (Sobel y Sadar 2005). Las células PC-3, no expresan este receptor y presentan una mayor agresividad que las líneas LNCaP-C4-2, lo que se puede observar al analizar las curvas de mayor proliferación. Por lo que la línea LNCaP es considerada como una línea sensible a los andrógenos y de baja agresividad y las células LNCaP-C4-2 y PC3 como líneas resistentes a la castración y más

agresivas. Los resultados obtenidos, reflejan lo obtenido por literatura y que estas líneas celulares responden a los andrógenos dependiendo del fenotipo correspondiente, lo que valida el uso de estas cuatro líneas celulares en nuestros estudios.

Comunicación paracrina de células endoteliales y líneas celulares de cáncer de próstata

El microambiente tumoral juega un rol en el desarrollo y progresión del cáncer que influye en las distintas etapas de su formación, desde el crecimiento tumoral hasta la diseminación a órganos distantes e incluso en la respuesta y resistencia a distintas terapias anticancerígenas (Levesque y Nelson 2018). Entre los componentes de este microambiente se encuentran las células endoteliales, que forman los vasos sanguíneos, las cuales han demostrado ser importantes debido al proceso conocido como angiogénesis (Folkman 2007). Por mucho tiempo, se creyó que el rol de los vasos sanguíneos se limitaba a formar conductos por los cuales el tumor recibía nutrientes y oxígeno. Sin embargo, debido al relativo fracaso de las terapias anti angiogénicas (Butler et al. 2010), se ha sugerido que las células endoteliales, podrían cumplir un rol independiente de la angiogénesis, como se ha visto en procesos fisiológicos, tales como la organogénesis (Rafii et al. 2016), mediante la secreción de distintos factores que promoverían el crecimiento y desarrollo tumoral (Butler et al. 2010).

Estudios en cáncer de mama y en modelos de leucemia mostraron que la comunicación paracrina, con contacto directo entre las CE y células cancerosas, es necesario para promover el crecimiento y las características metastásicas del tumor, así como también para la expansión de las células iniciadoras de leucemia (Ghiabi et al. 2014, Poulos et al. 2014). Por el contrario, otros estudios muestran que solo es necesario la secreción de factores paracrinos de CE, para producir el mismo efecto, como se observó en el carcinoma hepatocelular y cáncer colorrectal

(Wang et al. 2013, Wang et al. 2017). Por lo que el objetivo de nuestro trabajo se basó en entender el tipo de interacción que existe entre las CE y células de CaP, principalmente de tipo paracrina por sobre la interacción célula-célula, y el efecto de las CE sobre líneas celulares de epitelio benigno y de CaP. Para lo cual, utilizamos medios condicionados de CE, para realizar los estudios sin necesidad de una interacción directa entre ambos tipos celulares. Nuestros resultados mostraron que las CE ejercen un efecto paracrino sobre las células de CaP, solo por efecto del medio condicionado, y que este efecto es diferencial sobre las líneas celulares de CaP dependiendo de su grado de agresividad.

Así también, este efecto parece ser específico para líneas celulares de cáncer, ya que no se observó ningún efecto del medio condicionado de CE sobre la línea no maligna de próstata, tanto en proliferación, migración o invasión. Esto también se ha observado en modelos de cáncer de mama, que al utilizar líneas celulares de epitelio benigno (MCF10-A) en experimentos de co-cultivos con CE, no se observaron cambios en las características biológicas estudiadas como por ejemplo en la expresión de factores angiogénicos o formación de colonias epiteliales de tipo transición epitelial-mesenquimal. Esto difiere al realizar los experimentos con células de cáncer, las cuales sí responden al efecto paracrino de las CE en las características antes nombradas (Sigurdsson et al. 2011, Buchanan et al. 2012).

Durante la progresión, las células tumorales además presentar una alta proliferación, deben adquirir la capacidad de migrar fuera del tumor primario y de invadir la matriz extracelular para así poder diseminarse por todo el cuerpo para formar tumores metastásicos (Clark y Vignjevic 2015). Por esta razón, se realizó el análisis de la capacidad de migración e invasión en las células de CaP, estimuladas con medio condicionado de CE. Nuestros resultados muestran que existe una diferencia en la respuesta al medio condicionado, ya que solo las células más

agresivas, que corresponden a las células resistentes a la castración y metastásicas, mostraron un aumento significativo en la migración e invasión. Esto difiere de lo observado por Wang y colaboradores (2013), los cuales observaron en modelos de co-cultivos que las células de CaP, incluida la línea celular LNCaP, mostraban un incremento en la capacidad de invasión cuando se co-cultivaba con CE. La diferencia principal entre ambos trabajos se refiere justamente en el método de interacción permitida, y puede que en el caso de células de CaP de baja agresividad, sea necesario un mínimo de contacto celular para inducir la invasión, ya que se utilizaron experimentos de co-cultivo, por el método de transwell. Sin embargo, utilizando el modelo de xenoinjerto de pez cebra, el cual proporciona un método rápido para el análisis de la migración de células cancerosas (Teng et al. 2013), pudimos observar que aun co-inyectando células LNCaP con CE, no obtuvimos aumento en la migración de estas células. En consecuencia, esto apoya la idea de que el efecto de las CE es más amplio en las células de CaP más agresivas.

