UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

FACULTAD DE MEDICINA

BIOLOGÍA EXPERIMENTAL

EFECTO DE LA TESTOSTERONA SOBRE LA INDUCCIÓN DE CATELICIDINA MEDIADA POR LA VITAMINA D EN LA PLACENTA HUMANA

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

OLMOS ORTIZ ANDREA GUADALUPE

TUTORA PRINCIPAL DE TESIS: **DRA. LORENZA DÍAZ NIETO**
FACULTAD DE MEDICINA, UNAM

COMITÉ TUTOR: **DRA. CLAUDIA VERÓNICA ZAGA CLAVELLINA**
FACULTAD DE MEDICINA, UNAM

DRA. NORMA ANGÉLICA MORENO MENDOZA
INSTITUTO DE INVESTIGACIONES BIOMÉDICAS, UNAM

MÉXICO, CD. MX., ENERO, 2017.

UNAM
POSGRADO
Ciencias Biológicas

COORDINACIÓN

Lic. Ivonne Ramírez Wence
Directora General de Administración Escolar, UNAM
P r e s e n t e

Me permito informar a usted que el Subcomité de Biología Evolutiva y Sistemática del Posgrado en Ciencias Biológicas, en su sesión ordinaria del día 07 de noviembre de 2016, aprobó el jurado para la presentación del examen para obtener el grado de **DOCTORA EN CIENCIAS** de la alumna **OLMOS ORTIZ ANDREA GUADALUPE** con número de cuenta **510007908**, con la tesis titulada **"EFECTO DE LA TESTOSTERONA SOBRE LA INDUCCIÓN DE CATELICIDINA MEDIADA POR LA VITAMINA D EN LA PLACENTA HUMANA"**, realizada bajo la dirección de la **DRA. LORENZA DÍAZ NIETO:**

Presidente:	DR. HORACIO MERCHANT LARIOS
Vocal:	DRA. ANA ELENA LEMUS BRAVO
Secretario:	DRA. CLAUDIA VERÓNICA ZAGA CLAVELLINA
Suplente:	DRA. GUADALUPE DEL CARMEN ESTRADA GUTIÉRREZ
Suplente:	DRA. NORMA ANGÉLICA MORENO MENDOZA

Sin otro particular, me es grato enviarle un cordial saludo.

A T E N T A M E N T E
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Cd. Universitaria, Cd, Mx., a 13 de diciembre de 2016

POSGRADO EN CIENCIAS BIOLOGICAS
COORDINACIÓN

DRA. MARÍA DEL CORO ARIZMENDI ARRIAGA
COORDINADORA DEL PROGRAMA

AGRADECIMIENTOS

Agradezco al Posgrado en Ciencias Biológicas de la UNAM por los apoyos y orientaciones recibidos.

Agradezco al Consejo Nacional de Ciencia y Tecnología (CONACyT) por la beca recibida para llevar a cabo mis estudios de Doctorado durante el periodo agosto 2012 – julio 2016 (número de beca 231018). Y también por apoyar el financiamiento de los proyectos 153862 y 241034, correspondientes a la Dra. Lorenza Díaz Nieto y al Dr. Euclides Ávila Chávez, que facilitaron la compra de los insumos necesarios para la realización de este proyecto.

Finalmente le expreso honestamente mis agradecimientos a mi tutora principal, Dra. Lorenza Díaz Nieto, y a los miembros del Comité Tutor, Dra. Claudia Verónica Zaga Clavellina y Dra. Norma Angélica Moreno Mendoza, por su valiosa dirección y aportaciones, con lo que me permitieron mejorar mi formación profesional y la calidad del trabajo.

AGRADECIMIENTOS A TÍTULO PERSONAL

A mi madre, Esperanza Ortiz Mayagoitia, quien es mi pilar, mi sostén y mi guía. Porque nunca has dejado de creer en mí. Porque siempre me has alentado a seguir adelante, a confiar, luchar y creer en mí misma. Por todo tu apoyo, tu inspiración y tu entrega. Te agradezco todo porque por ti, yo soy quien soy. Te amo mucho mamá. Te lo agradeceré eternamente.

A toda mi familia. En especial a mis tíos Armando y Fausto, mis tías Mirna y Besy, y a mi hermana Bessie. Por todo lo que hemos compartido y que me hace sentir orgullosa de pertenecer a esta familia ¡Un abrazo a todos!

A Efrén Méndez. Por ser mi amigo y confidente. Porque hemos continuado creciendo como pareja. Por toda tu ayuda y tu apoyo, incluyendo las visitas nocturnas o dominicales al laboratorio. Te quiero Efrén.

A mis eternas amigas. Porque el tiempo sigue pasando, pero nuestra amistad sigue tan intacta como en nuestros años de Universidad. Les quiero mucho a todas: Susy, Diana, Carmen, Rebeca, Arlette, Selenita y Xóchitl. Y por supuesto, no podría faltar: ¡Arriba la cumpleañera!

Al Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán y en particular al Departamento de Biología de la Reproducción "Dr. Carlos Gual Castro" y al Dr. Fernando Larrea Gallo, jefe de este Departamento, por brindarme el apoyo para llevar a cabo la parte experimental de mi tesis en sus instalaciones.

A mi tutora. Querida Lore, no tengo cómo agradecerte por todo lo que me has brindado. Porque en estos años despertaste mi curiosidad científica, me enseñaste a mejorar mi trabajo diario y a no descuidar la ética profesional. Es decir, me enseñaste el camino para continuar creciendo como investigadora en el área biomédica. Y más aún, sembraste en mí el espíritu inagotable para crecer como un ser humano sensible, íntegro y en paz. Ha sido un largo caminar y me alentaste a seguir siempre adelante. Hoy al fin te puedo decir orgullosamente ¡Lo hicimos! Sí, tú y yo ¡Lo hicimos!

Al Dr. David Barrera. Por todas tus enseñanzas, por escucharme más allá de lo profesional. Yo sé que el camino ha sido más sinuoso de lo que esperábamos, pero hemos conseguido seguir adelante. Gracias por estar a mi lado todo este tiempo como investigador y como amigo.

Gracias al Dr. Ali Halhali por sembrar los cimientos de mi formación profesional. Hace 8 años me aceptó como su alumna y le agradezco por todo lo que de usted he aprendido. El día de hoy sólo espero continuar creciendo personal y profesionalmente, tal como me ha enseñado; es una promesa.

A los investigadores Dr. Euclides Ávila, Dra. Verónica Zaga y Dra. Marta Durand. Porque los considero mi ejemplo y admiración. Porque me demostraron lo valioso que es salpicar de risa y alegría nuestro trabajo diario. Gracias por cobijarme y por creer en mí.

A mis compañeras de jornada en el laboratorio, de quienes recibí mucho apoyo profesional y sobre todo su amistad. Queridas Nancy Noyola, Mitzi García, Vilma Trejo y Lorena Puente les agradezco sus horas de compañía, las pláticas en el comedor y las risas y sueños compartidos. Espero lo mejor para cada una de nosotras, y en verdad que así será. Mitzi te quiero mucho, fue un placer coincidir contigo en el laboratorio.

Finalmente les agradezco a todas las madres que decidieron participar y donar su placenta para la realización de este proyecto. A ustedes, infinitas gracias.

“Parece que la vida no es fácil para ninguno de los dos. Pero, ¿y qué? Debemos tener perseverancia y sobre todo confianza en nosotros mismos. Debemos creer que estamos dotados para algo, y que alcanzaremos ese objetivo cueste lo que cueste”

Carta de Marie Curie a su hermano Józef, 1894

Fragmento

ÍNDICE

ÍNDICE DE FIGURAS

ÍNDICE DE TABLAS

SIGLAS

AMPc: monofosfato de adenosina cíclico

AR: receptor de andrógenos

AREs: elementos de respuesta a andrógenos

BSA: albúmina bovina sérica

CK-7: citoqueratina 7

CREB: proteína de unión a los elementos de respuesta a AMPc

CREs: elementos de respuesta a AMPc

DAPI: 4',6-diamino-2-fenilindol

DBP: proteína de unión a la vitamina D

DHT: 5α-dihidrotestosterona

E_2: estradiol

HBD2: defensina 2 humana

HBD3: defensina 3 humana

HBSS: solución salina balanceada de Hank

hCTD: catelicidina humana

HRP: peroxidasa de rábano

PBS: amortiguador de fosfatos

PCOS: síndrome de ovario poliquístico

PTH: hormona paratiroidea

RXR: receptor a retinoides X

SHBG: globulina de unión a las hormonas sexuales

SFT: suero de feto de ternera

T: testosterona

TLRs: receptores tipo Toll

UPL: biblioteca de sondas de hidrólisis (*Universal probe library*).

VD: vitamina D

VDR: receptor de la vitamina D

VDRE: elementos de respuesta a la vitamina D

ABREVIATURAS

^{o}C: grados Celsius
g: gramos
h: horas
kDa: kilodaltons
L: litros
min: minutos
rpm: revoluciones por minuto

RESUMEN

Los fetos y los neonatos masculinos presentan mayor vulnerabilidad inmunológica en comparación con los femeninos, lo que posiciona en una situación de mayor riesgo a los varones ante el desarrollo de infecciones durante el periodo perinatal. Esta diferencia en el riesgo inmunológico podría atribuirse, al menos parcialmente, a la regulación diferencial que ejercen los esteroides sexuales sobre el metabolismo de la vitamina D (VD). Esta afirmación se construye a partir de evidencias que indican que el calcitriol, el metabolito más activo de la VD, fortalece la respuesta inmunológica innata particularmente al inducir transcripcionalmente al péptido antimicrobiano catelicidina en la placenta humana. La biodisponibilidad del calcitriol depende de la expresión de CYP27B1 y CYP24A1, citocromos involucrados en su síntesis y degradación, respectivamente. Sin embargo, los efectos de la testosterona sobre la regulación placentaria de estas enzimas y su efecto biológico final sobre el blanco inmunológico dependiente del calcitriol, la catelicidina, aún no han sido estudiados. En este trabajo demostramos que la testosterona inhibe significativamente la expresión de CYP27B1 mientras que estimula la de CYP24A1 en los trofoblastos en cultivo. Estos efectos se acompañaron de la activación de CREB a través de un mecanismo independiente de AMPc pero dependiente del receptor de andrógenos. Acorde a lo anterior, los cotiledones placentarios masculinos mostraron menor expresión génica basal de CYP27B1 y de catelicidina en comparación con cotiledones femeninos ($P < 0.05$). Además, la concentración de testosterona en sangre venosa umbilical fue mayor en los neonatos masculinos ($P = 0.007$) mientras que la concentración de catelicidina fue menor ($P = 0.002$). En conjunto, estos resultados sugieren que las placentas masculinas producen menos catelicidina debido a la menor biodisponibilidad de calcitriol. Por lo tanto, se propone que las diferencias dependientes del sexo que se observaron en el metabolismo de la vitamina D contribuyen en la respuesta fetal ante las infecciones, lo que podría parcialmente explicar por qué los fetos varones

presentan mayor vulnerabilidad inmunológica. Nuestros resultados sugieren también que la hiperandrogenemia fetal podría afectar adversamente el metabolismo placentario de la vitamina D, independientemente del sexo del feto, repercutiendo en la respuesta inmunológica.

Los resultados obtenidos de esta tesis doctoral se publicaron como artículo original en la revista indexada *Journal of Steroid Biochemistry & Molecular Biology* en el 2016 con el título “Evidence of sexual dimorphism in placental vitamin D metabolism: Testosterone inhibits calcitriol-dependent cathelicidin expression” (Anexo 1).

ABSTRACT

Male fetus and neonates show increased immune vulnerability compared to females, which results in a higher risk of perinatal infections. These differences could partially be due to sex steroids differential modulation of vitamin D metabolism; since calcitriol, the most active vitamin D metabolite, regulates immune responses and transcriptionally induces the antimicrobial peptide cathelicidin in the human placenta. Calcitriol availability depends on the expression of the citochromes CYP27B1 and CYP24A1 which are involved in its synthesis and degradation, respectively. However, the effects of testosterone upon these enzymes and the final biological outcome upon the calcitriol-dependent immune-target cathelicidin in the placenta have not been studied. In this study we show that testosterone significantly inhibited CYP27B1 while stimulated CYP24A1 gene expression in cultured trophoblasts. These effects were accompanied by CREB activation through cAMP-independent and androgen receptor-dependent mechanisms. Male placental cotyledons showed reduced basal CYP27B1 and cathelicidin gene expression compared to females ($P < 0.05$). Testosterone concentration was higher in the umbilical cord blood of male neonates ($P = 0.007$), whereas cathelicidin levels were lesser as compared to that of females ($P = 0.002$). Altogether our results suggest that male placentas produce less cathelicidin due to decreased calcitriol bioavailability. We propose that the observed sex-dependent differences in placental vitamin D metabolism contribute in fetal responses to infections and could partially explain why the increased male fetuses immune vulnerability. Moreover, gestational hyperandrogenemia could adversely affect placental vitamin D metabolism independently of fetal sex.

1. INTRODUCCIÓN

1.1. Dimorfismo sexual en el contexto inmunológico

Múltiples observaciones clínicas y epidemiológicas realizadas en los últimos 50 años muestran que la morbimortalidad neonatal es mayor en los hombres que en las mujeres. Más aún, uno de los primeros reportes históricos de la mayor mortalidad neonatal masculina se remonta a más de 200 años atrás, cuando el Dr. Joseph Clarke analizó poco más de 20 mil nacimientos ocurridos entre 1757 y 1784. Considerando los datos proporcionados por el Dr. Clarke, se puede hacer un cálculo de la mortalidad neonatal de esa época en alrededor de 160 hombres por cada 1000 recién nacidos vivos mientras que la tasa de mortalidad observada en las mujeres fue de 136 por cada 1000 [1]. Asombrosamente, y pese a los avances médicos que se han conseguido en los últimos dos siglos, este fenómeno continúa vigente pues según los datos del 2013 del Centro Nacional de las Estadísticas en Salud de Estados Unidos, la mortalidad neonatal masculina es de 6.51 en comparación con 5.39 muertes femeninas por cada 1000 recién nacidos vivos [2]. Estos datos fortalecen la idea de que existen mecanismos inherentes al sexo que condicionan la salud y la enfermedad neonatal.

Cabe agregar que las infecciones son una de las principales causas de la mortalidad neonatal en el mundo y se estima que alrededor del 26% de las muertes neonatales se asocian a procesos infecciosos como la neumonía o la sepsis [3]. En este sentido, los datos epidemiológicos sostienen que los hombres son más vulnerables inmunológicamente, pues el sexo masculino es el que se enferma en mayor proporción y severidad de padecimientos virales, bacterianos, fúngicos y parasitarios [4]. Las evidencias obtenidas de múltiples estudios clínicos y animales sugieren que los andrógenos debilitan la inmunocompetencia mientras que los estrógenos la fortalecen [4-6]. Sin embargo, los mecanismos subyacentes aún no se encuentran del todo esclarecidos.

El dimorfismo sexual en la respuesta inmunológica se aprecia a lo largo de todas las etapas de la vida y posiblemente inicia desde el periodo fetal. De hecho, se ha sugerido que durante la vida prenatal ocurren mecanismos dependientes del sexo que modulan diferencialmente la expresión de diversos factores inmunológicos, lo que, finalmente, se traduce en una respuesta diferencial ante las infecciones y los procesos inflamatorios *in útero* y probablemente también en la vida posnatal.

1.2. El privilegio inmunológico durante el embarazo

El sistema inmunológico comprende varios tipos celulares encargados de defender y proteger al organismo ante diversos agentes patógenos que llevarían al desarrollo de enfermedad. Éste a su vez se puede clasificar en sistema inmune innato y sistema inmune adaptativo. La respuesta inmune adaptativa es más fuerte y se caracteriza por la memoria inmunológica, lo cual le permite recordar a los patógenos de acuerdo a su firma antigénica. En cambio, la respuesta inmune innata aunque es más débil es la que se encuentra mejor conservada entre las especies y reconoce de manera general e inespecífica a un amplio espectro de microorganismos a través de los receptores de reconocimiento de patrones, como los receptores tipo Toll (TLRs), entre otros.

En este sentido, el embarazo representa todo un reto inmunológico. Hay que considerar que el feto presenta una carga genotípica diferente a la de la madre; sin embargo, esto no genera mecanismos de rechazo o ataque hacia el feto, sino que, por el contrario, la madre y el feto se encuentran en un fino equilibrio inmunológico que permite crecer, madurar y nutrirse a este organismo en formación. Para ello, el sistema inmune de la madre presenta cambios durante la gestación que permiten el desarrollo del feto a la vez que confiere las defensas necesarias para protegerlo en un ambiente potencialmente hostil. La supervivencia del feto, por tanto, es una consecuencia de las interacciones cooperativas entre el feto y la madre.

Para permitir el aloinjerto fetal, la madre sintetiza progesterona desde el comienzo del embarazo. Esta hormona esteroide inmuno-suprime la respuesta adaptativa, con lo cual el embarazo se desarrolla en un ambiente predominantemente de inmunidad innata. Por ejemplo, de manera posterior a la implantación, la decidua y el endometrio se convierten en sitios de alta actividad innata por parte de las células asesinas uterinas (NKu) y los macrófagos que ahí se encuentran [7]. Estas células proporcionan una línea de protección a la madre contra los patógenos; sin embargo, con un sistema inmune adaptativo relativamente debilitado, el 11% de las muertes maternas se relacionan con el desarrollo de infecciones en el líquido amniótico o en las membranas corioamnióticas que rodean al feto [8].

Por otra parte, la placenta en sí misma representa una barrera selectiva tanto inmune como mecánica, cuyas competencias innatas son esenciales para restringir el proceso infeccioso. Los trofoblastos secretan factores quimio-atrayentes, como el CXCL12/factor estromal 1 [9], que dirigen a los macrófagos y las NKu hacia el útero, el endometrio y la decidua. Además, los trofoblastos secretan progesterona y factores inmunosupresores, como el calcitriol, que favorecen la diferenciación tipo Th2 en el feto [10-12] . El perfil predominante Th2 parece ser un mecanismo protector natural *in-útero* ya que se sabe que el incremento en citocinas como interferón gamma (INF-γ) o el factor de necrosis tumoral alfa (TNF-α) puede inducir apoptosis de las células fetales, lo que favorece el riesgo de abortos o parto pretérmino [13, 14].

Otro mecanismo de defensa lo constituye la expresión de los antígenos leucocíticos humanos (HLA) tipo G y tipo E en la interfase materno-fetal. Estos antígenos se expresan en la membrana celular de los diferentes tejidos fetales, y se consideran una “bandera” que permite identificar y tolerar la presencia de células de distinto genotipo. Cuando los leucocitos uterinos, que poseen los receptores de HLA-E y HLA-G, se encuentran con las células fetales que expresan a estos antígenos, ocurre la unión ligando-receptor y se produce un efecto inhibidor de la actividad citotóxica de las NKs, lo que previene la lisis celular.

Finalmente, la placenta también secreta proteínas y péptidos antimicrobianos que actúan como efectores críticos en la defensa innata. Entre estos péptidos se incluye a la catelicidina (hCTD) y las defensinas humanas 2 y 3 (HBD2 y HBD3). Los péptidos antimicrobianos son un sistema evolutivamente conservado que proporciona una defensa rápida y efectiva ante una gran variedad de microorganismos.

1.2.1. La catelicidina

La hCTD es el único miembro de los péptidos antimicrobianos con dominio catelicidina. Inicialmente se pensaba que sólo los neutrófilos la sintetizaban, pero ahora se sabe que muchos tipos celulares lo hacen, entre ellos: linfocitos, macrófagos y NKs [15]; epitelios de la piel [16], del árbol bronquial [17] y de intestinos [18]; todo el tracto reproductor femenino [19], mamas [20, 21] y, especialmente durante el embarazo, el trofoblasto [22].

También conocida como LL-37, es un péptido catiónico de 37 aminoácidos que se genera por la ruptura del extremo carboxilo terminal de su precursor de 18 kDa, la hCAP18. La ruptura se realiza extracelularmente por actividad de la serina proteasa kalikreína o la proteinasa 3 [23, 24]. En su estructura presenta múltiples cargas positivas, lo que le permite interactuar con la carga negativa de las membranas bacterianas. Posterior a esta unión electroquímica, la hCTD adopta una estructura de alfa hélice que le permite penetrar y formar poros transmembranales que llevan a la lisis bacteriana [25]. Las células humanas se encuentran protegidas de la actividad lítica de la hCTD posiblemente por la presencia de múltiples cadenas membranales de colesterol, lo que genera una carga más neutra.

Además de la actividad antimicrobiana, la hCTD también tiene propiedades quimioatrayentes de neutrófilos y eosinófilos [26]; y paralelamente puede inducir la migración de los queratinocitos por lo que se ha propuesto que puede jugar un papel importante en la reparación de heridas [27]. Incluso, la hCTD puede modular la función del TLR-4 y generar un efecto anti-inflamatorio a través de su unión con el lipopolisacárido (LPS), ligando del TLR-4, con lo que se reduce la señalización

ejercida por este receptor y se disminuye entonces la producción de citocinas pro-inflamatorias [28].

Interesantemente, la expresión de hCTD en el humano es principalmente dependiente del calcitriol, metabolito más activo de la vitamina D (VD). Resultados *in vitro* han demostrado que el calcitriol es un potente agente inductor de la síntesis de hCTD en diversos tejidos, incluyendo las células trofoblásticas y deciduales [29-31]. Esta respuesta se debe a la regulación directa transcripcional del gen de hCTD por el calcitriol [32].

1.3. Síntesis y metabolismo de la vitamina D

La radiación UVB que proviene de la luz solar inicia la biosíntesis de VD al convertir el 7-dehidrocolesterol hacia previtamina D_3. De manera inmediata, la previtamina D_3 se isomeriza por una reacción térmica espontánea hacia VD_3. La VD también se puede obtener de la dieta en menor extensión. Las formas nutricionales de la VD incluyen tanto al colecalciferol (VD_3) de origen animal, como al ergocalciferol (VD_2) proveniente de algunos hongos y plantas. Una vez que se ha formado en la piel o que se ha absorbido vía intestinal, la VD se libera hacia la circulación y se transporta por la proteína de unión a la vitamina D (DBP) hacia el hígado, donde se convierte en 25-hidroxivitamina D (calcidiol o $25OHD_3$) por acción de la 25-hidroxilasa (CYP2R1). La $25OHD_3$ es el metabolito de la VD más abundante en la circulación y es el mejor indicador del estado nutricio en esta hormona. Sin embargo, la $25OHD_3$ no es la forma activa de la VD, para ello se requiere de la segunda hidroxilación catalizada por la enzima $25OHD_3$-1α-

hidroxilasa (CYP27B1) que da origen al calcitriol o 1,25$(OH)_2D_3$, el cual corresponde al metabolito con la mayor actividad hormonal de la VD. El CYP27B1 se expresa primordialmente en el riñón, sin embargo se le puede encontrar en diferentes tejidos, incluida la placenta [33]. La biodisponibilidad del calcitriol se encuentra rigurosamente controlada para regular las acciones biológicas de esta hormona y para mantener la homeostasis de calcio y fósforo en el organismo. Para ello, la enzima encargada de degradar al calcitriol hacia metabolitos hidrosolubles de menor actividad es la 1,25$(OH)_2D_3$-24-hidroxilasa (CYP24A1), también expresada en la placenta. Esta enzima es regulada a la alta por el propio calcitriol como mecanismo de retroalimentación negativa para controlar su disponibilidad [34, 35]. (Figura 1).

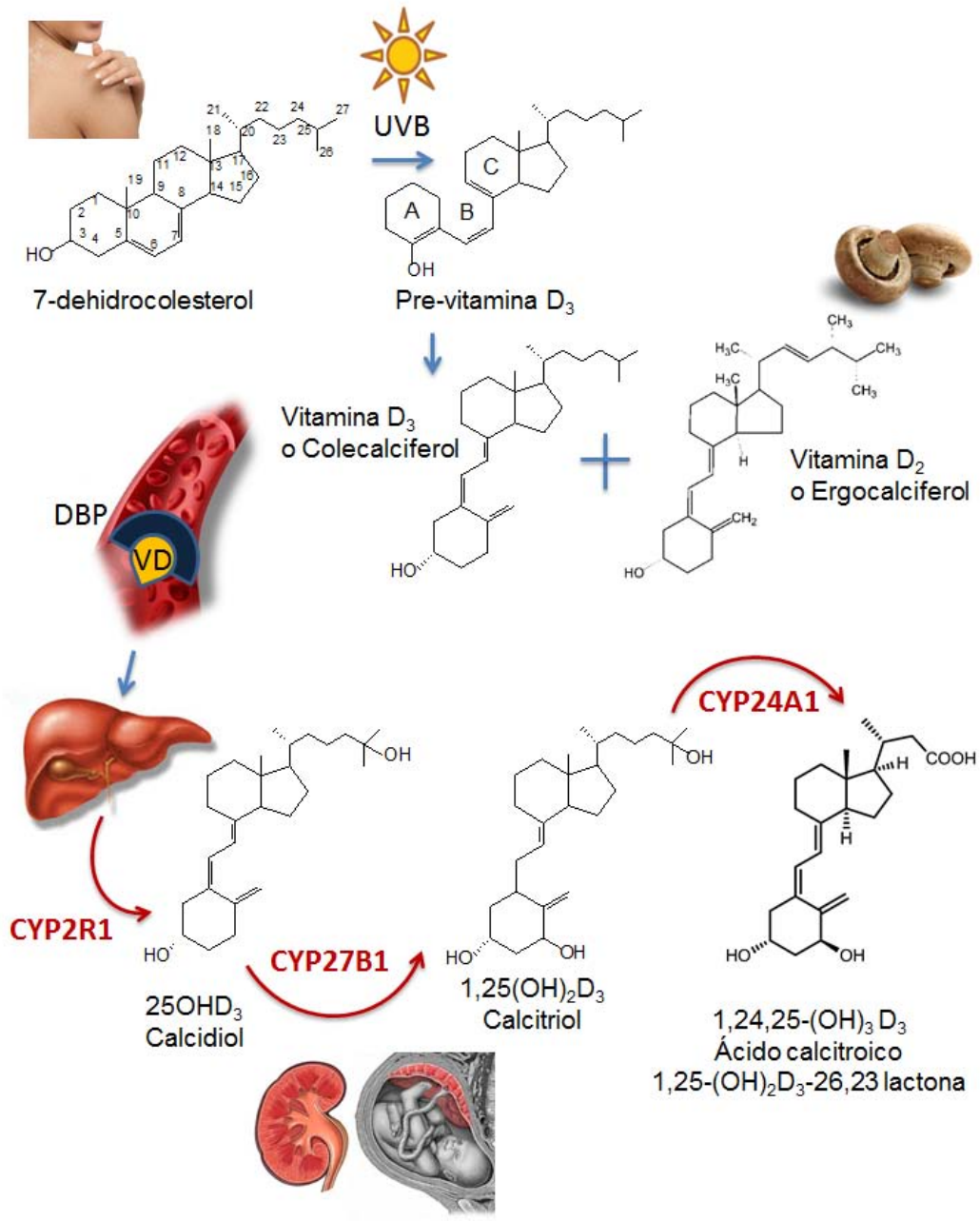

Figura 1. Metabolismo de la vitamina D.

Las formas de la vitamina D son el colecalciferol (D_3) y el ergocalciferol (D_2). Ambas son hidroxiladas en el hígado para dar origen al calcidiol ($25OHD_3$), posteriormente ocurre otra hidroxilación en el riñón o la placenta y se sintetiza el calcitriol ($1,25(OH)_2D_3$). La degradación de esta hormona es catalizada por el CYP24A1, el cual da origen a metabolitos hidrosolubles que serán desechados por la orina.

1.3.1. Funciones biológicas del calcitriol placentario y su mecanismo de acción

Una de las principales actividades atribuidas al calcitriol durante el embarazo es el incremento en la absorción intestinal de calcio y el aumento en el transporte placentario de este mineral. Sin embargo, dado que el receptor de la vitamina D (VDR) y el CYP27B1 también se expresan en tejidos reproductivos femeninos como el útero, ovario, endometrio, células del epitelio de las trompas de Falopio y la placenta [36-38], no se debe descartar la posibilidad de otros efectos potenciales del calcitriol de tipo paracrino o autocrino.

Los efectos biológicos del calcitriol producido por la placenta se relacionan con la regulación inmune, la hormonogénesis y en general con la fisiología placentaria. En su papel de regulación endocrina, el calcitriol placentario induce la decidualización del endometrio y la síntesis de progesterona y estradiol, así como también induce la expresión génica de la gonadotropina coriónica humana (hCG) y del lactógeno placentario [39-41].

En cuanto al papel inmunorregulador del calcitriol placentario, éste fortalece la respuesta inmune innata a la vez que contrarresta la inflamación exacerbada. El calcitriol consigue ambos propósitos a través de disminuir la producción de citocinas pro-inflamatorias como TNF-α, INF-γ y la interleucina (IL) -6, a la par que induce la síntesis del potente péptido antimicrobiano hCTD en la placenta [10, 11, 29, 31].

La actividad biológica del calcitriol es mediada por la unión de esta hormona con su receptor, el VDR. Una vez unido con su ligando, el VDR heterodimeriza con el receptor a retinoides X (RXR) y este complejo reconoce a los elementos de respuesta a la vitamina D (VDRE) en los promotores de los genes blancos de esta hormona. Posterior a la formación del complejo $1,25(OH)_2D_3$/VDR-RXR/VDRE, se atrae a co-activadores o co-represores con la finalidad de regular la transcripción de estos genes blanco [42]. Justamente, la caracterización del promotor de catelicidina en múltiples tipos celulares de origen humano demostró la presencia de un VDRE consenso [32], lo cual ayuda a explicar cómo el calcitriol induce la expresión de este blanco inmunológico (Figura 2).

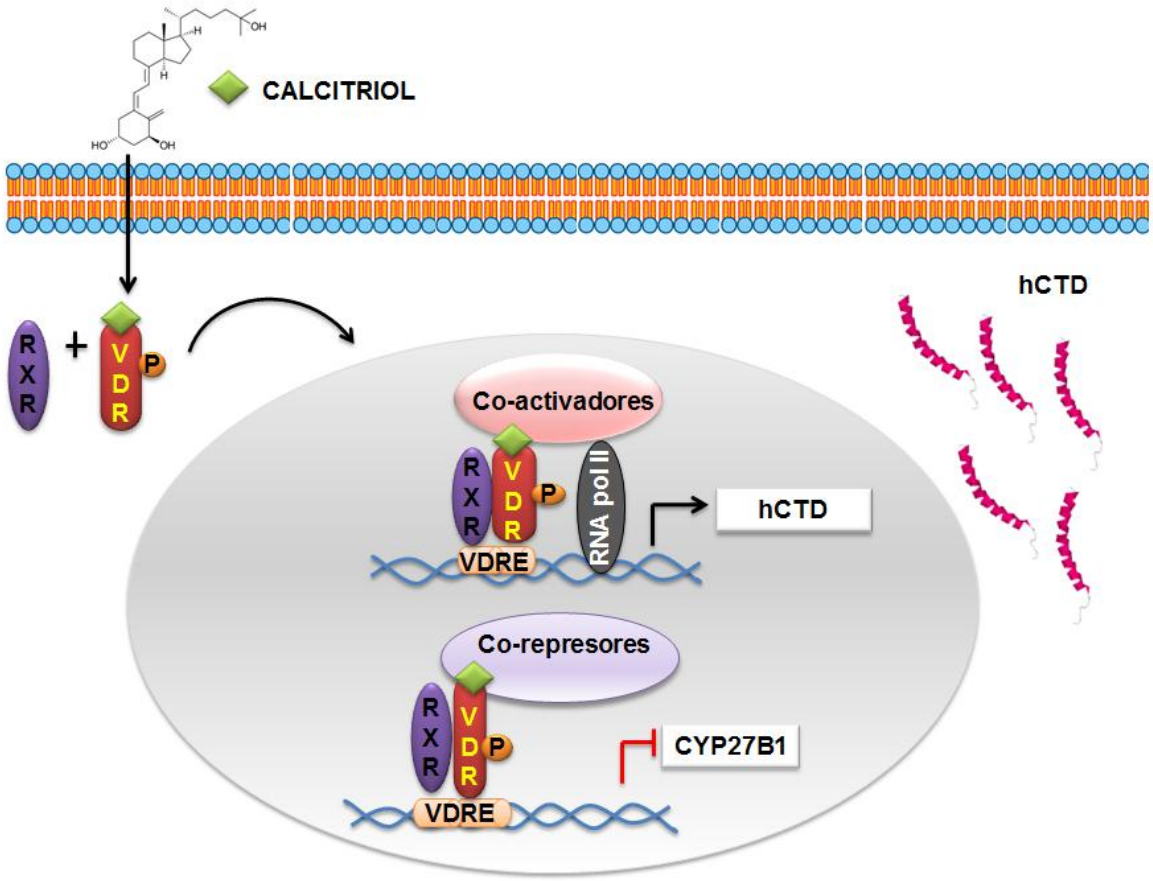

Figura 2. Mecanismo clásico de acción del calcitriol.

El calcitriol difunde a través de la membrana plasmática y se une a su receptor VDR en heterodímero con el RXR. Este complejo se transloca al núcleo y regula la expresión de genes que contienen VDREs en sus promotores. Por ejemplo, el calcitriol regula la expresión de catelicidina a través de este mecanismo.

1.3.2. Reguladores de la síntesis del calcitriol placentario

Pese a la importancia de los efectos biológicos del calcitriol placentario, la regulación de su producción ha sido poco estudiada. Se conoce que la regulación de la síntesis del calcitriol placentario difiere de la regulación que existe a nivel renal. En el riñón, la inducción transcripcional de CYP27B1 depende principalmente de la hormona paratiroidea (PTH); en contraste, en la placenta la expresión de CYP27B1 es independiente de la PTH [43].

Los trabajos realizados previamente en nuestro laboratorio en cultivos de trofoblasto humano han demostrado que el factor de crecimiento similar a la insulina, las citocinas, el calcitriol y el monofosfato de adenosina cíclico (AMPc) regulan diferencialmente a los citocromos involucrados en la síntesis y degradación del calcitriol, CYP27B1 y CYP24A1, respectivamente [10, 44, 45]. Interesantemente, se demostró que el AMPc inhibe significativamente la expresión de CYP27B1 mientras que estimula la de CYP24A1 de manera dependiente de la concentración [45]. Este estudio sugiere que las hormonas que modifican los niveles de AMPc en la placenta pueden regular la síntesis del calcitriol placentario. Además, de recientemente se demostró que la testosterona, hormona de tipo esteroide, puede actuar a través de una vía no clásica y también regular genes dependientes de AMPc en las células de Sertoli [46]. El estudio de los mecanismos reguladores de la vía de señalización del AMPc en la placenta podría ayudar a explicar, al menos parcialmente, el dimorfismo sexual en la respuesta inmune neonatal y la regulación de catelicidina dependiente de la VD en hombres y mujeres.

1.4. La testosterona y su mecanismo de acción

La testosterona es un esteroide del grupo de los andrógenos y se ha asociado con múltiples esferas de la sexualidad masculina. Este esteroide posee 19 átomos de carbono, un grupo cetónico en el carbono 3 y una doble ligadura en el carbono 4, por lo que, según el paradigma clásico, se considera un esteroide activo.

Durante el embarazo, la hormonogénesis esteroidea está cuidadosamente regulada por la interacción entre 3 compartimentos: la madre, la placenta y el feto. La síntesis de la testosterona inicia con la conversión placentaria del colesterol materno en pregnenolona. La pregnenolona se transfiere hacia el feto, en donde este último sulfoconjuga los compuestos esteroideos como mecanismo de protección para impedir la actividad biológica de estas hormonas. De esta forma, el feto forma secuencialmente sulfato de pregnenolona, sulfato de 17α-OH-pregnenolona y sulfato de dehidroepiandrosterona (DHEAS). El DHEAS se transfiere entonces hacia el compartimento placentario. La placenta convierte eficientemente este compuesto inactivo en dehidroepiandrosterona libre (DHEA) y posteriormente en androstendiona. En la placenta se puede interconvertir, a demanda, la androstendiona y la testosterona. La mayor parte de la testosterona sintetizada en la placenta se convierte de forma inmediata en estradiol, debido a que este órgano posee gran actividad del citocromo CYP19A1, enzima responsable de la conversión de andrógenos a estrógenos mediante el proceso de aromatización. En menor medida, la testosterona también puede pasar a la circulación acoplada a la globulina de unión a las hormonas sexuales (SHBG).

El complejo testosterona/SHBG se puede separar fácilmente y la testosterona libre puede entonces difundir a través de la membrana celular debido a la naturaleza lipídica de esta hormona. En el mecanismo clásico de acción, la testosterona se une al receptor de andrógenos (AR) que se localiza en el citoplasma e induce un cambio conformacional en el AR, lo que le permite fosforilarse y liberarse de las proteínas de choque térmico Hsp90. Posteriormente, el complejo testosterona/AR se dirige hacia el núcleo en donde se une en forma de dímero a los elementos de respuesta a andrógenos (AREs) localizados en los promotores de los genes regulados por la testosterona. De esta forma la testosterona puede regular la expresión de genes que presentan AREs en sus promotores, como el gen del antígeno prostático (Figura 3).

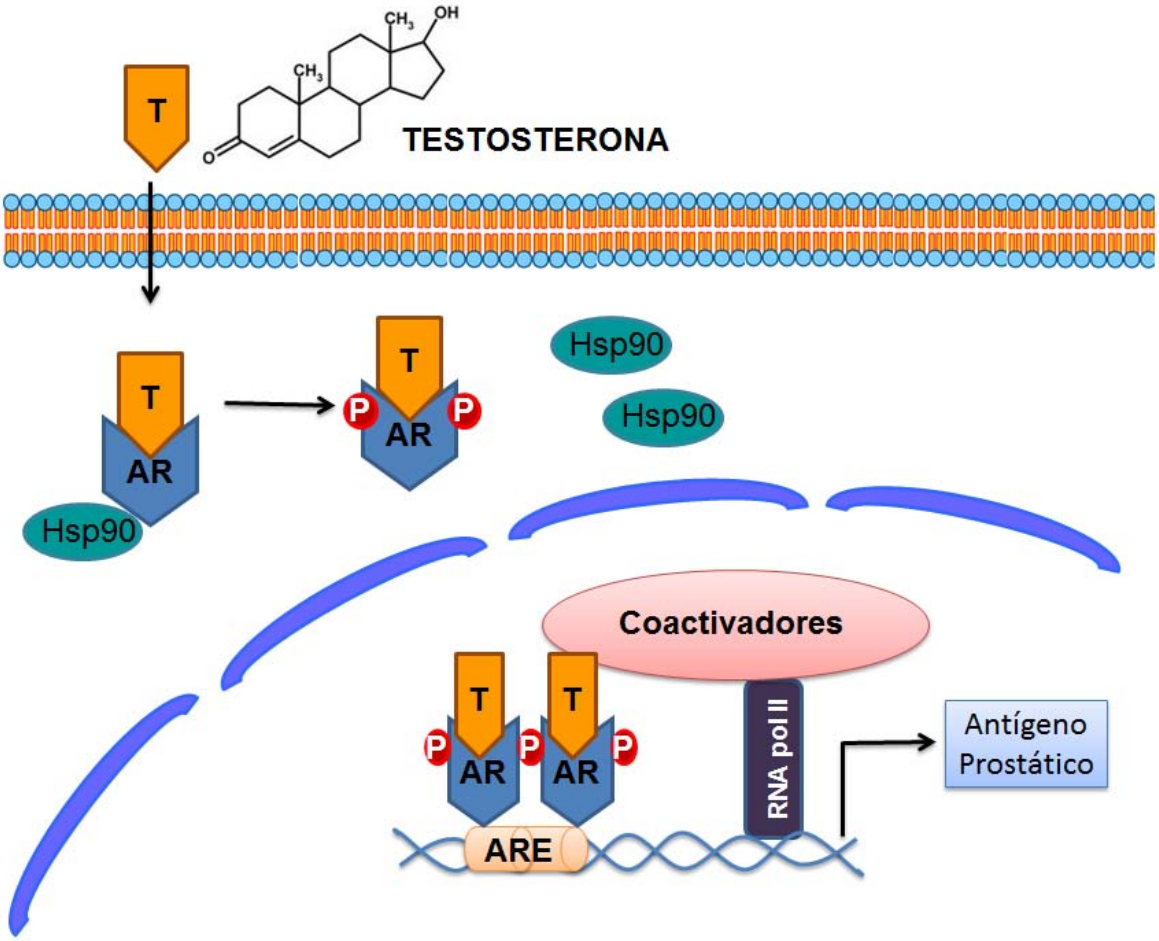

Figura 3. Mecanismo clásico de acción de la testosterona.

La testosterona difunde libremente a través de la membrana plasmática y se une a su receptor AR. Este complejo se transloca al núcleo y se une en homodímeros a los AREs. De esta forma se regula la expresión de genes dependientes de andrógenos.

Aunque los principales efectos de la testosterona son mediados por el mecanismo genómico clásico que involucra la regulación de la actividad transcripcional mediante la interacción directa entre el AR y el ADN, se ha confirmado que la testosterona también puede activar otras vías de señalización que culminan en la regulación de la expresión de genes que no presentan ningún AREs conocido, entre ellos algunos genes regulados por el AMPc [46-49]. En el estudio realizado por Fix y colaboradores en las células de Sertoli [46], tipo celular que expresa en mayor abundancia al AR, se demostró que la unión de la testosterona con su receptor resulta en la fosforilación de la proteína cinasa Src y posteriormente en la fosforilación del receptor del factor de crecimiento epidermal (EGFR). Este entrecruzamiento de las vías de señalización de la testosterona y del EGFR culmina con la activación de la vía de las MAPK y de la proteína de unión a los elementos de respuesta al AMPc (CREB).

El factor de transcripción CREB es el último efector de la cascada de señalización iniciada por el AMPc. La vía del AMPc es usada por múltiples ligandos para modular sus efectos biológicos; incluida la regulación de los citocromos de la vitamina D, como ya se había mencionado (sección 1.3.2.). Brevemente, la vía inicia cuando un ligando se une a algún receptor de la súper familia de receptores acoplados a proteínas G. Posteriormente se induce un cambio conformacional en el receptor, se separa la subunidad G alfa estimuladora (Gαs), se une la Gαs con la adenilato ciclasa y se activa la producción de AMPc. Este segundo mensajero se une a la proteína cinasa A (PKA), tetrámero constituido por dos subunidades catalíticas y dos subunidades reguladoras. La

unión del AMPc a la PKA libera a las subunidades catalíticas, las cuales se traslocan hacia el núcleo y fosforilan a CREB. Finalmente, CREB fosforilado se une a los elementos de respuesta al AMPc en el DNA (CREs) e induce la transcripción de genes regulados por este segundo mensajero [50] (Figura 4).

Como se mencionó anteriormente, existen reportes previos de que los andrógenos pueden modular la fosforilación del factor de transcripción CREB en distintos tipos celulares [46-49], sin embargo, esta vía de señalización no ha sido estudiada ni en la placenta humana ni en la de otras especies. El interés por estudiar el posible impacto de la testosterona sobre esta vía en la placenta reside en el hecho de que los dos citocromos que metabolizan al calcitriol, CYP27B1 y CYP24A1, son genes dependientes de AMPc [51, 52]. De ser así, la testosterona impactaría en la síntesis de calcitriol y por ende en genes dependientes de la vitamina D, como la catelicidina. La relación entre la testosterona y la función inmune fueron abordadas en la sección 1.1, por lo que a continuación se delimitará el impacto del sexo y la testosterona en la función inmunológica placentaria.

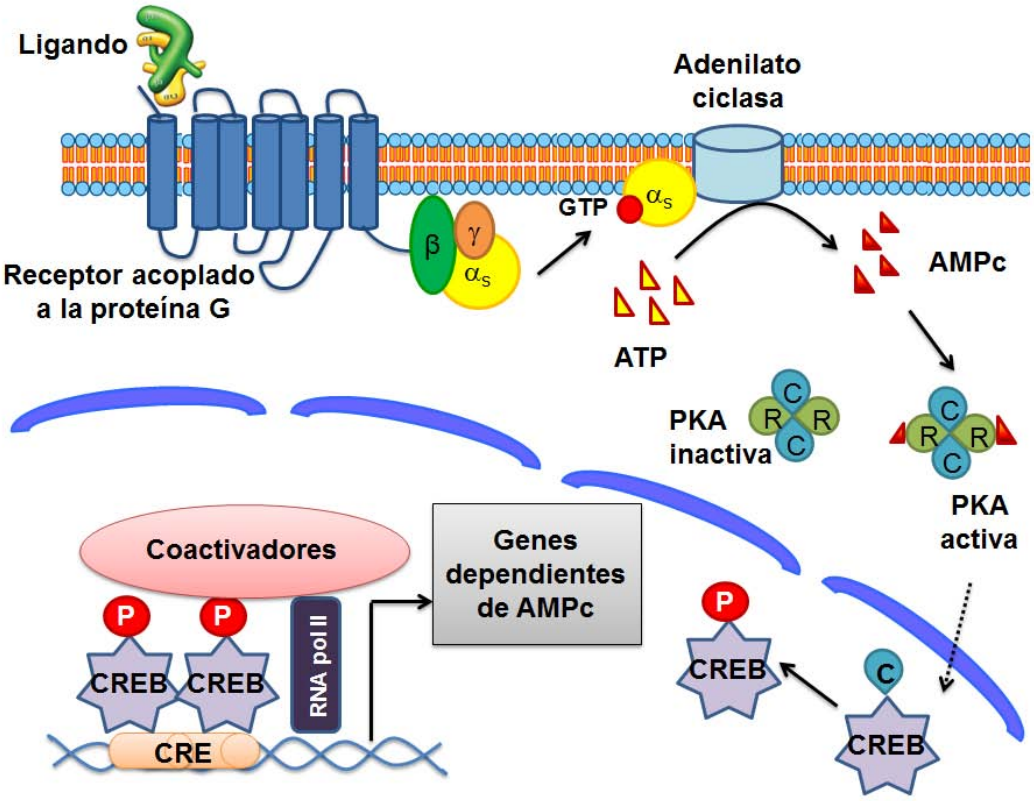

Figura 4. Vía de señalización del AMPc.

La vía inicia cuando un ligando activa a un receptor acoplado a proteínas G. Esto genera una serie secuencial de activaciones que involucran la síntesis de AMPc, la liberación de la subunidad catalítica de la PKA y la fosforilación de CREB. CREB fosforilado es el factor de transcripción que regula finalmente la expresión de genes dependientes de AMPc.

1.5. El sexo fetal como regulador de la función inmune placentaria

Las funciones placentarias pueden verse modificadas por varios factores ambientales incluyendo la oxigenación y el aporte de nutrientes, lo que finalmente lleva a modificar la expresión génica placentaria y, por lo tanto, su desarrollo y función [53, 54]. El sexo del feto, y por tanto de la placenta, es uno de los factores que influye en el epigenoma placentario. Se sabe que el sexo fetal modifica las funciones metabólicas e inmunológicas placentarias y define tanto el tamaño del propio feto como el de la placenta, siendo los hombres y sus placentas más largos y pesados [55, 56]. Varios estudios epidemiológicos han mostrado que el parto pretérmino, la ruptura prematura de membranas, las infecciones placentarias y la preeclampsia ocurren en una frecuencia 20% mayor en aquellas embarazadas que portan un feto masculino [57-60].

Sood y colaboradores (2006) evaluaron por microarreglos del DNA, el perfil de expresión de diferentes tejidos fetales en embarazos normoevolutivos a término [61]. Ellos observaron diferencias específicas asociadas al sexo y no sólo en los genes ligados a los cromosomas X o Y, sino también en genes derivados de autosomas. En particular, ellos observaron que las placentas de mujeres expresan en mayor proporción algunos factores relacionados con las vías de señalización implicadas en inmunidad, como JAK1, el receptor β de IL-2, *clusterin* o apolipoproteína J, la proteína latente de unión a TGF-β, la quimiocina ligando 1 y la proteína 1 similar al receptor de IL-1. Posiblemente, éstas y otras diferencias inmunológicas ligadas al sexo podrían ayudar al entendimiento de la respuesta fetal masculina y femenina ante la infección.

Por tal motivo, en este proyecto se plantea evaluar si la testosterona es capaz de modular la síntesis de la hCTD, como factor de defensa innata. Considerando nuestros antecedentes, creemos que la testosterona ejercerá una regulación negativa vía menor síntesis del calcitriol placentario a través de la señalización del AMPc.

2. JUSTIFICACIÓN

Los fetos y los neonatos hombres presentan mayor vulnerabilidad inmunológica en comparación con las mujeres, lo que posiciona en una situación de mayor riesgo a los hombres ante el desarrollo de infecciones durante el periodo perinatal [4-6]. Esta información concuerda con el reporte de que la tasa de mortalidad neonatal es mayor en los recién nacidos hombres, en especial durante los primeros 7 días de vida [62]. Además, se reconoce que alrededor del 26% de las muertes neonatales [3] y el 11% de las muertes maternas [8] se asocian a procesos infecciosos, lo que resalta la importancia de evaluar aquellos mecanismos y estrategias que confieren protección a la madre gestante, al feto en desarrollo y al recién nacido.

Durante el embarazo, uno de los factores relativamente recientes asociados a la protección inmunológica de la madre y del feto es el calcitriol. El calcitriol es el metabolito más activo de VD y es sintetizado por la placenta [33]. El calcitriol placentario, entre otras funciones, estimula la síntesis de factores antimicrobianos como la catelicidina [29, 63] y promueve el ambiente anti-inflamatorio necesario para la tolerancia al aloinjerto fetal [10, 64]. La asociación de la VD con la protección inmunológica es clara; por ejemplo, en un meta-análisis reciente se reportó que la deficiencia de VD aumenta en casi el doble el riesgo de sepsis [65]. Sin embargo, los datos epidemiológicos más recientes en México indican que el 28.4% de las mujeres mexicanas mayores de 20 años, y por lo tanto en edad fértil, presentan deficiencia o insuficiencia en el estado nutricio de la VD [66]. La vaginosis bacteriana es común en las madres deficientes en VD, lo cual podría

ser una posible consecuencia de la menor protección inmunológica conferida por el calcitriol; pero además las madres deficientes también presentan riesgo aumentado a desarrollar diabetes gestacional, partos pre-término, abortos recurrentes y preeclampsia [67-70].

Es así que en este proyecto de doctorado se propuso evaluar el efecto de la testosterona, como hormona sexual involucrada en la vulnerabilidad inmunológica masculina, sobre la síntesis placentaria de catelicidina mediada por la vitamina D. Los resultados de esta tesis doctoral podrían ayudar a explicar uno de los mecanismos por el que los recién nacidos masculinos son más susceptibles a presentar infecciones; y la posible asociación de esta regulación inmune con el metabolismo placentario de la vitamina D.

3. HIPÓTESIS

La testosterona, mediante la activación de CREB, disminuirá la biosíntesis de calcitriol placentario como consecuencia de la inhibición y estimulación de la expresión génica de CYP27B1 y CYP24A1, respectivamente. Lo anterior impactará negativamente en la expresión de catelicidina, ya que ésta es un blanco transcripcional del calcitriol en la placenta humana.

4. OBJETIVOS

4.1. Objetivo general

Estudiar el efecto de la testosterona sobre la inducción de catelicidina mediada por la vitamina D en la placenta humana.

4.2. Objetivos específicos

- Evaluar el efecto de la testosterona sobre la expresión génica de CYP27B1 y CYP24A1 en células placentarias en cultivo.
- Estudiar si la testosterona participa en la vía de señalización de AMPc/CREB en células placentarias en cultivo.
- Evaluar si la testosterona impacta en la biosíntesis de catelicidina, como blanco regulado por el calcitriol en la placenta humana.
- Evaluar si la concentración fisiológica de testosterona asociada a los recién nacidos hombres puede impactar en la expresión placentaria de CYP27B1, CYP24A1 y hCTD.

5. METODOLOGÍA

5.1. Criterios generales

Para la realización de este proyecto se procesaron placentas humanas obtenidas únicamente de operación cesárea en la Unidad Médica de Alta Especialidad Hospital de Gineco-Obstetricia No. 4 “Luis Castelazo Ayala” del IMSS. Las muestras se obtuvieron de mujeres que cursaron con embarazo normoevolutivo a término (de 37 a 41 semanas de gestación), previa firma de la madre del consentimiento informado para la donación de placenta (Anexo 2). El proyecto cuenta con la autorización de la Comisión Nacional de Investigación Científica del IMSS (número de registro R-2013-785-033).

Se incluyó a embarazadas clínicamente sanas entre 18 y 37 años de edad que dieron a luz a un recién nacido único con valoración de Apgar superior a 8. Se excluyó a aquellas mujeres que desarrollaron infecciones en el tracto genitourinario durante el periodo gestacional o que presentaron previamente o durante el embarazo en curso hipertensión, diabetes, enfermedades endocrinas o metabólicas, o que estuvieron bajo el consumo de algún medicamento. No se recolectó la placenta de mujeres en las que se detectó meconio ni en mujeres alérgicas a penicilina o estreptomicina. En todos los casos se registraron las características clínicas de la madre y de su recién nacido.

5.2. Recolección del suero de la vena umbilical y de explantes placentarios

Inmediatamente después del parto se punzó con una jeringa la vena umbilical para extraer la sangre. Se decidió recolectar la sangre venosa porque a través de este vaso circulan los factores que cruzaron la barrera placentaria y aquellos secretados por la propia placenta para poder cubrir los requerimientos fetales. Los sueros se obtuvieron por centrifugación a 2500 rpm durante 10 min a 4 ^{o}C y se almacenaron congelados a -70 ^{o}C hasta su procesamiento.

Adicionalmente se recolectaron cotiledones placentarios para su uso en cultivo o para el estudio de la expresión génica basal de *CYP27B1, CYP24A1* y hCTD. Para este fin, los cotiledones se lavaron exhaustivamente con solución salina estéril (NaCl 0.9%) y posteriormente se colocaron en la solución comercial RNAlater® que impide la actividad de ribonucleasas. Los cotiledones se transportaron al laboratorio y se guardaron con Trizol® en tubos que contenían perlas de cerámica para la homogenización del tejido. Los cotiledones se almacenaron a -70 ^{o}C hasta su procesamiento.

5.3. Cultivo primario de trofoblasto humano

Inmediatamente después del alumbramiento se colocó la placenta en solución salina en un recipiente de acero inoxidable con tapa y se transportó hacia el laboratorio. A continuación se describe la técnica para el aislamiento de citotrofoblastos que se desarrolla en nuestro laboratorio con base en algunas modificaciones a la técnica establecida por Kliman en 1986 [71]. Con ayuda de material quirúrgico, se eliminan las membranas corioamnióticas y el tejido decidual. Posteriormente los cotiledones placentarios se sumergen varias veces

en solución salina para eliminar el exceso de sangre. Los cotiledones se colocan sobre gasas estériles para cortar y retirar los restos de decidua así como los vasos sanguíneos presentes. Se separan 3 porciones de los cotiledones obtenidos con peso entre 35 y 45 g, y posteriormente se homogeniza el tejido con ayuda de unas tijeras y se coloca cada porción en un matraz Erlen Meyer de 1 L. En los matraces se realizan tres digestiones enzimáticas consecutivas de 20 min a 37 °C con agitación constante. Las soluciones de digestión contienen tripsina (T9201, Sigma) y desoxirribonucleasa I (DNAsa) de páncreas bovino (DN25, Sigma) diluidas en solución salina balanceada de Hank 1X (HBSS) libre de calcio y magnesio. En la siguiente tabla se describen las concentraciones de tripsina y DNAsa empleadas durante las digestiones.

Tabla 1. Soluciones enzimáticas empleadas para digerir los cotiledones placentarios

Digestión	HBSS	Tripsina (153.8 mg/mL)	DNAsa (100 mg/mL)
1era	150 mL	780 µL	85 µL
2da	100 mL	480 µL	45 µL
3era	75 mL	280 µL	45 µL

En cada digestión se colecta el sobrenadante de cada matraz, en el cual se encuentran las células disgregadas. El sobrenadante se deposita en tubos cónicos y posteriormente se adiciona suavemente una cama de suero fetal de ternera (SFT) inactivado. Los tubos recolectados se someten a centrifugación de 3000 rpm durante 10 minutos. Al término de la centrifugación se aspira el sobrenadante, y el botón celular resultante se resuspende en 600 µL de medio de cultivo DMEM.

En cada etapa, el botón celular obtenido se mantiene en baño maría a 37 ºC hasta colectar las tres digestiones. La suspensión celular total recolectada se coloca cuidadosamente sobre tres gradientes continuos de Percoll del 5 al 70 % de densidad. En la Tabla 2 se describe la preparación del gradiente de percoll. El gradiente se centrifuga a 3000 rpm durante 20 min. La obtención de citotrofoblastos se lleva a cabo recuperando la fase intermedia del Percoll con una densidad de entre 1.048 y 1.062 g/mL. Las células obtenidas se transfieren a nuevos tubos cónicos de 50 mL y se lavan nuevamente con medio DMEM.

Tabla 2. Preparación del gradiente de Percoll

Tubo	% Percoll	mL Percoll 90%	mL HBSS
1	70	2.33	0.67
2	65	2.17	0.83
3	60	2.00	1.00
4	55	1.83	1.17
5	50	1.67	1.33
6	45	1.50	1.50
7	40	1.33	1.67
8	35	1.17	1.83
9	30	1.00	2.00
10	25	0.83	2.17
11	20	0.67	2.33
12	15	0.50	2.50
13	10	0.33	2.67
14	5	0.17	2.83

Finalmente se realiza la última centrifugación a 3000 rpm por 10 min y se obtiene el botón de las células de interés. Los botones se transportan hacia la campana de cultivo celular y en ambiente de esterilidad se resuspenden en medio DMEM suplementado (DMEM + 10% SFT + penicilina 100 U/mL + estreptomicina 100 µg/mL). El total de células obtenidas se estima a partir del conteo en la cámara de Neubauer con ayuda del colorante azul de tripano.

Para los estudios de expresión génica y de citometría de flujo se sembraron 3 x 10^6 células en cajas de 25 cm^2 o en cajas de 6 pozos. Para el estudio de fosforilación se sembraron 9 x 10^6 células en cajas de Petri de 10 cm de diámetro. Para el estudio de biosíntesis de calcitriol placentario se sembraron 2.5 x 10^6 células en cajas de 6 pozos En todos los casos, la siembra se realizó en presencia de medio DMEM suplementado. Las células obtenidas se mantuvieron en un incubador a 37 °C con 80% de humedad y 5% de CO_2.

5.4. Línea celular HTR-8/SVneo

Además del cultivo celular de trofoblasto, también se eligió como modelo biológico a la línea celular HTR-8/SVneo (se abreviará como HTR-8 para facilitar su uso), que corresponde a una línea celular establecida de trofoblasto extravelloso del primer trimestre de embarazo. El linaje epitelial de esta línea se caracterizó por la presencia de las citoqueratinas 8 y 18 [72]. En este proyecto, las condiciones de incubación y de tratamientos experimentales con las células HTR-8 fueron exactamente las mismas que las empleadas para el manejo del cultivo primario de trofoblasto.

5.5. Tratamientos experimentales

Los reactivos utilizados para los tratamientos experimentales fueron adquiridos de Sigma®: testosterona, 5α-dihidrotestosterona (DHT), estradiol (E_2), flutamida, exemestano, toxina de cólera, calcidiol, calcitriol, 8-bromo monofosfato de adenosina 3'5' cíclico (8Br-AMPc) y gonadotropina coriónica humana (hCG). Los reactivos se diluyeron en etanol, cuya concentración final en el medio de estímulo fue 0.1%, excepto para la toxina de cólera y la hCG que se diluyeron en medio DMEM-F12 sin suero, y para el exemestano que se diluyó en DMSO y cuya concentración final en el medio de estímulo fue 0.05%. El SFT y los medios de cultivo DMEM y DMEM-F12 provienen de Invitrogen®.

Después de las primeras 24 h de siembra se lavaron las cajas o pozos en dos ocasiones con HBSS para eliminar las células no adheridas. Los tratamientos se realizaron con medio DMEM-F12 suplementado al 5% con SFT tratado con carbón-dextran, penicilina 100 U/mL y estreptomicina 100 µg/mL. Dependiendo del experimento, las células estuvieron entre 24, 48 y 72 h en cultivo; los detalles particulares se describen en cada sección de las técnicas experimentales empleadas. Al término del experimento se agregó el amortiguador de lisis correspondiente y/o se recolectaron los medios de cultivo.

5.6. Caracterización morfológica y funcional del cultivo primario de trofoblasto *in vitro*

Con la finalidad de evaluar la pureza y la funcionalidad endocrina de los trofoblastos separados en el gradiente de percoll, se analizó la expresión de la citoqueratina 7 (CK-7), un marcador clásico de la población trofoblástica [73] y la secreción de hCG al medio de cultivo.

La pureza del cultivo se evaluó por inmunofluorescencia mediante el marcaje de CK-7. Para este fin, se sembraron 5 x 10^5 células en laminillas de dos cámaras adaptadas para el cultivo celular. Después de 24, 48 y 72 horas de cultivo se procedió a fijar las células con solución comercial de zinc (BD Pharmigen, BD Biosciences) a 4 ^oC durante 24 h. Se permeó a las células con PBS + 1% Tritón X-100 durante 5 minutos y se lavó con PBS. En seguida se bloqueó con 1% PBS-BSA durante 1 hora. Se mantuvo en incubación a las laminillas toda la noche a 4 ^oC con el anticuerpo de ratón anti-CK7 (BSB-5412) en una dilución 1:1000 en 1% PBS-BSA. Los controles negativos se incubaron en ausencia del anticuerpo primario. Al día siguiente se procedió a marcar la CK-7 con el anticuerpo secundario de cabra anti-ratón IgG conjugado con Cy3 (Jackson Immuno Research 1:300) en 1% PBS-BSA. La incubación con el anticuerpo secundario se realizó durante 2 h a temperatura ambiente evitando la exposición de las muestras a la luz. Finalmente se lavaron las laminillas, se secaron y se agregó una gota del medio de montaje Ultracruz (Santa Cruz) que contiene 4',6-diamino-2-fenilindol (DAPI) para la visualización nuclear. Las muestras se cubrieron con el cubreobjetos y se procedió a fotografiar los sinciciotrofoblastos con el microscopio Olympus (Olympus Optical Co., LTD, Tokyo, Japón) equipado

con una cámara Olympus DP72 y una lámpara de mercurio de 100W, así como los filtros apropiados para Cy3 y DAPI. Se calculó la proporción de células positivas a CK-7 en el total de células (todos los núcleos) para determinar la pureza de la población trofoblástica.

Por último, se evaluó el fenotipo endocrino mediante la cuantificación de hCG en el medio de cultivo en condiciones basales y en estímulo con 8Br-AMPc a las 24, 48 y 72 h de cultivo. La cuantificación se realizó con ayuda de un estuche comercial de ELISA (DRG Instruments, EIA-1469) que ha sido previamente utilizado en el grupo de trabajo. El estuche permite detectar entre 5 y 1000 mUI/mL de hCG. El coeficiente de variación intraensayo es de 2.2 - 4.7 % y la variación interensayo es de 3.3 - 4.3 %. Se normalizó la secreción de hCG en función del contenido total de proteína celular cuantificada por el método del ácido bicinconínico [74].

5.7. Estudios de expresión génica por qPCR

Se utilizó la técnica de PCR en tiempo real (qPCR) para evaluar el efecto de la testosterona sobre la regulación de la expresión génica de CYP27B1, CYP24A1 y hCTD, y para evaluar la expresión basal de estos mismos marcadores dependiendo del sexo del recién nacido. Primero se realizó la extracción del RNA total con el uso del reactivo comercial Trizol®, de acuerdo a la técnica propuesta por Chomczynski y Sacchi con algunas modificaciones [75]. La cantidad y calidad de RNA se calculó espectrofotométricamente a 260 y 280 nm. Se retrotranscribió hacia DNA complementario (cDNA) una cantidad constante de 1 µg de RNA cuando se utilizaron células en cultivo, y de 3 µg de RNA cuando se utilizaron

explantes placentarios. La síntesis de cDNA se realizó con un estuche comercial de transcripción reversa (Roche). Se siguieron las mismas condiciones de PCR para todos los genes, y se normalizó contra la expresión del gen constitutivo gliceraldehído-3-fosfato-deshidrogenasa (GAPDH). Las amplificaciones se realizaron en el equipo LightCycler480 para qPCR (Roche), con el uso del estuche comercial Taqman master mix (Roche) y las sondas de hidrólisis de la biblioteca *Universal Probe Library* (Roche). En la siguiente tabla se muestra la secuencia de oligonucleótidos así como las sondas utilizadas para amplificar los genes de interés.

Tabla 3. Secuencia de oligonucleótidos y sondas de hidrólisis para los estudios de expresión génica

Gen	Número de acceso	Secuencia	Sonda UPL
GAPDH	NM_002046.3	agccacatcgctcagacac gcccaatacgaccaaatcc	60
CYP27B1	NM_000785.2	cttgcggactgctcactg cgcagactacgttgttcagg	63
CYP24A1	NM_000782.3	catcatggccatcaaaacaa gcagctcgactggagtgac	88
hCTD	NM_004345.3	tcggatgctaacctctaccg gtctgggtccccatccat	85

El número de acceso corresponde a la secuencia de referencia del GenBank del NCBI.

5.8. Estudios de Western blot

Con la finalidad de evaluar la fosforilación de CREB bajo diferentes condiciones experimentales, se mantuvo en incubación a cotiledones o células placentarias en medio DMEM-F12 sin SFT durante 12 horas y posteriormente se trataron durante 5 a 60 minutos en presencia de diferentes concentraciones de testosterona, DHT o estradiol. En algunos casos se pre-trató con flutamida (1 μM) o exemestano (5 μM). En los siguientes experimentos se eligió el tiempo de incubación de 15 minutos. En el caso de los explantes, después de los tratamientos se lavó y disgregó mecánicamente el tejido con la ayuda de perlas de cerámica y el homogeinizador MagNA Lyser (Roche) en presencia de solución de lisis (9.1 mM Na_2HPO_4, 1.7 mM H_2NaO_4P, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, pH 7.4, adicionado con 15 mM NaF y 1 mM Na_3VO_4). En el caso de las células, éstas fueron lavadas después de los tiempos de incubación y se rasparon con PBS frío, se centrifugaron a 3500 rpm durante 8 minutos y se resuspendió el botón en la solución de lisis. Adicionalmente, se realizaron 3 ciclos de congelación / descongelación con nitrógeno líquido y se volvió a centrifugar las muestras a 10,000 rpm durante 10 minutos. Finalmente se recolectó el sobrenadante en el que se encuentra la proteína solubilizada.

Se cuantificó la concentración de proteína por el método del ácido bicinconínico [74]. Las muestras se mezclaron con el buffer de carga y se calentaron a 96 °C durante 10 minutos. Se cargó la misma cantidad de proteína para separarla por SDS-PAGE 12% [76] y posteriormente se transfirieron los geles a membranas de nitrocelulosa de 0.45 μM para realizar la inmunodetección por Western blot. Las membranas se incubaron toda la noche a 4 °C con el anticuerpo

monoclonal hecho en conejo dirigido contra CREB fosforilado en la serina 133 (pCREB, Cell Signalling Technology) en dilución 1:1,500. Posteriormente se incubó 1 hora con el anticuerpo de cabra anti-conejo BioRad (75489) acoplado a HRP en dilución 1:8,000.

Adicionalmente, se evaluó la expresión proteica del VDR en los cotiledones placentarios obtenidos inmediatamente después del alumbramiento. El procesamiento del tejido fue similar al que se describió para el procesamiento de los explantes en cultivo. El anticuerpo primario utilizado para la detección del VDR fue el anticuerpo monoclonal hecho en ratón anti-VDR (sc-13133, Santa Cruz 1:250). Posteriormente se incubó 1 hora con el anticuerpo de cabra anti-ratón acoplado a HRP en dilución 1:6,000 (sc-2055, Santa Cruz). Cabe agregar que la abundancia de pCREB y del VDR se normalizaron contra la abundancia de la proteína constitutiva GAPDH. La detección de GAPDH se hizo con el anticuerpo monoclonal hecho en ratón anti-GAPDH (1:10,000, Millipore Research Reagents). Posteriormente, se ocupó el anticuerpo secundario de cabra anti-ratón acoplado a HRP en dilución 1:6,000 (sc-2055, Santa Cruz).

Para la visualización de CREB, VDR y GAPDH, las membranas se expusieron al reactivo luminiscente ECL+ Plus *Western blotting detection system* (GE Healthcare). Las imágenes se adquirieron y analizaron utilizando el fotodocumentador de imágenes ChemiDoc XRS+ (Bio-Rad) y el programa Image Lab (Bio-Rad).

5.9. Cuantificación de AMPc

La concentración total de AMPc (intra- y extra-celular) se evaluó por radioinmunoensayo mediante el antisuero CV-27 AMPc (*National Hormone & Peptide Program*) a dilución final de 1:75,000, como ha sido previamente descrito [77]. Brevemente, se sembraron las células placentarias a densidad de 200,000 por pozo en caja de 24 pozos. Se mantuvieron durante 12 horas en medio DMEM-F12 sin SFT y posteriormente se realizaron los tratamientos en medio DMEM-F12 sin SFT + IBMX (0.2 mM) para impedir la actividad de las fosfodiesterasas y de los factores crecimiento presentes en el suero. Los tratamientos en presencia de testosterona o de toxina del cólera (5 ng/mL) se mantuvieron durante 24 horas. Posteriormente se colectaron las células junto con medio de cultivo y se llevaron a ebullición durante 3 minutos. La sensibilidad del ensayo es de 2.0 fmol/mL.

5.10. Evaluación de la síntesis de calcitriol en células de trofoblasto en cultivo

Se incubó a las células de trofoblasto durante 24 horas para permitir su adhesión y diferenciación celular. Posteriormente se trató a las células con el vehículo de etanol o con 8Br-AMPc (0.6 mM) durante 24 horas para modular el cambio en la expresión génica de CYP27B1 y de CYP24A1 [45]. Después se adicionó el calcidiol ($25OHD_3$, 1 μM) y se mantuvo a las células en incubación durante 2 horas. Transcurrido este tiempo se mantuvo a las células a -70 ºC hasta su cuantificación.

La manipulación de las células se hizo en obscuridad para evitar la fotodegradación del calcitriol. Se descongeló y raspó las células con ayuda de

puntas estériles de 1 mL. Se recolectó el total de células y medio de cultivo. Posteriormente se realizaron 3 ciclos de congelación/descongelación en nitrógeno líquido. Las alícuotas se etiquetaron y se mandaron a analizar en el laboratorio LabcoNoûs ubicado en C/Alta Ribagorca 12 en Barcelona, España. La cuantificación se hizo por radioinmunoanálisis. El límite de detección es de 5 pg/mL. Los resultados obtenidos se expresaron en pg/mL/2.5 x 10^6 células.

5.11. Evaluación de la expresión de hCTD por citometría de flujo en trofoblastos en cultivo

Se eligió a la citometría de flujo como método para estudiar el efecto de los andrógenos en la síntesis del marcador inmunológico innato catelicidina, como resultado de la modulación del metabolismo placentario de la vitamina D. Para este fin se incubaron 3 x 10^6 trofoblastos en cajas de 6 pozos en presencia o ausencia de andrógenos durante 24 horas para modular la expresión de CYP27B1 y CYP24A1. Posteriormente se adicionó $25OHD_3$ en el medio de tratamiento y se mantuvo a las células durante otras 15 horas en incubación para evaluar la expresión de hCTD como marcador de la bioactividad del calcitriol. Se impidió la secreción de hCTD con el uso de monensina 2 µM, un inhibidor de la secreción proteica (BD GolgiStop, BD Biosciences).

Después del tiempo de incubación, se tripsinizó a las células y se centrifugó a 2,000 rpm durante 3 minutos para obtener el botón. Posteriormente, las células se fijaron y permeabilizaron con ayuda del estuche *BD Cytofix/Cytoperm Plus* (BD Biosciences). El marcaje intracelular de hCTD en las células CK-7 positivas se consiguió con el uso del anticuerpo monoclonal hecho en conejo (Abcam) que

reconoce tanto la forma completa como la escindida en su dominio C-terminal de la hCTD, en coincubación con el anticuerpo hecho en ratón contra CK-7 (BioSB), ambos en dilución 1:60. Después de 1 hora de incubación, las células se lavaron con PBS-albúmina 0.8% y se incubaron con los anticuerpos secundarios fluorescentes de cabra anti-ratón conjugado con FITC (verde) (Santa Cruz) y de cabra anti-conejo conjugado con Cy3 (rojo) (Jackson ImmunoResearch), diluidos 1:300 durante 1 hora a temperatura ambiente y protegiendo las muestras de la exposición a la luz. Después de lavar, las células se sometieron al análisis citométrico con el equipo Becton Dickinson FACSCalibur. Las células se analizaron por citometría de flujo de 2 colores para medir la expresión de hCTD en las células CK-7 positivas. El análisis se desarrolló con el programa *CellQuest Pro* (Becton Dickinson) y los resultados se expresaron como intensidad de fluorescencia, que resultó de multiplicar el número de eventos (número de células positivas por cada 1000) por el promedio de la fluorescencia.

5.12. Cuantificación de hCTD en el suero de cordón umbilical

Las concentraciones de hCTD en el suero umbilical se cuantificaron mediante ELISA utilizando el anticuerpo anti-hCAP18/LL-37 (policlonal hecho en cabra,1:500, Santa Cruz) para la captura y el anti-LL-37 (policlonal hecho en conejo, 1:1000, Abcam) como se describió previamente [78]. Previo al montaje de la ELISA, se filtraron las muestras con membranas de 10 KDa (*Amicon ultra centrifugal filters 10K*, Millipore) para asegurar que sólo la forma activa de la catelicidina fuera cuantificada. El estándar de hCTD purificado por HPLC fue

generosamente donado por el Dr. Robert Hancock de la Universidad British Columbia en Vancouver, Canadá.

5.13. Cuantificación de testosterona, estradiol y 25OHD$_3$ en el suero de cordón umbilical

La cuantificación de estradiol en el suero de cordón umbilical se evaluó por RIA con el equipo automático Siemens Immulite 2500 analyzer. La concentración de testosterona total se determinó por inmunoensayo enzimático quimioluminiscente también en el equipo Siemens Immulite 2500 analyzer. La concentración de 25OHD$_3$ se evaluó por inmunoensayo quimioluminiscente con el estuche de Liaison, DiaSorin Inc.

5.14. Análisis estadístico

Las diferencias entre los promedios de los datos normalizados analizados por qPCR, western blot o citometría de flujo se analizaron por ANOVA de una vía o la prueba *t* de Student, según fuera apropiado. Las diferencias en la concentración de las distintas hormonas y hCTD en el suero de cordón umbilical de recién nacidos hombres y mujeres se evaluaron por la prueba *U* de Mann-Whitney o la prueba *t* de Student dependiendo de la normalidad en la distribución de los datos. Las diferencias se consideraron estadísticamente significativas cuando el valor de $P < 0.05$.

6. RESULTADOS

6.1. Caracterización del cultivo primario de trofoblasto humano

Los trofoblastos placentarios en cultivo forman sincicios de manera dependiente del tiempo. Este proceso es resultado de la fusión entre 2 o más citotrofoblastos vecinos, de naturaleza mononononuclear, para dar origen a sinciciotrofoblastos multinucleados y de mayor tamaño. La morfología de los sinciciotrofoblastos se evaluó mediante el marcaje fluorescente de la CK-7, marcador específico del linaje trofoblástico. Como se observa en la figura 5, el marcaje de la CK-7 con rojo permitió visualizar la morfología característica de los sinciciotrofoblastos como grandes cúmulos multinucleados. La CK-7 es la única queratina que expresan todas las poblaciones trofoblásticas, pero está ausente en otras células placentarias mesenquimales como los fibroblastos y los macrófagos [73], por lo que es común que se utilice su abundancia como indicador de la pureza del cultivo de trofoblasto [63]. En promedio, el análisis de células CK-7 positivas en el cultivo primario de placenta demostró que alrededor del 85% de la población son trofoblastos.

Para evaluar el fenotipo endocrino, se analizó la secreción de la subunidad beta de hCG durante las primeras 72 horas de cultivo (Figura 6). Como se muestra en la figura, durante la diferenciación de los trofoblastos se observa un incremento significativo en la secreción de hCG, y este efecto se potenció cuando se incubó a las células con el análogo de AMPc, el 8Br-AMPc.

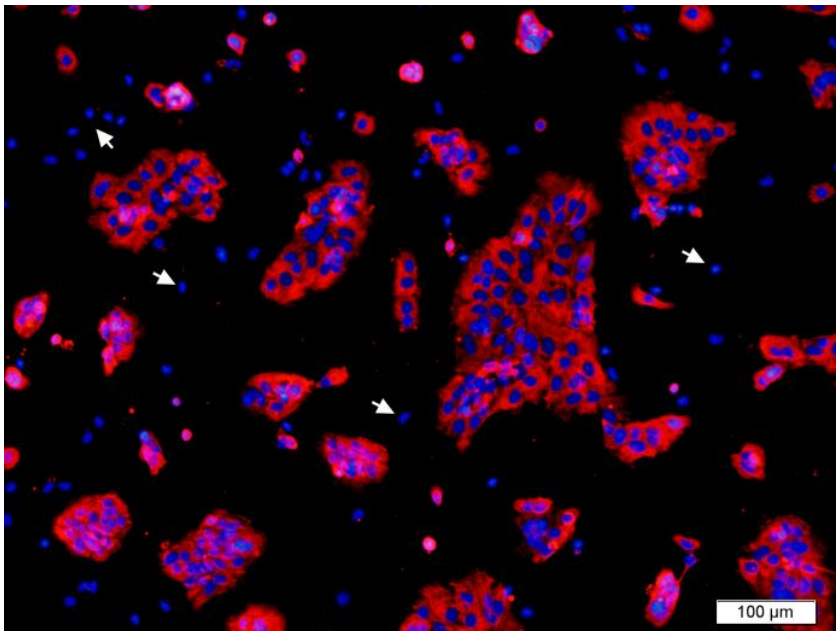

Figura 5. Inmunomarcaje de CK-7 en trofoblastos en cultivo.

La pureza del cultivo se evaluó por inmunofluorescencia. Después de 3 días de cultivo, las células fijadas y permeabilizadas se incubaron con el anticuerpo anti-CK-7 hecho en ratón. El anticuerpo secundario anti-ratón marcado con Cy3 permitió la visualización y conteo de las células CK-7 positivas (rojo). Las células CK-7 negativas se señalan con flechas. Los núcleos se tiñeron con DAPI (azul). El promedio de células CK-7 positivas fue 84 ± 4%.

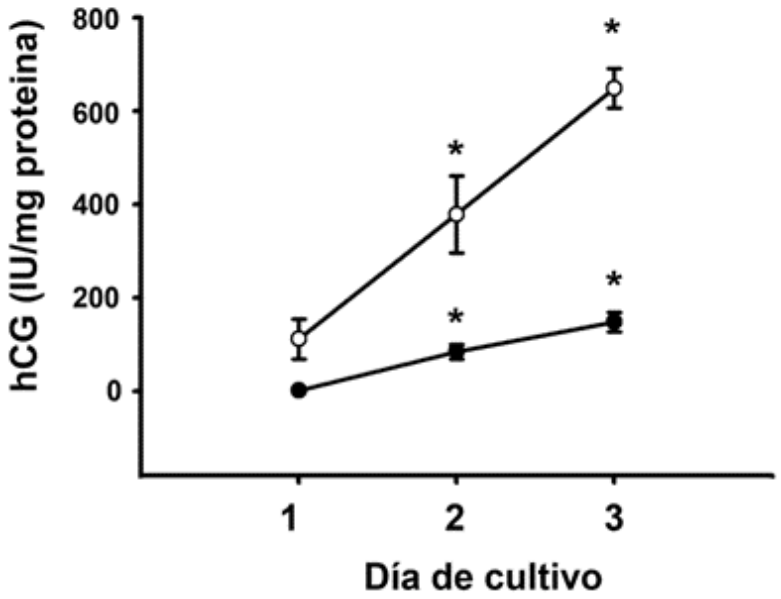

Figura 6. Secreción de hCG al medio por los trofoblastos en cultivo.

La funcionalidad del fenotipo endocrino se demostró mediante la cuantificación de hCG en el medio de cultivo. Conforme avanza el grado de diferenciación celular se aprecia mayor síntesis de hCG (●). El tratamiento con 8Br-AMPc (0.6 mM) estimuló la síntesis de hCG (○). *$P < 0.05$ *vs.* día 1. $n = 4$ cultivos independientes.

6.2. La testosterona induce la expresión de CYP24A1 mientras que inhibe la expresión de CYP27B1 en células placentarias

Además del cultivo primario de trofoblasto, también se utilizó la línea de trofoblasto extravelloso HTR-8/SVneo como modelo adicional de estudio en la placenta humana. Para investigar el posible efecto transcripcional de la testosterona sobre los citocromos del metabolismo de la vitamina D, los trofoblastos y las células HTR-8 se incubaron en presencia de diferentes concentraciones de testosterona o el andrógeno no aromatizable DHT durante 24 horas. Se contó también con el control de 8Br-AMPc. Como se muestra en las figuras 7 y 8, la testosterona, DHT y 8Br-AMPc disminuyeron la expresión de CYP27B1 en los trofoblastos y HTR-8 en cultivo.

Por el contrario, la expresión de CYP24A1 fue regulada a la alta por estos andrógenos y el 8Br-AMPc en los trofoblastos en cultivo (Figuras 9 y 10). La expresión de este citocromo no se puede evaluar en la línea HTR-8 ya que en estas células el gen CYP24A1 se encuentra altamente metilado y su expresión es nula.

El efecto observado por los andrógenos fue la inhibición en la expresión de CYP27B1 y el aumento en la expresión de CYP24A1; esto nos permitió hipotetizar que el efecto biológico de los andrógenos en el metabolismo de la vitamina D en la placenta humana impactaría en la disminución de la biodisponibilidad de calcitriol mediante mecanismos relacionados con su menor síntesis y/o con el aumento de su degradación.

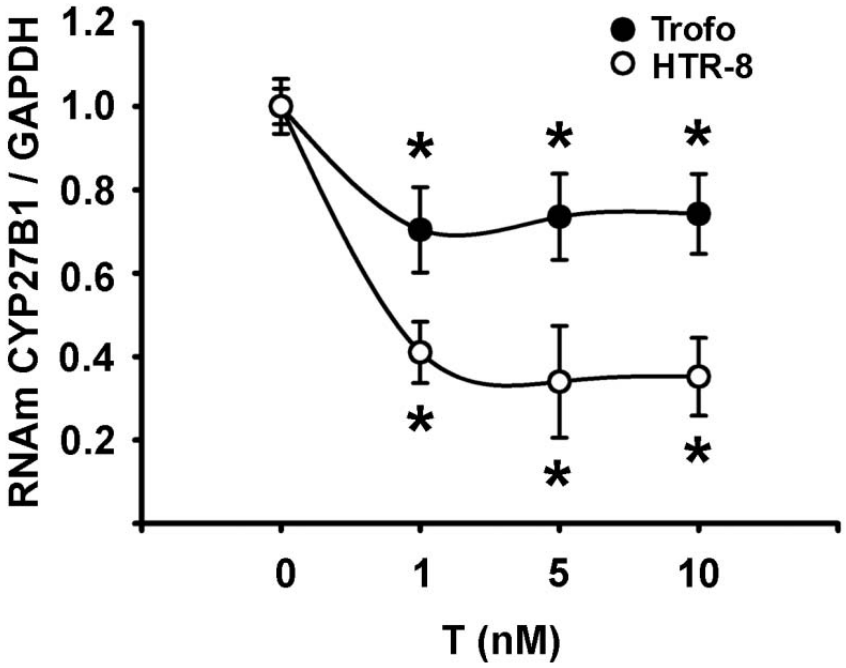

Figura 7. La testosterona disminuye la expresión génica de CYP27B1 en células placentarias en cultivo.

T: Testosterona. Trofoblastos en cultivo (círculos negros). Células HTR-8 (círculos blancos). * $P < 0.05$ *vs* células control. Los datos se presentan como promedio ± EE. n ≥ 3 experimentos independientes.

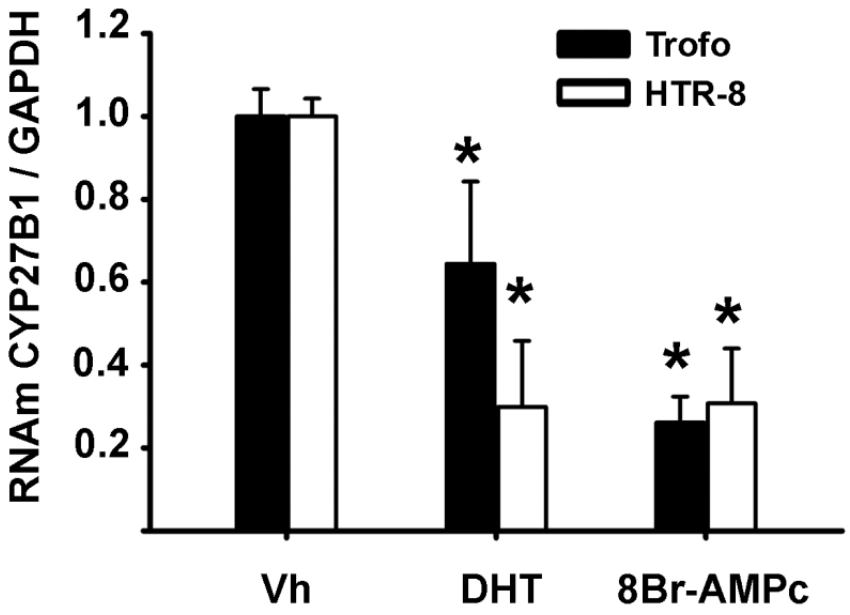

Figura 8. La DHT y el 8Br-AMPc disminuyen la expresión génica de CYP27B1 en células placentarias en cultivo.

DHT: 5nM. 8Br-AMPc: 0.6 mM. Trofoblastos (barras negras). Células HTR-8 (barras blancas). * $P < 0.05$ *vs* células tratadas con vehículo. Los datos se presentan como promedio ± EE. n ≥ 3 experimentos independientes.

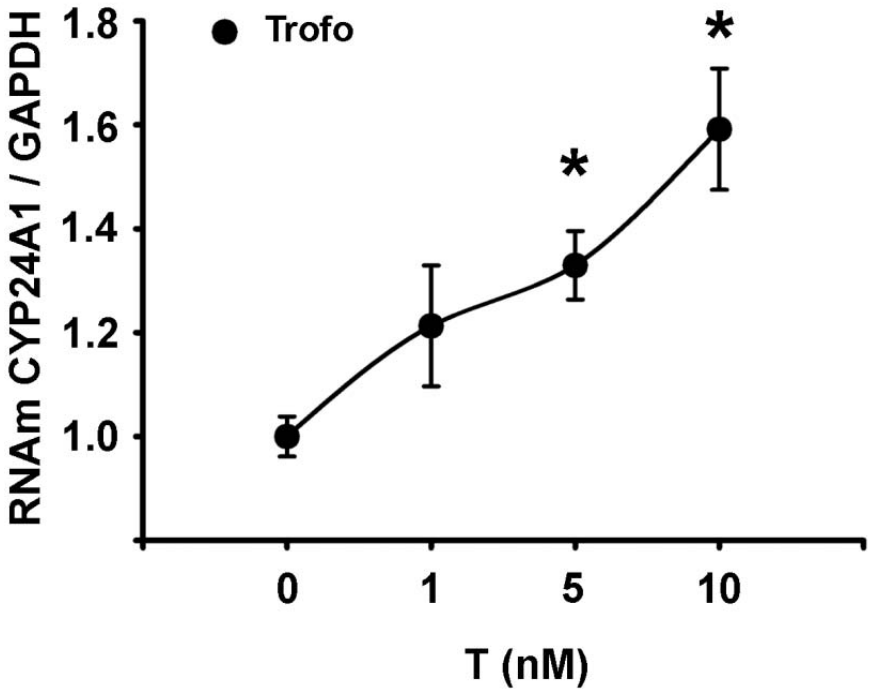

Figura 9. La expresión génica de CYP24A1 es estimulada por la testosterona en los trofoblastos en cultivo.

T: testosterona. Los datos se presentan como promedio ± EE. * $P < 0.05$ *vs* células control. n ≥ 3 experimentos independientes.

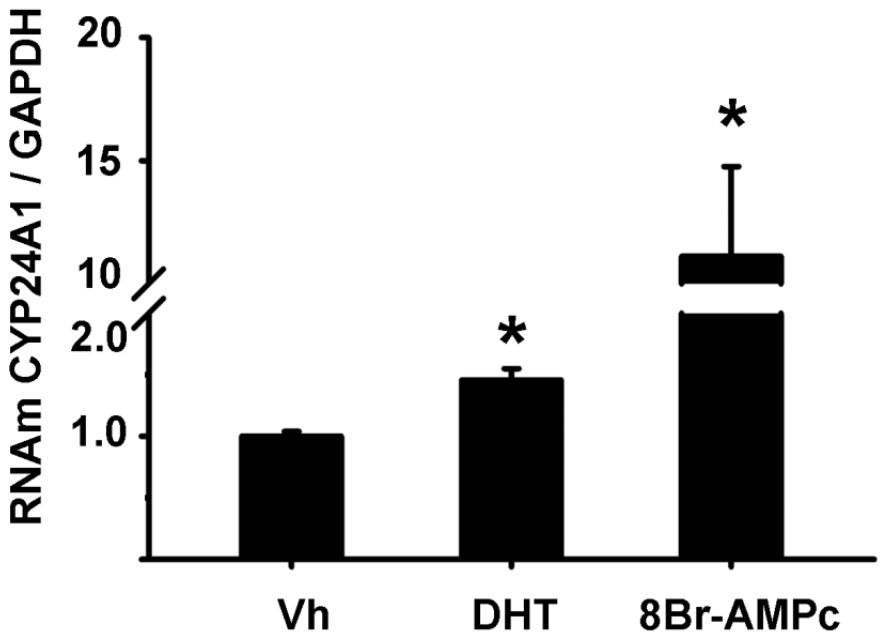

Figura 10. La DHT y el 8-Bromo AMPc estimulan la expresión génica de CYP24A1 en los trofoblastos en cultivo.

Los datos son presentados como promedio ± EE. n ≥ 3. * $P < 0.05$ *vs*. células tratadas con vehículo.

6.3. La testosterona activa a CREB de manera AMPc-independiente pero dependiente del AR

Previamente describimos que los genes CYP27B1 y CYP24A1 son regulados por la vía AMPc / PKA / CREB en la placenta [45], lo cual va de acuerdo con que estos citocromos presentan elementos CREs en sus promotores [51, 52]. Por lo tanto, y con el propósito de evaluar la implicación de esta vía de señalización en la regulación que ejerce la testosterona sobre las enzimas que metabolizan a la vitamina D, se decidió tratar los explantes placentarios con esta hormona durante 5 a 60 minutos. Primero, y para corroborar la especificidad del anticuerpo empleado, se expuso con hCG a los cotiledones placentarios ya que es conocido que esta hormona actúa a través de la vía AMPc/PKA/CREB e induce la fosforilación de CREB (pCREB por sus siglas en inglés). Como era esperado, esta glicoproteína indujo la fosforilación de CREB durante los 5 a 60 minutos post-estímulo. Sorprendentemente, el tratamiento con testosterona también resultó en la fosforilación significativa de CREB en los primeros 60 minutos, con un pico máximo alrededor de los 15 minutos (Figura 11). Considerando esto, los siguientes experimentos se desarrollaron bajo este tiempo de estímulo. Tanto en los explantes como en las células placentarias, la incubación con testosterona rápidamente activó a CREB (Figuras 12 y 13). Este efecto también se observó cuando se usó DHT, mientras que la preincubación con flutamida antagonizó la activación de CREB dependiente de los andrógenos. Aunque el estradiol también indujo a pCREB, el inhibidor de la aromatasa exemestano no antagonizó la fosforilación de CREB dependiente de la testosterona, lo que resalta la participación de los andrógenos en la inducción de pCREB. Finalmente, en las

células placentarias en cultivo el tratamiento con los andrógenos resultó también en la activación de CREB (Figura 12).

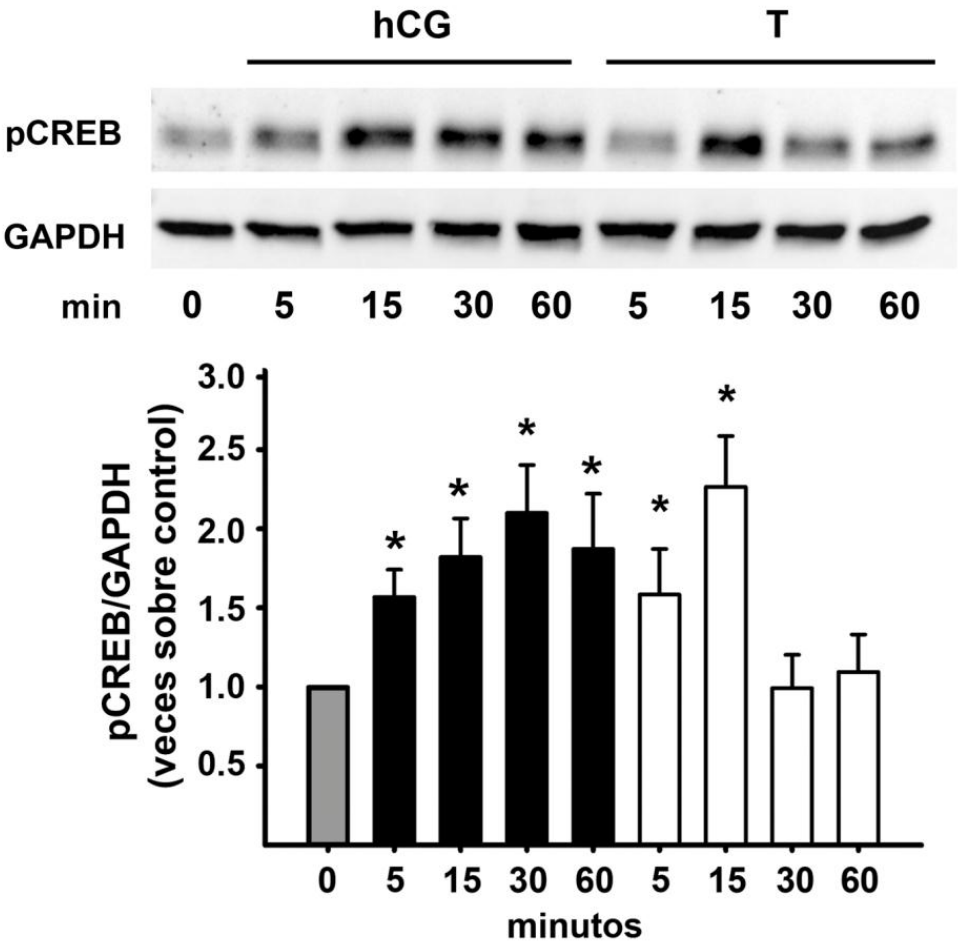

Figura 11. La testosterona rápidamente fosforila a CREB en explantes placentarios en cultivo.