La interacción de CE y de cáncer ha sido estudiada adicionalmente utilizando modelos *in vivo*, principalmente con modelos de xenoinjerto en ratones inmunocomprometidos, en los cuales se realiza una co- inyección de células de cáncer con CE en distintas proporciones (Wang et al. 2013, Ghiabi et al. 2014, Lee et al. 2014). Estos estudios han demostrado que las CE aumentan el crecimiento tumoral (Zhao et al. 2016) y que los tumores presentan más características agresivas (Wang et al. 2013, Ghiabi et al. 2014). En CaP, la co- inyección de CE con la línea celular CWR22Rv1, que corresponde a una línea celular resistente a la castración, mostró que hay un incremento en la incidencia de metástasis hacia distintos órganos (Wang et al. 2013). Nosotros utilizamos dos modelos *in vivo*, xenoinjertos en ratones inmunocomprometidos, e inyección en larvas de peces cebra, para estudiar la comunicación paracrina entre células PC-3, las cuales presentan el fenotipo más agresivo, y CE. Si bien el uso de ratones ha sido invaluable

para la comprensión del cáncer humano, el modelo del pez cebra, se ha propuesto como una alternativa que proporciona una solución a los costosos y extensos experimentos con ratones, ya que en muchos casos el ensayo se puede realizar dentro de las 24 a 36 horas posteriores al xenotrasplante, en grandes grupos de peces, lo que además proporciona un alto poder estadístico a los resultados (Moore y Langenau 2016). Además, el pez cebra posee características únicas que lo hacen un buen modelo de estudio en cáncer: 1) produce embriones transparentes y fertilizados externamente que facilitan en gran medida el uso de análisis genéticos y químicos y la visualización directa del interior del pez 2) Se puede lograr un injerto eficiente mediante el trasplante de células en el pez cebra en etapa larval temprana que aún no ha desarrollado un sistema inmunitario adquirido funcional. Y 3) La capacidad de manipular genéticamente el pez cebra a través de knockdowns, knockouts y transgénesis (Zhang et al. 2015, Moore y Langenau 2016). Por consiguiente, el pez cebra se ha propuesto como un modelo rápido, robusto y económico para evaluar el potencial metastásico de las células cancerosas humanas (Teng et al. 2013, Tobia et al. 2013). Nuestros resultados mostraron que al analizar la condición de co-inyección de células PC-3 con CE tanto en pez cebra, como en ratones se observa que existe un aumento tanto en la migración de las células PC-3 como un incremento en el tamaño de los tumores, respectivamente. Sin embargo, y de forma interesante, pudimos determinar que el efecto del medio condicionado en células PC-3 produce cambios a largo plazo, ya que, al inyectar estas células tratadas previamente con medio condicionado obtuvimos la misma respuesta, que al co-inyectar con células HUVEC. Estos resultados demuestran en primer lugar que el modelo de pez cebra podría ser utilizado para evaluar el potencial metastásico de líneas celulares, y a su vez, que la comunicación del endotelio mediante factores secretados con CaP podría ser un factor importante para el crecimiento tumoral.

La angiogénesis es un evento clave en el desarrollo de tumores y metástasis (Folkman 1971). El aumento de la vascularización a través de la angiogénesis aumenta el crecimiento del tumor primario al suministrar nutrientes y oxígeno y proporciona una vía para la metástasis (Hanahan y Weinberg 2011). Está bien establecido que las células tumorales pueden iniciar o propagar el interruptor angiogénico por la secreción de factores pro- angiogénicos (Taverna et al. 2013). Entre todos estos factores, el factor de crecimiento endotelial vascular (VEGF) parece ser fundamental principalmente para el desarrollo de vasos sanguíneos (Coultas et al. 2005). Varios estudios han determinado que en el CaP hay una mayor expresión de VEGF y una mayor densidad microvascular que en su contraparte normal (Eisermann y Fraizer 2017). En nuestros estudios, pudimos determinar que los tumores en ambas condiciones, co- inyectadas y tratadas previamente con medio condicionado, presentaban aumento en la proliferación de las células tumorales, un incremento en el número de vasos sanguíneos y de VEGF y un aumento en el número de metástasis en comparación con la condición control. Sin embargo, el aumento de VEGF no fue tan pronunciado como se esperaba, lo que podría reflejar que, en este modelo en particular, la angiogénesis tumoral podría estar modulada por probablemente más de un factor pro-angiogénico. Otros análisis proteómicos podrían ayudar a definir el perfil del factor pro-angiogénico estimulado por células endoteliales en células CaP.