Se realizó la cinética de tiempo (de 5 a 60 minutos) para evaluar los cambios en la fosforilación de CREB mediada por hCG (50 UI/mL) y testosterona (5 nM). Arriba, la imagen es representativa de 5 placentas independientes. Abajo, se muestra el histograma con las densitometrías obtenidas; los datos son presentados como promedio ± EE. * $P < 0.05$ *vs.* tiempo cero.

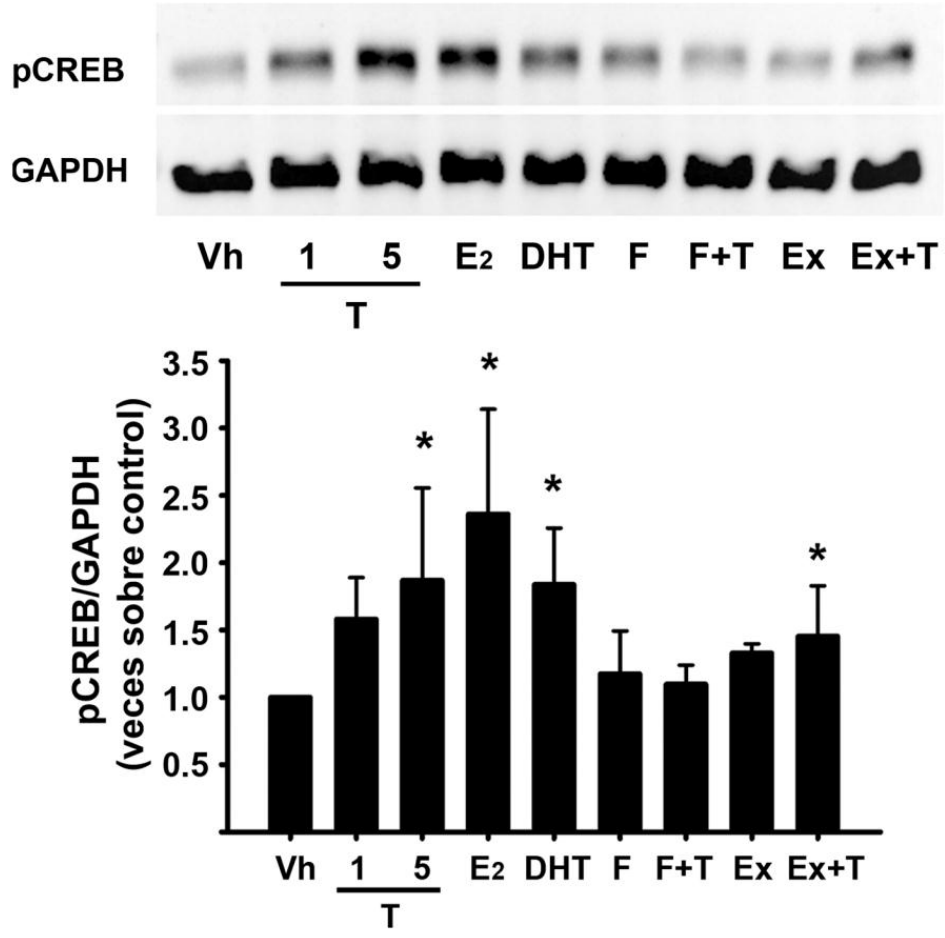

Figura 12. Los andrógenos inducen la fosforilación de CREB de forma dependiente del AR.

Los explantes placentarios se incubaron en presencia de testosterona (T, nM), estradiol (E_2, 10 nM), DHT 1 nM, Flutamida (F, 1 μM) y exemestano (Ex, 5 μM) solos o en combinación con T 5 nM para evaluar los cambios en la abundancia de pCREB. Arriba, la imagen es representativa de 4 placentas independientes. Abajo, se muestra el histograma con las densitometrías obtenidas; los datos son presentados como promedio ± EE. * $P < 0.05$ *vs.* vehículo.

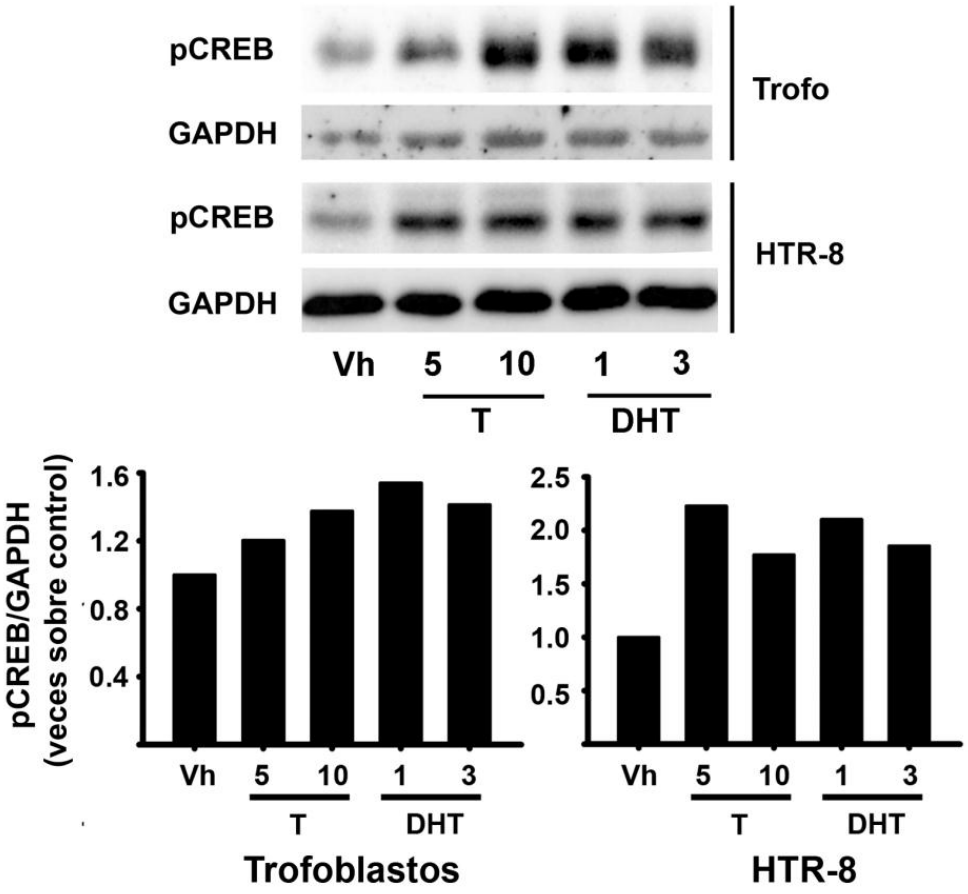

Figura 13. Los andrógenos fosforilan a CREB en el cultivo primario de trofoblasto y en HTR-8.

Se evaluó la fosforilación de CREB mediada por T y DHT (nM) en el cultivo primario de trofoblasto y en células HTR-8. Arriba, se muestra la imagen representativa de 2 placentas independientes y 2 cultivos de HTR-8. Abajo a la izquierda se presenta la densitometría de pCREB/GAPDH observada en el cultivo de trofoblasto, mientras que a la derecha se presenta la densitometría en HTR-8.

Posteriormente nos preguntamos si esta inducción de CREB dependiente de los andrógenos podría estar ligada al aumento en la producción de AMPc. Para ello, primero se corroboró la funcionalidad de la adenilato ciclasa en nuestros modelos de estudio mediante la activación constitutiva con toxina de cólera y el consiguiente esperado aumento en la síntesis de AMPc (Figuras 14 y 15). En efecto, tanto las células de trofoblasto como las HTR-8 respondieron activamente al estímulo con la toxina de cólera. Sin embargo, no se observó ningún cambio significativo en el cultivo de trofoblasto en presencia de testosterona sola o en coincubación con exemestano, un inhibidor de la aromatización (Figura 14). De manera similar, en las células HTR-8 tampoco se observó algún cambio en la producción de AMPc generado por la testosterona o por DHT (Figura 15).

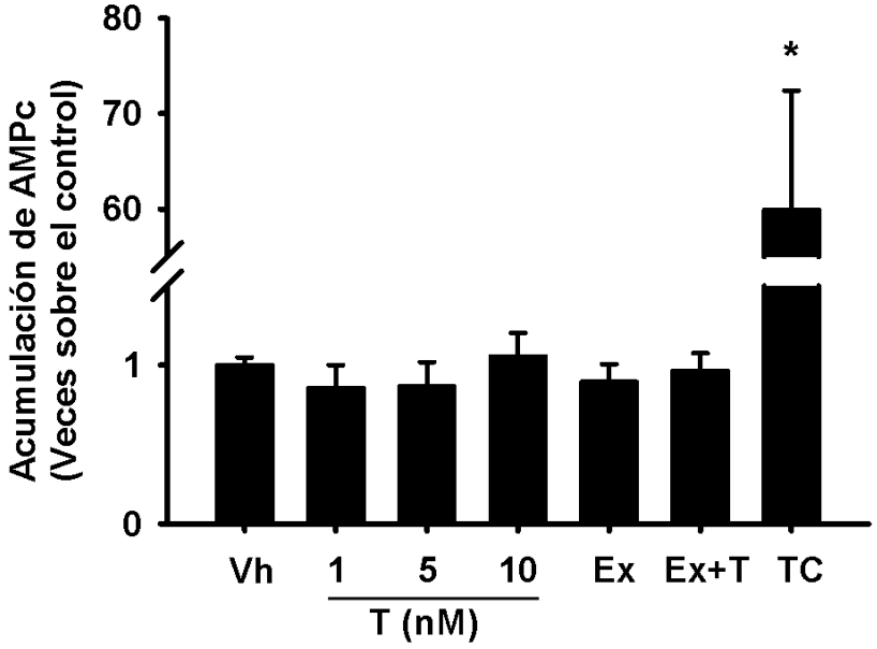

Figura 14. La testosterona no modifica la síntesis de AMPc en el cultivo de trofoblasto.

El tratamiento con testosterona (T) sola o en coincubación con exemestano (Ex) no modificó la síntesis de AMPc. Toxina de cólera (TC, 5 ng/mL). Los datos se presentan como promedio ± EE. * $P <$ 0.05 *vs.* vehículo. n = 3 cultivos independientes. La síntesis de AMPc se normalizó contra el vehículo, al cual se le asignó el valor de 1.

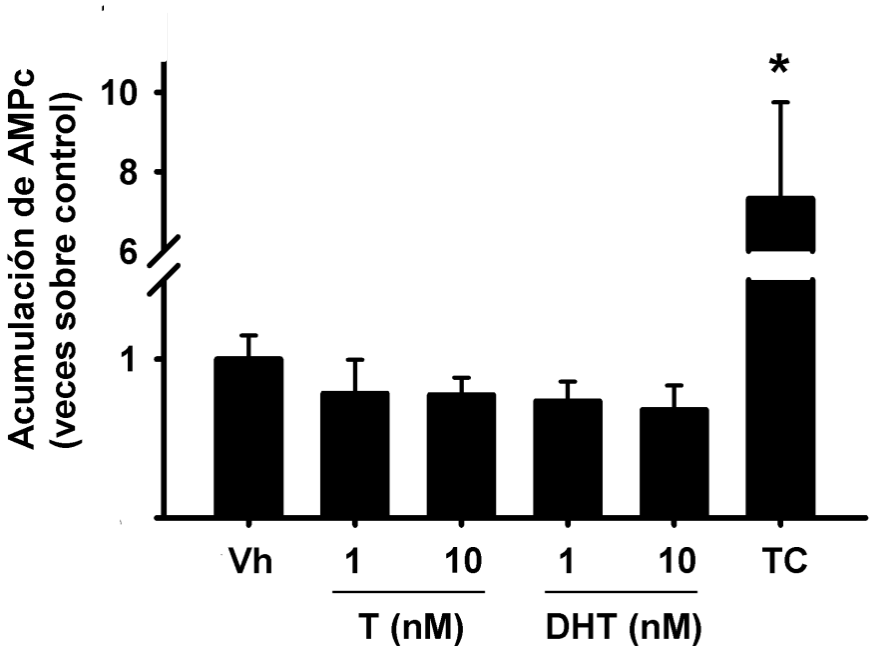

Figura 15. Los andrógenos no modifican la síntesis de AMPc en el cultivo de HTR-8.

El tratamiento con testosterona (T) o con DHT no modificó la síntesis de AMPc. Toxina de cólera (TC, 5 ng/mL). Los datos se presentan como promedio ± EE. * $P < 0.05$ vs vehículo. n = 3 experimentos independientes. La síntesis de AMPc se normalizó contra el vehículo, al cual se le asignó el valor de 1.

6.4. Los andrógenos inhiben indirectamente la síntesis de hCTD a través de modificar el metabolismo de la vitamina D en trofoblastos en cultivo

Para evaluar la capacidad enzimática de CYP27B1 y CYP24A1, se decidió hacer un bioensayo con calcidiol ($25OHD_3$) en los trofoblastos en cultivo. Es necesario recordar que el CYP27B1 actúa sobre el calcidiol para dar origen al calcitriol, mientras que la CYP24A1 participa en la degradación del calcitriol sintetizado (Figura 1). Por lo que el balance entre CYP27B1 y CYP24A1 se verá reflejado en la síntesis final del calcitriol placentario.

Como se observa en la Figura 16, las células incubadas únicamente con el medio de tratamiento no sintetizan calcitriol. Es decir, en ausencia de $25OHD_3$, la sola presencia de CYP27B1 no condiciona la síntesis de calcitriol. Posteriormente, cuando se adicionó 1 μM de calcidiol, se observó el claro aumento del calcitriol sintetizado (intra- y extra-celular). Por último, se adicionó 8Br-AMPc a las células en cultivo como control de la inhibición de CYP27B1 y del paralelo aumento de CYP24A1. Como era esperado, el tratamiento con 8Br-AMPc resultó en la disminución significativa del calcitriol sintetizado, en comparación con el observado en las células incubadas únicamente con $25OHD_3$.

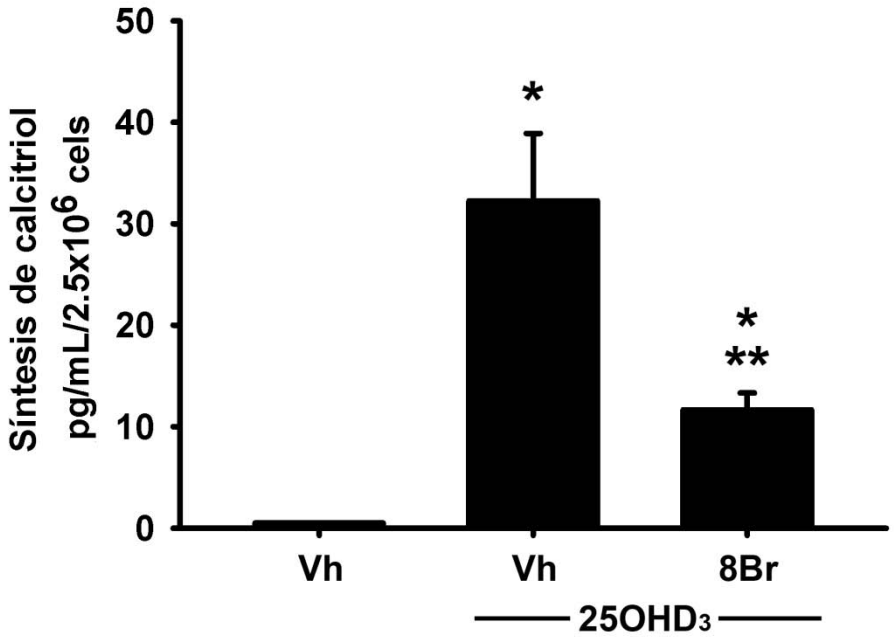

Figura 16. Síntesis de calcitriol en los trofoblastos en cultivo.

Los trofoblastos se incubaron en presencia de $25OHD_3$ (1 µM) durante 2 horas y se cuantificó el calcitriol total sintetizado: intracelular y en el medio de cultivo. Vh: vehículo; 8Br: 8Br-AMPc 0.6 mM. Cada barra representa el promedio de 4 cultivos de trofoblasto. Los datos se presentan como promedio ± EE. * $P < 0.05$ *vs*. vehículo. ** $P < 0.05$ *vs*. vehículo con $25OHD_3$

Después de corroborar que el trofoblasto en cultivo es capaz de sintetizar calcitriol debido a la actividad enzimática de CYP27B1 cuando se le proporciona el sustrato $25OHD_3$, se decidió evaluar la plausibilidad biológica de esta observación en los efectos inmunológicos mediados por el calcitriol. Para ello, decidimos investigar el impacto de la testosterona en la producción del calcitriol placentario utilizando a la hCTD como marcador dependiente del calcitriol y además como marcador de la defensa innata placentaria. La adición de $25OHD_3$ resultó en la síntesis de $1,25(OH)_2D_3$, tal como se demostró en la Figura 16, por lo que la intención de este bioensayo fue evaluar la consiguiente síntesis de catelicidina como blanco dependiente del calcitriol. Como ya se ha mencionado, es bien conocido que el calcitriol induce la expresión génica de hCTD en la placenta humana [29, 63], tal como también se observa en esta tesis. Cabe aclarar, que esta síntesis observada de hCTD es dependiente de la bioconversión *in vitro* de $25OHD_3$ a $1,25(OH)_2D_3$, siendo que a mayor calcitriol sintetizado, mayor inducción de catelicidina.

Primero se determinó el efecto del calcitriol biosintetizado en la expresión a nivel génico de hCTD, así como el posible efecto derivado de la preincubación con andrógenos. Como se observa en la Figura 17, la adición de $25OHD_3$ resultó en la potente inducción en la expresión génica de hCTD; un estímulo de más de 200 veces en comparación con la de células incubadas sin calcidiol. Interesantemente, cuando se preincubó a las células con testosterona, DHT o el 8Br-AMPc, se observó la significativa disminución en la expresión génica de hCTD derivada de la menor bioconversión de calcitriol.

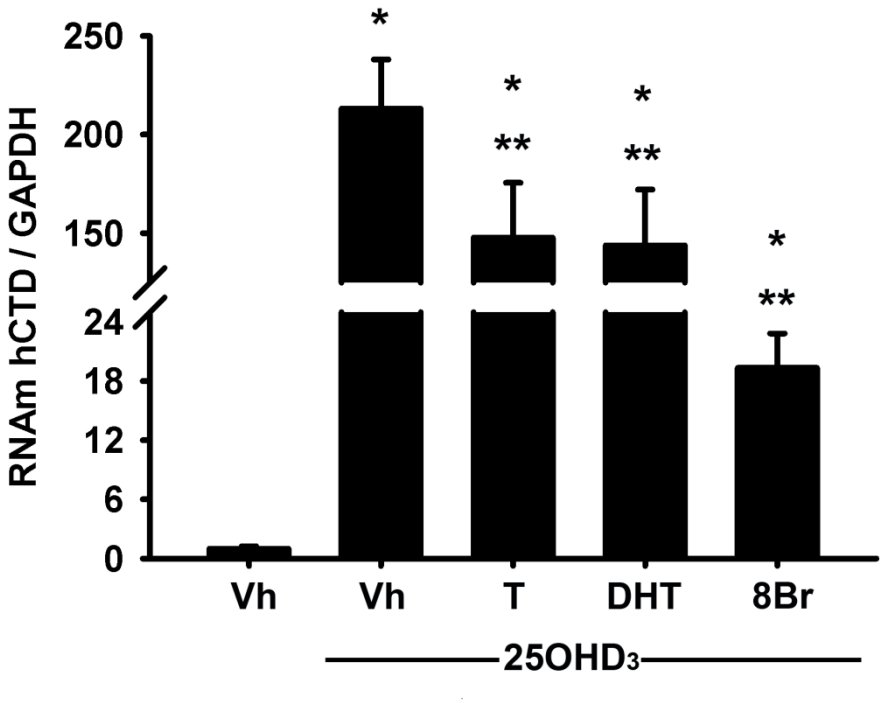

Figura 17. La testosterona, la DHT y el 8Br-AMPc inhiben la expresión génica de la catelicidina, secundaria a la menor biosíntesis de calcitriol.

Los trofoblastos se preincubaron en presencia de testosterona (T) 10 nM, 5α-dihidrotestosterona (DHT) 1 nM, 8Br-AMPc (8Br) 0.6 mM o su vehículo (Vh, etanol 0.1%) durante 24 horas. Posteriormente se adicionó $25OHD_3$ (1 μM) durante 24 horas más. Al cabo de este tiempo se cuantificó la expresión génica de hCTD. Cada barra representa el promedio de 4 cultivos de trofoblasto. Los datos se presentan como promedio ± EE. * $P < 0.05$ *vs*. vehículo. ** $P < 0.05$ *vs*. vehículo con $25OHD_3$

El siguiente objetivo fue evaluar la expresión proteica de hCTD dependiente de la síntesis de calcitriol. Como primer control, se incubó a las células con calcitriol (10 nM) y se corroboró el estímulo en la síntesis de hCTD (Figura 18). Como segundo control se incubó a las células con $25OHD_3$, lo que resultó en la bioconversión hacia $1,25(OH)_2D_3$ (como se demostró en la Figura 16) y posteriormente el calcitriol biosintetizado reguló a la alta la síntesis de hCTD de manera equiparable a la inducción observada con el tratamiento de calcitriol.

Interesantemente, y en referencia a nuestras preguntas experimentales en el bioensayo propuesto en este proyecto encontramos que la preincubación con testosterona o con DHT resultó en la menor expresión proteica de hCTD después de exponer a las células a $25OHD_3$, en comparación con la observada solamente en presencia de $25OHD_3$ (Figura 18, vehículo barra negra). Sorprendentemente, esta reducción en la síntesis de hCTD que se observó con el tratamiento con los andrógenos fue comparable a la síntesis basal de hCTD en ausencia de $25OHD_3$ (Figura 18, vehículo barra blanca). Es decir, los andrógenos prácticamente bloquearon por completo el efecto inductor de la $25OHD_3$ sobre la hCTD.

Por el contrario, el tratamiento con estradiol no modificó la síntesis de hCTD inducida por $25OHD_3$, con lo que se comprueba que los estrógenos no modifican la respuesta inmune mediada por la VD. En cuanto al tratamiento con 8Br-AMPc que inhibe la expresión de CYP27B1 y estimula la de CYP24A1, tal como era esperado, se observó que este mensajero disminuyó la expresión proteica de hCTD (Figura 18). Por último, el uso del antagonista del VDR, TEI-9647, bloqueó por completo el estímulo en la síntesis de hCTD. Con esto se comprueba que, en efecto, la regulación de la expresión de hCTD en la placenta humana es

dependiente del calcitriol y la consiguiente unión a su receptor, mediante la formación del complejo $1,25(OH)_2D_3$ / VDR-RXR / VDREs.

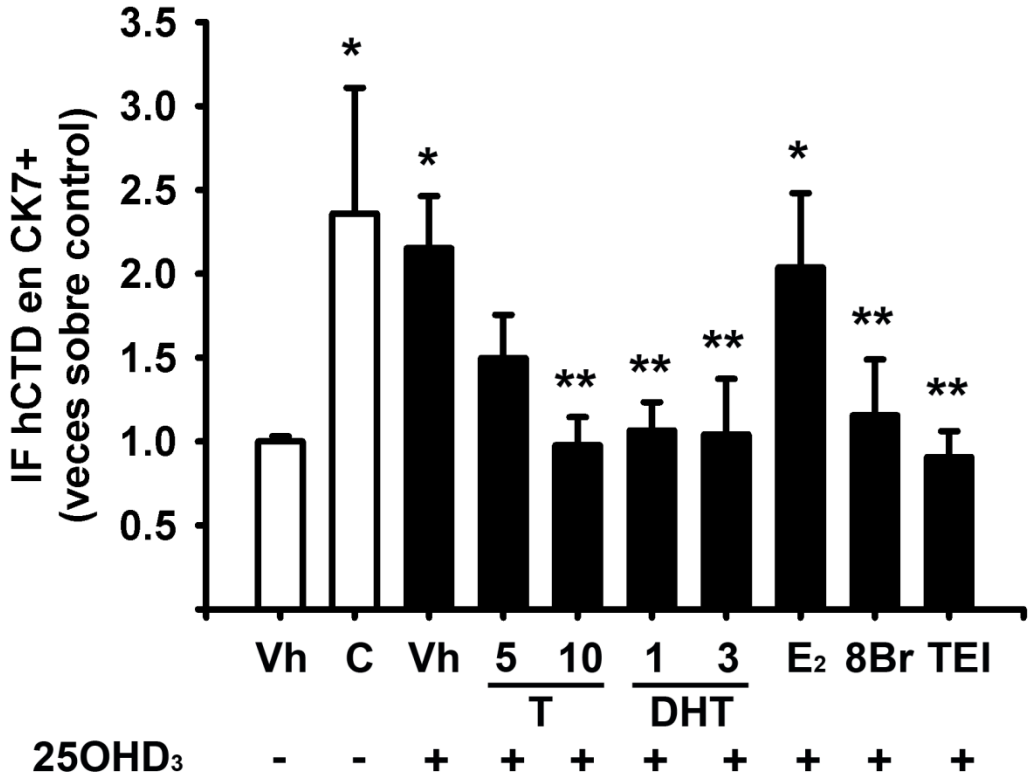

Figura 18. La inhibición del metabolismo de la vitamina D por los andrógenos resulta en la menor expresión de la proteína hCTD en los trofoblastos en cultivo.

Los trofoblastos se incubaron en presencia de testosterona (T, 5 o 10 nM), DHT (1 o 3 nM), estradiol (E_2, 10 nM) u 8Br-AMPc (8Br, 0.6 mM) durante 24 horas. Posteriormente las células se incubaron sin (barras blancas) o con $25OHD_3$ (barras negras), calcitriol (C, 10 nM) o TEI-9647 (TEI, 1 µM) en presencia de GolgiStop® durante 15 h. La expresión de hCTD se analizó por citometría de flujo en las células citoqueratina 7 positivas (CK-7+). El valor de la intensidad de fluorescencia (IF) en las células tratadas con vehículo en ausencia de $25OHD_3$ (Vh barra blanca) se estableció como 1, y los demás datos se normalizaron contra éste. Cada barra representa el promedio de al menos 4 cultivos de trofoblasto, excepto para 8Br y TEI. Los datos se presentan como promedio ± EE. * $P < 0.05$ vs vehículo sin $25OHD_3$ (Vh barra blanca); ** $P < 0.05$ vs vehículo con $25OHD_3$ (Vh barra negra).

6.5. Regulación transcripcional de hCTD por los metabolitos de la vitamina D y los andrógenos

Para corroborar el efecto transcripcional del calcitriol endógeno y exógeno sobre la expresión génica de hCTD y para descartar la posible regulación transcripcional directa de los andrógenos o del 8Br-AMPc sobre este péptido antimicrobiano, decidimos evaluar esta posibilidad mediante estudios de PCR en tiempo real. Los resultados que se muestran en la figura 19 confirman el robusto estímulo en la expresión génica de hCTD después de incubar a las células con calcitriol o con $25OHD_3$. Por otra parte, el tratamiento con testosterona o DHT no modificó de manera directa la expresión de hCTD, por lo que se puede descartar que los andrógenos regulen por sí mismos la expresión génica de este péptido. Interesantemente, la incubación con 8Br-AMPc inhibió significativamente la expresión de hCTD.

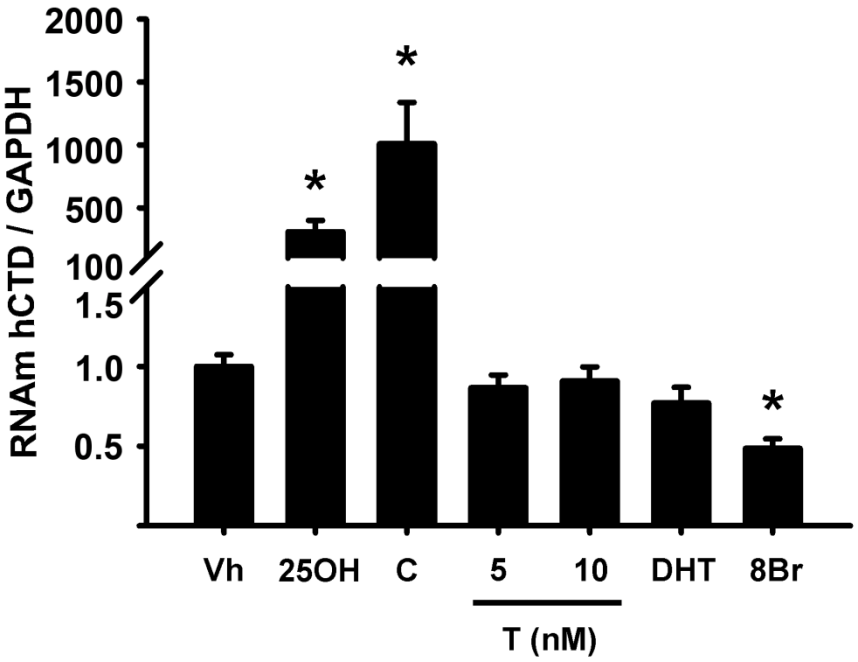

Figura 19. El calcitriol y $25OHD_3$, pero no los andrógenos, modifican la expresión génica placentaria de hCTD.

Se expuso a los trofoblastos en cultivo con $25OHD_3$ (25OH, 1 µM), calcitriol (C, 10 nM), testosterona (T 5 o 10 nM), DHT 1nM u 8Br-AMPc (8Br, 0,6 mM) durante 24 horas. Mediante qPCR se evaluó la expresión génica de hCTD. Los datos se normalizaron respecto a la expresión de GAPDH. Se le asignó al vehículo el valor de 1 y los demás datos se ajustaron respecto a éste. Los datos se presentan como promedio $\pm$ EE de n $\geq$ 3. *$P < 0.05$ *vs*. vehículo.

6.6. Diferencias dependientes del sexo en la expresión de hCTD y las enzimas placentarias que metabolizan a la vitamina D

Con la finalidad de evaluar la posible regulación dependiente del sexo de los citocromos que metabolizan a la vitamina D, decidimos analizar la expresión de CYP27B1 y CYP24A1 en el tejido placentario recolectado inmediatamente después del alumbramiento. Se demostró que los cotiledones placentarios procedentes de embarazos con recién nacidos hombres presentan significativamente menor expresión génica de CYP27B1 en comparación con los procedentes de recién nacidos mujeres (Figura 20A). Sin embargo, la expresión de CYP24A1 no mostró cambios significativos entre los cotiledones provenientes de ambos sexos (Figura 20B). Para investigar la posible disparidad basada en el sexo en la regulación de la respuesta inmune modulada por la vitamina D, se analizó la expresión de hCTD en el tejido placentario. De manera semejante a los resultados observados en el RNAm de CYP27B1, se demostró que la expresión génica de hCTD se encuentra significativamente reducida en los cotiledones placentarios masculinos en comparación con los cotiledones femeninos ($P < 0.01$) (Figura 20C).

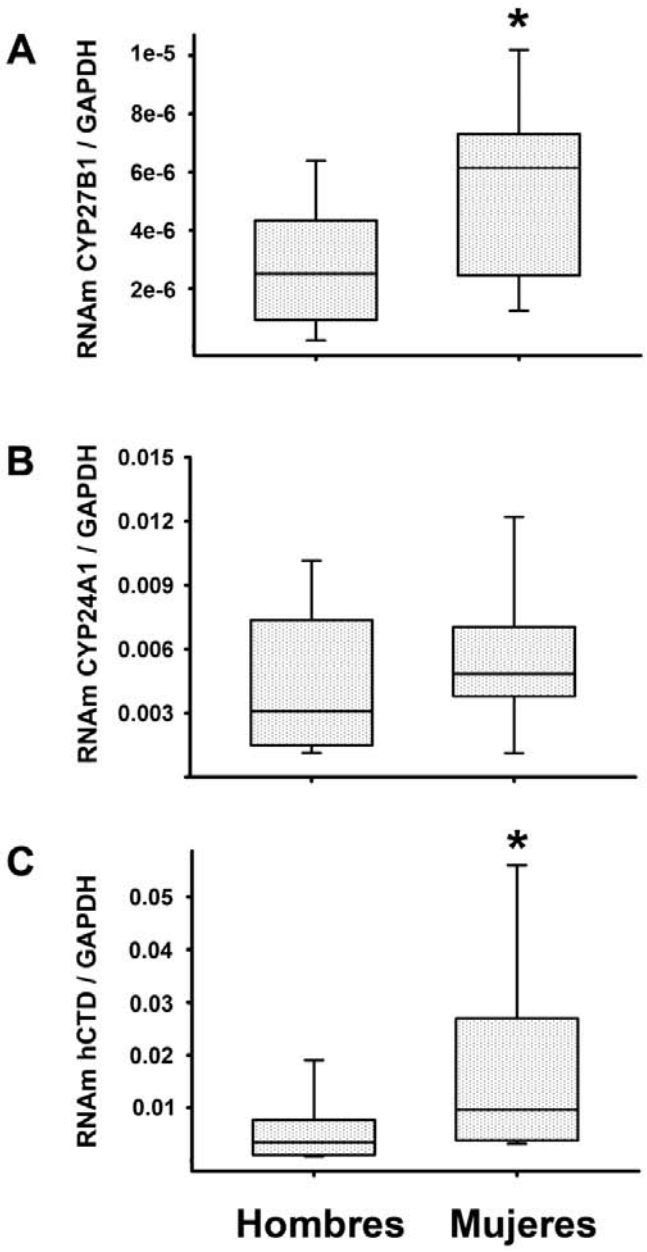

Figura 20. Expresión génica de CYP27B1 y hCTD dependiente del sexo en cotiledones placentarios.

Expresión génica de A) CYP27B1, B) CYP24A1 y C) hCTD en cotiledones placentarios según sexo del recién nacido. * $P < 0.05$ *vs.* hombres. n = 9. Se normalizó la expresión génica *vs*. GAPDH. Los datos se presentan como gráficas de cajas y bigotes (en la caja se indica la mediana y los percentiles 25% y 75%; en los bigotes se indican los percentiles 5% y 95%).

Adicionalmente, se exploró la posibilidad de evaluar si estas diferencias en la expresión de hCTD podrían estar relacionadas con la expresión diferencial del receptor de la vitamina D a nivel proteína. Esto dado que este receptor es el responsable de mediar la actividad biológica del calcitriol, y por ende, es el responsable de la actividad transcripcional mediada por el calcitriol. Como se observa en el Western blot de la Figura 21, no se observó diferencia en la expresión del VDR en los cotiledones placentarios masculinos *versus* los femeninos (P = 0.373).

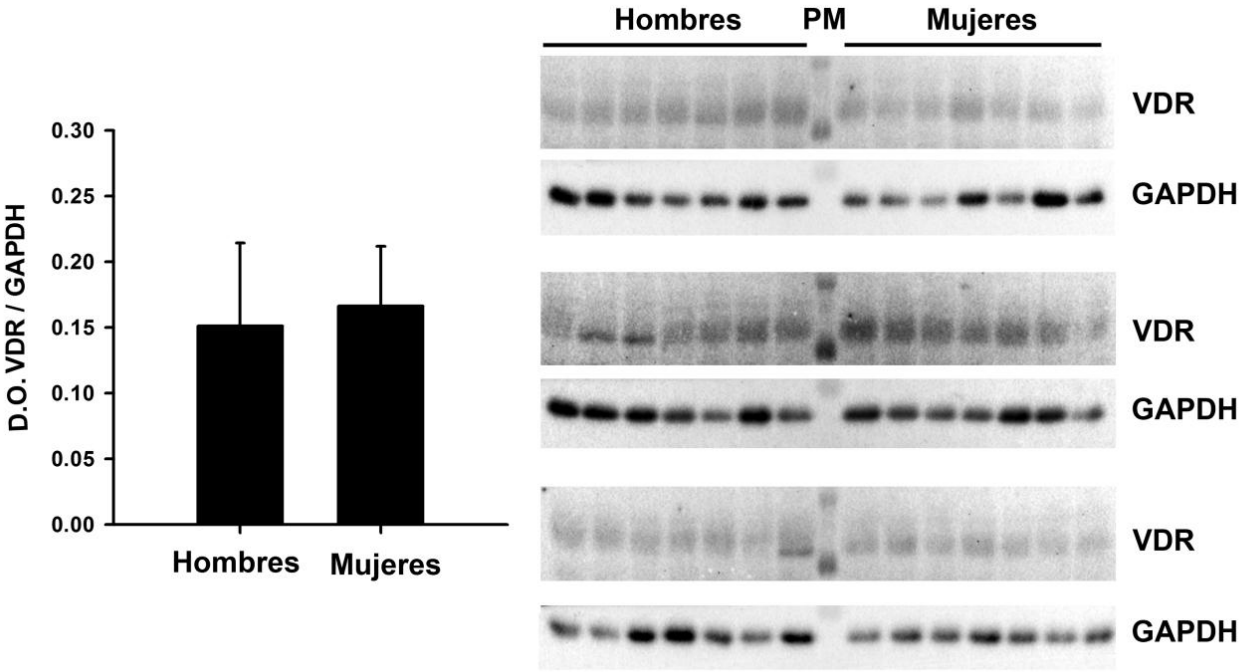

Figura 21. La expresión de VDR es similar entre los cotiledones placentarios masculinos y femeninos.

Por WB se observó la abundancia del VDR. A la izquierda se presenta el promedio de las densitometrías obtenidas en cada sexo. Las barras representan el promedio ± EE. A la derecha se muestran los WB del VDR en todos los cotiledones analizados. n = 21 hombres y 21 mujeres. PM: peso molecular.

6.7. Concentración de hCTD, $25OHD_3$, E_2 y testosterona en el suero de cordón umbilical de recién nacidos hombres y mujeres.

Siguiendo con la línea de las observaciones anteriores, se evaluó la concentración de hCTD, $25OHD_3$, testosterona y estradiol en el suero de la vena umbilical en recién nacidos (RN) hombres y mujeres (Tabla 4). Coincidentemente con lo demostrado en la figura 17 y 19C, se encontró que la concentración de hCTD se encuentra significativamente reducida en el suero de los RN hombres en comparación con el suero de las mujeres (P = 0.002). Como era esperado, la concentración de testosterona es mayor en el suero de cordón umbilical de RN hombres *versus* mujeres (P = 0.007). Sin embargo, no se encontraron diferencias significativas en la concentración de estradiol ni de $25OHD_3$ entre los grupos estudiados (Tabla 4).

Tabla 4. Concentración de hormonas esteroideas y hCTD en el suero de cordón umbilical según sexo del recién nacido.

Metabolito	RN hombres	RN mujeres	*P*
hCTD (ng/mL)	143.5 ± 117.7	342.2 ± 205.7	[a] 0.002
Testosterona (nmol/L)	5.5 (4.5 – 6.9)	4.2 (3.5 – 5.2)	[b] 0.007
Estradiol (ng/mL)	8.6 ± 5.1	11.2 ± 4.6	[a] 0.112
$25OHD_3$ (ng/mL)	16.4 ± 5.9	19.0 ± 6.0	[a] 0.250

Las diferencias estadísticas se evaluaron por [a] la prueba *t* de Student o [b] la prueba *U* de Mann-Whitney. Correspondientemente, los datos se presentan como promedio ± DE o mediana (percentiles 25% – 75%), respectivamente. n = 17 muestras de cada sexo. RN: recién nacidos.

7. DISCUSIÓN

De manera general, es bien conocido que la testosterona favorece la atenuación de la respuesta inmune en los hombres [4, 79], mientras que los estrógenos ayudan a contrarrestar las infecciones. Las evidencias actuales sugieren que estos cambios observados en la respuesta inmune podrían estar relacionados con el metabolismo diferencial de la vitamina D. existe evidencia de que los estrógenos, a través de mecanismos dependientes de su receptor, disminuyen la expresión génica de CYP24A1 mientras que inducen la de CYP27B1, lo que reduce el catabolismo del calcitriol a la vez que incrementa su síntesis [80, 81], lo que conlleva al desarrollo de una respuesta inmunológica innata más fuerte en las mujeres [80, 82, 83]. Sin embargo, hasta el momento se desconoce si la testosterona puede afectar un blanco inmunológico dependiente de la vitamina D en la placenta humana. En el presente estudio se demostró que la testosterona disminuye la expresión génica de CYP27B1 a la par que induce la de CYP24A1 a través de un mecanismo independiente de AMPc que se acompaña de la fosforilación de CREB, lo que resulta en la inhibición de la síntesis de hCTD dependiente de la VD. Hasta donde sabemos, éste es el primer reporte que demuestra que la testosterona, a través de un mecanismo no clásico, modula genes que contienen CREs en la placenta humana, de manera similar a como se ha reportado en células de Sertoli [46]. Éste es, probablemente, un efecto mayoritariamente androgénico, dado que la activación de CREB y la regulación de los citocromos fueron mimetizadas por la DHT, un andrógeno no aromatizable, y antagonizadas por la flutamida, un bloqueador del AR. En éste y en otros estudios

se ha observado que tanto el estradiol como los andrógenos activan rápidamente a CREB [46, 84]; sin embargo, aquí se observó por primera vez que la señalización de esta vía culminó en la inhibición de la expresión de hCTD dependiente de la VD.

En la práctica clínica es común que se utilice la concentración de los andrógenos en la sangre de cordón umbilical como marcador de la exposición fetal a los andrógenos. Normalmente, la concentración de testosterona total se encuentra significativamente aumentada en las muestras de sangre umbilical de los hombres *vs.* las mujeres [85], de manera semejante a como se observó en este proyecto. Como referencia, en el suero de los recién nacidos hombres la concentración de testosterona total varía entre 2.4 y 13.8 nM, mientras que en las recién nacidas mujeres la concentración se encuentra entre los 0.69 y 2.77 nM [86]. Considerando esto, en este trabajo hipotetizamos que la síntesis de calcitriol placentario sería menor en los recién nacidos masculinos en comparación con las mujeres. Nuestros resultados apoyaron esta hipótesis, ya que encontramos menor expresión de CYP27B1 y de hCTD en las placentas del sexo masculino *vs.* femenino. Con base en estas observaciones, diseñamos un bioensayo *in vitro* para demostrar el efecto de la testosterona sobre la regulación diferencial de los citocromos CYP27B1 y CYP24A1 que reflejara el metabolismo de la $25OHD_3$ hacia $1,25(OH)_2D_3$ y su impacto biológico en la expresión de hCTD. Tal como se explicó anteriormente, se eligió a la hCTD debido a su robusta inducción transcripcional dependiente del VDR, y por tanto del calcitriol, en la placenta humana a la vez que no es estimulada por mediadores inflamatorios [29, 30, 63, 87]. Los resultados obtenidos por citometría de flujo mostraron que los trofoblastos

expuestos previamente a los andrógenos, presentaron menor expresión proteica de hCTD, lo que sugiere menor tasa de bioconversión de $25OHD_3$ en calcitriol bioactivo. Además, la concentración de hCTD que se encontró en los trofoblastos tratados con andrógenos y $25OHD_3$ fue semejante a la que se encontró en los trofoblastos sin estímulo de $25OHD_3$, lo que fortalece la hipótesis del efecto represor de los andrógenos sobre la biosíntesis de calcitriol. Por otra parte, la observación de que el 8Br-AMPc disminuyó la expresión placentaria basal y dependiente de VD podría representar un posible mecanismo de acción de los microorganismos para facilitar la infección. Por ejemplo, diversas moléculas derivadas de patógenos pueden actuar a través de la vía AMPc/PKA e inhibir directamente la expresión de hCTD con lo que se favorece la colonización e infección bacteriana, tal como se ha demostrado en células del epitelio intestinal [88].

Para investigar el posible dimorfismo sexual placentario en la regulación de la catelicidina promovida por la VD, adicionalmente estudiamos la concentración sérica de hCTD en el cordón umbilical de recién nacidos de ambos sexos. Como se esperaba, la concentración de hCTD en el suero de cordón de los hombres se encontró significativamente reducida en comparación con el de las mujeres. Estos resultados apoyan nuestra hipótesis experimental de que las placentas masculinas producen menos hCTD debido a una disminución en la síntesis y/o por un aumento en la degradación del calcitriol. El hallazgo de que no hay diferencias ligadas al sexo en la concentración de $25OHD_3$ en el suero de cordón umbilical sugiere que bajo las mismas condiciones de disponibilidad de este precursor, la producción del calcitriol depende más de la expresión local de los reguladores de

CYP27B1 y CYP24A1, lo que coincide con la tasa observada de más del doble en la relación CYP24A1/CYP27B1 en las placentas masculinas. Aunque se conoce que la concentración de $25OHD_3$ en el suero umbilical es menor que la observada en el suero materno [89, 90], las concentraciones observadas en este proyecto indican deficiencia de VD, lo que podría representar una señal de alarma del estado nutricio de VD en las mujeres embarazadas de la ciudad de México.

Además de mostrar diferencias ligadas al sexo en la producción placentaria de hCTD, en conjunto estos resultados sugieren que aquellas patologías del embarazo que cursan con concentraciones anormalmente elevadas de andrógenos pueden acompañarse de alteraciones en el metabolismo de la VD y eventualmente esto podría resultar en la insuficiente producción de calcitriol placentario. Al respecto se ha descrito que las mujeres con preeclampsia, una condición hipertensiva del embarazo, presentan alta concentración de testosterona [91-94] y aumento en la expresión placentaria del AR [95, 96]. Interesantemente, en estas mujeres también se ha observado la disminución en la producción de calcitriol placentario secundario a la menor expresión de CYP27B1 en los sinciciotrofoblastos [97, 98]. Además, la preeclampsia se ha asociado con bajos niveles circulantes de calcitriol tanto en el compartimento materno como en el fetal [98-100]. Dado que al remover la placenta disminuyen considerablemente los síntomas de la preeclampsia, se cree que los principales factores desencadenantes de esta patología residen precisamente en este órgano como resultado de la desregulación de los procesos placentarios normales. Los resultados de esta tesis aportan información que puede ayudar a comprender, al menos en parte, cómo algunos factores producidos a nivel de la placenta impactan

en ciertas patologías gestacionales, ya que es bien conocida la capacidad del calcitriol de regular la presión arterial, la hormonogénesis y la respuesta inmunológica placentaria.

Al respecto, la insuficiencia de VD también se ha observado en mujeres con síndrome de ovario poliquístico (PCOS), otra patología asociada a hiperandrogenemia [101, 102]. Interesantemente, la prevalencia de corioamnionitis debido a infecciones bacterianas es significativamente mayor en embarazadas con PCOS en comparación con aquellas con embarazos normoevolutivos [103]. Esta observación, con base en nuestros hallazgos, podría estar relacionada con la alteración del metabolismo placentario de la VD y, consecuentemente, con la insuficiente producción de péptidos antimicrobianos, lo que merece ser explorado en un futuro.

Hasta donde tenemos conocimiento, ésta es la primera vez que se describe que la hCTD, importante marcador de la respuesta inmune innata, es indirectamente regulada por la testosterona. Sin embargo, existe información adicional que sugiere un papel inhibidor de los andrógenos sobre la hCTD, como la mayor concentración de este péptido en la saliva de mujeres *vs*. hombres [104] o los hallazgos en ratones que demuestran que los andrógenos atenúan la producción y el metabolismo de la VD en la piel [105]. Cabe mencionar que los elementos de respuesta a la vitamina D en el promotor del gen de hCTD son específicos para primates y por tanto no se encuentran en ratones o en mamíferos inferiores [32], lo que apremia la necesidad de realizar estudios en tejidos humanos.

Considerando los efectos potenciales del calcitriol sobre la fisiología placentaria y las defensas inmunes, nosotros creemos que los resultados aquí presentados pueden explicar en parte porqué los embarazos que llevan fetos hombres son más susceptibles de experimentar peores pronósticos asociados a un ambiente con defensas inmunes disminuidas. En este sentido, se puede tomar en consideración algunas evidencias complementarias del papel de los esteroides sexuales en el *imprinting* del genoma fetal y la programación de la respuesta inmune en el adulto, particularmente en condiciones que afectan la adecuada biodisponibilidad de la VD durante el embarazo [106, 107]. Dado que los factores del ambiente prenatal, incluida la VD, impactan en la salud a largo plazo del individuo mediante eventos de programación intrauterina [69], nosotros creemos que se debe tener especial cuidado para mantener la suficiencia de calcitriol *in útero*, especialmente en los embarazos que llevan un feto hombre o en aquellos con alguna condición que afecte el metabolismo de la VD.

8. CONCLUSIONES

En este estudio se evaluó la hipótesis de si la testosterona regular el metabolismo placentario de la VD y, de esta forma, impacta en la respuesta inmune innata dependiente de calcitriol. Nuestras conclusiones son las siguientes:

1) Se requiere del receptor de andrógenos para que la testosterona pueda activar a CREB de manera independiente del AMPc en la placenta humana;

2) La testosterona estimula la expresión placentaria de CYP24A1 a la par que inhibe la expresión de CYP27B1;

3) La expresión placentaria de hCTD dependiente de la vitamina D se reduce ante la exposición a andrógenos;

4) Los recién nacidos hombres presentan menor concentración de hCTD en el suero venoso de cordón umbilical comparado con el de mujeres.

En conjunto este estudio apoya y sustenta, al menos en parte, uno de los mecanismos involucrados en la mayor susceptibilidad de los recién nacidos hombres ante el desarrollo de infecciones debido a diferencias en el metabolismo de la vitamina D atribuibles al sexo, con lo cual se resalta el dimorfismo sexual placentario en la respuesta a la infección dependiente de la vitamina D (Figura 22).

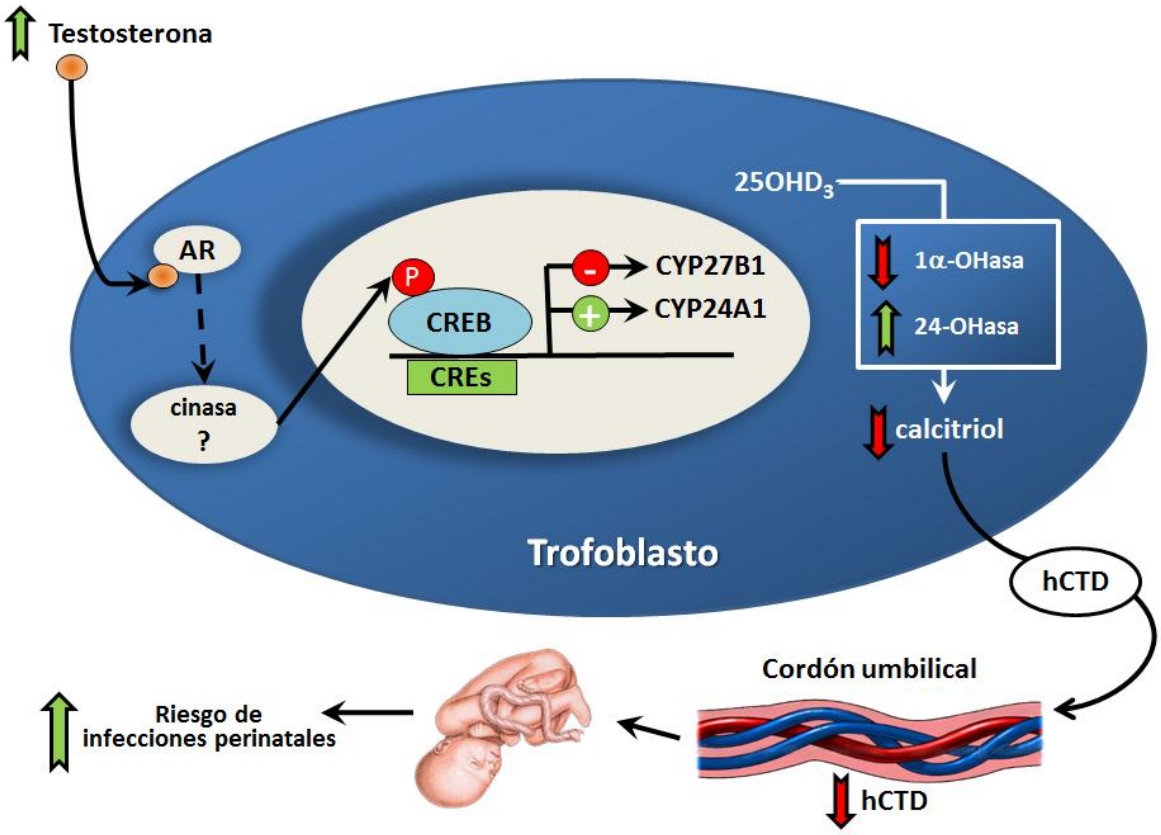

Figura 22. Propuesta de la regulación del metabolismo de la VD y la hCTD por la testosterona en la placenta humana.

La testosterona atraviesa libremente la membrana plasmática y se une al AR citoplasmático. Posteriormente, por una vía aún desconocida alguna cinasa fosforila a CREB, lo que afecta la expresión de aquellos genes que poseen CREs en sus promotores, como CYP27B1 y CYP24A1. Esto podría resultar en la disminución de la síntesis de calcitriol placentario debido al incremento en su degradación y/o a la menor bioconversión de $25OHD_3$ a $1,25(OH)_2D_3$. Esto podría reducir la expresión placentaria de hCTD, lo cual proporcionaría menos defensas innatas al feto a través de la vena umbilical, colocando al feto en un ambiente de mayor riesgo ante las infecciones perinatales.

9. LITERATURA CITADA

1. Clarke J. Observations on some causes of excess mortality of males above that of females. *Philos Trans R Soc Lond* 1786,**76**:349–364.
2. Mathews TJ, MacDorman MF, Thoma ME. Infant mortality statistics from the 2013 period linked birth/infant death data set. *National Vital Statistics Reports* 2015,**64**:1-29.
3. Lawn JE, Cousens S, Zupan J. 4 million neonatal deaths: when? Where? Why? *Lancet* 2005,**365**:891-900.
4. Klein SL. The effects of hormones on sex differences in infection: from genes to behavior. *Neurosci Biobehav Rev* 2000,**24**:627-638.
5. Angele MK, Pratschke S, Hubbard WJ, Chaudry IH. Gender differences in sepsis: cardiovascular and immunological aspects. *Virulence* 2014,**5**:12-19.
6. Sakiani S, Olsen NJ, Kovacs WJ. Gonadal steroids and humoral immunity. *Nat Rev Endocrinol* 2013,**9**:56-62.
7. Hunt JS. Stranger in a strange land. *Immunol Rev* 2006,**213**:36-47.
8. Desale M, Thinkhamrop J, Lumbiganon P, Qazi S, Anderson J. Ending preventable maternal and newborn deaths due to infection. *Best Pract Res Clin Obstet Gynaecol* 2016,**36**:116-130.
9. Wu X, Jin LP, Yuan MM, Zhu Y, Wang MY, Li DJ. Human first-trimester trophoblast cells recruit CD56brightCD16- NK cells into decidua by way of expressing and secreting of CXCL12/stromal cell-derived factor 1. *J Immunol* 2005,**175**:61-68.
10. Diaz L, Noyola-Martinez N, Barrera D, Hernandez G, Avila E, Halhali A, *et al.* Calcitriol inhibits TNF-alpha-induced inflammatory cytokines in human trophoblasts. *J Reprod Immunol* 2009,**81**:17-24.
11. Noyola-Martinez N, Diaz L, Avila E, Halhali A, Larrea F, Barrera D. Calcitriol downregulates TNF-alpha and IL-6 expression in cultured placental cells from preeclamptic women. *Cytokine* 2013,**61**:245-250.
12. Piccinni MP, Giudizi MG, Biagiotti R, Beloni L, Giannarini L, Sampognaro S, *et al.* Progesterone favors the development of human T helper cells producing Th2-type cytokines and promotes both IL-4 production and membrane CD30 expression in established Th1 cell clones. *J Immunol* 1995,**155**:128-133.
13. Kumar SK, Bhat BV. Distinct mechanisms of the newborn innate immunity. *Immunol Lett* 2016,**173**:42-54.
14. Zhang C, Deng X, Zhang X, Pan Z, Zhao W, Zhang Y, *et al.* Association between Serum TNF-alpha Levels and Recurrent Spontaneous Miscarriage: A Meta-analysis. *Am J Reprod Immunol* 2016,**75**:86-93.
15. Agerberth B, Charo J, Werr J, Olsson B, Idali F, Lindbom L, *et al.* The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations. *Blood* 2000,**96**:3086-3093.
16. Frohm M, Agerberth B, Ahangari G, Stahle-Backdahl M, Liden S, Wigzell H, *et al.* The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders. *J Biol Chem* 1997,**272**:15258-15263.

17. Yim S, Dhawan P, Ragunath C, Christakos S, Diamond G. Induction of cathelicidin in normal and CF bronchial epithelial cells by 1,25-dihydroxyvitamin D(3). *J Cyst Fibros* 2007,**6**:403-410.
18. Hase K, Eckmann L, Leopard JD, Varki N, Kagnoff MF. Cell differentiation is a key determinant of cathelicidin LL-37/human cationic antimicrobial protein 18 expression by human colon epithelium. *Infect Immun* 2002,**70**:953-963.
19. Frohm Nilsson M, Sandstedt B, Sorensen O, Weber G, Borregaard N, Stahle-Backdahl M. The human cationic antimicrobial protein (hCAP18), a peptide antibiotic, is widely expressed in human squamous epithelia and colocalizes with interleukin-6. *Infect Immun* 1999,**67**:2561-2566.
20. Armogida SA, Yannaras NM, Melton AL, Srivastava MD. Identification and quantification of innate immune system mediators in human breast milk. *Allergy Asthma Proc* 2004,**25**:297-304.
21. Murakami M, Dorschner RA, Stern LJ, Lin KH, Gallo RL. Expression and secretion of cathelicidin antimicrobial peptides in murine mammary glands and human milk. *Pediatr Res* 2005,**57**:10-15.
22. King AE, Paltoo A, Kelly RW, Sallenave JM, Bocking AD, Challis JR. Expression of natural antimicrobials by human placenta and fetal membranes. *Placenta* 2007,**28**:161-169.
23. Yamasaki K, Schauber J, Coda A, Lin H, Dorschner RA, Schechter NM*, et al.* Kallikrein-mediated proteolysis regulates the antimicrobial effects of cathelicidins in skin. *FASEB J* 2006,**20**:2068-2080.
24. Sorensen OE, Follin P, Johnsen AH, Calafat J, Tjabringa GS, Hiemstra PS*, et al.* Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. *Blood* 2001,**97**:3951-3959.
25. Lee CC, Sun Y, Qian S, Huang HW. Transmembrane pores formed by human antimicrobial peptide LL-37. *Biophys J* 2011,**100**:1688-1696.
26. Tjabringa GS, Ninaber DK, Drijfhout JW, Rabe KF, Hiemstra PS. Human cathelicidin LL-37 is a chemoattractant for eosinophils and neutrophils that acts via formyl-peptide receptors. *Int Arch Allergy Immunol* 2006,**140**:103-112.
27. Tokumaru S, Sayama K, Shirakata Y, Komatsuzawa H, Ouhara K, Hanakawa Y*, et al.* Induction of keratinocyte migration via transactivation of the epidermal growth factor receptor by the antimicrobial peptide LL-37. *J Immunol* 2005,**175**:4662-4668.
28. Rosenfeld Y, Papo N, Shai Y. Endotoxin (lipopolysaccharide) neutralization by innate immunity host-defense peptides. Peptide properties and plausible modes of action. *J Biol Chem* 2006,**281**:1636-1643.
29. Liu N, Kaplan AT, Low J, Nguyen L, Liu GY, Equils O*, et al.* Vitamin D induces innate antibacterial responses in human trophoblasts via an intracrine pathway. *Biol Reprod* 2009,**80**:398-406.
30. Wang TT, Nestel FP, Bourdeau V, Nagai Y, Wang Q, Liao J*, et al.* Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. *J Immunol* 2004,**173**:2909-2912.
31. Evans KN, Nguyen L, Chan J, Innes BA, Bulmer JN, Kilby MD*, et al.* Effects of 25-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 on cytokine production by human decidual cells. *Biol Reprod* 2006,**75**:816-822.
32. Gombart AF, Borregaard N, Koeffler HP. Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-

regulated in myeloid cells by 1,25-dihydroxyvitamin D3. *FASEB J* 2005,**19**:1067-1077.
33. Diaz L, Sanchez I, Avila E, Halhali A, Vilchis F, Larrea F. Identification of a 25-hydroxyvitamin D3 1alpha-hydroxylase gene transcription product in cultures of human syncytiotrophoblast cells. *J Clin Endocrinol Metab* 2000,**85**:2543-2549.
34. Bikle D. Highlights from the 16th Vitamin D Workshop, San Francisco, CA, June 11-14, 2013. *J Steroid Biochem Mol Biol* 2014,**144 Pt A**:1-4.
35. Wacker M, Holick MF. Sunlight and Vitamin D: A global perspective for health. *Dermatoendocrinol* 2013,**5**:51-108.
36. Johnson JA, Grande JP, Roche PC, Kumar R. Immunohistochemical detection and distribution of the 1,25-dihydroxyvitamin D3 receptor in rat reproductive tissues. *Histochem Cell Biol* 1996,**105**:7-15.
37. Shahbazi M, Jeddi-Tehrani M, Zareie M, Salek-Moghaddam A, Akhondi MM, Bahmanpoor M*, et al.* Expression profiling of vitamin D receptor in placenta, decidua and ovary of pregnant mice. *Placenta* 2011,**32**:657-664.
38. Zarnani AH, Shahbazi M, Salek-Moghaddam A, Zareie M, Tavakoli M, Ghasemi J*, et al.* Vitamin D3 receptor is expressed in the endometrium of cycling mice throughout the estrous cycle. *Fertil Steril* 2010,**93**:2738-2743.
39. Halhali A, Acker GM, Garabedian M. 1,25-Dihydroxyvitamin D3 induces in vivo the decidualization of rat endometrial cells. *J Reprod Fertil* 1991,**91**:59-64.
40. Barrera D, Avila E, Hernandez G, Halhali A, Biruete B, Larrea F*, et al.* Estradiol and progesterone synthesis in human placenta is stimulated by calcitriol. *J Steroid Biochem Mol Biol* 2007,**103**:529-532.
41. Barrera D, Avila E, Hernandez G, Mendez I, Gonzalez L, Halhali A*, et al.* Calcitriol affects hCG gene transcription in cultured human syncytiotrophoblasts. *Reprod Biol Endocrinol* 2008,**6**:3.
42. Dusso AS, Brown AJ, Slatopolsky E. Vitamin D. *Am J Physiol Renal Physiol* 2005,**289**:F8-28.
43. Pitkin RM. Endocrine regulation of calcium homeostasis during pregnancy. *Clin Perinatol* 1983,**10**:575-592.
44. Halhali A, Diaz L, Sanchez I, Garabedian M, Bourges H, Larrea F. Effects of IGF-I on 1,25-dihydroxyvitamin D(3) synthesis by human placenta in culture. *Mol Hum Reprod* 1999,**5**:771-776.
45. Avila E, Diaz L, Barrera D, Halhali A, Mendez I, Gonzalez L*, et al.* Regulation of Vitamin D hydroxylases gene expression by 1,25-dihydroxyvitamin D3 and cyclic AMP in cultured human syncytiotrophoblasts. *J Steroid Biochem Mol Biol* 2007,**103**:90-96.
46. Fix C, Jordan C, Cano P, Walker WH. Testosterone activates mitogen-activated protein kinase and the cAMP response element binding protein transcription factor in Sertoli cells. *Proc Natl Acad Sci U S A* 2004,**101**:10919-10924.
47. Kim J, Amano O, Wakayama T, Takahagi H, Iseki S. The role of cyclic AMP response element-binding protein in testosterone-induced differentiation of granular convoluted tubule cells in the rat submandibular gland. *Arch Oral Biol* 2001,**46**:495-507.
48. Shihan M, Bulldan A, Scheiner-Bobis G. Non-classical testosterone signaling is mediated by a G-protein-coupled receptor interacting with Gnalpha11. *Biochim Biophys Acta* 2014,**1843**:1172-1181.

49. Nguyen TV, Yao M, Pike CJ. Dihydrotestosterone activates CREB signaling in cultured hippocampal neurons. *Brain Res* 2009,**1298**:1-12.
50. Altarejos JY, Montminy M. CREB and the CRTC co-activators: sensors for hormonal and metabolic signals. *Nat Rev Mol Cell Biol* 2011,**12**:141-151.
51. Kong XF, Zhu XH, Pei YL, Jackson DM, Holick MF. Molecular cloning, characterization, and promoter analysis of the human 25-hydroxyvitamin D3-1alpha-hydroxylase gene. *Proc Natl Acad Sci U S A* 1999,**96**:6988-6993.
52. Zierold C, Darwish HM, DeLuca HF. Identification of a vitamin D-response element in the rat calcidiol (25-hydroxyvitamin D3) 24-hydroxylase gene. *Proc Natl Acad Sci U S A* 1994,**91**:900-902.
53. Green BB, Marsit CJ. Select Prenatal Environmental Exposures and Subsequent Alterations of Gene-Specific and Repetitive Element DNA Methylation in Fetal Tissues. *Curr Environ Health Rep* 2015,**2**:126-136.
54. Januar V, Desoye G, Novakovic B, Cvitic S, Saffery R. Epigenetic regulation of human placental function and pregnancy outcome: considerations for causal inference. *Am J Obstet Gynecol* 2015,**213**:S182-196.
55. Clifton VL. Review: Sex and the human placenta: mediating differential strategies of fetal growth and survival. *Placenta* 2010,**31 Suppl**:S33-39.
56. Gabory A, Roseboom TJ, Moore T, Moore LG, Junien C. Placental contribution to the origins of sexual dimorphism in health and diseases: sex chromosomes and epigenetics. *Biol Sex Differ* 2013,**4**:5.
57. Challis J, Newnham J, Petraglia F, Yeganegi M, Bocking A. Fetal sex and preterm birth. *Placenta* 2013,**34**:95-99.
58. Elsmen E, Kallen K, Marsal K, Hellstrom-Westas L. Fetal gender and gestational-age-related incidence of pre-eclampsia. *Acta Obstet Gynecol Scand* 2006,**85**:1285-1291.
59. Vatten LJ, Skjaerven R. Offspring sex and pregnancy outcome by length of gestation. *Early Hum Dev* 2004,**76**:47-54.
60. Goldenberg RL, Andrews WW, Faye-Petersen OM, Goepfert AR, Cliver SP, Hauth JC. The Alabama Preterm Birth Study: intrauterine infection and placental histologic findings in preterm births of males and females less than 32 weeks. *Am J Obstet Gynecol* 2006,**195**:1533-1537.
61. Sood R, Zehnder JL, Druzin ML, Brown PO. Gene expression patterns in human placenta. *Proc Natl Acad Sci U S A* 2006,**103**:5478-5483.
62. NCHS. Infant Mortality Statistics from the Period Linked Birth/Infant Death Data Set. In: *year*. August 6, 2015 ed: Centers for Disease Control and Prevention; 2016.
63. Olmos-Ortiz A, Noyola-Martinez N, Barrera D, Zaga-Clavellina V, Avila E, Halhali A, *et al.* IL-10 inhibits while calcitriol reestablishes placental antimicrobial peptides gene expression. *J Steroid Biochem Mol Biol* 2015,**148**:187-193.
64. Barrera D, Noyola-Martinez N, Avila E, Halhali A, Larrea F, Diaz L. Calcitriol inhibits interleukin-10 expression in cultured human trophoblasts under normal and inflammatory conditions. *Cytokine* 2012,**57**:316-321.
65. Upala S, Sanguankeo A, Permpalung N. Significant association between vitamin D deficiency and sepsis: a systematic review and meta-analysis. *BMC Anesthesiol* 2015,**15**:84.
66. Flores M, Sánchez-Romero LM, Macías N, Lozada A, Díaz E, Barquera S. Concentraciones séricas de vitamina D en niños, adolescentes y adultos mexicanos.

Resultados de la ENSANUT 2006. *Cuernavaca, México: Instituto Nacional de Salud Pública.* 2011.
67. Lu M, Xu Y, Lv L, Zhang M. Association between vitamin D status and the risk of gestational diabetes mellitus: a meta-analysis. *Arch Gynecol Obstet* 2016,**293**:959-966.
68. Qin LL, Lu FG, Yang SH, Xu HL, Luo BA. Does Maternal Vitamin D Deficiency Increase the Risk of Preterm Birth: A Meta-Analysis of Observational Studies. *Nutrients* 2016,**8**.
69. Olmos-Ortiz A, Avila E, Durand-Carbajal M, Diaz L. Regulation of calcitriol biosynthesis and activity: focus on gestational vitamin D deficiency and adverse pregnancy outcomes. *Nutrients* 2015,**7**:443-480.
70. Bodnar LM, Catov JM, Simhan HN, Holick MF, Powers RW, Roberts JM. Maternal vitamin D deficiency increases the risk of preeclampsia. *J Clin Endocrinol Metab* 2007,**92**:3517-3522.
71. Kliman HJ, Nestler JE, Sermasi E, Sanger JM, Strauss JF, 3rd. Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. *Endocrinology* 1986,**118**:1567-1582.
72. Graham CH, Hawley TS, Hawley RG, MacDougall JR, Kerbel RS, Khoo N*, et al.* Establishment and characterization of first trimester human trophoblast cells with extended lifespan. *Exp Cell Res* 1993,**206**:204-211.
73. Blaschitz A, Weiss U, Dohr G, Desoye G. Antibody reaction patterns in first trimester placenta: implications for trophoblast isolation and purity screening. *Placenta* 2000,**21**:733-741.
74. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD*, et al.* Measurement of protein using bicinchoninic acid. *Anal Biochem* 1985,**150**:76-85.
75. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987,**162**:156-159.
76. Chrambach A, Rodbard D. Polyacrylamide gel electrophoresis. *Science* 1971,**172**:440-451.
77. Olivares A, Mendez JP, Zambrano E, Cardenas M, Tovar A, Perera-Marin G*, et al.* Reproductive axis function and gonadotropin microheterogeneity in a male rat model of diet-induced obesity. *Gen Comp Endocrinol* 2010,**166**:356-364.
78. Castaneda-Delgado JE, Miranda-Castro NY, Gonzalez-Amaro R, Gonzalez-Curiel I, Montoya-Rosales A, Rivas-Calderon B*, et al.* Production of antimicrobial peptides is preserved in aging. *Clin Immunol* 2013,**148**:198-205.
79. Rettew JA, Huet-Hudson YM, Marriott I. Testosterone reduces macrophage expression in the mouse of toll-like receptor 4, a trigger for inflammation and innate immunity. *Biol Reprod* 2008,**78**:432-437.
80. Correale J, Ysrraelit MC, Gaitan MI. Gender differences in 1,25 dihydroxyvitamin D3 immunomodulatory effects in multiple sclerosis patients and healthy subjects. *J Immunol* 2010,**185**:4948-4958.
81. Lechner D, Bajna E, Adlercreutz H, Cross HS. Genistein and 17beta-estradiol, but not equol, regulate vitamin D synthesis in human colon and breast cancer cells. *Anticancer Res* 2006,**26**:2597-2603.

82. Pike JW, Spanos E, Colston KW, MacIntyre I, Haussler MR. Influence of estrogen on renal vitamin D hydroxylases and serum 1alpha,25-(OH)2D3 in chicks. *Am J Physiol* 1978,**235**:E338-343.
83. Cheema C, Grant BF, Marcus R. Effects of estrogen on circulating "free" and total 1,25-dihydroxyvitamin D and on the parathyroid-vitamin D axis in postmenopausal women. *J Clin Invest* 1989,**83**:537-542.
84. Aronica SM, Kraus WL, Katzenellenbogen BS. Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. *Proc Natl Acad Sci U S A* 1994,**91**:8517-8521.
85. Keelan JA, Mattes E, Tan H, Dinan A, Newnham JP, Whitehouse AJ, *et al.* Androgen concentrations in umbilical cord blood and their association with maternal, fetal and obstetric factors. *PLoS One* 2012,**7**:e42827.
86. Mayo Clinic. Mayo medical laboratories. Testosterone total, bioavailable and free Serum. Clinical and interpretative. In. http://www.mayomedicallaboratories.com/test-catalog/Clinical+and+Interpretive/83686: Mayo Foundation for Medical Education and Research; Consultado el 3 de diciembre de 2016.
87. Klaffenbach D, Friedrich D, Strick R, Strissel PL, Beckmann MW, Rascher W, *et al.* Contribution of different placental cells to the expression and stimulation of antimicrobial proteins (AMPs). *Placenta* 2011,**32**:830-837.
88. Chakraborty K, Ghosh S, Koley H, Mukhopadhyay AK, Ramamurthy T, Saha DR, *et al.* Bacterial exotoxins downregulate cathelicidin (hCAP-18/LL-37) and human beta-defensin 1 (HBD-1) expression in the intestinal epithelial cells. *Cell Microbiol* 2008,**10**:2520-2537.
89. Ron M, Menczel J, Schwartz L, Palti Z, Kidroni G. Vitamin D3 metabolites in amniotic fluid in relation with maternal and fetal sera in term pregnancies. *J Perinat Med* 1987,**15**:282-290.
90. Gupta MM, Kuppuswamy G, Subramanian AR. Transplacental transfer of 25-hydroxy-cholecalciferol. *Postgrad Med J* 1982,**58**:408-410.
91. Salamalekis E, Bakas P, Vitoratos N, Eleptheriadis M, Creatsas G. Androgen levels in the third trimester of pregnancy in patients with preeclampsia. *Eur J Obstet Gynecol Reprod Biol* 2006,**126**:16-19.
92. Carlsen SM, Romundstad P, Jacobsen G. Early second-trimester maternal hyperandrogenemia and subsequent preeclampsia: a prospective study. *Acta Obstet Gynecol Scand* 2005,**84**:117-121.
93. Troisi R, Potischman N, Roberts JM, Ness R, Crombleholme W, Lykins D, *et al.* Maternal serum oestrogen and androgen concentrations in preeclamptic and uncomplicated pregnancies. *Int J Epidemiol* 2003,**32**:455-460.
94. Tache V, Baer RJ, Currier RJ, Li CS, Towner D, Waetjen LE, *et al.* Population-based biomarker screening and the development of severe preeclampsia in California. *Am J Obstet Gynecol* 2014,**211**:377 e371-378.
95. Hsu TY, Lan KC, Tsai CC, Ou CY, Cheng BH, Tsai MY, *et al.* Expression of androgen receptor in human placentas from normal and preeclamptic pregnancies. *Taiwan J Obstet Gynecol* 2009,**48**:262-267.
96. Sathishkumar K, Balakrishnan M, Chinnathambi V, Chauhan M, Hankins GD, Yallampalli C. Fetal sex-related dysregulation in testosterone production and their

receptor expression in the human placenta with preeclampsia. *J Perinatol* 2012,**32**:328.
97. Diaz L, Arranz C, Avila E, Halhali A, Vilchis F, Larrea F. Expression and activity of 25-hydroxyvitamin D-1 alpha-hydroxylase are restricted in cultures of human syncytiotrophoblast cells from preeclamptic pregnancies. *J Clin Endocrinol Metab* 2002,**87**:3876-3882.
98. Halhali A, Diaz L, Barrera D, Avila E, Larrea F. Placental calcitriol synthesis and IGF-I levels in normal and preeclamptic pregnancies. *J Steroid Biochem Mol Biol* 2014,**144 Pt A**:44-49.
99. Halhali A, Tovar AR, Torres N, Bourges H, Garabedian M, Larrea F. Preeclampsia is associated with low circulating levels of insulin-like growth factor I and 1,25-dihydroxyvitamin D in maternal and umbilical cord compartments. *J Clin Endocrinol Metab* 2000,**85**:1828-1833.
100. Halhali A, Diaz L, Avila E, Ariza AC, Garabedian M, Larrea F. Decreased fractional urinary calcium excretion and serum 1,25-dihydroxyvitamin D and IGF-I levels in preeclampsia. *J Steroid Biochem Mol Biol* 2007,**103**:803-806.
101. Thys-Jacobs S, Donovan D, Papadopoulos A, Sarrel P, Bilezikian JP. Vitamin D and calcium dysregulation in the polycystic ovarian syndrome. *Steroids* 1999,**64**:430-435.
102. Li HW, Brereton RE, Anderson RA, Wallace AM, Ho CK. Vitamin D deficiency is common and associated with metabolic risk factors in patients with polycystic ovary syndrome. *Metabolism* 2011,**60**:1475-1481.
103. Koster MP, de Wilde MA, Veltman-Verhulst SM, Houben ML, Nikkels PG, van Rijn BB, *et al.* Placental characteristics in women with polycystic ovary syndrome. *Hum Reprod* 2015,**30**:2829-2837.
104. Davidopoulou S, Diza E, Menexes G, Kalfas S. Salivary concentration of the antimicrobial peptide LL-37 in children. *Arch Oral Biol* 2012,**57**:865-869.
105. Xue Y, Ying L, Horst RL, Watson G, Goltzman D. Androgens Attenuate Vitamin D Production Induced by UVB Irradiation of the Skin of Male Mice by an Enzymatic Mechanism. *J Invest Dermatol* 2015,**135**:3125-3132.
106. Rajan TV, Nelson FK, Shultz LD, Shultz KL, Beamer WG, Yates J, *et al.* Influence of gonadal steroids on susceptibility to Brugia malayi in scid mice. *Acta Trop* 1994,**56**:307-314.
107. Klein SL, Bird BH, Glass GE. Sex differences in Seoul virus infection are not related to adult sex steroid concentrations in Norway rats. *J Virol* 2000,**74**:8213-8217.

ANEXO 1

Artículo requisito para obtener el grado de Doctora en Ciencias

Journal of Steroid Biochemistry & Molecular Biology 163 (2016) 173–182

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

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

Evidence of sexual dimorphism in placental vitamin D metabolism: Testosterone inhibits calcitriol-dependent cathelicidin expression

Andrea Olmos-Ortiz[a,g], Janice García-Quiroz[a], Rebeca López-Marure[b], Irma González-Curiel[c], Bruno Rivas-Santiago[d], Aleida Olivares[e], Euclides Avila[a], David Barrera[a], Ali Halhali[a], Felipe Caldiño[f], Fernando Larrea[a], Lorenza Díaz[a,*]

[a] Departamento de Biología de la Reproducción, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Av. Vasco de Quiroga 15, Col. Sección XVI, Tlalpan 14080, México City, Mexico
[b] Departamento de Biología Celular, Instituto Nacional de Cardiología "Ignacio Chávez", Juan Badiano 1, Col. Sección XVI, Tlalpan 14080,Mexico City, Mexico
[c] Unidad Académica de Ciencias Químicas, Universidad Autónoma de Zacatecas, Carr. Guadalajara Km. 6, Ejido la Escondida 98160, Zacatecas, Zacatecas, Mexico
[d] Unidad de Investigación Médica–Zacatecas, Instituto Mexicano del Seguro Social, UIMZ-IMSS, Interior de la Alameda 45, Zacatecas, Zacatecas, Mexico
[e] Unidad de Investigación Médica en Medicina Reproductiva, UMAE, Mexico
[f] División de Obstetricia, Hospital de Gineco Obstetricia No. 4 Luis Castelazo Ayala, IMSS, Río Magdalena No. 289, Col. Tizapán, Álvaro Obregón 01090,Mexico City, Mexico
[g] Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Av. Universidad 3000, Coyoacán 04360,.Mexico City, Mexico

ARTICLE INFO

Article history:
Received 19 February 2016
Received in revised form 11 May 2016
Accepted 13 May 2016
Available online 19 May 2016

Keywords:
LL-37
Cord blood
Male
cAMP
Androgen

ABSTRACT

Male fetus and neonates show increased immune vulnerability compared to females, which results in a higher risk of perinatal infections. These differences could partially be due to sex steroids differential modulation of vitamin D metabolism; since calcitriol, the most active vitamin D metabolite, regulates immune responses and transcriptionally induces the antimicrobial peptide cathelicidin in the human placenta. Calcitriol availability depends on CYP27B1 and CYP24A1 expression, the cytochromes involved in its synthesis and degradation, respectively. However, the effects of testosterone upon these enzymes and the final biological outcome upon the calcitriol-dependent immune-target cathelicidin in the placenta have not been studied. In this study we show that testosterone significantly inhibited CYP27B1 while stimulated CYP24A1 gene expression in cultured trophoblasts. These effects were accompanied by CREB activation through cAMP–independent and androgen receptor-dependent mechanisms. Male placental cotyledons showed reduced basal CYP27B1 and cathelicidin gene expression compared to females ($P < 0.05$). Testosterone concentration was higher in the cord blood of male neonates ($P = 0.007$), whereas cathelicidin levels were lesser compared to females ($P = 0.002$). Altogether our results suggest that male placentas produce less cathelicidin due to decreased calcitriol bioavailability. We propose that the observed sex-dependent differences in placental vitamin D metabolism contribute in fetal responses to infections and could partially explain why the increased male fetuses immune vulnerability. Moreover, gestational hyperandrogenemia could adversely affect placental vitamin D metabolism independently of fetal sex.

1. Introduction

Diseases affect men and women differently; specifically, males are more prone than females to viral, bacterial, fungal and parasitic diseases. Whereas estrogens seem to enhance immune responses, androgens weaken immunocompetence [1]. The mechanisms underlying these differences are largely not understood. During prenatal life, sex-dependent differences in immune gene-expression are also suggested to contribute in fetal responses to infection and inflammation. Indeed, sex-specific feto-placental adaptations normally take place in human pregnancies, and interestingly, the male fetus is more likely to experience poorer outcomes under complicated conditions [2]. In this regard, male fetal gender has been associated with an overall increased risk of preeclampsia, premature rupture of membranes, placental infection, preterm

Abbreviations: 25OHD$_3$, 25-hydroxyvitamin D$_3$; AR, Androgen Receptor; cAMP, cyclic AMP; CRE, cAMP response elements; CREB, cAMP response element binding protein; DHT, 5α-dihydrotestosterone; E$_2$, 17β-estradiol; hCTD, human Cathelicidin; VD, vitamin D; VDR, vitamin D receptor.

* Corresponding author.
E-mail address: lorenzadiaz@gmail.com (L. Díaz).

http://dx.doi.org/10.1016/j.jsbmb.2016.05.017
0960-0760/

birth and increased placental expression of inflammatory, hypoxic and apoptotic molecules [3–7]. In contrast to testosterone immunosuppressive and proinflammatory effects, another hormone, calcitriol, the most active vitamin D_3 (VD) metabolite, inhibits placental inflammatory cytokines synthesis [8,9] and fortifies the innate immune response *via* stimulation of antimicrobial peptides expression. Among these natural antibiotics, cathelicidin (hCTD) stands as an essential component of the host innate immune response with strong microbicidal activity against a wide range of microorganisms [10]. The human placenta is able to produce an array of bioactive VD metabolites using VD as substrate by a recently discovered pathway catalyzed by CYP11A1 [11–14]. In the classic VD-activation pathway operating in placental syncytiotrophoblasts, VD is biotransformed by either CYP27A1 or CYP2R1 to 25-hydroxyvitamin D_3 ($25OHD_3$), which is also available from maternal pool. This metabolite is then hydroxylated by the enzyme $25OHD_3$-1α-hydroxylase (CYP27B1) to produce calcitriol (1,25 $(OH)_2D_3$) [11]. Importantly, the activation of the VD receptor (VDR) by calcitriol drives hCTD expression and antibacterial activity in human placenta [15,16]. Bioactive calcitriol is catabolized by the calcitriol-degrading enzyme 1,25-$(OH)_2D_3$-24-hydroxylase (CYP24A1), which is also expressed in human placenta; therefore, an imbalance in the CYP27B1/CYP24A1 ratio might directly affect calcitriol bioavailability. The regulation of CYP27B1 and CYP24A1 expression is tissue specific. In contrast to kidney, cyclic AMP (cAMP) in the placenta decreases CYP27B1 while increases CYP24A1 gene transcription [17,18]. This suggests that hormones that modify cAMP levels may also regulate placental calcitriol synthesis, as shown with calcitonin and parathyroid hormone [17]. Classically, cAMP-mediated signaling depends on the binding of hormones to their cell-surface receptors increasing cAMP production and activating protein kinase A (PKA), which in turn phosphorylates the cAMP response element binding protein (CREB). This activated transcription factor binds to cAMP response elements (CRE) in target genes thereby increasing or decreasing transcription [19]. On the other hand, testosterone actions are mediated by a canonical genomic mechanism involving its binding to the androgen receptor (AR); however, it may also activate CREB by a non-classic mechanism of action not requiring the interaction between the AR and the DNA and independently of cAMP generation, as has been previously described in Sertoli cells [20]. This particular testosterone signal transduction pathway has not been described in placenta. Considering that both CYP27B1 and CYP24A1 display putative CREs in their promoters [21,22], the objective of this study was to investigate: (1) if testosterone was able to modify these cytochromes gene expression through the cAMP/CREB signaling pathway in cultured placental cells, and (2) if physiological testosterone concentrations, which are associated to a male product, could affect endogenous calcitriol-mediated transcription of hCTD in placenta. Considering the increased susceptibility of male fetuses and neonates to infections, we have hypothesized that the presence of higher testosterone levels associated to male-carrying placentas modify local calcitriol bioavailability resulting in less hCTD in cord blood of male compared to female newborns.

2. Material and methods

2.1. Ethical approval

This protocol was approved and registered with the number R-2013-785-033 by the applicable Medical Ethical Comitee from the Instituto Mexicano del Seguro Social and a written informed consent was obtained from each mother before delivery. The study methodologies conformed to the standards set by the Declaration of Helsinki.

2.2. Reagents

Culture media, fetal bovine serum (FBS) and Trizol were from Invitrogen (CA, USA). The cAMP analogue 8-Bromo-cAMP (8Br-cAMP), testosterone, 5α-dihydrotestosterone (DHT), 17β-estradiol (E_2), exemestane, flutamide, calcitriol and $25OHD_3$ were purchased from Sigma (St. Louis, MO). The VDR antagonist TEI-9647 was kindly donated by Teijin Pharma Limited (Tokyo, Japan). The placental cell line HTR-8/SVneo was kindly donated by Dr. Fabian Arechavaleta-Velasco, from UMAE "Luis Castelazo Ayala" IMSS, México D. F.

2.3. Sample collection and preparation

Patients with chronic hypertension, diabetes mellitus, cervicovaginal infections, renal, and other systemic illnesses were excluded from the study. All placentas in this study were obtained by cesarean section from term uncomplicated pregnancies. Samples of cord blood were obtained immediately after birth and were collected exclusively from the umbilical vein, which provides information about blood directed from the placenta to the fetus. The cords were clamped, resected and punctured with a syringe. The plasma was separated by centrifugation (3000 rpm, 10 min, 4 °C) and stored at −70 °C until analysis. The placentas were extensively washed with 0.9% NaCl solution and further processed for cell or explant culture. In addition, samples of placental cotyledons were immediately obtained after delivery and preserved in RNAlater (Sigma) in order to study basal *CYP24A1*, *CYP27B1* and hCTD gene expression from male and female newborns.

2.4. Placental cell cultures and experimental conditions

Trophoblast cells were obtained from placental cotyledons as previously described with some modifications [23]. Briefly: Villous tissue was enzymatically dispersed and cells were separated on density percoll gradients. Cell viability was estimated by dye exclusion (0.4% trypan-blue) and were plated at a density of 3×10^6 cells in supplemented medium (DMEM-HG, 100 U/mL penicillin, 100 mg/mL streptomycin, 10% heat-inactivated-FBS). Incubations were performed in humidified 5% CO_2-95% air at 37 °C. Cells were allowed to attach overnight and further washed in order to remove all non-adherent cells [24]. In addition, an established placental cell line, the HTR-8/SVneo was also used. For experiments, cells were incubated during 24 h in treatment medium (DMEM-F12, antibiotics and 5% charcoal-stripped heat-inactivated-FBS) in the presence or absence of testosterone or its 5α-reduced metabolite DHT, a non-aromatizable androgen. After incubations, total RNA was extracted for gene expression studies.

2.5. Trophoblast cell culture characterization: human chorionic gonadotropin (hCG) secretion and cytokeratin-7 (CK-7) immunostaining

In order to estimate the purity and endocrine functionality of the percoll-purified trophoblasts, we analyzed the secretion of hCG by a commercial ELISA kit (DRG, Germany) as well as the expression by immunofluorescence of the well-known marker of trophoblastic cell population CK-7 [25].

2.6. PCR amplifications

Samples of placental cotyledons from male and female newborns were collected immediately after delivery and stored in RNAlater. The regulatory effects of androgens on gene expression were studied

by extracting total RNA from cultured treated cells or placental cotyledons using TRIzol reagent. The amount and quality of RNA were estimated spectrophotometrically at 260/280 nm and a constant amount of RNA was reverse transcribed using a reverse transcription assay (Roche Diagnostics, Basel, Switzerland). Identical PCR conditions were performed for all genes, which were normalized against the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as internal control. The primers sequences were the following: for hCTD (NM_004345.3): tcggatgctaacctctaccg/gtctgggtccccatccat; for *CYP24A1* (NM_000782.3): catcatggccatcaaaacaa/gcagctcgactggagtgac; for *CYP27B1* (NM_000785.2): cttgcggactgctcactg/cgcagactacgttgttcagg; and for *GAPDH* (NM_002046.3): agccacatcgctcagacac/gcccaatacgaccaaatcc. Amplifications were carried on the LightCycler 480 real-time PCR instrument (Roche) using the Roche master mix in combination with hydrolysis probes (Roche, Universal Probe Library), following a standard cycling protocol.

2.7. Preparation of cells and explants for Western blots

In order to evaluate CREB activation under different experimental conditions, placental explants or cells were incubated in fresh serum-free medium during 12 h and then treated during 5–60 min in the presence of testosterone. In the following experiments an incubation time of 15 min was thereafter used. In the case of explants, after treatments the tissue was washed and mechanically disrupted using ceramic beads and the MagNA Lyser homogenizer (Roche) in the presence of lysis buffer [9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, pH 7.4, a protease inhibitor cocktail (Roche) plus 15 mM NaF and 1 mM Na_3VO_4]. In the case of cells, after the incubation time they were washed, scrapped with cold PBS, pelleted and resuspended in lysis buffer. The cell or explant homogenates were boiled during 10 min and an equal amount of protein was assayed by Western immunoblot. The primary rabbit-antibody was directed against CREB phosphorylated on serine 133 (1:1000, Cell Signaling Technology, Inc. MA, USA). Phosphorylated CREB (pCREB) levels were normalized against GAPDH by using a mouse monoclonal antibody (1:8000, Millipore Research Reagents, CA, USA). In addition, we evaluated VDR protein expression in placental samples obtained immediately after delivery. Tissue process was similar as described for cultured explants. Primary VDR antibody was: mouse monoclonal anti-VDR (sc-13133, Santa Cruz 1:250). For visualization of CREB, VDR and GAPDH, membranes were then incubated with respective secondary antibodies: Goat anti-rabbit IgG-HRP (170–6515, BioRad, 1:8000) or goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, Inc, sc-2055, 1:6000). Afterwards membranes were revealed with the ECL + Plus Western blotting detection system (GEHealthcare, UK) and densitometric analysis were performed by using the Molecular Imager ChemiDoc XRS System and the Image Lab Software (Bio-Rad, Hercules, California, USA).

2.8. Quantification of cAMP

Total cAMP (intra- and extracellular) was measured by radioimmunoassay (RIA) employing the CV-27 cAMP antiserum (National Hormone & Peptide Program) at a final dilution of 1:75,000 as previously described [26]. Briefly, placental cells were plated at a density of 2×10^5 cells per well in 24 well boxes with supplemented medium. Cells were incubated in the presence of testosterone or cholera toxin (5 ng/mL) in serum-free treatment medium conditioned with 3-isobutyl-1-methylxanthine (0.2 mM). After 24 h incubations were stopped and samples (cells and culture media) were boiled during 3 min. The sensitivity of the assay was 2.0 fmol/mL.

2.9. Evaluation of hCTD expression in cultured trophoblasts by flow cytometry

To study how androgens affect a calcitriol-induced innate immune marker as a result of modulating VD metabolism in placental cells, we first incubated 3×10^6 trophoblasts in the presence of androgens during 24 h to allow the modulation of CYP27B1 and CYP24A1 expression. Then, cells were exposed to $25OHD_3$ in treatment medium and after 15 h we evaluated hCTD protein expression as a marker of calcitriol bioactivity. Secretion of hCTD was avoided by using a protein secretion inhibitor (BD GolgiStop, BD Biosciences, CA, USA). After the incubation time, cells were trypsinized, pelleted at 2000 rpm for 3 min, fixed and permeabilized with BD Cytofix/Cytoperm Plus (BD Biosciences). Intracellular staining of hCTD in CK-7 positive cells was achieved using a rabbit antibody (Abcam, Cambridge, MA, USA) that recognizes both the full length and cleaved C terminus of hCTD, conjointly with a mouse anti-CK-7 antibody (BioSB), both at 1:60 dilution. After 1 h of incubation, cells were washed with PBS-albumin (0.8%) and further incubated with a FITC conjugated goat anti mouse (Santa Cruz Biothechnology, Santa Cruz, CA, USA) and a Cy3 conjugated goat anti rabbit antibodies (Jackson ImmunoResearch) diluted 1:300 for 1 h. After washing, cells were subjected to cytometric analysis with a Becton Dickinson FACSCalibur instrument. Cells were analyzed by two-color flow cytometry to measure expression of hCTD in CK-7 positive cells. Analysis was performed with the CellQuest Pro Software (Becton Dickinson) and results are expressed as fluorescence intensity, calculated multiplying the number of events by the mean of the fluorescence value.