Para entender los mecanismos moleculares que podrían mediar los efectos biológicos de las CE con las células CaP, realizamos análisis proteómicos para buscar factores extracelulares involucradas en la señalización paracrina célula-célula. Las CE secretan citoquinas, quimiocinas y factores de crecimiento que podrían modular la biología de las células de cáncer (Butler et al. 2010). Entre los factores secretados por las células HUVEC y posterior a un análisis de interacciones proteína-proteína, encontramos que CCL2, IL-6, CXCL1, FGF2 y HGF eran los

nodos centrales de toda la red de interacción. FGF2 y HGF se han relacionado con un aumento en el crecimiento de células de CaP (Giri et al. 1999, Han et al. 2016). Por otro lado, las proteínas IL-6 y CXCL1, y CCL2 son proteínas que se han asociado con una mayor migración e invasión de células de CaP (Zhang et al. 2010, Kuo et al. 2012, Wang et al. 2013). Al analizar los factores secretados por las células CaP en presencia de los medios condicionados, encontramos diferencias notables entre las citoquinas, las quimiocinas y los factores de crecimiento secretados por las células LNCaP y PC-3. Nuestro análisis proteómico indicó que las células LNCaP expuestas a medio condicionado secretan principalmente proteínas relacionadas con la modulación inmune, como la proteína IL-8, IL17A, IL-11, Lipocalina-2 y SERPIN1 (PAI-1). Las proteínas NGAL, PAI-1 y IL-8 están sobre expresadas en células de fenotipo agresivo como PC-3 y en tejidos de pacientes con CaP pero no se encuentran, o en niveles muy bajos, expresadas en la línea celular LNCaP (Araki et al. 2007, Hagelgans et al. 2013, Ding et al. 2015). Sin embargo, se ha observado que la expresión de estas proteínas puede ser inducida por citoquinas proinflamatorias, tanto en enfermedades inflamatorias agudas y crónicas para la proteína NGAL (Chakraborty et al. 2012), como al estimular líneas celulares de CaP para las proteínas PAI-1 y IL-8 (Araki et al. 2007, Hagelgans et al. 2013). La sobreexpresión de IL-8 en células LNCaP, dio como resultado un incremento en la proliferación celular reduciendo su dependencia a los andrógenos (Araki et al. 2007). Esto sugiere que las CE podrían, a través de la expresión de estas proteínas en las células de CaP, promover una progresión de esta enfermedad.

En el caso de las células PC-3, nuestro análisis indicó que estas células secretan factores relacionados con la inflamación, como la IL-10 y la IL-22, de las cuales existen pruebas que las relacionan con las funciones tumorigénicas en otros tipos de cáncer (Shao et al. 2011, Hernandez

et al. 2018). PC-3 también secreta quimioquinas como CCL20 y CXCL5. CCL20 promueve, de manera autocrina, la supervivencia, proliferación y adhesión de las células cancerosas in vitro y aumenta el crecimiento de la vascularización de las células tumorales xenoinjertadas y la invasión in vivo. En tejidos de CaP humano, CCL20 se sobre expresa, y se ha informado que promueve el crecimiento de tumores en modelos de xenoinjerto (Beider et al. 2009). Se ha observado que CXCL5 induce fenotipos malignos de células CaP de forma autocrina / paracrina in vitro. Además, la sobreexpresión de CXCL5 promueve la formación y el crecimiento de xenoinjertos de células CaP en modelos murinos (Qi et al. 2018). Finalmente, al analizar las proteínas relacionadas con la angiogénesis, pudimos corroborar lo que se obtuvo en el experimento in vivo, que las CE pueden aumentar la capacidad pro-angiogénica de las células cancerosas. Nuestro análisis proteómico indicó que las células PC-3, estimuladas con medio condicionado de células HUVEC, secretaban niveles más altos de los factores pro-angiogénicos VEGF, ANG-2, PDGF-A y THBS1 que las células LNCaP, lo que podría explicar, al menos en parte, por qué los tumores de xenoinjerto PC3 estimulados con medio condicionado tienen una gran densidad microvascular y, probablemente, una mayor capacidad metastásica, que los tumores sin estimulación de las células endoteliales.