2.10. Quantification of cord serum hCTD by ELISA

Levels of hCTD in cord blood serum were determined with a sandwich ELISA using a rabbit anti- hCTD polyclonal antibody (1:1000, Abcam) for capture and a goat anti human hCTD antibody (1:500 Santa Cruz) for detection, as previously described [27]. Serum samples were filtered by using Amicon Ultra centrifugal filters with a 10 K membrane (Millipore, Tullagreen, Carrigtwohill, CO, USA) before analysis, assuring that only the active form of hCTD was quantified. HPLC purified hCTD was kindly provided by Dr. Robert Hancock (British Columbia University, Vancouver, Canada) and was used as standard.

2.11. Quantification of testosterone, E_2 and $25OHD_3$

Estradiol levels in cord blood serum samples were determined by a commercial RIA kit, while total testosterone concentrations were measured using competitive chemiluminescent enzyme immunoassays on a Siemens Immulite 2500 analyzer (Siemens Healthcare Diagnostics Inc, Deerfield, IL). The concentration of serum $25OHD_3$ was analyzed by a chemiluminescent immunoassay (Liaison, DiaSorin Inc., Stillwater, MN).

2.12. Statistical analysis

Differences between mean values from qPCR analyses were analyzed by One-way ANOVA or Student's *t*-tests as appropriate. Differences in serum umbilical cord metabolites and hCTD concentrations between male and female neonates were evaluated by Mann-Whitney *U* test or Student's *t*-test. Association studies between cord umbilical serum variables were done using Spearman rank correlation test. Differences were considered statistically significant at $P < 0.05$.

3. Results

In the present study one of the biological models used was the primary trophoblasts cell culture. Our previous characterization of this model showed that cultured cells formed functional syncytia in a time-dependent manner and were able to secrete basal and cAMP-stimulated hCG-β [17,25]. The percentage of CK-7-positive cells was ~85%. In this study we also used HTR-8/SVneo, a human placenta trophoblasts cell line.

3.1. Testosterone induced CYP24A1 while inhibited CYP27B1 gene expression in placental cells

In order to investigate a possible transcriptional effect of testosterone upon the VD- metabolizing cytochromes, placental cells were incubated in the presence of different concentrations of this androgen during 24 h. Both 8Br-cAMP and DHT were used as controls. As depicted in Fig. 1A, *CYP27B1* was significantly downregulated by testosterone in cultured HTR-8/SVneo and trophoblast cells. On the contrary, *CYP24A1* was upregulated ($P < 0.05$) by this hormone only in trophoblasts (Fig. 1B), since the HTR-8/SVneo cell line does not express *CYP24A1*. DHT and 8Br-cAMP regulated the two VD cytochromes expression in a similar manner as testosterone (Fig. 1C and 1D).

3.2. Testosterone activates CREB in a cAMP-independent but AR-dependent manner

Previously, we described that *CYP27B1* and *CYP24A1* genes were regulated by the cAMP/PKA pathway in the placenta [17], which was in accordance with the description that these two genes contain putative CREs in their promoters [21,22]. Herein, in order to corroborate the role of CREB/CRE signaling pathway in the regulation of the VD-metabolizing enzymes by testosterone, placental explants were treated with this androgen for 5–60 min. As a control, we used hCG, a well-known CREB-activator in placenta (Fig. 2A). As expected, hCG induced CREB phosphorylation within the first 60 min. Similarly, the incubation of placental explants in the presence of testosterone resulted in the significant phosphorylation of CREB, with a maximum peak around 15 min (Fig. 2A); therefore, all the following experiments were performed during this period of time. As depicted in Fig. 2B, phosphorylation of CREB was also observed when DHT was used, while preincubation in the presence of flutamide, an AR antagonist, blocked the testosterone-dependent CREB activation. Although E_2 also induced pCREB, the aromatase inhibitor exemestane did not antagonize the testosterone-dependent activation of CREB (Fig. 2B), highlighting the participation of androgens in pCREB induction. Testosterone and DHT also activated CREB in cultured placental cells (Fig. 2C).

We then asked if the observed androgen-dependent CREB induction was due to increased accumulation of cAMP. First, the adenylate cyclase functionality in our cultured trophoblasts was corroborated by experiments in the presence of cholera toxin. In these experiments, a significant cAMP increase of more than 60 folds over control was observed. Then, we analyzed if androgens could modify cAMP cell content. In contrast to the observed hCG-mediated stimulation of cAMP (1.66 ± 0.23 folds over control using 100 IU hCG/mL, $P = 0.024$), which we used as control, testosterone (1–50 nM) did not significantly affect cAMP content in placental cells (data not shown).

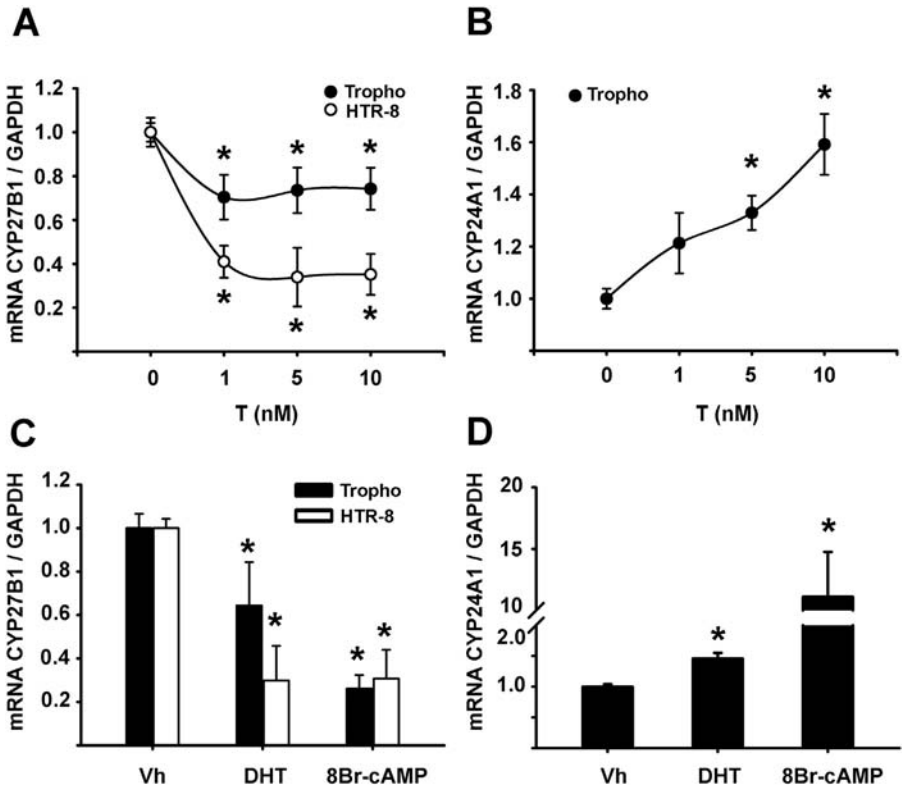

Fig. 1. Regulation of CYP27B1 and CYP24A1 gene expression by androgens and 8Br-cAMP in placental cells. (A) The gene expression of *CYP27B1* was downregulated by testosterone (T) in both cultured trophoblasts (black circles) and HTR-8/SVneo cells (white circles). (B) The gene expression of *CYP24A1* was up-regulated by testosterone in cultured trophoblasts. (C) The gene expression of *CYP27B1* was downregulated by DHT (5 nM) and 8Br-cAMP (0.6 mM) in trophoblasts (black bars) and HTR-8 cells (white bars). (D) *CYP24A1* gene expression was up-regulated by the same treatments in cultured trophoblasts. *$P < 0.05$ *vs* vehicle-treated cells (Vh or 0). Data is depicted as the mean ± SEM. n ≥ 3 different individual experiments. Results were normalized against control values which were set to 1.

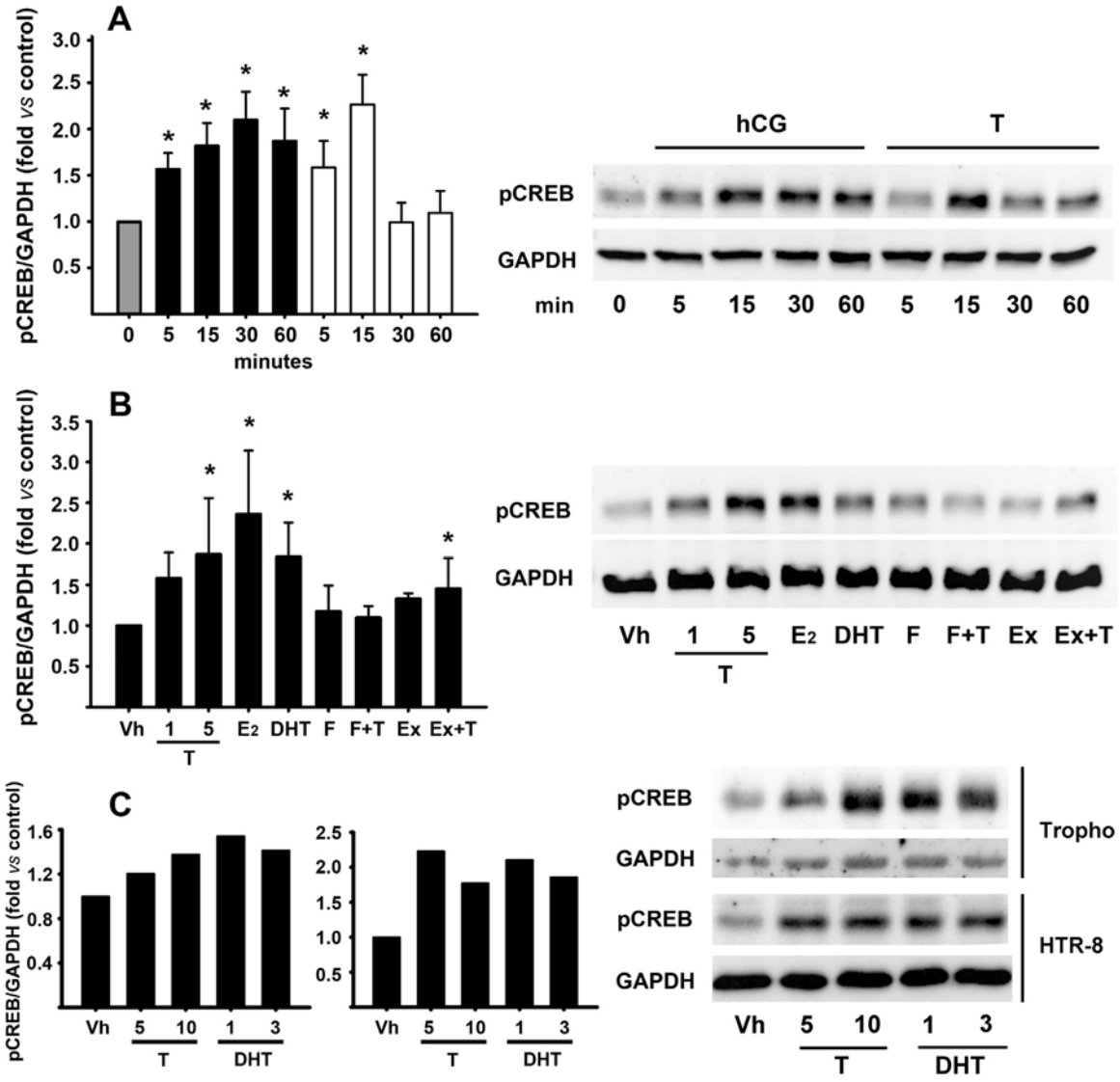

Fig. 2. Androgens stimulated CREB phosphorylation in an AR-dependent manner.
(A) Placental explants were treated with hCG (50 IU/mL, black bars) or testosterone (T, 5 nM, white bars) during 5–60 min. Time zero is depicted in gray. Densitometric analysis of pCREB/GAPDH from 5 different placentas is shown in left panel. A representative Western blot is shown in right panel. (B) Cultured placental explants were exposed for 15 min to testosterone (T, 1 or 5 nM), estradiol (E_2, 10 nM), DHT (1 nM), flutamide (F, 1 μM), exemestane (Ex, 5 μM) or the indicated combinations with 5 nM of T. Densitometric analysis of pCREB/GAPDH from 4 different placentas is shown in left panel. A representative Western blot is shown in right panel. (C) Trophoblasts were exposed to testosterone (T, 5 or 10 nM) or DHT (1 or 3 nM) during 15 min and a representative densitometric analysis of pCREB/GAPDH in trophoblasts is shown in left panel, whereas HTR-8 in middle panel. Right panel shows representative Western blots of trophoblasts (Tropho) and HTR-8/SVneo cells. Two independent experiments were performed for each cell type. $^*P < 0.05$ *vs* vehicle-treated cells (Vh or 0). Data is depicted as the mean ± SEM.

3.3. Androgens inhibit hCTD protein expression by affecting VD-metabolism in cultured trophoblasts

Considering the effects of androgens on *CYP27B1* and *CYP24A1*, and in order to find a biological plausibility of this observation on VD-mediated immune effects, we investigated the impact of testosterone on intracrine calcitriol using hCTD as a marker of placental innate defense. In the bioassay used in this study, and as expected, the addition of the CYP27B1 substrate, $25OHD_3$, to cultured trophoblasts doubled hCTD protein expression (Fig. 3). This stimulatory effect was similar when testing increasing concentrations of substrate: 1.0 μM, 2.5 μM and 6.0 μM of $25OHD_3$. Interestingly, we found a significant downregulating effect of both testosterone and DHT upon $25OHD_3$-induced hCTD expression. Surprisingly, the levels of hCTD in cells exposed to androgens and $25OHD_3$ were not significantly different as compared to cells incubated in the absence of substrate. On the other hand, estradiol, at the dose tested, did not affect $25OHD_3$-induced hCTD expression in our cultured trophoblasts. In a similar manner as observed with androgens, the treatment with 8Br-cAMP, which was used as a control, decreased hCTD protein expression, compared to substrate only ($P = 0.04$), while the use of the VDR antagonist TEI-9647 reversed the stimulatory effect of substrate addition on hCTD expression, indicating the participation of the VDR (Fig. 3). Since the magnitude of the stimulatory effect of substrate and the inhibitory effect of androgens, TEI and 8 Br-cAMP was similar independently of the concentration of $25OHD_3$ used, we decided to integrate all these data in a single

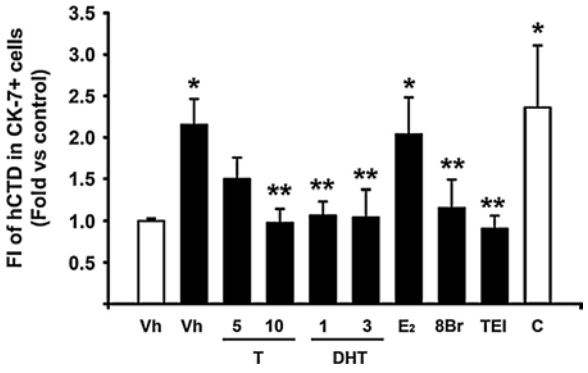

Fig. 3. Androgen inhibition of VD metabolism resulted in decreased cathelicidin protein expression in cultured trophoblasts.
Trophoblasts were incubated in the presence of testosterone (T, 5 or 10 nM), DHT (1 or 3 nM), estradiol (E_2, 10 nM) or 8Br-cAMP (8Br, 0.6 mM) during 24 h. Then, cells were incubated without (white bars) or with $25OHD_3$ (black bars), calcitriol (C, 10 nM) or TEI-9647 (TEI, 1 μM) in the presence of GolgiStop during 15 h. The expression of cathelicidin (hCTD) was analyzed by flow cytometry in cytokeratin-7 positive cells (CK-7+). The value of fluorescence intensity (FI) units of vehicle (Vh)-treated cells in the absence of $25OHD_3$ was set to 1 and results were normalized against this value. Each bar represents averaged results of at least 4 trophoblast cultures, except for 8Br and TEI. Data is depicted as the mean ± SEM. $^{*}P < 0.05$ *vs* Vh-treated cells without $25OHD_3$ (white Vh); $^{**}P < 0.05$ *vs* Vh-treated cells with $25OHD_3$ (black Vh).

figure. The control calcitriol, incubated in the absence of $25OHD_3$, stimulated hCTD expression in a similar manner as observed in the presence of $25OHD_3$ only (Fig. 3).

3.4. Transcriptional regulation of hCTD by VD metabolites and androgens

To corroborate the transcriptional effect of endogenous and exogenous calcitriol on hCTD gene expression and to discard a possible direct regulation of androgens or 8Br-cAMP upon this antimicrobial peptide, we incubated placental trophoblasts in the presence of $25OHD_3$, calcitriol, testosterone, DHT or 8Br-cAMP during 24 h and further analyzed hCTD gene expression by qPCR. Results shown in Fig. 4 depict the robust stimulation of hCTD gene expression after exposing cultured trophoblast to calcitriol. Likewise, the bioconversion of $25OHD_3$ to calcitriol evoked a similar and significant effect. No modification of hCTD gene transcription was observed when cells were incubated in the presence of testosterone or DHT. A significant downregulation of hCTD gene expression was observed when incubating the cells in the presence of 8Br-cAMP (Fig. 4).

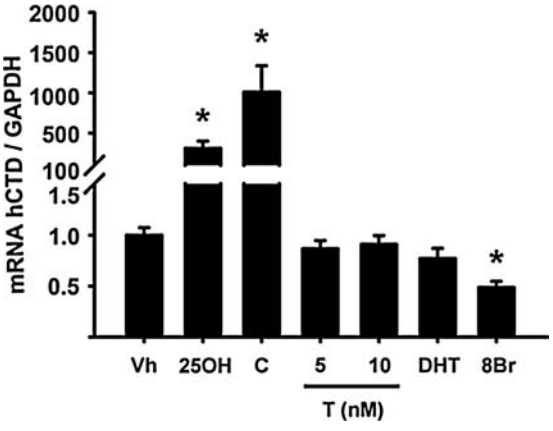

Fig. 4. The gene expression of cathelicidin is induced by $25OHD_3$ and calcitriol but not by androgens in the placenta. Cultured trophoblasts were exposed to $25OHD_3$ (25OH, 1 μM), calcitriol (C, 10 nM), testosterone (T, 5 or 10 nM), DHT (1 nM) or 8Br-cAMP (8Br, 0.6 mM) for 24 h. The transcriptional regulation of hCTD was studied by qPCR. Results are the mean ± SEM of at least 3 different placental cultures. $^{*}P < 0.05$ *vs* vehicle (Vh)-treated cells, which was normalized to 1.

3.5. Sex-dependent differences in placental VD-metabolizing enzymes and hCTD expression

In order to study a possible sex-dependent regulation of the VD metabolizing cytochromes, we analyzed the expression of *CYP24A1* and *CYP27B1* in placental tissue collected immediately after birth. In accordance to our observations in cultured placental cells, male placental cotyledons showed significantly less *CYP27B1* gene expression compared to female samples (Fig. 5A), while the expression of *CYP24A1* was not statistically different (Fig. 5B). However, the *CYP24A1/CYP27B1* ratio was on average 2.43 folds higher in males than in females, which might be explained by the lower *CYP27B1* gene expression found in males.

To further investigate a sex-based dimorphism in placental VD immune regulation, hCTD gene expression, which in humans

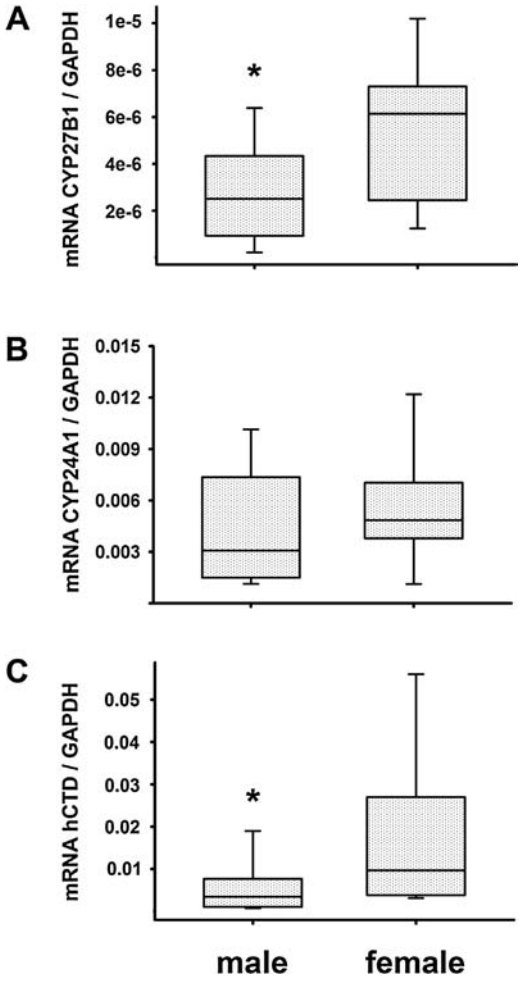

Fig. 5. Gender differences in *CYP27B1*, *CYP24A1* and hCTD gene expression in placental cotyledons. Placental cotyledons from male and female neonates were collected immediately after delivery. Tissue samples were processed by qPCR and the expression of *CYP27B1* (A), *CYP24A1* (B) and hCTD (C) was analyzed. Male cotyledons showed significantly less *CYP27B1* and hCTD gene expression compared to female. $^{*}P < 0.05$ *vs* female; n = 12 males and 9 females. Data are presented as the relative mRNA concentration of each individual gene *vs GAPDH*, and are depicted as box plots (box median; 25%, 75% percentiles; whiskers 5th and 95th percentiles).

depends on calcitriol stimuli, was also analyzed in placental tissue. In a similar manner as CYP27B1 gene expression, hCTD mRNA showed to be significantly reduced in placental cotyledons from male newborns, compared to those from females ($P < 0.01$, Fig. 5C).

Of note, no significant difference in VDR protein expression was detected in male *vs.* female samples (Supplementary Fig. 1); thus corroborating that the observed dissimilarities in cathelicidin and CYP27B1 gene expression are not attributable to gender-specific changes in VDR abundance.

3.6. Cord serum levels of hCTD, 25OHD$_3$, E$_2$ and testosterone in male and female newborns

In line with the previous observations, the analysis of cord blood hCTD concentration was found to be significantly reduced in males compared to females ($P = 0.002$, Table 1). In the case of testosterone and as expected, its concentration was higher in the cord blood of male neonates ($P = 0.007$) with no statistical differences in estradiol or 25OHD$_3$ concentrations between groups (Table 1). In these serum samples, neither significant correlation was found between the concentration of testosterone and hCTD (rho = −0.029, $P = 0.912$ in female samples and rho = −0.131, $P = 0.615$ in male samples) nor between 25OHD$_3$ and hCTD (rho = −0.271, $P = 0.348$ in female samples and rho = −0.387, $P = 0.154$ in male samples).

4. Discussion

It is known that testosterone participates in male-biases during infection causing an overall attenuation of the immune response [1,28]. Estrogens, on the contrary, help to fight against infections. Current evidence suggests that these differential effects could partially be explained by estrogen-promoted differences in VD metabolism, which favor slower calcitriol inactivation and consequently, a stronger innate immune response in females [29–31]. However, it is not known if testosterone may affect a VD-dependent immune target in human placenta. In the present study, we showed that testosterone inhibited placental CYP27B1 while stimulated CYP24A1 expression *via* a cAMP-independent process accompanied by CREB phosphorylation, resulting in the inhibition of VD-dependent hCTD expression. To our knowledge, this is the first report showing that testosterone, acting through a non-classical mechanism, modulates CRE-carrying genes in the placenta, in a similar fashion as previously reported in Sertoli cells [20]. This was probably a pure androgenic effect, since CREB activation and cytochromes regulation were mimicked by DHT, a non-aromatizable androgen, and antagonized by flutamide, an AR blocker. In this and other studies both estradiol and androgens rapidly activated CREB, [20,32]; however, herein a different outcome on VD-dependent stimulation of hCTD expression was observed. This difference could be explained on the bases of how estrogens and androgens differentially act upon calcitriol biogenesis. Indeed, in clear contrast to androgens, estrogens, acting through a receptor-signaling dependent mechanism, decrease CYP24A1 while stimulate CYP27B1 gene expression, thus reducing calcitriol catabolism while increasing its synthesis [29,33]. In this regard, androgen concentration in umbilical cord blood has been widely used as a marker of prenatal androgen exposure, and total testosterone levels have been found to be significantly elevated in male *vs.* female cord blood samples [34], in a similar manner as observed in this study. As a reference, in newborn serum, total testosterone concentration ranges from 2.4 to 13.8 nM (75–400 ng/dL) in males, while from 0.69 to 2.77 nM (20–80 ng/dL) in females. Considering this, we *a priori* assumed that placental calcitriol synthesis was lower in male than females. In line with this, we found lower CYP27B1 and hCTD gene expression in male-carrying placentas, as compared with those obtained from female newborns. On the bases of this observation, we used an *in vitro* bioassay that would reflect differences in 25OHD$_3$ metabolism due to differential testosterone CYPs regulation, using hCTD as an immune-biological marker of locally synthesized calcitriol. As explained before, hCTD was chosen due to its robust transcriptional VDR-dependent induction by calcitriol in the human placenta, while it is not stimulated by inflammatory mediators [16,25,35,36]. The results by flow cytometry analysis showed significantly reduced hCTD protein expression in trophoblasts previously exposed to androgens, suggesting a reduced bioconversion rate of 25OHD$_3$ into biologically active calcitriol. Furthermore, hCTD protein levels in the presence of androgens and 25OHD$_3$ were similar to those observed in the absence of the latter; thus, supporting the effects of androgens on calcitriol biosynthesis. On the other hand, the observation that 8Br-cAMP downregulated basal and VD-dependent placental hCTD expression might represent a mechanism used by microorganisms to facilitate infection. Indeed, pathogen-derived molecules may activate the cAMP/PKA pathway directly inhibiting hCTD expression, as seen in intestinal epithelial cells [37].

To further investigate a sex-based disparity in placental VD immune regulation, we additionally studied hCTD serum concentrations in umbilical cords obtained from newborns of different sex. As expected, hCTD levels were significantly reduced in male when compared to females. These results further support our experimental hypothesis that male placentas produce less hCTD due to decreased calcitriol synthesis and/or increased degradation. The finding of no significant gender-differences in 25OHD$_3$ cord blood concentrations suggested that under a similar precursor availability condition, calcitriol production depends more on local regulators of CYP24A1 and CYP27B1, which agreed with the more than two folds higher CYP24A1/CYP27B1 ratio observed in the male placenta. Even though cord blood 25OHD$_3$ levels are known to be reduced compared to maternal serum [38,39], herein we observed an overall reduced concentration of this metabolite in the cord blood samples independently of fetal sex, which might explain why we did not find a significant correlation between 25OHD$_3$ and hCTD levels.

Altogether these results suggest that pregnancy pathologies characterized by abnormally high androgen levels could be accompanied by altered VD metabolism resulting in insufficient calcitriol production. In this regard, high testosterone concentrations have been described in women with preeclampsia, a hypertensive disease of pregnancy [40–43] also associated with increased placental AR expression [44,45]. Interestingly, decreased placental calcitriol production has been observed in preeclampsia, secondary to decreased CYP27B1 expression in syncytiotrophoblasts cells [46,47]. Moreover, preeclampsia is associated with low circulating levels of calcitriol in maternal and umbilical cord compartments [47–49]. VD insufficiency has also been observed in women with polycystic ovary syndrome (PCOS), another

Table 1
Steroid hormones and hCTD levels in human umbilical vein serum by newborn gender.

Analyte	Male newborn (ng/mL)	Female newborn (ng/mL)	*P*
hCTD	143.5 ± 117.7	342.2 ± 205.7	[a]0.002
Testosterone	1.6 (1.3–2.0)	1.2 (1.0–1.5)	[b]0.007
Estradiol	8.6 ± 5.1	11.2 ± 4.6	[a]0.112
25OHD$_3$	16.4 ± 5.9	19.0 ± 6.0	[a]0.250

Statistical differences were evaluated by [a]Student's *t*-test or by [b]Mann-Whitney *U* test. Accordingly, data is presented as mean ± standard deviation or the median (25–75th percentile), respectively. n = 17 samples from each sex.

hyperandrogenemia-associated pathology [50,51]. Interestingly, the prevalence of chorioamnionitis due to bacterial infection is significantly higher in PCOS pregnant women compared to normal pregnancies [52]. This observation, based on our findings, could be related to altered placental VD metabolism and consequently, insufficient antimicrobial peptide production, which deserves to be further explored.

To our knowledge, this is the first time that hCTD, an important marker of the innate immune response, is found to be indirectly regulated by testosterone. However, there is additional information suggesting an inhibitory role of androgens on hCTD, such as the higher concentration of this peptide in saliva from female *vs* male subjects [53] and the findings in mice showing that androgens attenuate VD production and metabolism in the skin [54]. It is noteworthy to mention that the VD response element in the hCTD gene is specific for primates and therefore is not conserved in mice and other lower mammals [55], highlighting the need of studies in human tissues. In this regard, it is widely accepted that the classic activation pathway of VD operating in the whole body implies the cutaneous photosynthesis of VD followed by hepatic production of $25OHD_3$ and renal synthesis of calcitriol [56]. However, whether human placental VD metabolism is affected by androgens in a similar manner as previously shown in mice skin remains unknown. Recently, it has been demonstrated that human placenta is able to biotransform VD to $25OHD_3$ [11] and that $25OHD_3$ and calcitriol are not the predominant VD metabolites in placental cells, but rather several hydroxylated VD compounds such as 20-hydroxyvitamin D_3. Although these CYP11A1-derived VD metabolites are known to be bioactive, no studies have been undertaken to explore their possible participation as physiological regulators of placental immuno-endocrine functions. Interestingly, the VD-hydroxyderivatives generated by CYP11A1 are also modified by CYP27B1 activity [11], which suggests that an imbalance between CYP27B1/CYP24A1 expression might affect overall VD metabolism and actions in placental cells.

Considering the potential beneficial effects of calcitriol upon placental biology and immune defenses, we believe that the results presented herein may in part explain why male fetus-bearing pregnancies are more likely to experience poorer outcomes generally associated to a decreased immune defense condition. In this regard, some complementary evidence of the role of sex steroids on genome fetal imprinting and programming of adult immune response should also be taken into consideration, particularly in conditions affecting adequate VD availability in pregnancy [57,58]. Given that prenatal environment factors, including VD among them [56], are recognized to significantly impact adult health, we believe that special care should be taken in maintaining calcitriol sufficiency *in utero*, especially in pregnancies carrying a male fetus or those with a particular VD metabolism deficient trait.

5. Conclusion

In summary, in this study we tested and probed the hypothesis that testosterone regulates placental VD metabolism and that by this mechanism impacts on calcitriol-dependent immune actions during development. We concluded that: (1) Testosterone stimulates whilst inhibits placental *CYP24A1* and *CYP27B1* gene expression, respectively. (2) The AR is required for testosterone to induce CREB activation in a cAMP independent manner in the placenta. (3) The placental VD-dependent expression of hCTD protein is reduced by androgen exposure or 8Br-cAMP. (4) Male placental cotyledons express less *CYP27B1* and hCTD mRNA compared to female samples. (5) Male newborns had lower serum umbilical cord hCTD concentration compared to females. Overall, this study supports that male fetuses might be more prone to infections and inflammation due to sex-dependent differences in placental VD metabolism, highlighting sexually-dimorphic placental VD-dependent responses to infection (Fig. 6).

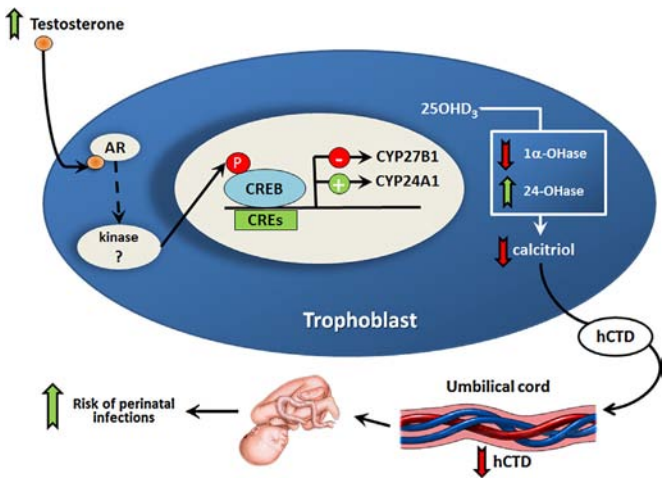

Fig. 6. Proposed mechanism of VD metabolism and hCTD regulation by testosterone in the human placenta. Testosterone freely crosses the cell membrane to bind the cytoplasmic androgen receptor (AR). Then, by a yet unknown pathway that activates a kinase, CREB is phosphorylated, thereby affecting the expression of the CRE containing genes *CYP27B1* and *CYP24A1*. This may result in diminished placental calcitriol biosynthesis due to increased degradation and/or reduced bioconversion from $25OHD_3$. In turn, the trophoblastic expression of hCTD is reduced, providing less innate defenses to the fetus through the cord umbilical vein, placing the fetus in a greater risk for perinatal infections.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

We acknowledge with thanks to Roberto Chavira for performing the radioimmunoassays, to Horacio Reyes Vivas for helping to validate antibodies specificity and to Nancy Noyola-Martínez for helping in samples collection and technical assistance. We also acknowledge with thanks to Hospital de Gineco-Obstetricia "Luis Castelazo Ayala" for assistance in obtaining the placentas and Teijin Pharma Limited Tokyo, Japan, for the donation of TEI-9647. AOO is a Doctoral student from Programa de Doctorado en Ciencias Biológicas, Universidad Nacional Autónoma de México (UNAM) and received fellowship 231018 from the Consejo Nacional de Ciencia y Tecnología (CONACyT). This work was supported by CONACyT, México, grant numbers 153862 and 241034 to LD and EA, respectively. The funder had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2016.05.017.

References

[1] S.L. Klein, The effects of hormones on sex differences in infection: from genes to behavior, Neurosci. Biobehav. Rev. 24 (2000) 627–638.
[2] V.L. Clifton, Review: sex and the human placenta: mediating differential strategies of fetal growth and survival, Placenta (Suppl. 31) (2010) S33–S39.
[3] J. Challis, J. Newnham, F. Petraglia, M. Yeganegi, A. Bocking, Fetal sex and preterm birth, Placenta 34 (2013) 95–99.

[4] E. Elsmen, K. Kallen, K. Marsal, L. Hellstrom-Westas, Fetal gender and gestational-age-related incidence of pre-eclampsia, Acta Obstet. Gynecol. Scand. 85 (2006) 1285–1291.
[5] L.J. Vatten, R. Skjaerven, Offspring sex and pregnancy outcome by length of gestation, Early Hum. Dev. 76 (2004) 47–54.
[6] R.L. Goldenberg, W.W. Andrews, O.M. Faye-Petersen, A.R. Goepfert, S.P. Cliver, J. C. Hauth, The Alabama preterm birth study: intrauterine infection and placental histologic findings in preterm births of males and females less than 32 weeks, Am. J. Obstet. Gynecol. 195 (2006) 1533–1537.
[7] S. Muralimanoharan, A. Maloyan, L. Myatt, Evidence of sexual dimorphism in the placental function with severe preeclampsia, Placenta 34 (2013) 1183–1189.
[8] L. Diaz, N. Noyola-Martinez, D. Barrera, G. Hernandez, E. Avila, A. Halhali, F. Larrea, Calcitriol inhibits TNF-alpha-induced inflammatory cytokines in human trophoblasts, J. Reprod. Immunol. 81 (2009) 17–24.
[9] N. Noyola-Martinez, L. Diaz, E. Avila, A. Halhali, F. Larrea, D. Barrera, Calcitriol downregulates TNF-alpha and IL-6 expression in cultured placental cells from preeclamptic women, Cytokine 61 (2013) 245–250.
[10] K. Bandurska, A. Berdowska, R. Barczynska-Felusiak, P. Krupa, Unique features of human cathelicidin LL-37, Biofactors 41 (2015) 289–300.
[11] A.T. Slominski, T.K. Kim, H.Z. Shehabi, I. Semak, E.K. Tang, M.N. Nguyen, H.A. Benson, E. Korik, Z. Janjetovic, J. Chen, C.R. Yates, A. Postlethwaite, W. Li, R.C. Tuckey, In vivo evidence for a novel pathway of vitamin D(3) metabolism initiated by P450scc and modified by CYP27B1, FASEB J. 26 (2012) 3901–3915.
[12] A. Slominski, I. Semak, J. Zjawiony, J. Wortsman, W. Li, A. Szczesniewski, R.C. Tuckey, The cytochrome P450scc system opens an alternate pathway of vitamin D3 metabolism, FEBS J. 272 (2005) 4080–4090.
[13] A.T. Slominski, T.K. Kim, W. Li, A. Postlethwaite, E.W. Tieu, E.K. Tang, R.C. Tuckey, Detection of novel CYP11A1-derived secosteroids in the human epidermis and serum and pig adrenal gland, Sci. Rep. 5 (2015) 14875.
[14] A.T. Slominski, W. Li, T.K. Kim, I. Semak, J. Wang, J.K. Zjawiony, R.C. Tuckey, Novel activities of CYP11A1 and their potential physiological significance, J. Steroid Biochem. Mol. Biol. 151 (2015) 25–37.
[15] K.N. Evans, L. Nguyen, J. Chan, B.A. Innes, J.N. Bulmer, M.D. Kilby, M. Hewison, Effects of 25-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 on cytokine production by human decidual cells, Biol. Reprod. 75 (2006) 816–822.
[16] N. Liu, A.T. Kaplan, J. Low, L. Nguyen, G.Y. Liu, O. Equils, M. Hewison, Vitamin D induces innate antibacterial responses in human trophoblasts via an intracrine pathway, Biol. Reprod. 80 (2009) 398–406.
[17] E. Avila, L. Diaz, D. Barrera, A. Halhali, I. Mendez, L. Gonzalez, U. Zuegel, A. Steinmeyer, F. Larrea, Regulation of vitamin D hydroxylases gene expression by 1,25-dihydroxyvitamin D3 and cyclic AMP in cultured human syncytiotrophoblasts, J. Steroid Biochem. Mol. Biol. 103 (2007) 90–96.
[18] E. Avila, L. Diaz, A. Halhali, F. Larrea, Regulation of 25-hydroxyvitamin D3 1alpha-hydroxylase 1,25-dihydroxyvitamin D3 24-hydroxylase and vitamin D receptor gene expression by 8-bromo cyclic AMP in cultured human syncytiotrophoblast cells, J. Steroid Biochem. Mol. Biol. 89-90 (2004) 115–119.
[19] M.S. Weedon-Fekjaer, K. Tasken, Review: spatiotemporal dynamics of hCG/cAMP signaling and regulation of placental function, Placenta (Suppl. 33) (2012) S87–S91.
[20] C. Fix, C. Jordan, P. Cano, W.H. Walker, Testosterone activates mitogen-activated protein kinase and the cAMP response element binding protein transcription factor in Sertoli cells, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 10919–10924.
[21] X.F. Kong, X.H. Zhu, Y.L. Pei, D.M. Jackson, M.F. Holick, Molecular cloning, characterization, and promoter analysis of the human 25-hydroxyvitamin D3-1alpha-hydroxylase gene, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 6988–6993.
[22] C. Zierold, H.M. Darwish, H.F. DeLuca, Identification of a vitamin D-response element in the rat calcidiol (25-hydroxyvitamin D3) 24-hydroxylase gene, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 900–902.
[23] H.J. Kliman, J.E. Nestler, E. Sermasi, J.M. Sanger, J.F. Strauss 3rd, Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae, Endocrinology 118 (1986) 1567–1582.
[24] C. Tscherning-Casper, N. Papadogiannakis, M. Anvret, L. Stolpe, S. Lindgren, A. B. Bohlin, J. Albert, E.M. Fenyo, The trophoblastic epithelial barrier is not infected in full-term placentae of human immunodeficiency virus-seropositive mothers undergoing antiretroviral therapy, J. Virol. 73 (1999) 9673–9678.
[25] A. Olmos-Ortiz, N. Noyola-Martinez, D. Barrera, V. Zaga-Clavellina, E. Avila, A. Halhali, B. Biruete, F. Larrea, L. Diaz, IL-10 inhibits while calcitriol reestablishes placental antimicrobial peptides gene expression, J. Steroid Biochem. Mol. Biol. 148 (2015) 187–193.
[26] A. Olivares, J.P. Mendez, E. Zambrano, M. Cardenas, A. Tovar, G. Perera-Marin, A. Ulloa-Aguirre, Reproductive axis function and gonadotropin microheterogeneity in a male rat model of diet-induced obesity, Gen. Comp. Endocrinol. 166 (2010) 356–364.
[27] J.E. Castaneda-Delgado, N.Y. Miranda-Castro, R. Gonzalez-Amaro, I. Gonzalez-Curiel, A. Montoya-Rosales, B. Rivas-Calderon, B. Rivas-Santiago, Production of antimicrobial peptides is preserved in aging, Clin. Immunol. 148 (2013) 198–205.
[28] J.A. Rettew, Y.M. Huet-Hudson, I. Marriott, Testosterone reduces macrophage expression in the mouse of toll-like receptor 4, a trigger for inflammation and innate immunity, Biol. Reprod. 78 (2008) 432–437.
[29] J. Correale, M.C. Ysrraelit, M.I. Gaitan, Gender differences in 1,25-dihydroxyvitamin D3 immunomodulatory effects in multiple sclerosis patients and healthy subjects, J. Immunol. 185 (2010) 4948–4958.
[30] J.W. Pike, E. Spanos, K.W. Colston, I. MacIntyre, M.R. Haussler, Influence of estrogen on renal vitamin D hydroxylases and serum 1alpha 25-(OH)2D3 in chicks, Am. J. Physiol. 235 (1978) E338–343.
[31] C. Cheema, B.F. Grant, R. Marcus, Effects of estrogen on circulating free and total 1,25-dihydroxyvitamin D and on the parathyroid-vitamin D axis in postmenopausal women, J. Clin. Invest. 83 (1989) 537–542.
[32] S.M. Aronica, W.L. Kraus, B.S. Katzenellenbogen, Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 8517–8521.
[33] D. Lechner, E. Bajna, H. Adlercreutz, H.S. Cross, Genistein and 17beta-estradiol but not equol, regulate vitamin D synthesis in human colon and breast cancer cells, Anticancer Res. 26 (2006) 2597–2603.
[34] J.A. Keelan, E. Mattes, H. Tan, A. Dinan, J.P. Newnham, A.J. Whitehouse, P. Jacoby, M. Hickey, Androgen concentrations in umbilical cord blood and their association with maternal, fetal and obstetric factors, PLoS One 7 (2012) e42827.
[35] T.T. Wang, F.P. Nestel, V. Bourdeau, Y. Nagai, Q. Wang, J. Liao, L. Tavera-Mendoza, R. Lin, J.W. Hanrahan, S. Mader, J.H. White, Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression, J. Immunol. 173 (2004) 2909–2912.
[36] D. Klaffenbach, D. Friedrich, R. Strick, P.L. Strissel, M.W. Beckmann, W. Rascher, A. Gessner, J. Dotsch, U. Meissner, M. Schnare, Contribution of different placental cells to the expression and stimulation of antimicrobial proteins (AMPs), Placenta 32 (2016) 830–837.
[37] K. Chakraborty, S. Ghosh, H. Koley, A.K. Mukhopadhyay, T. Ramamurthy, D.R. Saha, D. Mukhopadhyay, S. Roychowdhury, T. Hamabata, Y. Takeda, S. Das, Bacterial exotoxins downregulate cathelicidin (hCAP-18/LL-37) and human beta-defensin 1 (HBD-1) expression in the intestinal epithelial cells, Cell. Microbiol. 10 (2008) 2520–2537.
[38] M. Ron, J. Menczel, L. Schwartz, Z. Palti, G. Kidroni, Vitamin D3 metabolites in amniotic fluid in relation with maternal and fetal sera in term pregnancies, J. Perinat. Med. 15 (1987) 282–290.
[39] M.M. Gupta, G. Kuppuswamy, A.R. Subramanian, Transplacental transfer of 25-hydroxy-cholecalciferol, Postgrad. Med. J. 58 (1982) 408–410.
[40] E. Salamalekis, P. Bakas, N. Vitoratos, M. Eleptheriadis, G. Creatsas, Androgen levels in the third trimester of pregnancy in patients with preeclampsia, Eur. J. Obstet. Gynecol. Reprod. Biol. 126 (2006) 16–19.
[41] S.M. Carlsen, P. Romundstad, G. Jacobsen, Early second-trimester maternal hyperandrogenemia and subsequent preeclampsia: a prospective study, Acta Obstet. Gynecol. Scand. 84 (2005) 117–121.
[42] R. Troisi, N. Potischman, J.M. Roberts, R. Ness, W. Crombleholme, D. Lykins, P. Siiteri, R.N. Hoover, Maternal serum oestrogen and androgen concentrations in preeclamptic and uncomplicated pregnancies, Int. J. Epidemiol. 32 (2003) 455–460.
[43] V. Tache, R.J. Baer, R.J. Currier, C.S. Li, D. Towner, L.E. Waetjen, L.L. Jelliffe-Pawlowski, Population-based biomarker screening and the development of severe preeclampsia in California, Am. J. Obstet. Gynecol. 211 (2014) 377.e1–377.e8.
[44] T.Y. Hsu, K.C. Lan, C.C. Tsai, C.Y. Ou, B.H. Cheng, M.Y. Tsai, H.Y. Kang, Y.H. Tung, Y. H. Wong, K.E. Huang, Expression of androgen receptor in human placentas from normal and preeclamptic pregnancies, Taiwan J. Obstet. Gynecol. 48 (2009) 262–267.
[45] K. Sathishkumar, M. Balakrishnan, V. Chinnathambi, M. Chauhan, G.D. Hankins, C. Yallampalli, Fetal sex-related dysregulation in testosterone production and their receptor expression in the human placenta with preeclampsia, J. Perinatol. 32 (2012) 328–335.
[46] L. Diaz, C. Arranz, E. Avila, A. Halhali, F. Vilchis, F. Larrea, Expression and activity of 25-hydroxyvitamin D-1 alpha-hydroxylase are restricted in cultures of human syncytiotrophoblast cells from preeclamptic pregnancies, J. Clin. Endocrinol. Metab. 87 (2002) 3876–3882.
[47] A. Halhali, L. Diaz, D. Barrera, E. Avila, F. Larrea, Placental calcitriol synthesis and IGF-I levels in normal and preeclamptic pregnancies, J. Steroid Biochem. Mol. Biol. 144 (Pt. A) (2014) 44–49.
[48] A. Halhali, A.R. Tovar, N. Torres, H. Bourges, M. Garabedian, F. Larrea, Preeclampsia is associated with low circulating levels of insulin-like growth factor I and 1,25-dihydroxyvitamin D in maternal and umbilical cord compartments, J. Clin. Endocrinol. Metab. 85 (2000) 1828–1833.
[49] A. Halhali, L. Diaz, E. Avila, A.C. Ariza, M. Garabedian, F. Larrea, Decreased fractional urinary calcium excretion and serum 1,25-dihydroxyvitamin D and IGF-I levels in preeclampsia, J. Steroid Biochem. Mol. Biol. 103 (2007) 803–806.
[50] S. Thys-Jacobs, D. Donovan, A. Papadopoulos, P. Sarrel, J.P. Bilezikian, Vitamin D and calcium dysregulation in the polycystic ovarian syndrome, Steroids 64 (1999) 430–435.
[51] H.W. Li, R.E. Brereton, R.A. Anderson, A.M. Wallace, C.K. Ho, Vitamin D deficiency is common and associated with metabolic risk factors in patients with polycystic ovary syndrome, Metabolism 60 (2011) 1475–1481.
[52] M.P. Koster, M.A. de Wilde, S.M. Veltman-Verhulst, M.L. Houben, P.G. Nikkels, B.B. van Rijn, B.C. Fauser, Placental characteristics in women with polycystic ovary syndrome, Hum. Reprod. 30 (2015) 2829–2837.
[53] S. Davidopoulou, E. Diza, G. Menexes, S. Kalfas, Salivary concentration of the antimicrobial peptide LL-37 in children, Arch. Oral Biol. 57 (2012) 865–869.
[54] Y. Xue, L. Ying, R.L. Horst, G. Watson, D. Goltzman, Androgens attenuate Vitamin D production induced by UVB irradiation of the skin of male mice by an enzymatic mechanism, J. Invest. Dermatol. 135 (2015) 3125–3132.

[55] A.F. Gombart, N. Borregaard, H.P. Koeffler, Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3, FASEB J. 19 (2005) 1067–1077.
[56] A. Olmos-Ortiz, E. Avila, M. Durand-Carbajal, L. Diaz, Regulation of calcitriol biosynthesis and activity: focus on gestational vitamin D deficiency and adverse pregnancy outcomes, Nutrients 7 (2015) 443–480.
[57] T.V. Rajan, F.K. Nelson, L.D. Shultz, K.L. Shultz, W.G. Beamer, J. Yates, D.L. Greiner, Influence of gonadal steroids on susceptibility to Brugia malayi in scid mice, Acta Trop. 56 (1994) 307–314.
[58] S.L. Klein, B.H. Bird, G.E. Glass, Sex differences in Seoul virus infection are not related to adult sex steroid concentrations in Norway rats, J. Virol. 74 (2000) 8213–8217.

ANEXO 2

Carta de consentimiento informado para la donación de placenta

“2016, Año del Bicentenario de la Declaración de la Independencia de México”

CARTA DE CONSENTIMIENTO INFORMADO PARA PARTICIPACIÓN EN PROTOCOLOS DE INVESTIGACIÓN

Nombre del estudio: **Regulación hormonal de la síntesis de calcitriol y sus efectos en cultivos de trofoblastos humanos**

El objetivo de este estudio es investigar el efecto de algunas hormonas del embarazo sobre la síntesis y acciones del calcitriol, que es un derivado de la vitamina D, en células de placenta humana en cultivo. Su participación en este estudio consistirá únicamente en donar la placenta ya expulsada, para la investigación. La placenta se utilizará exclusivamente para los fines que fueron señalados. Es obligación del personal que participa en este proyecto aclarar todas las dudas presentes e informarle que su participación no le generará ningún riesgo, gasto ni obligación alguna, así como tampoco recibirá nada a cambio de la donación. En el caso de que se considere conveniente, Usted puede negar su participación en el estudio, sin que ello afecte la atención que recibe en este Hospital. No se identificará el nombre de la donadora en ninguna de las presentaciones o publicaciones que deriven de este estudio.

Los datos clínicos relacionados serán manejados en forma confidencial.

En caso de dudas o aclaraciones relacionadas con el estudio podrá dirigirse a:

Investigador Responsable: **Dr. Felipe Caldiño Soto**. Tel.: 55 50 64 22 ext. 28001, 28057 y 28083.

Investigadores Asociados: **Dra. Lorenza Díaz Nieto.** Tel 54 87 09 00 ext. 2417 y M. en C. **Andrea Olmos Ortiz.** Tel 54 87 09 00 ext. 2417.

En caso de dudas o aclaraciones sobre sus derechos como participante podrá dirigirse a: Comisión de Ética de Investigación de la CNIC del IMSS: Avenida Cuauhtémoc 330 4° piso Bloque “B” de la Unidad de Congresos, Colonia Doctores. México, D.F., CP 06720. Teléfono 56 27 69 00 extensión 21230, Correo electrónico: comision.etica@imss.gob.mx

México D.F. a _____ de _____ de ____.

_____ Nombre y firma de la paciente

_____ Nombre y firma de quien obtiene el consentimiento

_____ Nombre y firma del Testigo 1

_____ Nombre y firma del Testigo 2

ANEXO 3

Artículos publicados durante el periodo de estudios de Doctorado

Journal of Steroid Biochemistry & Molecular Biology 148 (2015) 187–193

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

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

IL-10 inhibits while calcitriol reestablishes placental antimicrobial peptides gene expression

Andrea Olmos-Ortiz [a], Nancy Noyola-Martínez [a], David Barrera [a], Verónica Zaga-Clavellina [b], Euclides Avila [a], Ali Halhali [a], Benjamín Biruete [c], Fernando Larrea [a], Lorenza Díaz [a,*]

[a] Departamento de Biología de la Reproducción, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga No. 15, México City, Tlalpan 14000, Mexico
[b] Departamento de Inmunobioquímica, Instituto Nacional de Perinatología Isidro Espinosa de los Reyes, Montes Urales No. 800, México City, Col. Lomas de Virreyes 11000, Mexico
[c] División de Obstetricia, Hospital de Ginecología y Obstetricia del IMSS "Luis Castelazo Ayala", Río Magdalena No. 289, México City, Tizapán 01090, Mexico

ARTICLE INFO

Article history:
Received 8 July 2014
Received in revised form 25 July 2014
Accepted 29 July 2014
Available online 1 August 2014

Keywords:
Cathelicidin
β-Defensin
Innate defense
Placenta

ABSTRACT

IL-10 and calcitriol help to achieve a successful pregnancy by suppressing active maternal immunity; however, these factors exert opposite effects upon microbial infections. In the skin and immune cells, IL-10 downregulates β-defensins while calcitriol induces cathelicidin gene expression in various tissues including placenta. Though, the regulation of human placental β-defensins by IL-10 and calcitriol has not been studied. Therefore, we explored the regulation of these antimicrobial peptides expression in cultured placental cells by calcitriol and IL-10 alone and combined. Real time PCR showed that calcitriol stimulated, while IL-10 inhibited, β-defensins and cathelicidin gene expression ($P < 0.05$). In coincubations studies, calcitriol was able to maintain antimicrobial peptides gene expression above control values, overriding IL-10 inhibitory effects. Calcitriol downregulated endogenous IL-10 secretion. Interestingly, calcitriol and TNF-α cooperatively enhanced β-defensins, while TNF-α reduced basal and calcitriol-stimulated cathelicidin gene expression. In summary, calcitriol and IL-10 exerted opposite effects on antimicrobial peptides expression in the human placenta, suggesting that unbalanced production of IL-10 and calcitriol could be deleterious to innate immune responses during gestation. Our results suggest that calcitriol enhancement of placental defenses involves two mechanisms: (1) downregulation of IL-10 secretion and (2) direct upregulation of β-defensins and cathelicidin gene expression. Considering that IL-10 and calcitriol differentially regulate the innate immune response in the placenta, in the case of an infection, calcitriol might restrict IL-10 permissive actions towards microbial invasion while restrains inflammation, allowing for pregnancy to continue in quiescence. These results strongly advice maternal vitamin D sufficiency during pregnancy.

1. Introduction

Clinical studies have shown a strong association between intrauterine bacterial or viral infections and pregnancy complications such as abortion, preterm labor, intrauterine growth restriction, and preeclampsia [1–3]. During intrauterine infection, the placenta represents a mechanical and immunological barrier whose innate immune competences are essential to restrict infection progress. Calcitriol, the most potent metabolite of vitamin D_3 (VD), is a pregnancy supporting factor particularly during early fetal allograft acceptation and pathological inflammatory and infectious conditions. Indeed, pregnant women with sub-optimal VD levels are at risk of developing inflammatory conditions as well as recurrent infections such as preeclampsia and bacterial vaginosis, respectively [4–6]. This might be explained by the fact that calcitriol stimulates innate immunity by enhancing microbial killing while prevents exacerbated inflammation by regulating adaptive immunity. Given that the VD-activating enzyme 25-hydroxyvitamin D_3-1α-hydroxylase (CYP27B1) is expressed in many tissues including placenta [7], adequate VD intake and/or enough sun exposure are essential for maternal and

Abbreviations: VD, Vitamin D_3; AMP, Antimicrobial peptide; HBD, Human β-defensin; hCTD, Cathelicidin; ETC, Enriched trophoblastic cell preparations; CK-7, Cytokeratin 7.

* Corresponding author. Tel.: +52 5 54 87 09 00x2417.
E-mail address: lorenzadiaz@gmail.com (L. Díaz).

http://dx.doi.org/10.1016/j.jsbmb.2014.07.012
0960-0760/

fetal health, since local calcitriol production depends on the bioavailability of 25-hydroxyvitamin D (25-OHD_3), the CYP27B1 substrate. Calcitriol exhibits two key immunomodulatory properties that help sustain a quiescent and healthy pregnancy: its ability to inhibit inflammatory responses, and its capacity to orchestrate antimicrobial defenses [8–10]. Although calcitriol and interleukin (IL)-10 both act as immunosuppressive and anti-inflammatory factors produced by trophoblasts and immune cells, they diverge in their effects in response to a microbial insult. Indeed, while calcitriol stimulates antimicrobial responses in many cell types including human trophoblasts [8,11], IL-10 expression is associated with a poor or absent response against infection [12]. In fact, *in vivo* and *in vitro* studies involving intervention with IL-10 suggested that this cytokine can be deleterious to situations in which a strong immune defense is required [13–15]. The mechanism comprises inhibition of several macrophage functions, including microbicidal properties and presentation of antigens to Th1 cells. Moreover, IL-10 downregulates the expression of important antimicrobial peptides (AMPs) such as human β-defensins (HBD) in the skin and human peripheral blood cells [16–18]. Interestingly, regulation of AMPs gene expression by IL-10 in the human placenta has not been addressed. AMPs are small endogenous antibiotics with a positive charge and an amphipathic structure that enables them to interact with bacterial membranes. They participate in multiple aspects of immunity such as inflammation, wound repair, and regulation of the adaptive immune system. In contrast to acquired adaptive immunity, AMPs provide a fast and energy-effective mechanism as front-line defense since they have the capacity to kill a wide variety of microorganisms. AMPs are expressed throughout the female reproductive tract, and specifically during pregnancy, placental trophoblasts are a major source of AMPs [19]. AMPs expression is tightly regulated. The expression of HBD1 has been found to be constitutive; however, HBD2 (also known as DEFB4) expression is upregulated by infectious and inflammatory stimuli [20]. Indeed, HBD2 expression is stimulated by IL-1α, IL-1β, tumor necrosis factor-α (TNF-α), Gram-positive, and Gram-negative bacteria, as well as *Candida albicans* and lipopolysaccharides (LPS). *Escherichia coli*,*Streptococcus agalactiae*, and *Gardnerella vaginalis* have also shown to stimulate HBD2 and HBD3 secretion in human fetal membranes [21–23], while VD-mediated intracrine induction of cathelicidin (LL37, hCTD) gene expression has been demonstrated in different human tissues including trophoblastic and decidual cells [8,24,25]. In human keratinocytes, calcitriol also induces HBD3 and hCTD expression [26]. Moreover, both hCTD and HBD2 promoters contain VD-response elements (VDREs), suggesting that calcitriol directly regulates their expression, as shown in keratinocytes, lung, squamous adenocarcinoma and monocytes [24,27]. However, HBD's regulation by calcitriol in fetal placental cells has not yet been studied. In human placenta, calcitriol seems to suppress both Th1- and Th2-cytokines, including IL-10 [28–30]. Knowing that IL-10 inhibits AMPs expression in certain tissues while calcitriol downregulates IL-10 secretion in human trophoblasts, we hypothesized that IL-10 could downregulate placental AMPs expression while calcitriol would revert this effect. This postulate was corroborated herein, where we show that IL-10 and calcitriol exerted opposed effects on placental innate defenses.

2. Materials and methods

2.1. Reagents

Culture media, fetal bovine serum (FBS), and Trizol were from Invitrogen (CA, USA). TaqMan Master reaction, TaqMan probes, capillaries and the reverse transcription (RT) system were from Roche (Roche Applied Science, IN, USA); calcitriol (1α,25-dihydroxycholecalciferol) was kindly donated from Hoffmann-La Roche Ltd. (Basel, Switzerland). Enzymes used for cell cultures and recombinant cytokines were from Sigma–Aldrich (MO, USA). The concentration of IL-10 and AMPs in the culture media was measured by using specific enzyme-linked immunosorbent sandwich assays (ELISA) (Duo Set R&D systems, MN, USA; Pepro Tech, Rock Hill, NJ; for IL-10 and AMPs, respectively).

2.2. Placenta donors

Patients with chronic hypertension, diabetes mellitus, cervicovaginal infections, renal, and other systemic illnesses were excluded from the study. All placentas in this study were obtained by cesarean section. This study was approved by the Institutional Human Ethical Committee (Comisión Nacional de Investigación Científica del Instituto Mexicano del Seguro Social, protocol number: R-2013-785-033). Placentas were acquired after obtaining written informed consent from each donor.

2.3. Placental cell cultures and experimental conditions

Enriched trophoblastic cell preparations (ETC) were obtained from placental cotyledons as previously described with some modifications [31]. Briefly, villous tissue was enzymatically dispersed, and cells were separated on density percoll gradients. An additional purification step after percoll-separation was performed, which consists in allowing cells to attach to the surface of the flasks for 18 h before aspirating the cell culture media in order to remove all non-adherent cells (which include T-lymphocytes and granulocytes). As determined by Tscherning-Casper et al., by using this method at least 85% of cells correspond to trophoblasts [32]; therefore, our cell cultures are further referred as ETCs. Before plating, the viability of percoll-enriched cells was estimated by dye exclusion (0.4% trypan-blue). Cells were plated at a density of 3×10^6 cells per 25 cm^2 flasks with 4 mL each of supplemented medium (DMEM-HG, 100 U/mL penicillin, 100 mg/mL streptomycin), containing 10% heat-inactivated-FBS. Incubations were performed in humidified 5% CO_2-95% air at 37 °C. Cells were allowed to acclimate overnight and after washing the incubations continued in treatment medium (DMEM-HG, antibiotics and 10% charcoal-stripped heat-inactivated-FBS) in the presence or absence of different calcitriol and/or IL-10 concentrations. Additional experiments were performed incubating the cells with or without TNF-α (10 ng/mL) during 24 h in order to study a possible synergistic effect of this cytokine and calcitriol on AMPs regulation. Medium was collected and stored at −70 °C for AMPs determination by ELISA, while RNA was extracted from cells for gene expression studies.

2.4. PCR amplifications

Regulatory effects on gene expression were studied by extracting total RNA from treated cells using TRIzol reagent. In all cases, the amount and quality of RNA were estimated spectrophotometrically at 260/280 nm, and a constant amount of RNA (1 μg) was reverse transcribed using a RT assay. Primers and probes for PCR amplifications were designed on line using the Universal ProbeLibrary Assay Design Center from Roche, and the sequences are shown in Table 1. Identical PCR conditions were performed for all genes and in all cases normalized against TATA-binding protein (TBP), used as housekeeping gene internal control. Amplifications were carried on the LightCycler® 2.0 instrument (Roche), according to the following protocol: activation of Taq DNA polymerase and DNA denaturation at 95 °C for 10 min, proceeded by 45 amplification cycles of 10 s at 95 °C, 30 s at 60 °C, and 1 s at 72 °C. In this study, the endocrine functionality of our cultured

Table 1
Primers and probes.

Gene/accession number	Upper primer	Lower primer	Amplicon (nt)	Probe number[a]
HBD2/NM_004942.2	tcagccatgagggtcttgta	ggatcgcctataccaccaaa	89	35
HBD3/AJ237673.1	ctgtttttggtgcctgtcc	cacttgccgatctgttcctc	128	8
hCTD/NM_004345.3	tcggatgctaacctctaccg	gtctgggtccccatccat	66	85
CYP24A1/NM_000782.3	catcatggccatcaaaacaa	gcagctcgactggagtgac	65	88
TBP/NM_003194.3	gctggcccatagtgatcttt	cttcacacgccaagaaacagt	60	3
hCGβ/NM_000737.2	gctcaccccagcatcctat	cagcagcaacagcagcag	131	79

[a] From the universal probe library (Roche).

syncytiotrophoblasts was corroborated by human chorionic gonadotropin (hCG) gene expression.

2.5. Quantification of HBD2, HBD3, IL-10 and hCTD by ELISA

HBD2, HBD3, hCTD and IL-10 were quantified by ELISA in the culture media following manufacturer's directions. Assays were performed in 96-well plates and optical density of each well was determined by using a microplate reader (Multiskan MS photometer type 352, Labsystems, Helsinki, Finland). Assay sensitivity was as follows: for HBD2: 15.6 pg/mL; for HBD3: 62.5 pg/mL; for hCTD: 53 pg/mL and for IL-10: 30 pg/mL.

2.6. Characterization of the cell culture by cytokeratin-7 immunofluorescence

In order to evaluate the purity of our cell culture system, we analyzed the percentage of cytokeratin-7 (CK-7) positive cells, since CK-7 is the only keratin expressed in all trophoblast subpopulations but is absent in placental mesenchymal cells [33], allowing its use for adequate identification of cells from the trophoblastic lineage. For this, ETCs were seeded onto sterile two-well chamber slides and incubated during 48 h. Afterwards the cells were washed and fixed for 24 h with freshly prepared zinc fixative (BD Pharmingen, BD Biosciences, CA, USA) at 4 °C. After washing, cells were permeabilized with a 5-min treatment of 1% Triton X-100, washed with PBS, and blocked with 1% bovine serum albumin (BSA) for 1 h. Slides were then incubated overnight at 4 °C with a 1:500 dilution of a mouse anti-CK-7 antibody (BSB5412, Bio SB Inc., Santa Barbara, CA) in 1% BSA. Controls were incubated in the absence of primary antibody. After a washing step, CK-7 was labeled with goat anti-mouse-Cy3 (Life Technologies Inc. 1:500) for 2 h. A drop of UltraCruz mounting medium (Santa Cruz), which contains 4′,6′-diamidino-2-phenylindole (DAPI) for nuclear visualization, was applied to air-dried slides, which were then coverslipped. Syncytiotrophoblasts were photographed with a conventional fluorescence microscope, and the percentage of CK-7-positive cells was calculated.

2.7. Statistical analysis

All data are expressed as the mean ± standard error (SEM) of at least three different cell cultures. Statistical differences for dose-response assays were determined by one-way ANOVA followed by appropriate *ad hoc* tests. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Characterization of the cell culture system

ETCs formed syncytia in a time-dependent manner. Gene expression of hCG-β, used as a marker of endocrine villous cell phenotype, was 8.33 and 61.21 folds higher in days 2 and 3, respectively, compared to day 1 ($P < 0.05$, $n = 3$). Syncytiotrophoblasts were strongly positive for CK-7, which is characteristic of cytoskeletal proteins of epithelial cells and a highly specific trophoblastic marker, as depicted in Fig. 1 in red color (Fig. 1A). Nuclei are shown in blue stained with DAPI. Controls in the absence of first antibody did not show fluorescent signal (Fig. 1B). The percentage of CK-7 positive cells was ~85%.

3.2. AMPs gene expression is inhibited by IL-10 while calcitriol exerts the opposite effect and antagonizes IL-10

Real time PCR analyses showed that calcitriol up-regulated HBD2, HBD3 and hCTD gene expression in a concentration-dependent

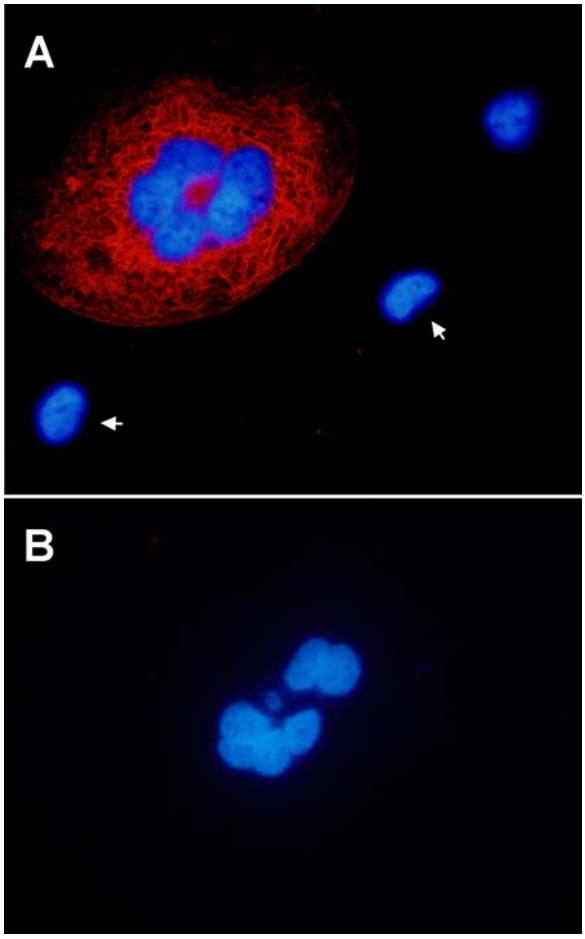

Fig. 1. Characterization of the cell culture system by CK-7 protein expression. ETCs were photographed after 48 h incubation. CK-7, labeled with Cy3 (red, A) was localized in the cytoplasmic skeleton. Nuclei were stained with DAPI (blue, A, B). Negative control (absence of first antibody) is shown in B. All images are 40×. Arrows show some CK-7-negative cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

manner (Fig. 2A and B). In contrast, IL-10 evoked the opposite effect (Fig. 3A and B). Interestingly, coincubations of both IL-10 and calcitriol resulted in a counterbalancing effect. In fact, calcitriol antagonized IL-10 inhibitory effects upon AMPs, maintaining their expression equal or above control values in all cases (Fig. 4A).

We also studied the possibility that IL-10 directly affected calcitriol actions. Therefore, we focused on a well characterized VD-target gene, the VD-24-hydroxylase (CYP24A1). As expected, calcitriol significantly stimulated CYP24A1 gene expression in a dose-response manner, further corroborating the presence and functionality of the VD receptor in our cell cultures (Fig. 4B). Nevertheless, coincubations in the presence of IL-10 resulted in a 50% reduction of the calcitriol-dependent CYP24A1 induction ($P < 0.05$, Fig. 4B).

3.3. TNF-α and calcitriol cooperatively stimulate HBD2 and HBD3 while TNF-α inhibits hCTD gene expression

In order to simulate an inflammatory scenario similar to that of a pathogen threat, we used the proinflammatory cytokine TNF-α. Incubation of cells in the presence of recombinant TNF-α stimulated only HBD2 gene expression (Fig. 5A). Nevertheless, when coincubating the cells in the presence of calcitriol and TNF-α, a cooperative stimulatory action was observed upon both HBD2 and HBD3 gene expression. Interestingly, TNF-α suppressed both basal and calcitriol-stimulated hCTD gene expression by 50% and 70%, respectively ($P < 0.05$, Fig. 5A).

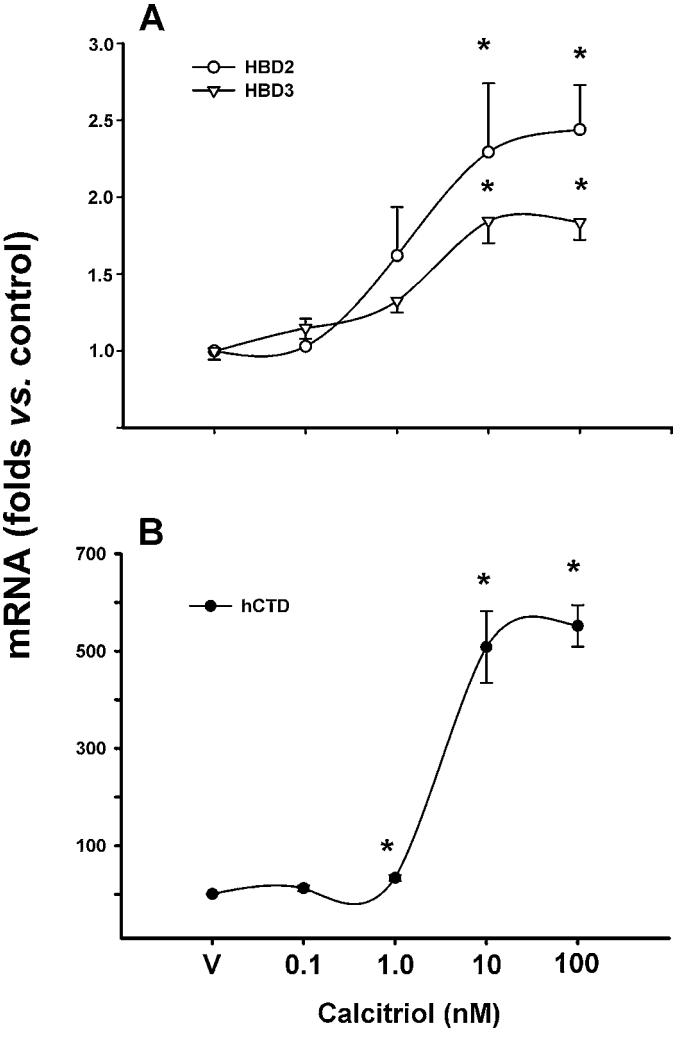

Fig. 2. Calcitriol stimulates HBD2, HBD3 and hCTD gene expression in cultured placental cells. ETCs were cultured in the presence of different calcitriol concentrations or its vehicle (V) during 24 h. AMPs mRNA was normalized against TBP mRNA; HBD2 and HBD3, circles and triangles, respectively (A); cathelicidin, black circles (B). The mean value obtained from vehicle-treated cells was arbitrarily set to 1. $^{*}P < 0.05$ *vs.* V.

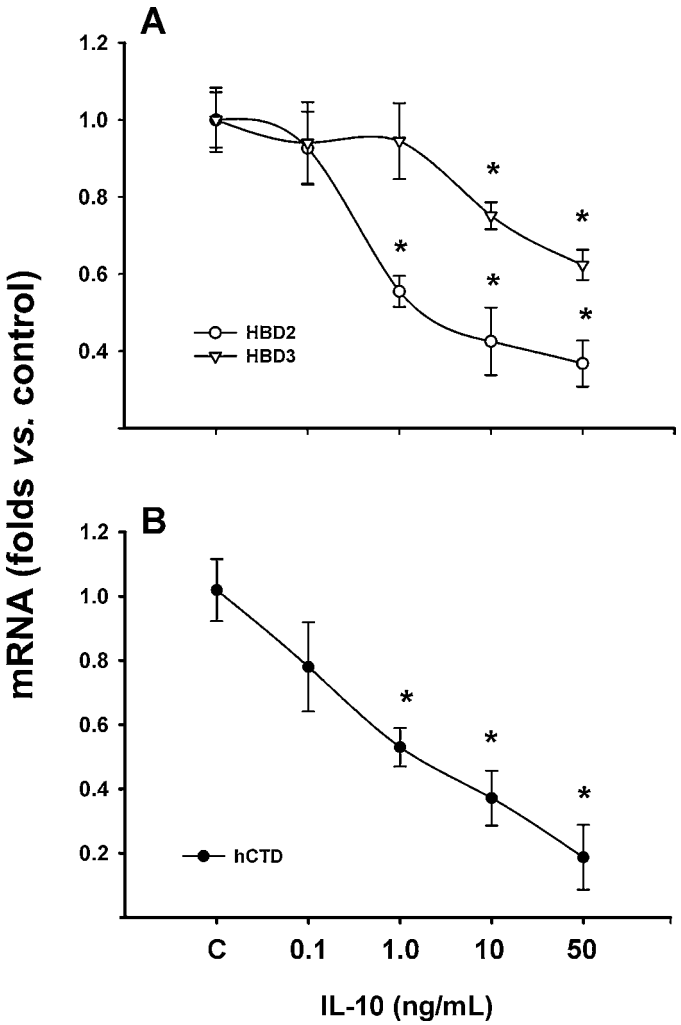

Fig. 3. IL-10 inhibits HBD2, HBD3 and hCTD gene expression in cultured placental cells. ETCs were cultured in the absence (C) or presence of different IL-10 concentrations during 24 h. AMPs mRNA was normalized against TBP mRNA. (A) HBD2 and HBD3, circles and triangles, respectively. (B) Cathelicidin, black circles. The mean value of control was arbitrarily set to 1. $^{*}P < 0.05$ *vs.* C.

3.4. Protein expression studies

As expected and as previously shown [28], IL-10 secretion was significantly downregulated by calcitriol (Fig. 5B).

Our efforts to detect AMPs protein in the culture media by ELISA were unsuccessful, since the levels of these peptides were below the detection limits of the assays. We also assayed cell homogenates; however, this was also ineffective. The latter may be related to incubation times or peptides degradation. It is noteworthy to mention that a similar difficulty to detect AMPs in the culture media of placental cells has been previously reported by others [34].

4. Discussion

The present study was aimed at investigating the regulation of placental AMPs by calcitriol and IL-10, as well as the interaction

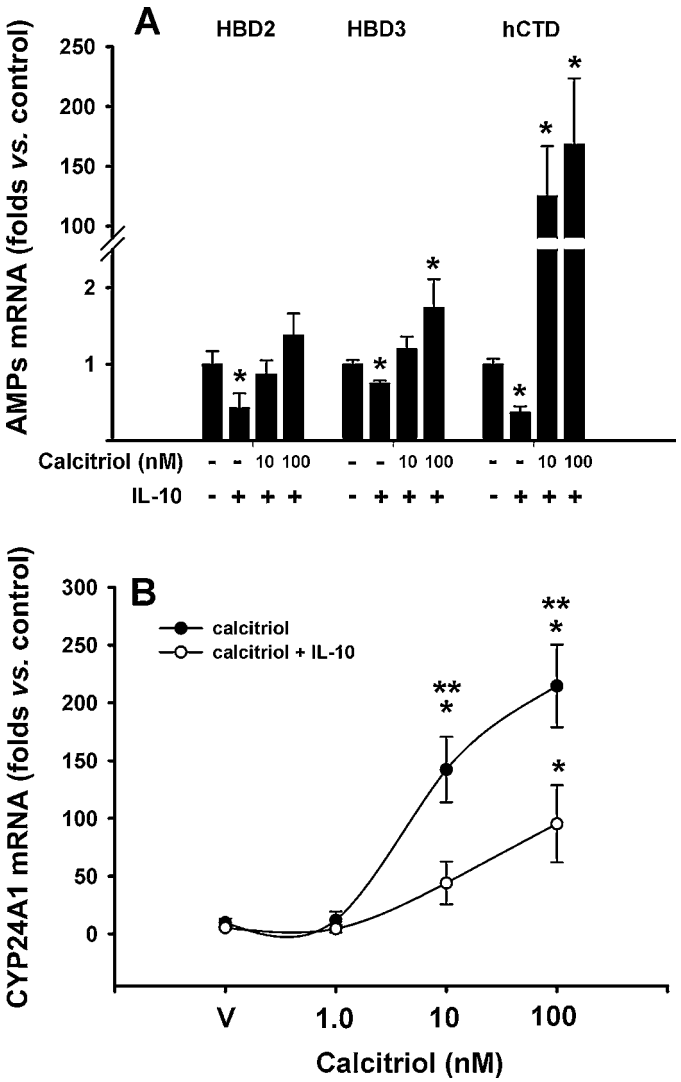

Fig. 4. Antagonistic effects of IL-10 and calcitriol upon placental antimicrobial peptides expression. (A) ETCs were cultured in the absence or presence of IL-10 (10 ng/mL) with or without different calcitriol concentrations during 24 h. Amplifications were performed using oligonucleotides for HBD2, HBD3 or hCTD as indicated, and results were normalized against TBP mRNA. (B) Induction of CYP24A1 gene expression by calcitriol is downregulated by IL-10. Calcitriol alone (closed circles) significantly stimulated CYP24A1 gene expression. Coincubations in the presence of IL-10 (10 ng/mL, open circles) partially antagonized this effect. The mean value obtained from vehicle-treated cells was arbitrarily set to 1. $^{*}P < 0.05$ *vs.* control, $^{**}P < 0.05$ *vs.* same calcitriol concentration in the presence of IL-10.

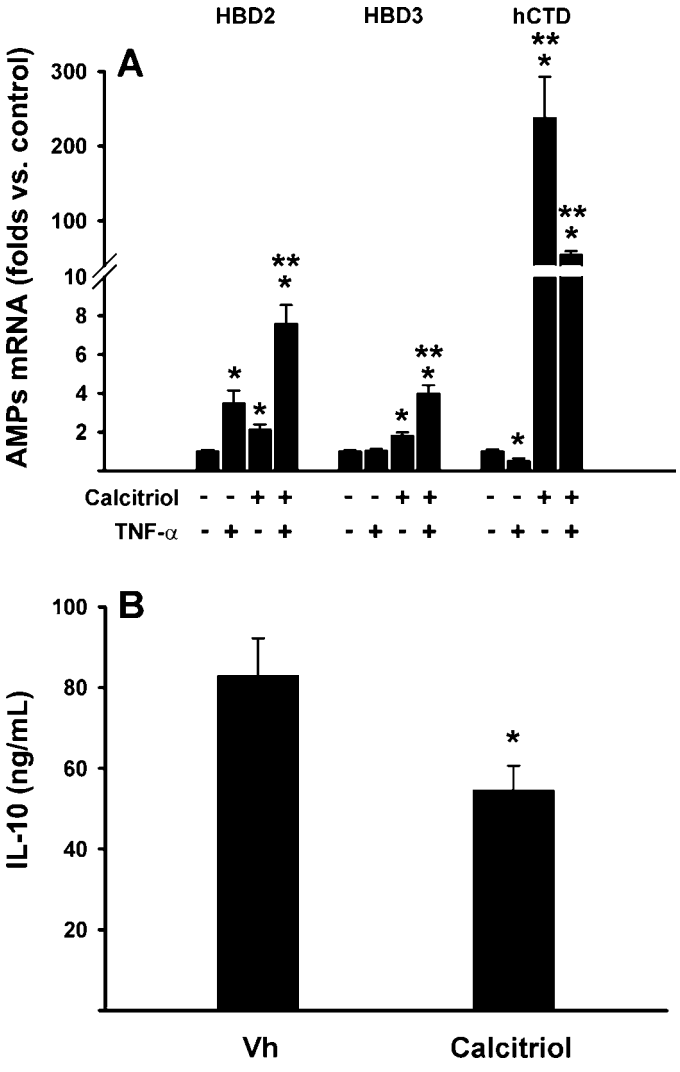

Fig. 5. Calcitriol differentially regulates AMPs and IL-10 gene expression and secretion, respectively. (A) TNF-α and calcitriol cooperatively stimulate HBD2 and HBD3 while TNF-α inhibits hCTD gene expression. ETCs were cultured in the absence or presence of TNF-α (10 ng/mL) with or without calcitriol (10 nM) during 24 h. RT-qPCR amplifications were performed with oligonucleotides for HBD2, HBD3, or hCTD as indicated. AMPs mRNA was normalized against TBP mRNA. The mean value obtained from vehicle-treated cells was arbitrarily set to 1. (B) IL-10 secretion is inhibited by calcitriol. ETCs were cultured in the absence or presence of calcitriol (10 nM) during 24 h. Cell culture media was assayed for IL-10 ELISA. $^{*}P < 0.05$ *vs.* control, $^{**}P < 0.05$ *vs.* calcitriol alone.

between these two immunomodulatory molecules. Our working hypothesis was that IL-10 would damper placental AMPs expression while calcitriol would restore them. Calcitriol is an outstanding hormone given the wide variety of beneficial actions it elicits in different sites of the body, from regulating calcium homeostasis to fighting cancer and infection. In the female reproductive physiology, calcitriol modulates many important functions [35], particularly during the perinatal period [36]. Mammalian gestation creates an immunological paradox wherein the body must balance tolerance of an allogeneic fetus with protection against invading pathogens [37]. A solid body of evidence highlights calcitriol as the hormone in charge of maintaining this balance in the fetoplacental unit. Indeed, calcitriol restrains abnormal inflammation, while stimulates host defense by improving the innate immune response [29,38]. Maintaining a healthy innate immune system is paramount during gestation, since subversion of placental defenses by pathogens can lead to pregnancy complications such as preterm labor or vertical transmission with fetal morbidity or mortality [2]. Placental barriers to infection can be breached by pathogens using different strategies. One of these strategies is embracing IL-10 immunosuppressive properties. It is widely accepted that IL-10 diminishes the capacity of innate immune cells to kill pathogens, as well as reduces their capacity to generate and maintain responsive antigen-specific T cells. The ability of IL-10 to suppress immune responses against intracellular agents is so efficient that some viruses have included a genomic insert homologue to the human IL-10 gene, with the apparent purpose of avoiding host defense [39]. Indeed, the immunosuppressive properties of IL-10 can be exploited by pathogens to facilitate their own survival [12]. In this study, we showed for the first time that IL-10 consistently inhibited HBD2, HBD3 and hCTD gene expression in cultured placental cells, suggesting that in the placenta, IL-10 is involved in restraining innate immune responses, as seen elsewhere. The inhibitory effect of IL-10 upon HBDs was

demonstrated previously in human skin and peripheral blood cells, and the treatment of skin explants with an antibody against IL-10 augmented the expression of HBD2 and hCTD. These effects were attributed to the known ability of IL-10 to reduce inflammatory cytokines that induce AMPs [16–18]. This mechanistic explanation, however, might not be the case in the placenta, since in this study only HBD2 was stimulated by TNF-α. Alternatively, other proinflammatory cytokines inhibited by IL-10 could be participating in placental AMPs induction, or IL-10 could be directly inhibiting AMPs gene expression, which is actually suggested by the results in this study.

It is noteworthy to mention that the skill of IL-10 to dampen AMPs expression may be detrimental if not moderated. In fact, high levels of IL-10 may result in chronic infection [40]. Interestingly, our results showed that in the placenta, calcitriol antagonized IL-10 inhibitory effects upon AMPs, maintaining their expression equal or above control values. Moreover, no inhibitory effect of IL-10 upon AMPs gene expression in the presence of calcitriol was observed, strongly suggesting a compensatory mechanism between the stimulatory *vs.* inhibitory effects of calcitriol and IL-10, respectively, on these genes. This might be explained by the fact that calcitriol induces HBD2, HBD3 and hCTD gene expression, as shown herein.

From a previous study from this laboratory showing the inhibition of IL-10 expression and secretion by calcitriol in cultured placental cells [28], which agreed with the results presented herein, we suggest that calcitriol reestablishment of AMPs expression could involve two different and complementary mechanisms: a direct upregulation of AMPs gene expression and the downregulation of IL-10 secretion. Downregulation of IL-10 may be beneficial to resolve a pathogen threat, as demonstrated in various *in vivo* and *in vitro* experimental settings showing that neutralization of IL-10, either by knockout or by an antibody blockade of the IL-10 receptor, resulted in a faster clearance of intracellular infections than in the presence of IL-10 [12]. However, in some instances, the removal of IL-10 during an infection results in inflammation-associated death, highlighting the critical immunosuppressive role of IL-10 *in vivo*[12]. Nevertheless, in the placenta, the anti-inflammatory effects of calcitriol could prevent this situation, as shown in placental cells cultured under natural and experimental inflammatory conditions and in pregnant mice exposed to bacterial products [29,30,38]. Therefore, considering that IL-10 and calcitriol differentially regulate innate immune responses in the placenta, in the presence of an immunological/inflammatory threat during pregnancy IL-10 permissive actions towards infections might be antagonized by calcitriol while, at the same time, the anti-inflammatory effects of IL-10 are maintained by calcitriol, allowing pregnancy to continue in quiescence and maternal tolerance. Certainly, it is likely that both calcitriol and IL-10 work in concert to sustain gestation, exerting anti-inflammatory actions redundantly.

Herein, we also studied the effects of TNF-α upon AMPs. TNF-α is a master proinflammatory cytokine capable to induce other proinflammatory cytokines including itself, by activation of the nuclear factor kappa B (NFκB). Induction of AMPs by inflammatory mediators is likely since an adequate innate immune response is needed in the event of a pathogen threat. Indeed, HBD2 promoter contains functional NF-κB-binding sites and is induced by proinflammatory cytokines and bacterial products in respiratory epithelia, amnion and endometrial cells [41–45]. Similarly, HBD3 expression in amnion increases in response to LPS or whole bacteria [21–23,46]. In this study, we observed differential regulation exerted by TNF-α upon all three AMPs studied: it stimulated HBD2, inhibited hCTD and did not affect HBD3 gene expression. This might be due to differences in their respective promoter regions, since HBD2 contains three NF-κB sites, HBD3 only one and the hCTD promoter none [47]. Moreover, coincubations with TNF-α and calcitriol further stimulated both β-defensins gene expression, in a similar fashion as previously reported in squamous cell carcinoma cells and monocytes, where coincident signaling by NFκB enhanced calcitriol-mediated induction of HBD2 and hCTD expression [24,48]. Certainly, in some cases, enhancement of NFκB seems to be required in order to potentiate calcitriol antimicrobial effects against intrusion of harmful microorganisms [11]. This fact may shed some light to understand the surprising antagonistic activity exerted by IL-10 upon calcitriol effects found in this study, since IL-10 is able to transcriptionally induce IκBα, the cytoplasmic inhibitor of NFκB [49]. Therefore, the ability of IL-10 to suppress NFκB by preserving IκBα, as seen in macrophages and whole lungs [49], deserves to be further investigated in the placenta. The observation in this study showing that TNF-α and calcitriol work synergistically to induce β-defensins expression indicates that under a pathogen threat, adequate VD levels are very important to mount a strong placental immune defense.

In contrast to the β-defensins, hCTD expression was not directly or concomitantly influenced by the inflammatory stimuli. Moreover, TNF-α significantly inhibited hCTD while reduced calcitriol-mediated induction of hCTD gene expression. Similar results were obtained by Liu and co-workers with primary trophoblasts incubated in the presence of LPS [8]. The physiological meaning of this effect deserves to be further investigated.

Several lines of evidence suggest that the majority of preterm births are caused by bacterial infections, with the organisms originating in the vagina. Preterm delivery is the major problem in obstetrics today, accounting for 70% of perinatal mortality [1]. Considering the results reported herein together with the high prevalence of VD insufficiency during pregnancy (<80 nM serum 25-OHD_3) [50–52], it is imperative to adjust maternal VD levels to reach sufficiency in order to improve host defense.

5. Conclusions

In summary, the results in this study show that IL-10 reduces antimicrobial peptides gene expression in the placenta, an effect that was significantly abrogated by calcitriol. In addition, calcitriol and TNF-α synergistically induced β-defensins expression, indicating that under an infection, adequate VD levels are crucial to enhance innate immunity. Our data suggest that calcitriol induces placental innate immune responses by directly stimulating AMPs gene expression, while at the same time downregulates IL-10 secretion. Altogether this study supports that under a scenario of microbial colonization and infection of placental tissues, maternal VD sufficiency should help deploy a robust innate immune response while restraining exaggerated inflammation.

Acknowledgments

We acknowledge with thanks to Hoffmann-La Roche Ltd. for calcitriol donation and to Hospital de Gineco-Obstetricia “Luis Castelazo Ayala” for assistance in obtaining the placentas. This work was supported by the Consejo Nacional de Ciencia y Tecnología (CONACyT), México. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. AOO is a Ph.D. student from Universidad Nacional Autónoma de México (UNAM) supported by the grant number 231018 from CONACyT.

References

[1] R.L. Goldenberg, J.C. Hauth, W.W. Andrews, Intrauterine infection and preterm delivery, N. Engl. J. Med. 342 (2000) 1500–1507.