Conclusiones

El propósito de esta tesis fue estudiar la interacción paracrina entre las celulas endoteliales y las células de CaP, principalmente el efecto paracrino de las CE sobre las celulas de CaP. Por lo que, en primer lugar, determinamos que las células endoteliales HUVEC expresan funcionalmente el receptor de andrógenos y que la presencia de andrógenos modula su proliferación lo que las convierte en un modelo valido en el estudio de las interacciones entre celulas endoteliales y células de cáncer de próstata.

Los estudios de comunicación entre ambos tipos celulares mostraron que existiría una comunicación paracrina entre las CE y las células de CaP, para la cual no es necesario el contacto directo entre ambos tipos celulares, solo la secreción de distintos factores por parte de las CE al microambiente tumoral. De manera interesante, el papel angiocrino de las células endoteliales en la biología celular CaP fue más amplio en los fenotipos de CaP más agresivos, lo que sugiere que estos fenotipos de células de CaP podrían adaptarse aumentando la sensibilidad a las señales extracelulares producidas por el microambiente tumoral. Estos hallazgos sugieren que los efectos sobre la proliferación, crecimiento tumoral y metástasis por parte de las CE en los fenotipos más agresivos podría ser un indicativo de que las CE podrían contribuir a la adquisición de características más agresivas y/o metastásicas de esta enfermedad. Por lo que realizar estudios sobre citoquinas y/o factor(es) de crecimiento liberados por las CE, así como las vías moleculares reguladas en las células de CaP, como resultado de esta interacción, podrían culminar en la identificación de nuevos biomarcadores u objetivos terapéuticos para contrarrestar esta enfermedad, en especial el cáncer de próstata resistente a la castración.

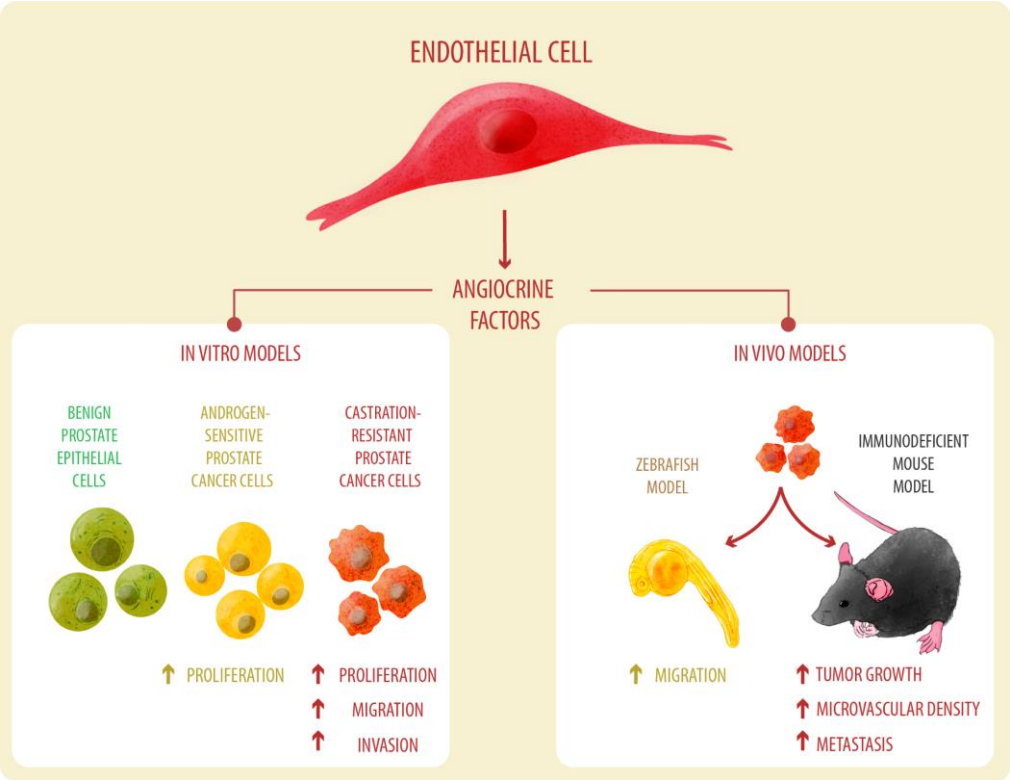


Figura 1. Resumen de los resultados obtenidos en esta tesis. Efecto de los factores angiocrinos secretados por las celulas endoteliales sobre las celulas de cáncer de próstata en ensayos in vitro e in vivo.

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