[2] R. Romero, J. Espinoza, L.F. Goncalves, J.P. Kusanovic, L. Friel, S. Hassan, The role of inflammation and infection in preterm birth, Semin. Reprod. Med. 25 (2007) 21–39.
[3] F. Arechavaleta-Velasco, L. Gomez, Y. Ma, J. Zhao, C.M. McGrath, M.D. Sammel, D.B. Nelson, S. Parry, Adverse reproductive outcomes in urban women with adeno-associated virus-2 infections in early pregnancy, Hum. Reprod. 23 (2008) 29–36.
[4] K.J. Hensel, T.M. Randis, S.E. Gelber, A.J. Ratner, Pregnancy-specific association of vitamin D deficiency and bacterial vaginosis, Am. J. Obstet. Gynecol. 204 (2011) e41–49 41.
[5] L.M. Bodnar, J.M. Catov, H.N. Simhan, M.F. Holick, R.W. Powers, J.M. Roberts, Maternal vitamin D deficiency increases the risk of preeclampsia, J. Clin. Endocrinol. Metab. 92 (2007) 3517–3522.
[6] L.M. Bodnar, M.A. Krohn, H.N. Simhan, Maternal vitamin D deficiency is associated with bacterial vaginosis in the first trimester of pregnancy, J. Nutr. 139 (2009) 1157–1161.
[7] L. Diaz, I. Sanchez, E. Avila, A. Halhali, F. Vilchis, F. Larrea, Identification of a 25-hydroxyvitamin D3 1alpha-hydroxylase gene transcription product in cultures of human syncytiotrophoblast cells, J. Clin. Endocrinol. Metab. 85 (2000) 2543–2549.
[8] N. Liu, A.T. Kaplan, J. Low, L. Nguyen, G.Y. Liu, O. Equils, M. Hewison, Vitamin D induces innate antibacterial responses in human trophoblasts via an intracrine pathway, Biol. Reprod. 80 (2009) 398–406.
[9] M.T. Cantorna, Y. Zhu, M. Froicu, A. Wittke, Vitamin D status, 1,25-dihydroxyvitamin D3, and the immune system, Am. J. Clin. Nutr. 80 (2004) 1717S–1720S.
[10] P.T. Liu, S. Stenger, H. Li, L. Wenzel, B.H. Tan, S.R. Krutzik, M.T. Ochoa, J. Schauber, K. Wu, C. Meinken, D.L. Kamen, M. Wagner, R. Bals, A. Steinmeyer, U. Zugel, R.L. Gallo, D. Eisenberg, M. Hewison, B.W. Hollis, J.S. Adams, B.R. Bloom, R.L. Modlin, Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response, Science 311 (2006) 1770–1773.
[11] M. Hewison, Antibacterial effects of vitamin D, Nat. Rev. Endocrinol. 7 (2011) 337–345.
[12] J.C. Cyktor, J. Turner, Interleukin-10 and immunity against prokaryotic and eukaryotic intracellular pathogens, Infect. Immun. 79 (2011) 2964–2973.
[13] A. Maroof, L. Beattie, S. Zubairi, M. Svensson, S. Stager, P.M. Kaye, Posttranscriptional regulation of Il10 gene expression allows natural killer cells to express immunoregulatory function, Immunity 29 (2008) 295–305.
[14] R.T. Gazzinelli, I.P. Oswald, S.L. James, A. Sher, IL-10 inhibits parasite killing and nitrogen oxide production by IFN-gamma-activated macrophages, J. Immunol. 148 (1992) 1792–1796.
[15] A. Oberholzer, C. Oberholzer, L.L. Moldawer, Interleukin-10: a complex role in the pathogenesis of sepsis syndromes and its potential as an anti-inflammatory drug, Crit. Care Med. 30 (2002) S58–S63.
[16] M.D. Howell, M. Boguniewicz, S. Pastore, N. Novak, T. Bieber, G. Girolomoni, D. Y. Leung, Mechanism of HBD-3 deficiency in atopic dermatitis, Clin. Immunol. 121 (2006) 332–338.
[17] M.D. Howell, N. Novak, T. Bieber, S. Pastore, G. Girolomoni, M. Boguniewicz, J. Streib, C. Wong, R.L. Gallo, D.Y. Leung, Interleukin-10 downregulates anti-microbial peptide expression in atopic dermatitis, J. Invest. Dermatol. 125 (2005) 738–745.
[18] Q.X. Chen, J.L. Zhou, M. Book, [IL-10 inhibits inducible expression pattern of beta-defensin-2 in human peripheral blood cells], Zhejiang Da Xue Xue Bao Yi Xue Ban 35 (2006) 596–599 604.
[19] A.E. King, A. Paltoo, R.W. Kelly, J.M. Sallenave, A.D. Bocking, J.R. Challis, Expression of natural antimicrobials by human placenta and fetal membranes, Placenta 28 (2007) 161–169.
[20] R. Bals, Epithelial antimicrobial peptides in host defense against infection, Respir. Res. 1 (2000) 141–150.
[21] G. Garcia-Lopez, P. Flores-Espinosa, V. Zaga-Clavellina, Tissue-specific human beta-defensins (HBD) 1HBD2, and HBD3 secretion from human extra-placental membranes stimulated with *Escherichia coli*, Reprod. Biol. Endocrinol. 8 (2010) 146.
[22] V. Zaga-Clavellina, G. Garcia-Lopez, P. Flores-Espinosa, Evidence of in vitro differential secretion of human beta-defensins-1, -2, and -3 after selective exposure to Streptococcus agalactiae in human fetal membranes, J. Matern. Fetal Neonatal Med. 25 (2012) 358–363.
[23] V. Zaga-Clavellina, R.V. Martha, P. Flores-Espinosa, In vitro secretion profile of pro-inflammatory cytokines IL-1beta, TNF-alpha, IL-6, and of human beta-defensins (HBD)-1HBD-2, and HBD-3 from human chorioamniotic membranes after selective stimulation with *Gardnerella vaginalis*, Am. J. Reprod. Immunol. 67 (2012) 34–43.
[24] T.T. Wang, F.P. Nestel, V. Bourdeau, Y. Nagai, Q. Wang, J. Liao, L. Tavera-Mendoza, R. Lin, J.W. Hanrahan, S. Mader, J.H. White, Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression, J. Immunol. 173 (2004) 2909–2912.
[25] K.N. Evans, L. Nguyen, J. Chan, B.A. Innes, J.N. Bulmer, M.D. Kilby, M. Hewison, Effects of 25-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 on cytokine production by human decidual cells, Biol. Reprod. 75 (2006) 816–822.
[26] X. Dai, K. Sayama, M. Tohyama, Y. Shirakata, Y. Hanakawa, S. Tokumaru, L. Yang, S. Hirakawa, K. Hashimoto, PPARgamma mediates innate immunity by regulating the 1alpha,25-dihydroxyvitamin D3 induced hBD-3 and cathelicidin in human keratinocytes, J. Dermatol. Sci. 60 (2010) 179–186.
[27] S.R. Krutzik, M. Hewison, P.T. Liu, J.A. Robles, S. Stenger, J.S. Adams, R.L. Modlin, IL-15 links TLR2/1-induced macrophage differentiation to the vitamin D-dependent antimicrobial pathway, J. Immunol. 181 (2008) 7115–7120.
[28] D. Barrera, N. Noyola-Martinez, E. Avila, A. Halhali, F. Larrea, L. Diaz, Calcitriol inhibits interleukin-10 expression in cultured human trophoblasts under normal and inflammatory conditions, Cytokine 57 (2012) 316–321.
[29] L. Diaz, N. Noyola-Martinez, D. Barrera, G. Hernandez, E. Avila, A. Halhali, F. Larrea, Calcitriol inhibits TNF-alpha-induced inflammatory cytokines in human trophoblasts, J. Reprod. Immunol. 81 (2009) 17–24.
[30] N. Noyola-Martinez, L. Diaz, E. Avila, A. Halhali, F. Larrea, D. Barrera, Calcitriol downregulates TNF-alpha and IL-6 expression in cultured placental cells from preeclamptic women, Cytokine 61 (2013) 245–250.
[31] H.J. Kliman, J.E. Nestler, E. Sermasi, J.M. Sanger, J.F. Strauss 3rd, Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae, Endocrinology 118 (1986) 1567–1582.
[32] C. Tscherning-Casper, N. Papadogiannakis, M. Anvret, L. Stolpe, S. Lindgren, A. B. Bohlin, J. Albert, E.M. Fenyo, The trophoblastic epithelial barrier is not infected in full-term placentae of human immunodeficiency virus-seropositive mothers undergoing antiretroviral therapy, J. Virol. 73 (1999) 9673–9678.
[33] A. Blaschitz, U. Weiss, G. Dohr, G. Desoye, Antibody reaction patterns in first trimester placenta: implications for trophoblast isolation and purity screening, Placenta 21 (2000) 733–741.
[34] D. Klaffenbach, D. Friedrich, R. Strick, P.L. Strissel, M.W. Beckmann, W. Rascher, A. Gessner, J. Dotsch, U. Meissner, M. Schnare, Contribution of different placental cells to the expression and stimulation of antimicrobial proteins (AMPs), Placenta 32 (2011) 830–837.
[35] M. Grundmann, F. von Versen-Hoynck, Vitamin D – roles in women's reproductive health? Reprod. Biol. Endocrinol. 9 (2011) 146.
[36] N.Q. Liu, M. Hewison, Vitamin D, the placenta and pregnancy, Arch. Biochem. Biophys. 523 (2012) 37–47.
[37] V.B. Zeldovich, A.I. Bakardjiev, Host defense and tolerance: unique challenges in the placenta, PLoS Pathog. 8 (2012) e1002804.
[38] N.Q. Liu, A.T. Kaplan, V. Lagishetty, Y.B. Ouyang, Y. Ouyang, C.F. Simmons, O. Equils, M. Hewison, Vitamin D and the regulation of placental inflammation, J. Immunol. 186 (2011) 5968–5974.
[39] K.W. Moore, P. Vieira, D.F. Fiorentino, M.L. Trounstine, T.A. Khan, T.R. Mosmann, Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein–Barr virus gene BCRFI, Science 248 (1990) 1230–1234.
[40] K.N. Couper, D.G. Blount, E.M. Riley, IL-10: the master regulator of immunity to infection, J. Immunol. 180 (2008) 5771–5777.
[41] S.J. Stock, R.W. Kelly, S.C. Riley, A.A. Calder, Natural antimicrobial production by the amnion, Am. J. Obstet. Gynecol. 196 (2007) e251–256 255.
[42] J. Harder, U. Meyer-Hoffert, L.M. Teran, L. Schwichtenberg, J. Bartels, S. Maune, J.M. Schroder, Mucoid *Pseudomonas aeruginosa*, TNF-alpha, and IL-1beta but not IL-6, induce human beta-defensin-2 in respiratory epithelia, Am. J. Respir. Cell Mol. Biol. 22 (2000) 714–721.
[43] C.Y. Kao, C. Kim, F. Huang, R. Wu, Requirements for two proximal NF-kappaB binding sites and IkappaB-zeta in IL-17A-induced human beta-defensin 2 expression by conducting airway epithelium, J. Biol. Chem. 283 (2008) 15309–15318.
[44] S. Matsuo, S. Yamazaki, K. Takeshige, T. Muta, Crucial roles of binding sites for NF-kappaB and C/EBPs in IkappaB-zeta-mediated transcriptional activation, Biochem. J. 405 (2007) 605–615.
[45] A.E. King, D.C. Fleming, H.O. Critchley, R.W. Kelly, Regulation of natural antibiotic expression by inflammatory mediators and mimics of infection in human endometrial epithelial cells, Mol. Hum. Reprod. 8 (2002) 341–349.
[46] I.A. Buhimschi, M. Jabr, C.S. Buhimschi, A.P. Petkova, C.P. Weiner, G.M. Saed, The novel antimicrobial peptide beta3-defensin is produced by the amnion: a possible role of the fetal membranes in innate immunity of the amniotic cavity, Am. J. Obstet. Gynecol. 191 (2004) 1678–1687.
[47] C. Albanesi, H.R. Fairchild, S. Madonna, C. Scarponi, O. De Pita, D.Y. Leung, M.D. Howell, IL-4 and IL-13 negatively regulate TNF-alpha- and IFN-gamma-induced beta-defensin expression through STAT-6, suppressor of cytokine signaling (SOCS)-1, and SOCS-3, J. Immunol. 179 (2007) 984–992.
[48] P.T. Liu, M. Schenk, V.P. Walker, P.W. Dempsey, M. Kanchanapoomi, M. Wheelwright, A. Vazirnia, X. Zhang, A. Steinmeyer, U. Zugel, B.W. Hollis, G. Cheng, R.L. Modlin, Convergence of IL-1beta and VDR activation pathways in human TLR2/1-induced antimicrobial responses, PLoS One 4 (2009) e5810.
[49] A.B. Lentsch, T.P. Shanley, V. Sarma, P.A. Ward, In vivo suppression of NF-kappa B and preservation of I kappa B alpha by interleukin-10 and interleukin-13, J. Clin. Invest. 100 (1997) 2443–2448.
[50] L.M. Bodnar, H.N. Simhan, R.W. Powers, M.P. Frank, E. Cooperstein, J.M. Roberts, High prevalence of vitamin D insufficiency in black and white pregnant women residing in the northern United States and their neonates, J. Nutr. 137 (2007) 447–452.
[51] A.L. Ponsonby, R.M. Lucas, S. Lewis, J. Halliday, Vitamin D status during pregnancy and aspects of offspring health, Nutrients 2 (2010) 389–407.
[52] B.W. Hollis, C.L. Wagner, Vitamin D deficiency during pregnancy: an ongoing epidemic, Am. J. Clin. Nutr. 84 (2006) 273.

Nutrients **2015**, *7*, 443-480; doi:10.3390/nu7010443

OPEN ACCESS

nutrients

ISSN 2072-6643

www.mdpi.com/journal/nutrients

Review

Regulation of Calcitriol Biosynthesis and Activity: Focus on Gestational Vitamin D Deficiency and Adverse Pregnancy Outcomes

Andrea Olmos-Ortiz, Euclides Avila, Marta Durand-Carbajal and Lorenza Díaz *

Department of Reproductive Biology, National Institute of Medical Sciences and Nutrition Salvador Zubirán, Vasco de Quiroga No. 15, Tlalpan 14000, Mexico City, Mexico;
E-Mails: nut.aolmos@gmail.com (A.O.-O.); euclides.avilac@incmnsz.mx (E.A.); mdurand65@gmail.com (M.D.-C.)

***** Author to whom correspondence should be addressed; E-Mail: lorenzadiaz@gmail.com; Tel.: +525-54-87-09-00 (ext. 2417).

Received: 31 October 2014 / Accepted: 16 December 2014 / Published: 9 January 2015

Abstract: Vitamin D has garnered a great deal of attention in recent years due to a global prevalence of vitamin D deficiency associated with an increased risk of a variety of human diseases. Specifically, hypovitaminosis D in pregnant women is highly common and has important implications for the mother and lifelong health of the child, since it has been linked to maternal and child infections, small-for-gestational age, preterm delivery, preeclampsia, gestational diabetes, as well as imprinting on the infant for life chronic diseases. Therefore, factors that regulate vitamin D metabolism are of main importance, especially during pregnancy. The hormonal form and most active metabolite of vitamin D is calcitriol. This hormone mediates its biological effects through a sp ecific nuclear receptor, which is found in many tissues including the placenta. Calcitriol synthesis and degradation depend on the expression and activity of CYP27B1 and CYP24A1 cytochromes, respectively, for which regulation is tissue specific. Among the factors that modify these cytochromes expression and/or activity are calcitriol itself, parathyroid hormone, fibroblast growth factor 23, cytokines, calcium and phosphate. This review provides a current overview on t he regulation of vitamin D metabolism, focusing on vitamin D deficiency during gestation and its impact on pregnancy outcomes.

Keywords: micronutrient; placenta; maternal diet; gestational pathologies

1. Vitamin D Synthesis and Metabolism

UVB radiation from sunlight initiates vitamin D (VD) biosynthesis in the skin by bioconverting 7-dehydrocholesterol to previtamin D_3, which is thermally isomerized to VD_3. VD may also be obtained from the diet to a lesser extent. The nutritional forms of VD include both VD_3 (cholecalciferol) from animal origin and VD_2 (ergocalciferol) from fungi and plant origin. Once formed in the skin or absorbed in the intestine, VD is released into the circulation and transported by the VD-binding protein (DBP) to the liver, where it is converted by the VD-25-hydroxylase (CYP2R1) into 25-hydroxyvitamin D (calcidiol, 25OHD). Calcidiol is the major vitamin D metabolite present in the circulation and the best indicator of VD nutritional status. Nevertheless, this metabolite is not the active form of VD, and therefore needs further activation by a second hydroxylation step catalyzed by the enzyme 25OHD-1-α-hydroxylase (CYP27B1) in order to generate $1,25\text{-}(OH)_2D_3$ (calcitriol), which is the hormonal form and most active VD metabolite. CYP27B1 is primarily expressed in the kidney but also may be found in different tissues including the placenta. The bioavailability of calcitriol is tightly regulated to restrict the biological actions of this hormone in target cells while maintaining calcium and phosphate homeostasis, and the enzyme in charge of degrading calcitriol to water-soluble and less active metabolites is the $1,25\text{-}(OH)_2D_3$-24-hydroxylase (CYP24A1), which in turn is highly upregulated by calcitriol itself, as a negative feedback mechanism [1,2].

Importantly, in order for CYP27B1 to fulfill its duty of producing calcitriol, there must be enough substrate available. This requirement is readily accomplished by the endocytic receptors megalin/cubilin and the adaptor protein disabled-2 (Dab2) present in the kidney, where 25OHD in complex with DBP is filtered through the glomerulus and reabsorbed in the proximal tubules by the cooperative action of these proteins [3–5]. Inside the cell, DBP is degraded and 25OHD is released and hydroxylated by C YP27B1. This specialized mechanism allowing renal uptake and activation of 25OHD is paramount for calcitriol production, as demonstrated by Nykjaer A. *et al.* [3] in rats infused with ^{3}H-25OHD/DBP and where megalin activity was blocked. In these animals, no c onversion products (*i.e.*, ^{3}H-calcitriol) were recovered from serum samples. In contrast, in animals with intact and active megalin that were also infused, ^{3}H-calcitriol was readily detected in their plasma samples. Similarly, the importance of cubilin in the 25OHD reabsorption process is evident in human patients carrying inactivating mutations in cubilin gene (Imerslund–Gräsbeck disease), which exhibit abnormal urinary excretion of 25OHD and DBP [4].

2. Calcitriol Biological Effects

Calcitriol actions are mediated through the vitamin D receptor (VDR), a high-affinity ligand-activated transcription factor. Once bound to its ligand, the VDR heterodimerizes with the retinoid X receptor (RXR). This complex recognizes vitamin D response elements (VDRE) in the promoter regions of VD target genes and recruits co-activators or co-repressors in order to induce or repress gene transcription [6]. In addition, non-genomic calcitriol-dependent biological effects can also take place in cells, involving second messengers generated by membrane-initiated signaling pathways [7,8]. Indeed, the classic VDR and the membrane-associated rapid response steroid-binding protein (MARRS) found in the cell membrane may bind calcitriol and initiate the activation of numerous pathways involving protein

kinase C (PKC) [9], mitogen-activated protein kinase (MAPK) [10], protein kinase A (PKA) [11,12], phosphatidyl inositol phosphate [13] and Ca^{2+} and chloride channels [8,14]. Very important processes are mediated through these rapid signaling cascades, such as insulin secretion and transcaltachia [15–17]. In general, the known VDRE-containing genes can be grouped in very diverse biological networks including bone and mineral metabolism (*i.e.*, osteopontin [18]); cell life and death (comprising proliferation, differentiation, migration and apoptosis, *i.e.*, p21 [19]), metabolism and cardiovascular health (*i.e.*, cystathionine β-synthase [20], renin [21] and VEGF [22]), immune function (*i.e.*, cathelicidin hCTD, LL-37 [23,24]) and detoxification (*i.e.*, CYP3A4, CYP24A1 [25]).

3. Regulation of the VD-Metabolizing Enzymes

Renal CYP27B1, which is responsible for the most part of circulating calcitriol, is mainly regulated by parathyroid hormone (PTH) -as a signal of calcium status-, fibroblast growth factor 23 (FGF23) -as a signal of serum phosphate levels- and calcitriol itself -as a negative feedback regulatory loop- [26]. When serum calcium levels are low, PTH is secreted by the parathyroid gland in order to stimulate renal CYP27B1 by a cyclic AMP (cAMP)-dependent mechanism [27]. PTH also causes CYP24A1 mRNA degradation in the kidney [28]. Calcitriol production is then boosted, thereby increasing blood calcium levels by promoting absorption of dietary calcium from the gastrointestinal tract, increasing renal tubular calcium reabsorption and stimulating the release of calcium from bone. As feedback effects, the synthesis and secretion of PTH are then inhibited by calcitriol and FGF23, the latter being produced in the bone [29]. FGF23 also potently inhibits CYP27B1 in response to elevated phosphate levels [30]. Finally, calcitriol inhibits its own production by different mechanisms comprising PTH inhibition, direct transcriptional repression of the CYP27B1 gene, and FGF23 and CYP24A1 induction [31]. Other factors that stimulate renal CYP27B1 activity and/or expression are insulin-like growth factor type I (IGF-I) and calcitonin [32–34].

4. Extra-Renal Regulation of Vitamin D-Hydroxylases

Extra-renal CYP27B1 is regulated in different ways than those observed in the kidney, in a tissue-specific manner. For instance, macrophage and monocyte CYP27B1 is not stimulated by PTH but rather by cytokines such as interferon-γ (INF-γ) and tumor necrosis factor-α (TNF-α) [35–38]. In addition, CYPB7B1 in immune cells is not readily negatively regulated by calcitriol. Likewise, the negative regulatory loop exerted by CYP24A1 is not very effective; therefore, 25OHD availability is the limiting factor for calcitriol synthesis in these cells [38]. In a similar manner as in immune cells, in keratinocytes, TNF-α and INF-γ potently induce CYP27B1 whereas calcitriol does not directly inhibit this gene [39–41]. Nevertheless, CYP24A1 is induced by calcitriol and efficiently degrades bioactive calcitriol, thus regulating calcitriol availability in the epidermis [39]. The transcriptional induction of CYP24A1 by calcitriol is feasible considering the existence of multiple VDREs within its promoter. However, other regulatory mechanisms can also play a role in CYP24A1 regulation, such as alternative splicing, epigenetic gene silencing and metabolism of its mRNA [42–45].

In a comparable fashion as immune cells and keratinocytes, proinflammatory cytokines induce CYP27B1 gene expression in the human placenta [46]. Interestingly, in this tissue, CYP24A1 expression is also stimulated by these factors, suggesting that both synthesis and catabolism of

placental calcitriol are locally affected by inflammatory cytokines [46]. Nevertheless, we have shown that TNF-α increased significantly the expression of CYP24A1 over CYP27B1, whereas IFN-γ preferentially stimulated CYP27B1. These observations, together with those showing that cultured trophoblasts secrete significantly more TNF-α than INF-γ, suggest that in the placenta, increased TNF-α secretion may limit calcitriol bioavailability [46]. The latter agrees with *in vivo* evidence showing low serum calcitriol and low placental CYP27B1 expression and calcitriol production in preeclampsia (PE), a pregnancy-associated condition characterized by an exacerbated pro-inflammatory profile.

In contrast to immune and epidermal cells, calcitriol in the placenta is able to transcriptionally inhibit CYP27B1 expression, which is mediated by both the VDR and a cAMP-dependent mechanism [12]. In this tissue, CYP24A1 gene expression is also potently induced by calcitriol [12]. However, it is also recognized that the CYP24A1 gene is methylated in the human placenta, suggesting that epigenetic decoupling of VD feedback catabolism may play an important role in maximizing calcitriol bioavailability at the feto-maternal interface [43]. Nevertheless, as discussed previously by Rosen *et al.* [47], this assumption is not supported by functional studies, since there is unequivocal evidence for human placental synthesis of 24,25-dihydroxyvitamin D_3, the main metabolic product of CYP24A1 [48]. Moreover, in this seminal article by Rubin and colleagues, under physiologic concentrations of 25OHD, placental trophoblasts preferentially synthesize 24,25-dihydroxyvitamin D_3 over calcitriol [48], which may explain why fetal levels of 24-hydroxylated VD metabolites are 40-fold higher than calcitriol [47,49,50]. These studies strongly suggested that placental CYP24A1 expression and activity, together with substrate availability are important limiting factors for calcitriol synthesis in the feto-maternal interface. Other hormonal factors affecting placental VD-metabolism are IGF-I and the natural occurring calciotropic hormones calcitonin and PTH [12,51]. IGF-I stimulates biotransformation of 25OHD into calcitriol [51], while both calcitonin and PTH regulate the placental VD-hydroxylases in the same manner as calcitriol and cAMP, inducing CYP24A1 while repressing CYP27B1 gene expression, thereby favoring VD catabolism [12].

5. Physiological Changes of 25OHD, Calcitriol, DBP and VDR during Pregnancy

During pregnancy, significant changes in maternal serum calcitriol, DBP and placental VDR take place and interact to acquire extra calcium for adequate fetal bone mineralization (for a recent review see Brannon PM and Picciano MF [52]). Indeed, the fetus may accumulate up to 30 g of calcium at term, and to satisfy this demand, VD metabolism is boosted in order to increase calcium intestinal absorption [53]. Collectively, these changes include increased maternal serum calcitriol, DBP, placental VDR and renal and placental CYP27B1 activity, without changes in serum 25OHD or calcium levels. In fact, maternal ionized calcium does not increase despite higher circulating calcitriol [54–57]; instead, serum calcium remains normal as calcium is transferred to the fetus. This equilibrium could be attributed to the concomitant normal rise in calciuria [55–59], precluding the risk of hypercalcemia. Fetal serum calcium levels are higher than those observed in the mother, a situation that requires specific mechanisms for calcium transport against a concentration gradient. In this regard, the active transplacental passage of calcium to the fetus is mediated by placental expression of calcium binding proteins such as calbindin-D9k and 28k [60–62]. Interestingly, placental VDR has shown to be a positive predictor of fetal femur length and is positively correlated with maternal-to-fetal transfer of

calcium [63], suggesting that fetal skeletal growth could be affected by VDR-dependent mechanisms; and therefore, the relative VDR placental abundance would be a preponderant feature for fetal bone health.

Maternal 25OHD serum levels remain constant across pregnancy [56,57,59,64], suggesting that the increment observed in serum calcitriol levels is independent of changes in its precursor synthesis. Maternal 25OHD crosses the placental barrier and represents the main pool of VD in the fetus. In fact, serum fetal (cord blood) 25OHD levels are on average 25% reduced compared to maternal serum and correlate well with mother 25OHD levels [65,66]; therefore, VD deficiency in the mother could be vertically transmitted to the fetus.

During pregnancy, serum calcitriol rises from the first trimester, doubling its concentration compared to non-gravid women by the end of the third trimester and returning to normal values after delivery [54,56,58,59,64,67]. This physiological rise in calcitriol levels observed during pregnancy could be related to increased synthesis rather than decreased clearance [68]. Increased synthesis of calcitriol is linked to higher CYP27B1 activity in maternal kidney, placental trophoblasts and decidua [69–71].

The mechanisms underlying improved CYP27B1 activity during pregnancy remain elusive, partly because its known regulatory factors stay unchanged during this period, such as PTH [54–56,59], which may be even lower with respect to non-gravid women [58,64]. Also, a murine PTH null model supports that PTH does not contribute to increased calcitriol levels during pregnancy [72]. It has been hypothesized [64,73] that a potential regulatory factor for CYP27B1 could be the PTH analog PTH-related peptide (PTH-rP), which is synthesized by fetal parathyroids and placenta [74] and increases throughout pregnancy. PTH-rP could reach the maternal circulation and, after binding to PTH type 1 receptor (PTHR1), it may induce gene expression of CYP27B1 in the kidney and catalyze calcitriol synthesis for endocrine actions. However, this mechanism could not completely explain the higher activity of CYP27B1 in placenta because, as previously described, this tissue has its own mechanisms governing VD metabolism. In fact, PTH downregulates placental CYP27B1 gene expression [12].

The placental contribution to maternal calcitriol levels was demonstrated in 1978 by Weisman and colleagues in a nephrectomized pregnant rat model. In that study, the authors showed that anephric rats can synthesize calcitriol and 24,25-dihydroxyvitamin D_3 from 25OHD and that the fetoplacental unit is the most likely site of production of such metabolites [75]. Later, the *in vitro* synthesis of 24,25-dihydroxyvitamin D_3 and calcitriol by the human placenta was demonstrated [69]. Interestingly, in a model of chronic renal failure by Blum and colleagues [76], nephrectomized rats had lower calcitriol levels in comparison with normal rats, but during pregnancy the nephrectomized group reached similar levels to those observed in pregnant controls, despite kidney absence, which emphasized the important contribution of the placenta [76]. In 2000, studies in cultured human placental syncytiotrophoblasts showed that the synthesis of calcitriol from its endogenous precursor was driven by an enzymatic 1α-hydroxylation mechanism, since these cells expressed a CYP27B1 gene transcription product with a nucleotide sequence identical to that of transcripts previously characterized in the human kidney [70]. Later, the CYP27B1 protein localization was demonstrated in human placental decidual and trophoblastic cells [77].

In relation to VDR, it has been shown that its gene and protein expression is higher in placental and decidual tissue during the first and second trimesters in comparison with term placentas [78,79], in a

similar manner as CYP27B1, with highest levels of expression occurring in first trimester decidua [78], thus suggesting a more preponderant role for calcitriol during the first part of pregnancy. It is likely that this is related to the importance of maintaining an anti-inflammatory setting for the acceptance of the fetal allograft. This is discussed further in Section 6.

Regarding DBP, two longitudinal studies indicate that this protein increases 25% to 56% during pregnancy [59,67], but the mechanisms leading to this increment are still unknown. It has been hypothesized that the DBP rise may be due to increased calcitriol concentration in gestation. In support of this assumption, serum DBP levels in pregnant women correlated with serum calcitriol levels [80].

The rise in DBP during pregnancy is intriguing and should be taken into consideration for the analysis of VD homeodynamics and physiological impact during this period. Actually, a great debate has been taking place regarding which form of 25OHD is the more suitable for activation, the "free" or the DBP-bound form. In this regard, the "free hormone" hypothesis sustains that the free steroid, by diffusing freely through the cell membrane, is the one available for activation and thereafter capable of performing biological effects, while the hormone bound to its carrier protein is considered to be sequestered and therefore, not bioavailable. However, this hypothesis has been confronted in the last years, given the important transporting and interacting mechanisms existing between the megalin/cubilin complex and DBP/25OHD, together with the observed disparity between the expected amounts of free hormone available for passive diffusion and the levels required to efficiently occupy intracellular target receptors. For an excellent review see: Chun *et al.* [81]. Interestingly, Chun and colleagues have recently proposed a viable hypothesis considering a role for DBP in tissue discrimination of $25OHD_2$ and $25OHD_3$. Given that $25OHD_2$ binds to DBP with lower affinity than $25OHD_3$, the kidney would preferentially use the latter metabolite, while cells in the immune system might profit of a greater pool of $25OHD_2$ for antimicrobial peptide induction [81].

It is noteworthy mentioning that megalin/cubilin-mediated 25OHD/DBP endocytosis may also take place in the placenta, since these receptors are expressed in this tissue [82–84] and placental calcitriol production has been proved [69,75], suggesting that circulating 25OHD is accessible to placental CYP27B1 by active transport. However, trophoblastic production of calcitriol may also be explained by the steroid freely diffusing through the cell membrane, and consequently both free and bound-25OHD might represent a bioavailable material for placental CYP27B1. Therefore, even if the uptake of 25OHD by proximal tubule cells evidently depends on the internalization of DBP, the functional significance of the placental megalin/cubilin endocitic complex expression and functionality remains far from clear.

Interestingly, mice with genetic ablation of the DBP gene only presented classic VD-associated problems when maintained under a low VD diet, supporting a role for DBP in maintaining stable serum stores of VD metabolites while modulating the rates of its bioavailability, activation, and end-organ responsiveness [85].

6. Calcitriol Effects during Pregnancy

One of the main activities attributed to calcitriol during pregnancy is to increase calcium absorption and to upregulate placental calcium transport. However, since VDR and CYP27B1 are also expressed in reproductive feminine tissues like the uterus, ovary, endometrium, fallopian epithelial cells and

placenta [86–88], other potential paracrine and autocrine actions of calcitriol cannot be discarded. In this regard, during gestation, VD-dependent regulation of the immune function is paramount, since an adequate balance of the cytokine profile is necessary for pregnancy success. Specifically, calcitriol plays a dual role aimed at improving the innate immune response while restraining exacerbated inflammation. Calcitriol achieves this by inhibiting pro-inflammatory cytokines such as TNF-α, IFN-γ and interleukin-6 (IL-6), while at the same time induces the potent antimicrobial peptide hCTD in the fetoplacental unit [89–92]. In this tissue, calcitriol also stimulates other antimicrobial peptides known as β-defensins (HBDs), in particular HBD2 and HBD3 [93].

Calcitriol biological effects in the placenta are also related to hormonogenesis and overall placental physiology. In particular, calcitriol induces endometrial decidualization and estradiol and progesterone synthesis, but also regulates the expression of human chorionic gonadotropin and placental lactogen expression [94–97]. Table 1 lists currently known targets regulated by calcitriol in human placenta.

Considering these important effects of calcitriol on human pregnancy, it is not surprising that VD inadequacy may contribute to many gestation-associated disorders. Herein, we revise some common adverse pregnancy outcomes associated with maternal VD deficiency.

7. Vitamin D Deficiency and Adverse Pregnancy Outcomes

As previously mentioned, circulating 25OHD represents the best indicator of VD status. This metabolite was eligible instead of calcitriol for two principal reasons [98]: (1) 25OHD is present in a higher concentration than calcitriol (ng/mL *vs.* pg/mL); and (2) in a deficient 25OHD scenario, PTH is stimulated and consequently induces renal CYP27B1 expression and, therefore, calcitriol synthesis. Consequently, this could derivate in a relative and transient "normality" in calcitriol levels, given its short half-life (few hours for calcitriol instead of 2–3 weeks for 25OHD [99,100]).

The spectrum of VD status has been established considering the known risk for adverse consequences [101]. 25OHD values lesser than 25 nmol/L are related to rickets and osteomalacia and therefore are labeled as "severe deficiency". 25OHD values lesser than 50 nm ol/L may sustain long-term adverse health consequences and are classified as "deficiency." The 75 nmol/L cut-off is the point above which there is no uppe r stimulation of PTH [102] and was thereafter designated as "sufficient." However, Heaney pointed out that 25OHD concentrations < 80 nmol/L are associated with reduced calcium absorption and osteoporosis risk [103]; therefore, values lesser than 75 nmol/L are considered as "insufficient." The conversion factor between 25OHD nmol/L and ng/mL is 2.5 (Table 2).

Table 1. Targets modulated by calcitriol in the human placenta (+ = Stimulation; − = Inhibition).

Target	Biological Network	Regulation	Bioeffect	Cell Type	Reference
Placental lactogen	Cell life and death	+	Growth control	Trophoblast	[97]
TNF-α	Immune function	−	Restraining inflammation	Trophoblast	[91,92]
		−	Immunosupression	Decidual cells	[89]
IL-6	Immune function	−	Restraining inflammation	Trophoblast	[91,92]
		−	Immunosupression	Decidual cells	[89]
CSF2 (colony stimulating factor 2)	Immune function	−	Immunosupression	Decidual cells	[89]
hCTD	Immune function	+	Restraining infection	Trophoblast	[90]
				Decidual cells	[89]
CYP24A1	Bone and mineral metabolism	+	Calcitriol catabolism	Trophoblast	[12]
				Decidual cells	[104]
CYP27B1	Bone and mineral metabolism	−	Calcitriol synthesis	Trophoblast	[12]
IL-10 (Interleukin 10)	Immune function	−	Reducing risk of infection	Trophoblast	[105]
KCNH1 (Potassium voltage-gated channel)	Cell life and death	−	Unknown	Trophoblast	[106]
Calbindin-D 28 kDa	Bone and mineral metabolism	+	Calcium transfer	Trophoblast	[61,62]
Calbindin-D 9 kDa	Bone and mineral metabolism	+	Calcium transfer	Trophoblast	[61]
hCG (human chorionic gonadotrophin)	Cell life and death	+, −	Maintenance of pregnancy	Trophoblast	[96]
3β-HSD (3β-hydroxysteroid dehydrogenase) *	Cell life and death	+	Progesterone synthesis	Trophoblast	[95]
CYP19 (aromatase) *	Cell life and death	+	Estradiol synthesis	Trophoblast	[95]
Prolactin	Cell life and death	+	Establishment of pregnancy	Decidual cells	[107]
VDR	Bone and mineral metabolism	+	Allowing calcitriol actions	Trophoblast	[62]
Platelet-activating factor acetylhydrolase	Cell life and death	−	Inactivation of platelet-activating factor	Decidual macrophages	[108]
HOXA10 (Homeobox A10)	Cell life and death	+	Embryo implantation	Decidual cells	[109]
Osteopontin	Bone and mineral metabolism	+	Embryo implantation	Decidual cells	[104]
HBD2	Immune function	+	Restraining infection	Trophoblast	[93]
HBD3	Immune function	+	Restraining infection	Trophoblast	[93]

* Only observed at the enzyme activity level.

Table 2. Cut-offs in vitamin D status according to the Endocrine Society [110].

Vitamin D Status	25OHD (nmol/L)	25OHD (ng/mL)
Severe deficiency	<25	<10
Deficiency	<50	<20
Insufficiency	<75	<30
Sufficiency	75–110	30–44
Toxicity	>250	>100

Table 2 shows the criteria for VD status as proposed by the Endocrine Society. We believe this criteria is more applicable to this review since it provides guidance for clinicians caring for patients, as compared to the reference proposed by the Institute of Medicine, more likely intended for normal healthy populations only to ensure skeletal health [111].

Observational studies have described an association between insufficiency or deficiency in 25OHD levels and adverse pregnancy and neonatal outcomes including PE, gestational diabetes, bacterial vaginosis, recurrent abortion, premature rupture of membranes (PROM), preterm delivery, cesarean section, intrauterine growth restriction and also impaired fertility treatment.

The VD literature is growing rapidly and there are some recent and detailed reviews and meta-analyses about adverse pregnancy outcomes and VD [52,112–119]. The purpose of this section is therefore to show a broad overview of VD status and its relationship with epidemiological and observational data in adverse pregnancy outcomes.

7.1. VD and Preeclampsia (PE)

PE is a hypertensive disease associated to gestation. It is clinically diagnosed by new-onset hypertension (140/90 mmHg) and proteinuria (300 mg/24 h or protein dipstick 1+ or greater) after the 20th gestational week [120,121]. The major cause related to this disease is abnormal placentation secondary to insufficient throphoblastic invasion that disrupts the endocrine, immunologic and angiogenic environment, resulting in the clinical manifestation of PE [122,123]. Interestingly, a multicenter study with 2030 pregnant women of an American cohort from 1959 to 1966 showed that higher maternal circulating 25OHD levels were associated with a significantly lower risk of placental vascular pathology (hemorrhage, infarcts, microinfarcts, decidual atheromas or thrombosis of cord vessels) in pregnant women carrying male fetuses [124]. From this perspective, VD could be a protective factor for the correct development of placental vasculature.

There is a general consensus in the literature about preeclamptic women having lower 25OHD and calcitriol serum levels compared to normotensive normoevolutive pregnant women [125–129]. Indeed, VD deficiency is more common among preeclamptic women [130–135]. This could partially be explained by significantly lower CYP27B1 placental expression and therefore, lower calcitriol biosynthesis in preeclamptic *versus* normal placentas [136].

The relationship between VD, especially 25OHD serum levels, and the risk of PE development has been extensively analyzed in the medical literature. Herein, we resume the studies that positively correlate VD deficiency and PE risk (Table 3).

Table 3. Observational studies on 25OHD serum levels and the risk for PE development.

Reference	Sample Size (PE Cases *vs.* Controls)	Weeks of Gestation for Blood Sampling	25OHD Cut off	Risk for PE Development (OR (95% CI))
[130]	100 PE and 100 controls	>24	<75 nmol/L	3.26 (1.12–9.54)
			<37.5 nmol/L	4.23 (1.4–12.8)
[131]	33 PE, 79 eclamptic and 76 control	≥20 weeks, prior to magnesium sulfate therapy	<5 nmol/L	3.9 (1.18–12.87) for PE
				5.14 (1.98–13.37) for eclampsia
[132]	32 PE and 665 controls	24–26	<50 nmol/L	3.24 (1.37–7.69)
[133]	51 severe PE and 204 controls	15–20	<50 nmol/L	3.63 (1.52–8.65)
[134]	55 PE and 219 controls	22	<37.5 nmol/L	5.0 (1.7–14.1)

PE = Preeclampsia; OR = odds ratio; CI = confidence interval.

Interestingly, Robinson and colleagues [129] found that a 10 ng/mL increase in 25OHD levels yields a 63% decrease in the risk of severe PE, strongly suggesting that pregnant women should have VD sufficiency in order to lower the risk for PE development. In support of this postulation, recently, Bodnar and coworkers [137] studied a large cohort with 717 PE women and 2,986 control women, concluding that maternal VD deficiency is a clear risk factor for severe PE development. Specifically, they found that maternal 25OHD levels ≥ 50 nmol/L reduce in 40% the risk of severe PE development in comparison to women with 25OHD < 30 nmol/L. Another study reports that both 25OHD and soluble VEGF receptor type 1/placental growth factor (sFlt-1/PlGF) ratio at 15–20 weeks of gestation were significant predictors of severe PE [138]. Similarly, a Norwegian study [139] revealed that increased VD intake (15–20 μg/day) decreases about 25% the risk for PE development, and four independent meta-analyses showed a significant association between PE and 25OHD insufficiency or deficiency compared with control groups [112,119,140,141].

Despite all these reports, other authors inform they did not find any relation between maternal 25OHD levels and risk of PE development [142,143].

In summary, this data supports that maintaining VD sufficiency is a relatively simple measure (by cholecalciferol supplementation or reasonable skin sun exposure) for preventing one of the major causes in mother and baby morbidity-mortality, namely, preeclampsia.

7.2. VD and Bacterial Vaginosis

Bacterial vaginosis (BV) is a common infectious disease in reproductive-aged women. It is caused by the replacement of normal vaginal flora (especially Lactobacillus) for mixed anaerobic bacteria [144]. Its importance during pregnancy resides in the association of BV with adverse gynecologic and obstetric outcomes such as PROM, which can cause spontaneous or induced preterm delivery [145].

Interestingly, the National Health and Nutrition Examination Survey (NHANES) 2001–2004 reported that black women suffer two-fold more BV cases in comparison to white women (51.6% *vs.* 23.2%, respectively) [146]. This proportion could be explained by the fact that high melanin levels in darkly pigmented skin blocks ultraviolet radiation reducing cutaneous VD photosynthesis and consequently, decreasing the well-known antimicrobial activity of endogenous calcitriol [147,148].

Observational studies support this hypothesis. A prospective cohort study [149] developed at Pittsburgh University followed 469 pregnant women from <16th gestational week to term. It was observed that mothers with 25OHD serum levels <20 nmol/L had a 65% increased risk of developing BV compared to 25OHD sufficient women (>80 nmol/L), and similarly, in a subsample of the Nashville Birth Cohort [150], 25OHD serum levels were lower in women who developed BV during pregnancy. In a secondary analysis of data from the NHANES 2001–2004, Hensel and coworkers [151] described that VD insufficiency or deficiency had a statistically significant association with BV only among pregnant women (adjusted OR 2.87, 95% CI 1.13–7.28). Interestingly, a meta-analysis comprising 16 independent studies showed that women with bacterial or viral infections presented 2.1 increased risk (95% CI: 1.6–2.7) of PE development [152], suggesting a probable common risk factor which could be VD deficiency.

Besides BV, calcitriol can prevent other kinds of infections during pregnancy. In human bladder biopsies and established bladder cell lines, 25OHD treatment induced hCTD gene expression, which diminished uropathogenic *E. coli* infection, suggesting that adequate 25OHD serum levels could help prevent urinary tract infections [153].

In addition, a case-control study [154] reports that pregnant women with 25OHD insufficiency at 14–16 gestational week had 2.1-fold increased risk in developing severe to moderate periodontal disease (95% CI: 0.99–4.5).

The physiological mechanisms underlying these observations are possibly related to immune responses regulated by calcitriol. In fact, as previously mentioned, calcitriol can induce innate immune responses by activating hCTD in placenta, macrophages and dendritic cells [90,155]. The antimicrobial hCTD is an active peptide with broad spectrum antimicrobial activity (anti-Gram-positive and Gram-negative bacteria, mycobacterias, spirochetas and yeasts). Its mechanism of action includes bacterial membrane disruption and activation of toll-like receptors and macrophage and neutrophil chemotaxis [156]. Recently, it was also found that calcitriol can induce hCTD expression, multivesicular endosomes and phagolysosome biogenesis in macrophages, leading to microbial killing through autophagy [157–159]. Other important antimicrobial peptides induced by calcitriol in the placenta are HBD2 and HBD3, which also play a shielding role upon infections. Conjointly, these endogenous antibiotics provide an efficient mechanism of front-line defense since they have the capacity to kill a wide variety of microorganisms throughout the female reproductive tract [160]. Interestingly, it has been shown that IL-10, a physiological suppressor of maternal active immunity, downregulates placental antimicrobial peptides expression, which may be permissive for microbial invasion, since the placenta represents a mechanical and immunological barrier essential to restrict infection progress. However, calcitriol is able to antagonize IL-10 suppressive effects upon placental innate defenses by downregulating IL-10 expression, while at the same time restraining exacerbated inflammation and subsequently helping pregnancy to continue in quiescence [93,105]. These data indicate that adequate VD levels are crucial to enhance immunity.

By reducing infections, calcitriol may exert protective effects upon PROM. However, another mechanism might be involved in PROM prevention: calcitriol, either alone or combined with lipopolysaccharide endotoxin, decreases oxitocin and connexin 43 expression in myometrial smooth muscle cells, both proteins are associated with uterine contractions [161]. These results suggest that calcitriol can modulate uterine quiescence even under bacterial infection and therefore can prevent the

abnormal uterine contractions that favor PROM and preterm delivery. In this sense, 25OHD could exert a protective role in preterm birth but this still remains unclear, since in a multicenter American cohort with twin-gestation women [162], it was observed that women with sufficiency in 25OHD (>75 nmol/L) had a 60% lower risk of preterm labor in comparison to those <75 nmol/L (OR 0.4, 95% CI 0.2–0.8). However, in a case-control study with women at high risk for prior preterm birth, VD status at 16–22 gestational weeks was not associated with recurrent preterm birth [163].

7.3. Vitamin D and Gestational Diabetes

Gestational diabetes mellitus (GDM) is defined as carbohydrate intolerance resulting in hyperglycemia of variable severity with new-onset or first recognition during pregnancy [164,165]. GDM, one of the most common complications of pregnancy, is related to adverse outcomes that increase morbidity and mortality in mothers and neonates, including hypertension, PE, urinary tract infection, caesarean delivery, fetal macrosomia, neonatal hypoglycemia and high long-term risk for metabolic syndrome or diabetes mellitus type 2 (DM2) development [165–167].

The classic risk factors known for GDM include maternal overweight status or obesity, prior history of GDM, family history of DM2, antecedent of macrosomic infant, and increased maternal age [165]. However, since 1980 [168], there is a constantly growing body of evidence that supports a connection between VD and insulin or glucose metabolism.

Some observational studies reported lower 25OHD levels in pregnant women with GDM in comparison with normal pregnant women [169–176]. In Table 4, studies that evaluated serum 25OHD levels and the risk for GDM are shown.

Table 4. Observational studies on 25OHD serum levels and risk for GDM development.

Reference	Sample Size (GDM Cases *vs.* Controls)	Weeks of Gestation for Blood Sampling	25OHD Cut off	Risk for GDM Development (OR (95% CI))
[169]	20 GDM and 40 controls	At delivery	<50 nmol/L	30.78 (4.65–203.90)
[177]	68 GDM and 1,246 controls	26–28	<25 nmol/L	3.6 (1.7–7.8)
[171]	116 GDM and 219 controls	15–18	<73.5 nmol/L	2.21 (1.19–4.13)
[178]	200 GDM and 200 controls	26–28	<25 nmol/L	1.80 (1.209–2.678)
[172]	54 GDM and 111 controls	24–28	<37.5 nmol/L	2.66 (1.26–5.6)
			<50 nmol/L	2.02 (0.88–4.6)
[173]	57 GDM and 114 controls	16	<50 nmol/L	3.74 (1.47–9.50)
[170]	81 GDM and 226 controls	Between 2nd and 3rd trimester	<50 nmol/L	1.92 (0.89–4.17)

GDM = Gestational diabetes mellitus; OR = odds ratio; CI = confidence interval.

In a meta-analysis made by Poel and coworkers [179], the mean odds ratio calculated for GDM from 7 independent observational studies about 25OHD deficiency was 1.61 (95% CI 1.19–2.17). Similarly, a review on VD status and GDM risk concluded that maternal VD deficiency and insufficiency are associated with markers of altered glucose homeostasis [180].

Other studies correlated serum 25OHD levels with a significant inverse association with glucose metabolic response in women with GDM: higher serum 25OHD was found to be associated with at

least one of the following parameters: lower fasting glucose, lower 2 h glucose (*post* oral glucose tolerance test), lower glycosylated hemoglobin, lower serum insulin, lower insulin resistance or lower homeostasis model of assessment of insulin resistance HOMA-IR [170,171,174,175,178,181–184].

In the future, the VD and lifestyle intervention (DALI) protocol could provide interesting information about VD supplementation and GDM risk. DALI is a multicenter European protocol in which the risk of GDM is being evaluated in 880 pregnant women divided into eight intervention groups considering physical activity, healthy eating habits and VD_3 supplementation (1600 IU/day or placebo). Unfortunately, the results from this study are still unpublished [185]. To our knowledge, there is only one published double-blind randomized controlled clinical trial which supplemented VD_3 on pregnant women with GDM [186]. In this study, pregnant women received two oral doses of 50,000 IU (baseline and day 21) or placebo capsules after 24 weeks of gestation. Women treated with VD_3 had a significant increase in 25OHD serum levels and a significant decrease in fasting glucose, insulin serum and HOMA-IR, which supports positive glucose metabolic effects on mothers by VD_3 supplementation. Similarly, Mozzafari and coworkers [187] administered a single intramuscular VD_3 dose (300,000 IU) post-partum in 45 women with GDM. After three months of intervention, treated women presented significantly higher 25OHD levels and lower HOMA-IR than women with GDM in the control group.

Contradictorily to this background, there are some studies which concluded that there are no significant differences between VD status in women with GDM or controls [182,188,189], or conclude that VD deficiency is not a risk factor for GDM [190,191].

There is little consensus about the physiological mechanisms governing calcitriol and glucose metabolism connections. The classic mechanism known is that calcitriol can regulate intracellular calcium flux on β-pancreatic cells and therefore can modulate depolarization-stimulated insulin release [17]. However, there are recent evidences that include other VD-dependent mechanisms: (a) diminished inflammatory state in obesity and enhanced expression of genes involved in glucose and lipid metabolism like peroxisome proliferator-activated receptor gamma (PPARγ) or its coactivator (PGC1α) in peripheral blood mononuclear cells [192]; (b) weight reduction and muscle insulin receptor substrate 1 (IRS-1) upregulation [193]; (c) upregulation of adipocyte glucose transporter 4 (GLUT4) protein and its translocation to the cell surface [194]; and d) returning to normal liver activity of glucose metabolic enzymes hexokinase, fructose 1,6-bisphosphatase and glucose 6-phosphatase [195]. In GDM, these VD-modulated beneficial mechanisms could be altered, at least in part, because placentas from GDM mothers have higher CYP24A1 protein and gene expression which can derivate in lower calcitriol bioavailability [169].

7.4. Vitamin D and Low Birth Weight or Small for Gestational Age

Newborns small for gestational age (SGA) are defined as those with a birth weight for gestational age below to the 10th percentile in standard growth curves (<5th and 3rd percentiles are also used), whereas low birth weight is defined as newborn weight lower than 2500 g independently of gestational age [196,197]. These complications are associated with substantially higher rates of perinatal morbidity and mortality, including cerebral palsy, neonatal polycythemia, hyperbilirubinemia, and hypoglycemia [197].

The associations between serum maternal 25OHD levels and birth weight of SGA newborns have not been extensively studied. Epidemiological analyses support that black infants had lower birth weight than white infants, which suggests a possible role of VD [198]. However, there are still controversial opinions about lower VD levels failing to modulate fetal growth or if external parameters as maternal obesity, lower socioeconomic status or poor nutrition contribute to lower 25OHD levels and also to SGA and low birth weight development [199]. Herein, we present the major findings in this area. Few studies did not find a significant relation between birth weight for SGA proportion and 25OHD levels [190,200–202]; however, many observational studies did. A multicenter cohort study indicated that maternal 25OHD levels > 37.5 nmol/L are associated with higher birth weight infants in comparison to newborns from women with lesser than 37.5 nmol/L [203]. Another study indicates that pregnant women who delivered SGA infants had lower serum 25OHD levels at 11–13 gestational weeks [204]. Also, umbilical cord serum calcitriol concentrations were lower in SGA than in adequate weight for gestational age infants [205]. In this study, maternal 25OHD levels were also lower in the SGA group but did not reach statistical significance. Similarly, in a birth cohort study, women with 25OHD levels between 8.5 and 48 nmol/L were more likely to give birth to SGA offspring (OR 1.57, 95% CI 1.03–2.39) [206].

Dietary analysis of total VD_3 intake was a significant predictor of infant birth weight adjusted for gestation [207], whereas milk or VD intake during pregnancy were significant independent predictors of birth weight [208]. However, it seems that placental weight is not related to 25OHD levels [203].

In a case-control study, Bodnar and colleagues [209] also observed that pregnant women with 25OHD deficiency (<37.5 nmol/L) had an increased significant risk of SGA development in offspring. Interestingly, this effect was more evident in white women (OR 7.5, 95% CI 1.8–31.9) in comparison to black women (OR 1.5, 95% CI 0.6–3.5). Unexpectedly, the women with 25OHD insufficiency (37.5–75 nmol/L) presented a lower risk of SGA than women with 25OHD sufficiency (>75 nmol/L) in both black and white women. The authors discuss that the potential mechanisms which can explain this U-shaped SGA risk remain uncertain, but the risk of other diseases such as allergic responses or atopic disorders present a similar pattern.

We only found one interventional study about VD and birth weight. In a partially randomized assay on pregnant women, the intervention with one single oral dose of 1500 μg VD_3 (equal to 60,000 IU) or two doses of 3000 μg each (equal to 120,000 IU) in the 2nd and 3rd trimesters resulted in higher birth weight and length in comparison to newborns of mothers treated with usual care [210].

Interestingly, interventional studies with calcium and VD_3 performed in pregnant adolescents showed that both nutrients positively influenced fetal bone growth *in utero*, and that even if both factors were needed for fetal bone health, one could partially compensate for the other [211]. This study suggests that special attention should be paid to pregnant adolescents in order to fulfill adequate VD and calcium requirements, since not only fetal but also maternal bone health may be at risk. This is supported by other studies showing that lactating adolescents lose more bone mineral density when suffering from VD deficiency or low calcium intake [212].

Interestingly, Morley and colleagues suggested that studies on maternal VD status and birth weight should consider neonatal VDR polymorphism, since differences in this feature could help explain why findings from different populations regarding maternal VD status and neonate birth weight have been inconsistent [213].

8. Vitamin D Expenditure and Homeodynamics: Considerations for VD Supplementation

8.1. Endogenous and Exogenous Factors Affecting the VD Status Equation

Considering the high risk of adverse events during pregnancy associated to VD deficiency, it is also important to analyze additional factors that may significantly modify the bioavailability of VD and its metabolites in our body. Indeed, the VD endocrine system is not in constant equilibrium; instead, it is under dynamic regulation and interaction with different factors. For example, the half-life of 25OHD is strongly influenced by DBP concentration, since 25OHD binds to DBP with high affinity [81,214]. Moreover, 25OHD is also affected by DBP genotype [214]. Indeed, the genetic variations that occur in DBP modify its binding affinity for VD metabolites, and the lesser affinity, a shorter half-life is expected. Similarly, genetic variations in CYP27B1, CYP24A1, CYP2R1 and the VDR differentially impact on VD metabolism and biological effects. Single nucleotide polymorphisms have been reported for each of these proteins and may be found as population-specific variants that result in modification of the final VD status. For reviews on this issue please see [215–217]. Considering this, and as previously suggested [215], it is feasible that optimal concentrations of 25OHD required to reduce disease outcomes may vary according to genotype. Other endogenous elements that may predict serum 25OHD half-life are factors known to affect 25OHD metabolism, such as PTH, plasma phosphate and albumin-adjusted calcium [218].

Besides the endogenous factors previously described, exogenous aspects affecting 25OHD plasma concentration include dietary intake, type of clothing and sunshine protection [219], lifestyle and geophysical conditions, this last one interpreted as UVB exposure. In this regard, a study performed with pregnant women in Germany showed that during the winter months, 98% of the maternal blood samples and 94% of the cord blood samples had 25OHD levels < 50 nmol/L, while in the summer months, only 49% of the women and 35% of the cord blood samples were vitamin D deficient [220]. Interestingly, in the same study, the authors found that a significant risk factor for maternal VD deficiency was physical inactivity (adjusted OR 2.67, 95% CI 1.06–6.69, $p = 0.032$), which might be related to less sun exposure. However, a sedentary lifestyle may also be associated with obesity, which has been found to be linked to VD deficiency. Indeed, obese subjects normally have lower basal 25OHD serum concentrations than lean individuals [221], which is possibly explained by the fact that VD is readily stored in adipose tissue due to its fat-soluble nature. In this manner, VD may be sequestered in the greater body pool of fat present in obese individuals, which is supported by previous studies showing that after equal whole-body irradiation or VD supplementation, the increase in serum VD was more than 50% lower in obese than in non-obese subjects [221]. While this occurs in body fat, the muscle cells protect 25OHD from degradation by binding it to actin fibers, in a megalin-dependent process [222]. This may explain why 25OHD concentrations are usually positively associated to muscle-related parameters such as lean body mass and exercise [223].

Unfortunately, during pregnancy, the toll of VD deficiency in obese mothers affects also their child's VD status and health [224,225]. Many studies have shown an array of adverse health outcomes in the offspring of obese women; for example, lower maternal VD status may be linked to programmed differences in offspring fat mass [224,226]. Among the more important adverse effects of maternal VD deficiency upon their offspring, impaired fetal growth and bone development, altered growth and bone

mass later in childhood, neonatal hypocalcemia or tetany and respiratory tract infections have been reported [227].

8.2. Dose Regimens and Vitamin D Supplementation in Pregnant Women

Despite the health advantages associated with a sufficient VD status during pregnancy, as described previously, at this time general consensus supporting a guideline for VD supplementation in pregnant population has not yet been reached. In the literature, a broad spectrum for dosage and periodicity in cholecalciferol supplementation schemes has been reported: 2000 IU, 4000 IU, 14,000 IU, 60,000 IU, 120,000 IU or 200,000 IU administered daily, weekly, monthly or in a single mega-dose. Herein, we resume the more recent randomized clinical trials on V D supplementation in pregnancy. Table 5 includes doses and frequency for supplementation together with the outcome as percentage of women who achieved VD sufficiency at the end of intervention and those who developed hypercalcemia.

Based on these data, it seems that 4000 IU given daily results in the highest proportion of pregnant women reaching VD sufficiency without developing hypercalcemia. An exception to this observation is the study by Hossain *et al.* [228], in which only 15% women reached sufficiency under this regimen. Regarding this, we should mention that the group of Pakistani women included in the Hossain study were severely VD deficient (<25 nmol/L) and were an ethnic group in which particular genetic variants might be affecting VD metabolism, which remains to be further studied. Moreover, the cases of hypercalcemia were comparable in both control and supplemented groups, while hypercalcemia persisted despite VD deficiency, suggesting independence of the pharmacological intervention. On the other hand, it should be noted that in the study by Wagner *et al.* [229], the percentage of women considered to attain sufficiency might be underestimated, since their cut-off value was 25OHD serum levels > 100 nmol/L. As in the case of 4000 IU given daily, the weekly regimen of 50,000 IU seems to be also a good therapeutic strategy, since 100% women reached sufficiency without hypercalcemia. However, more studies are needed to confirm these findings.

In contrast, monthly and unique dose regimens do not seem to adequately fulfill VD sufficiency. It is noteworthy that under the single-dose regimen, some authors included in their analyses serum levels below 75 nmol/L (identified with * and ** in Table 5), which under the Endocrine Society parameters is still considered deficiency. Indeed, in the study by Sahu *et al.* [230], which considered a cut-off of 75 nmol/L, only 34.2% of women reached sufficiency with the highest dose, which is very low. Similarly, in the study by Yu *et al.* [231], the observed proportion of 93% in the supplemented group might be misleading, since this number includes women with serum 25OHD levels > 25 nmol/L.

The recent evidence discussed in Section 8.1 may help us to understand the differences in VD expenditure and homeodynamics in order to reach a general consensus on VD supplementation in a tailored manner. We believe, that at this moment, the ideal scheme for VD supplementation will depend on particular endogenous and exogenous factors, on the available formulations of VD (*i.e.*, in Mexico only tablets with 400 IU are available), and on the personal VD metabolism, which should be monitored periodically by serum 25OHD analyses. By taking all these considerations into account, and under medical counseling and supervision, every woman may modulate their VD levels for acquiring sufficiency and avoiding possible toxicity that could lead to hypercalcemia.

Table 5. Cholecalciferol supplementation and VD status in randomized clinical trials in healthy pregnant women.

Reference	Sample Size	Period of Supplementation	Cholecalciferol Supplemented (IU)	% Women with Serum 25OHD > 75 nmol/L at Delivery (No Asterisk)	Hypercalcemia
			Unique Dose		
[210]	97	2nd trimester	60,000	27% **	No evaluated
		Two doses at 2nd and 3rd trimester	120,000	62.5% **	
[230]	84	No supplementation	-	7%	No evaluated
		5th month	60,000	5.7%	
		Two doses at 5th and 7th month	120,000	34.2%	
[231]	180	No supplementation	-	60% *	No indicated
		27 week	200,000	93% *	
			Daily		
[232]	228	27 weeks to term	0	50%	No
			1000	89%	
			2000	91%	
[228]	175	Less than 20 weeks to term	0	1%	3 cases
			4000	15%	9 cases
[233]	162 deficient women	12–16 weeks to term	400	9.5%	No
			2000	24.4%	
			4000	65.1%	
[229]	257	12–16 weeks to term	2000	37.4% ***	No
			4000	46.2% ***	
[234]	350	12–16 weeks to term	400	50%	No
			2000	70.8%	
			4000	82%	
[231]	180	27 weeks to term	800	86% *	No indicated

Table 5. *Cont.*

Reference	Sample Size	Period of Supplementation	Cholecalciferol Supplemented (IU)	% Women with Serum 25OHD > 75 nmol/L at Delivery (No Asterisk)	Hypercalcemia
			Weekly		
[235]	109 deficient women	26–28 weeks to term (8 weeks)	400	3.70%	No
			50,000	100%	
[236]	28	26–28 weeks to term	Basement 70,000 + 35,000 weekly	90%	No
			14,000 weekly	56%	
			Monthly		
[237]	51 deficient women	From 2nd month to term	50,000	35% ****	No evaluated
			100,000	59% ****	

* 25OHD serum > 25 nmol/L; ** 25OHD serum > 50 nmol/L; *** 25OHD serum > 100 nmol/L; **** in cord blood.

9. Methods of Serum VD Measurement

Since four decades ago, numerous analytical methods have been developed for 25OHD measurement, including competitive protein binding assay, enzyme-linked immunoassay (ELISA), radioimmunoassay (RIA), chemiluminescence assay, gas chromatography-mass spectrometry (GC-MS), high performance liquid chromatography (HPLC) and, more recently, liquid chromatography coupled with mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS). A good review of accuracy, sensibility and technical description of these methods was made by Hollis [238].

LC-MS/MS is the most promising technique for VD analysis since it is a highly specific, reliable, reproducible and robust method and is considered the new gold standard for 25OHD quantification [239,240]. Despite its major field of application being research, LC-MS/MS technology is also currently being applied in clinical laboratories [241]. In addition, LC-MS/MS offers the possibility for quantifying other metabolites of VD in serum samples, such as $25OHD_2$, $25OHD_3$, 3-epi-$25OHD_2$, 3-epi-$25OHD_3$, 24R,25 dihydroxyvitamin D_3 [242,243] and $25OHD_3$ 3-sulfate [244]. Other metabolites as VD_2, VD_3, $1\alpha,25(OH)_2D_2$ and $1\alpha,25(OH)_2D_3$ may also be detected and discriminated.

One limitation of this technique is the interference with 3-epi $25(OH)D_3$ that can lead to 25OHD overestimation. This could be a problem especially in pediatric samples which are known to have significant amounts of this epimer. Recently, van den Ouweland and colleagues developed a LC-MS/MS method which eliminates this limitation [245].

Interestingly, new technology developed to be used in our daily life will allow us to measure 25OHD easily at home. The proposed system is a gold-nanoparticle-immunoassay developed at Cornell University, adapted to a device that couples to smartphones allowing them to calculate in a small drop of blood 25OHD serum concentrations with 10 nM sensitivity [246].

In the aftermath of what is discussed in Sections 5 and 8.1, it seems that 25OHD should not be exclusively considered for the assessment of VD, but rather, the equation for VD status may also consider DBP levels. Powe and coworkers [247] recently suggested that free 25OHD (25OHD minus DBP) is a better indicator for VD status since free 25OHD offers a strong correlation with PTH than total 25OHD. However, this article has been criticized by other authors [248]. Indeed, as previously discussed, Weintraub [248] pointed out that the 25OHD/DBP complex is necessary for the endocytosis by megalin/cubilin in the kidney, so 25OHD bound to DBP may be the real substrate mediating final calcitriol biosynthesis. Nevertheless, this would only apply to those cells expressing megalin/cubilin, and definitive further studies are needed in order to clarify the participation of other mechanisms of 25OHD storage and internalization into the cell.

10. Final Considerations

Maintaining adequate VD serum concentrations within the recommended levels is mandatory during pregnancy, since it is involved in many important biological processes, including fetal programming and development. Indeed, the benefits of maintaining adequate VD serum levels are not circumscribed to the mother, but also to the offspring. It is noteworthy mentioning that epidemiological studies have shown controversial results about the benefits of prescribing VD supplements to prevent adverse pregnancy outcomes associated with maternal deficiency. We believe

that the controversy may be explained because the biological phenomena can be affected not only by serum concentrations, but also by many factors, such as racial, climatological, or genetic reasons; nutritional status; lifestyle; physical activity; or health status during pregnancy. On the other hand, the few studies that do not corroborate benefits lack sustained clinical evidence of real risks due to VD supplementation. Conversely, systematic reviews and meta-analyses demonstrate a strong association between VD adequate levels and health benefits. Regarding this controversy, recently, two debated articles concluded that the proposed adverse health outcomes related to VD deficiency might be, in fact, the result of reverse causation, understanding by this that low VD levels are a consequence of ill health rather than a cause, and that the evidence does not really support VD supplementation for prevention of disease [249–251]. In response to these articles, Gillie, with straightforward arguments, demonstrated that the aforementioned articles had made a type 2 statistical error, and that VD deficiency, especially at critical times such as pregnancy and early childhood, could derive in serious health harm [252]. An example of the arguments exposed by Gillie is rickets, a disease characterized by bone deformation in children caused by VD deficiency. This illness may be corrected by adequate VD supplementation during childhood, but the alterations in bones cannot be reversed by this intervention once adulthood is reached. Another example discussed by Gillie is diabetes type 1, which occurs in children and is thought to be caused by VD deficiency in the womb, causing irreversible changes to biochemistry, immune status or organ structure.

A final consideration is the fact that VD supplementation is useful to prevent adverse pregnancy outcomes, but it might not always be necessary, especially when lifestyle recommendations are good enough to prevent them. In order to take the adequate decision about VD supplementation, every clinical individual situation must be analyzed and placed in the correct balance of risk and benefit before prescribing VD supplementation. However, when controversies about clinical decisions are involved, scientists must avoid creating medical barriers about the use of preventive strategies in medicine.

11. Conclusions

Although the importance of VD in the regulation of calcium homeostasis in pregnant women is well established, there is now increasing evidence that calcitriol is also important for the prevention of several adverse scenarios that could potentially threaten pregnancy such as infection and preeclampsia. Even though more interventional and basic studies are needed in order to understand the role of VD in pregnancy health and disease, through the information resumed herein it is clear that many of the beneficial effects of calcitriol during gestation involve its immunomodulatory properties as well as its capacity to regulate hormonogenesis. Despite the protective role of VD in pregnancy outcomes and that several epidemiological studies have documented highly prevalent gestational hypovitaminosis D around the world, routine VD screening is still not mandatory and not enough interventional studies have been undertaken to achieve a consensus for VD supplementation in pregnant women, highlighting the need for further studies and establishment of screening guidelines during pregnancy. Given that the human placenta expresses CYP27B1, which catalyzes the local synthesis of calcitriol, the supplementation with VD during pregnancy might be an accessible and safe way to reduce the incidence of some adverse events associated with mother and baby morbidity-mortality, such as PE, GDM, PROM and infections; while, at the same time, both the mother and the child will profit from

the physiological benefits of calcitriol. Notably, adequate sun exposure, a VD-rich diet and physical activity should always be considered as the first recommendation, while supplementation with cholecalciferol may be advised for persistent VD deficient women.

Acknowledgments

This work was supported by t he Consejo Nacional de Ciencia y Tecnología (CONACyT), México grant number 153862 to LD. AOO is a PhD. student from Posgrado en Ciencias Biológicas at Universidad Nacional Autónoma de México (UNAM) and is receiving a scholarship from CONACyT (231018).

Author Contributions

All of the authors contributed in the writing and proof-reading of this review article.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Bikle, D. Highlights from the 16th vitamin D workshop, San Francisco, CA, June 11–14, 2013. *J. Steroid Biochem. Mol. Biol.* **2014**, *144*, 1–4.
2. Wacker, M.; Holick, M.F. Sunlight and vitamin D: A global perspective for health. *Derm. Endocrinol.* **2013**, *5*, 51–108.
3. Nykjaer, A.; Dragun, D.; Walther, D.; Vorum, H.; Jacobsen, C.; Herz, J.; Melsen, F.; Christensen, E.I.; Willnow, T.E. An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3. *Cell* **1999**, *96*, 507–515.
4. Nykjaer, A.; Fyfe, J.C.; Kozyraki, R.; Leheste, J.R.; Jacobsen, C.; Nielsen, M.S.; Verroust, P.J.; Aminoff, M.; de la Chapelle, A.; Moestrup, S.K.; *et al*. Cubilin dysfunction causes abnormal metabolism of the steroid hormone 25(OH) vitamin D(3). *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 13895–13900.
5. Morris, S.M.; Tallquist, M.D.; Rock, C.O.; Cooper, J.A. Dual roles for the dab2 adaptor protein in embryonic development and kidney transport. *EMBO J.* **2002**, *21*, 1555–1564.
6. Dusso, A.S.; Brown, A.J.; Slatopolsky, E. Vitamin D. *Am. J. Physiol. Ren. Physiol.* **2005**, *289*, 8–28.
7. Costa, J.L.; Eijk, P.P.; van de Wiel, M.A.; ten Berge, D.; Schmitt, F.; Narvaez, C.J.; Welsh, J.; Ylstra, B. Anti-proliferative action of vitamin D in MCF7 is still active after siRNA-VDR knock-down. *BMC Genomics* **2009**, *10*, 499.
8. Haussler, M.R.; Jurutka, P.W.; Mizwicki, M.; Norman, A.W. Vitamin D receptor (VDR)-mediated actions of 1alpha,25(OH)(2)vitamin D(3): Genomic and non-genomic mechanisms. *Best Pract. Res. Clin. Endocrinol. Metab.* **2011**, *25*, 543–559.

9. Sylvia, V.L.; Schwartz, Z.; Ellis, E.B.; Helm, S.H.; Gomez, R.; Dean, D.D.; Boyan, B.D. Nongenomic regulation of protein kinase C isoforms by the vitamin D metabolites 1 alpha,25-(OH)2D3 and 24R,25-(OH)2D3. *J. Cell. Physiol.* **1996**, *167*, 380–393.
10. Beno, D.W.; Brady, L.M.; Bissonnette, M.; Davis, B.H. Protein kinase C and mitogen-activated protein kinase are required for 1,25-dihydroxyvitamin D3-stimulated EGR induction. *J. Biol. Chem.* **1995**, *270*, 3642–3647.
11. Berg, J.P.; Haug, E. Vitamin D: A hormonal regulator of the cAMP signaling pathway. *Crit. Rev. Biochem. Mol. Biol.* **1999**, *34*, 315–323.
12. Avila, E.; Diaz, L.; Barrera, D.; Halhali, A.; Mendez, I.; Gonzalez, L.; Zuegel, U.; Steinmeyer, A.; Larrea, F. Regulation of vitamin D hydroxylases gene expression by 1,25-dihydroxyvitamin D3 and cyclic AMP in cultured human syncytiotrophoblasts. *J. Steroid Biochem. Mol. Biol.* **2007**, *103*, 90–96.
13. Morelli, S.; de Boland, A.R.; Boland, R.L. Generation of inositol phosphates, diacylglycerol and calcium fluxes in myoblasts treated with 1,25-dihydroxyvitamin D3. *Biochem. J.* **1993**, *289*, 675–679.
14. Zanello, L.P.; Norman, A.W. 1 alpha,25(OH)2 vitamin D3-mediated stimulation of outward anionic currents in osteoblast-like ROS 17/2.8 cells. *Biochem. Biophys. Res. Commun.* **1996**, *225*, 551–556.
15. Nemere, I.; Dormanen, M.C.; Hammond, M.W.; Okamura, W.H.; Norman, A.W. Identification of a specific binding protein for 1 alpha,25-dihydroxyvitamin D3 in basal-lateral membranes of chick intestinal epithelium and relationship to transcaltachia. *J. Biol. Chem.* **1994**, *269*, 23750–23756.
16. Quesada, J.M.; Martin-Malo, A.; Santiago, J.; Hervas, F.; Martinez, M.E.; Castillo, D.; Barrio, V.; Aljama, P. Effect of calcitriol on insulin secretion in uraemia. *Nephrol. Dial. Transplant.* **1990**, *5*, 1013–1017.
17. Sergeev, I.N.; Rhoten, W.B. 1,25-dihydroxyvitamin D3 evokes oscillations of intracellular calcium in a pancreatic beta-cell line. *Endocrinology* **1995**, *136*, 2852–2861.
18. Noda, M.; Vogel, R.L.; Craig, A.M.; Prahl, J.; DeLuca, H.F.; Denhardt, D.T. Identification of a DNA sequence responsible for binding of the 1,25-dihydroxyvitamin D3 receptor and 1,25-dihydroxyvitamin D3 enhancement of mouse secreted phosphoprotein 1 (spp-1 or osteopontin) gene expression. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 9995–9999.
19. Liu, M.; Lee, M.H.; Cohen, M.; Bommakanti, M.; Freedman, L.P. Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. *Genes Dev.* **1996**, *10*, 142–153.
20. Kriebitzsch, C.; Verlinden, L.; Eelen, G.; van Schoor, N.M.; Swart, K.; Lips, P.; Meyer, M.B.; Pike, J.W.; Boonen, S.; Carlberg, C.; *et al*. 1,25-dihydroxyvitamin D3 influences cellular homocysteine levels in murine preosteoblastic MC3T3-E1 cells by direct regulation of cystathionine beta-synthase. *J. Bone Miner. Res.* **2011**, *26*, 2991–3000.
21. Yuan, W.; Pan, W.; Kong, J.; Zheng, W.; Szeto, F.L.; Wong, K.E.; Cohen, R.; Klopot, A.; Zhang, Z.; Li, Y.C. 1,25-dihydroxyvitamin D3 suppresses renin gene transcription by blocking the activity of the cyclic AMP response element in the renin gene promoter. *J. Biol. Chem.* **2007**, *282*, 29821–29830.

22. Garcia-Quiroz, J.; Rivas-Suarez, M.; Garcia-Becerra, R.; Barrera, D.; Martinez-Reza, I.; Ordaz-Rosado, D.; Santos, N.; Villanueva, O.; Santos-Cuevas, C.L.; Avila, E.; *et al*. Calcitriol reduces thrombospondin-1 and increases vascular endothelial growth factor in breast cancer cells: Implications for tumor angiogenesis. *J. Steroid Biochem. Mol. Biol.* **2014**, *144*, 215–222.
23. Liu, P.T.; Stenger, S.; Tang, D.H.; Modlin, R.L. Cutting edge: Vitamin D-mediated human antimicrobial activity against mycobacterium tuberculosis is dependent on t he induction of cathelicidin. *J. Immunol.* **2007**, *179*, 2060–2063.
24. Wang, T.T.; Nestel, F.P.; Bourdeau, V.; Nagai, Y.; Wang, Q.; Liao, J.; Tavera-Mendoza, L.; Lin, R.; Hanrahan, J.W.; Mader, S.; *et al*. Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. *J. Immunol.* **2004**, *173*, 2909–2912.
25. Thompson, P.D.; Jurutka, P.W.; Whitfield, G.K.; Myskowski, S.M.; Eichhorst, K.R.; Dominguez, C.E.; Haussler, C.A.; Haussler, M.R. Liganded VDR induces CYP3A4 in small intestinal and colon cancer cells via DR3 and ER6 vitamin D responsive elements. *Biochem. Biophys. Res. Commun.* **2002**, *299*, 730–738.
26. Henry, H.L. Regulation of vitamin D metabolism. *Best Pract. Res. Clin. Endocrinol. Metab.* **2011**, *25*, 531–541.
27. Rost, C.R.; Bikle, D.D.; Kaplan, R.A. In vitro stimulation of 25-hydroxycholecalciferol 1 alpha-hydroxylation by parathyroid hormone in chick kidney slices: Evidence for a role for adenosine 3′,5′-monophosphate. *Endocrinology* **1981**, *108*, 1002–1006.
28. Zierold, C.; Mings, J.A.; DeLuca, H.F. Parathyroid hormone regulates 25-hydroxyvitamin D(3)-24-hydroxylase mRNA by a ltering its stability. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 13572–13576.
29. Shimada, T.; Kakitani, M.; Yamazaki, Y.; Hasegawa, H.; Takeuchi, Y.; Fujita, T.; Fukumoto, S.; Tomizuka, K.; Yamashita, T. Targeted ablation of FGF23 demonstrates an essential physiological role of FGF23 in phosphate and vitamin D metabolism. *J. Clin. Invest.* **2004**, *113*, 561–568.
30. Shimada, T.; Hasegawa, H.; Yamazaki, Y.; Muto, T.; Hino, R.; Takeuchi, Y.; Fujita, T.; Nakahara, K.; Fukumoto, S.; Yamashita, T. FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis. *J. Bone Miner. Res.* **2004**, *19*, 429–435.
31. Bikle, D.D. Vitamin D metabolism, mechanism of action, and clinical applications. *Chem. Biol.* **2014**, *21*, 319–329.
32. Nesbitt, T.; Drezner, M.K. Insulin-like growth factor-I regulation of renal 25-hydroxyvitamin D-1-hydroxylase activity. *Endocrinology* **1993**, *132*, 133–138.
33. Menaa, C.; Vrtovsnik, F.; Friedlander, G.; Corvol, M.; Garabedian, M. Insulin-like growth factor I, a unique calcium-dependent stimulator of 1,25-dihydroxyvitamin D3 production. Studies in cultured mouse kidney cells. *J. Biol. Chem.* **1995**, *270*, 25461–25467.
34. Kawashima, H.; Torikai, S.; Kurokawa, K. Calcitonin selectively stimulates 25-hydroxyvitamin D3-1 alpha-hydroxylase in proximal straight tubule of rat kidney. *Nature* **1981**, *291*, 327–329.
35. Overbergh, L.; Stoffels, K.; Valckx, D.; Giulietti, A.; Bouillon, R.; Mathieu, C. Regulation of 25-hydroxyvitamin D-1alpha-hydroxylase by IFNgamma in human monocytic THP1 cells. *J. Steroid Biochem. Mol. Biol.* **2004**, *89–90*, 453–455.

36. Gyetko, M.R.; Hsu, C.H.; Wilkinson, C.C.; Patel, S.; Young, E. Monocyte 1 alpha-hydroxylase regulation: Induction by inflammatory cytokines and suppression by dexamethasone and uremia toxin. *J. Leukoc. Biol.* **1993**, *54*, 17–22.
37. Pryke, A.M.; Duggan, C.; White, C.P.; Posen, S.; Mason, R.S. Tumor necrosis factor-alpha induces vitamin D-1-hydroxylase activity in normal human alveolar macrophages. *J. Cell. Physiol.* **1990**, *142*, 652–656.
38. Adams, J.S.; Gacad, M.A. Characterization of 1 alpha-hydroxylation of vitamin D3 sterols by cultured alveolar macrophages from patients with sarcoidosis. *J. Exp. Med.* **1985**, *161*, 755–765.
39. Xie, Z.; Munson, S.J.; Huang, N.; Portale, A.A.; Miller, W.L.; Bikle, D.D. The mechanism of 1,25-dihydroxyvitamin D(3) autoregulation in keratinocytes. *J. Biol. Chem.* **2002**, *277*, 36987–36990.
40. Bikle, D.D.; Pillai, S.; Gee, E.; Hincenbergs, M. Tumor necrosis factor-alpha regulation of 1,25-dihydroxyvitamin D production by human keratinocytes. *Endocrinology* **1991**, *129*, 33–38.
41. Bikle, D.D.; Pillai, S.; Gee, E.; Hincenbergs, M. Regulation of 1,25-dihydroxyvitamin D production in human keratinocytes by interferon-gamma. *Endocrinology* **1989**, *124*, 655–660.
42. Ren, S.; Nguyen, L.; Wu, S.; Encinas, C.; Adams, J.S.; Hewison, M. Alternative splicing of vitamin D-24-hydroxylase: A novel mechanism for the regulation of extrarenal 1,25-dihydroxyvitamin D synthesis. *J. Biol. Chem.* **2005**, *280*, 20604–20611.
43. Novakovic, B.; Sibson, M.; Ng, H.K.; Manuelpillai, U.; Rakyan, V.; Down, T.; Beck, S.; Fournier, T.; Evain-Brion, D.; Dimitriadis, E.; *et al*. Placenta-specific methylation of the vitamin D 24-hydroxylase gene: Implications for feedback autoregulation of active vitamin D levels at the fetomaternal interface. *J. Biol. Chem.* **2009**, *284*, 14838–14848.
44. Chung, I.; Karpf, A.R.; Muindi, J.R.; Conroy, J.M.; Nowak, N.J.; Johnson, C.S.; Trump, D.L. Epigenetic silencing of CYP24 in tumor-derived endothelial cells contributes to selective growth inhibition by calcitriol. *J. Biol. Chem.* **2007**, *282*, 8704–8714.
45. Matilainen, J.M.; Malinen, M.; Turunen, M.M.; Carlberg, C.; Vaisanen, S. The number of vitamin D receptor binding sites defines the different vitamin D responsiveness of the CYP24 gene in malignant and normal mammary cells. *J. Biol. Chem.* **2010**, *285*, 24174–24183.
46. Noyola-Martinez, N.; Diaz, L.; Zaga-Clavellina, V.; Avila, E.; Halhali, A.; Larrea, F.; Barrera, D. Regulation of CYP27B1 and CYP24A1 gene expression by recombinant pro-inflammatory cytokines in cultured human trophoblasts. *J. Steroid Biochem. Mol. Biol.* **2014**, *144*, 106–109.
47. Rosen, C.J.; Adams, J.S.; Bikle, D.D.; Black, D.M.; Demay, M.B.; Manson, J.E.; Murad, M.H.; Kovacs, C.S. The nonskeletal effects of vitamin D: An endocrine society scientific statement. *Endocr. Rev.* **2012**, *33*, 456–492.
48. Rubin, L.P.; Yeung, B.; Vouros, P.; Vilner, L.M.; Reddy, G.S. Evidence for human placental synthesis of 24,25-dihydroxyvitamin D3 and 23,25-dihydroxyvitamin D3. *Pediatr. Res.* **1993**, *34*, 98–104.
49. Higashi, T.; Mitamura, K.; Ohmi, H.; Yamada, N.; Shimada, K.; Tanaka, K.; Honjo, H. Levels of 24,25-dihydroxyvitamin D3, 25-hydroxyvitamin D3 and 25-hydroxyvitamin D3 3-sulphate in human plasma. *Ann. Clin. Biochem.* **1999**, *36* , 43–47.
50. Lester, G.E.; Gray, T.K.; Lorenc, R.S. Evidence for maternal and fetal differences in vitamin D metabolism. *Proc. Soc. Exp. Biol. Med.* **1978**, *159*, 303–307.

51. Halhali, A.; Diaz, L.; Sanchez, I.; Garabedian, M.; Bourges, H.; Larrea, F. Effects of IGF-I on 1,25-dihydroxyvitamin D(3) synthesis by human placenta in culture. *Mol. Hum. Reprod.* **1999**, *5*, 771–776.
52. Brannon, P.M.; Picciano, M.F. Vitamin D in pregnancy and lactation in humans. *Ann. Rev. Nutr.* **2011**, *31*, 89–115.
53. Kumar, R.; Cohen, W.R.; Silva, P.; Epstein, F.H. Elevated 1,25-dihydroxyvitamin D plasma levels in normal human pregnancy and lactation. *J. Clin. Invest.* **1979**, *63*, 342–344.
54. Seki, K.; Makimura, N.; Mitsui, C.; Hirata, J.; Nagata, I. Calcium-regulating hormones and osteocalcin levels during pregnancy: A longitudinal study. *Am. J. Obstet. Gynecol.* **1991**, *164*, 1248–1252.
55. Dahlman, T.; Sjoberg, H.E.; Bucht, E. Calcium homeostasis in normal pregnancy and puerperium. A longitudinal study. *Acta Obstet. Gynecol. Scand.* **1994**, *73*, 393–398.
56. Cross, N.A.; Hillman, L.S.; Allen, S.H.; Krause, G.F.; Vieira, N.E. Calcium homeostasis and bone metabolism during pregnancy, lactation, and postweaning: A longitudinal study. *Am. J. Clin. Nutr.* **1995**, *61*, 514–523.
57. Kovacs, C.S. Calcium and bone metabolism disorders during pregnancy and lactation. *Endocrinol. Metab. Clin. North Am.* **2011**, *40*, 795–826.
58. Moller, U.K.; Streym, S.; Mosekilde, L.; Heickendorff, L.; Flyvbjerg, A.; Frystyk, J.; Jensen, L.T.; Rejnmark, L. Changes in calcitropic hormones, bone markers and insulin-like growth factor I (IGF-I) during pregnancy and postpartum: A controlled cohort study. *Osteoporos. Int.* **2013**, *24*, 1307–1320.
59. Ritchie, L.D.; Fung, E.B.; Halloran, B.P.; Turnlund, J.R.; Van Loan, M.D.; Cann, C.E.; King, J.C. A longitudinal study of calcium homeostasis during human pregnancy and lactation and after resumption of menses. *Am. J. Clin. Nutr.* **1998**, *67*, 693–701.
60. Tuan, R.S.; Moore, C.J.; Brittingham, J.W.; Kirwin, J.J.; Akins, R.E.; Wong, M. In vitro study of placental trophoblast calcium uptake using JEG-3 human choriocarcinoma cells. *J. Cell Sci.* **1991**, *98*, 333–342.
61. Halhali, A.; Figueras, A.G.; Diaz, L.; Avila, E.; Barrera, D.; Hernandez, G.; Larrea, F. Effects of calcitriol on calbindins gene expression and lipid peroxidation in human placenta. *J. Steroid Biochem. Mol. Biol.* **2010**, *121*, 448–451.
62. Belkacemi, L.; Zuegel, U.; Steinmeyer, A.; Dion, J.P.; Lafond, J. Calbindin-D28k (CaBP28k) identification and regulation by 1,2 5-dihydroxyvitamin D3 in human choriocarcinoma cell line JEG-3. *Mol. Cell. Endocrinol.* **2005**, *236*, 31–41.
63. Young, B.E.; Cooper, E.M.; McIntyre, A.W.; Kent, T.; Witter, F.; Harris, Z.L.; O'Brien, K.O. Placental vitamin D receptor (VDR) expression is related to neonatal vitamin D status, placental calcium transfer, and fetal bone length in pregnant adolescents. *FASEB J.* **2014**, *28*, 2029–2037.
64. Ardawi, M.S.; Nasrat, H.A.; HS, B.A.A. Calcium-regulating hormones and parathyroid hormone-related peptide in normal human pregnancy and postpartum: A longitudinal study. *Eur. J. Endocrinol.* **1997**, *137*, 402–409.
65. Ron, M.; Menczel, J.; Schwartz, L.; Palti, Z.; Kidroni, G. Vitamin D3 metabolites in amniotic fluid in relation with maternal and fetal sera in term pregnancies. *J. Perinat. Med.* **1987**, *15*, 282–290.

66. Gupta, M.M.; Kuppuswamy, G.; Subramanian, A.R. Transplacental transfer of 25-hydroxy-cholecalciferol. *Postgrad. Med. J.* **1982**, *58*, 408–410.
67. Wilson, S.G.; Retallack, R.W.; Kent, J.C.; Worth, G.K.; Gutteridge, D.H. Serum free 1,25-dihydroxyvitamin D and the free 1,25-dihydroxyvitamin D index during a longitudinal study of human pregnancy and lactation. *Clin. Endocrinol. (Oxf.)* **1990**, *32*, 613–622.
68. Paulson, S.K.; Ford, K.K.; Langman, C.B. Pregnancy does not alter the metabolic clearance of 1,25-dihydroxyvitamin D in rats. *Am. J. Physiol.* **1990**, *258*, E158–E162.
69. Weisman, Y.; Harell, A.; Edelstein, S.; David, M.; Spirer, Z.; Golander, A. 1 a lpha, 25-dihydroxyvitamin D3 and 24,25-dihydroxyvitamin D3 *in vitro* synthesis by human decidua and placenta. *Nature* **1979**, *281*, 317–319.
70. Diaz, L.; Sanchez, I.; Avila, E.; Halhali, A.; Vilchis, F.; Larrea, F. Identification of a 25-hydroxyvitamin D3 1alpha-hydroxylase gene transcription product in cultures of human syncytiotrophoblast cells. *J. Clin. Endocrinol. Metab.* **2000**, *85*, 2543–2549.
71. Whitsett, J.A.; Ho, M.; Tsang, R.C.; Norman, E.J.; Adams, K.G. Synthesis of 1,25-dihydroxyvitamin D3 by human placenta *in vitro*. *J. Clin. Endocrinol. Metab.* **1981**, *53*, 484–488.
72. Kirby, B.J.; Ma, Y.; Martin, H.M.; Buckle Favaro, K.L.; Karaplis, A.C.; Kovacs, C.S. Upregulation of calcitriol during pregnancy and skeletal recovery after lactation do not require parathyroid hormone. *J. Bone Miner. Res.* **2013**, *28*, 1987–2000.
73. Sanz-Salvador, L.; Garcia-Perez, M.A.; Tarin, J.J.; Cano, A. Endocrinology in pregnancy: Bone metabolic changes during pregnancy: A period of vulnerability to osteoporosis and fracture. *Eur. J. Endocrinol.* **2015**, *172*, 53–65.
74. Simmonds, C.S.; Kovacs, C.S. Role of parathyroid hormone (PTH) and PTH-related protein (PTHrP) in regulating mineral homeostasis during fetal development. *Crit. Rev. Eukaryot. Gene Expr.* **2010**, *20*, 235–273.
75. Weisman, Y.; Vargas, A.; Duckett, G.; Reiter, E.; Root, A.W. Synthesis of 1,25-dihydroxyvitamin D in the nephrectomized pregnant rat. *Endocrinology* **1978**, *103*, 1992–1996.
76. Blum, M.; Weisman, Y.; Turgeman, S.; Cabili, S.; Wollman, Y.; Peer, G.; Stern, N.; Silverberg, D.; Schwartz, D.; Iaina, A. Pregnancy decreases immunoreactive parathyroid hormone level in rats with chronic renal failure. *Clin. Sci. (Lond.)* **1999**, *96*, 427–430.
77. Zehnder, D.; Bland, R.; Williams, M.C.; McNinch, R.W.; Howie, A.J.; Stewart, P.M.; Hewison, M. Extrarenal expression of 25-hydroxyvitamin D(3)-1 alpha-hydroxylase. *J. Clin. Endocrinol. Metab.* **2001**, *86*, 888–894.
78. Zehnder, D.; Evans, K.N.; Kilby, M.D.; Bulmer, J.N.; Innes, B.A.; Stewart, P.M.; Hewison, M. The ontogeny of 25-hydroxyvitamin D(3) 1alpha-hydroxylase expression in human placenta and decidua. *Am. J. Pathol.* **2002**, *161*, 105–114.
79. Evans, K.N.; Bulmer, J.N.; Kilby, M.D.; Hewison, M. Vitamin D and placental-decidual function. *J. Soc. Gynecol. Investig.* **2004**, *11*, 263–271.
80. Bikle, D.D.; Gee, E.; Halloran, B.; Haddad, J.G. Free 1,25-dihydroxyvitamin D levels in serum from normal subjects, pregnant subjects, and subjects with liver disease. *J. Clin. Invest.* **1984**, *74*, 1966–1971.

81. Chun, R.F.; Peercy, B.E.; Orwoll, E.S.; Nielson, C.M.; Adams, J.S.; Hewison, M. Vitamin D and DBP: The free hormone hypothesis revisited. *J. Steroid Biochem. Mol. Biol.* **2014**, *144*, 132–137.
82. Burke, K.A.; Jauniaux, E.; Burton, G.J.; Cindrova-Davies, T. Expression and immunolocalisation of the endocytic receptors megalin and cubilin in the human yolk sac and placenta across gestation. *Placenta* **2013**, *34*, 1105–1109.
83. Akour, A.A.; Gerk, P.; Kennedy, M.J. Megalin expression in human term and preterm placental villous tissues: Effect of gestational age and sample processing and storage time. *J. Pharmacol. Toxicol. Methods* **2014**, doi:10.1016/j.vascn.2014.10.001.
84. Christensen, E.I.; Birn, H. Megalin and cubilin: Multifunctional endocytic receptors. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 256–266.
85. Safadi, F.F.; Thornton, P.; Magiera, H.; Hollis, B.W.; Gentile, M.; Haddad, J.G.; Liebhaber, S.A.; Cooke, N.E. Osteopathy and resistance to vitamin D toxicity in mice null for vitamin D binding protein. *J. Clin. Invest.* **1999**, *103*, 239–251.
86. Johnson, J.A.; Grande, J.P.; Roche, P.C.; Kumar, R. Immunohistochemical detection and distribution of the 1,25-dihydroxyvitamin D3 receptor in rat reproductive tissues. *Histochem. Cell Biol.* **1996**, *105*, 7–15.
87. Shahbazi, M.; Jeddi-Tehrani, M.; Zareie, M.; Salek-Moghaddam, A.; Akhondi, M.M.; Bahmanpoor, M.; Sadeghi, M.R.; Zarnani, A.H. Expression profiling of vitamin D receptor in placenta, decidua and ovary of pregnant mice. *Placenta* **2011**, *32*, 657–664.
88. Zarnani, A.H.; Shahbazi, M.; Salek-Moghaddam, A.; Zareie, M.; Tavakoli, M.; Ghasemi, J.; Rezania, S.; Moravej, A.; Torkabadi, E.; Rabbani, H.; *et al*. Vitamin D3 receptor is expressed in the endometrium of cycling mice throughout the estrous cycle. *Fertil. Steril.* **2010**, *93*, 2738–2743.
89. Evans, K.N.; Nguyen, L.; Chan, J.; Innes, B.A.; Bulmer, J.N.; Kilby, M.D.; Hewison, M. Effects of 25-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 on cytokine production by human decidual cells. *Biol. Reprod.* **2006**, *75*, 816–822.
90. Liu, N.; Kaplan, A.T.; Low, J.; Nguyen, L.; Liu, G.Y.; Equils, O.; Hewison, M. Vitamin D induces innate antibacterial responses in human trophoblasts via an intracrine pathway. *Biol. Reprod.* **2009**, *80*, 398–406.
91. Diaz, L.; Noyola-Martinez, N.; Barrera, D.; Hernandez, G.; Avila, E.; Halhali, A.; Larrea, F. Calcitriol inhibits TNF-alpha-induced inflammatory cytokines in human trophoblasts. *J. Reprod. Immunol.* **2009**, *81*, 17–24.
92. Noyola-Martinez, N.; Diaz, L.; Avila, E.; Halhali, A.; Larrea, F.; Barrera, D. Calcitriol downregulates TNF-alpha and IL-6 expression in cultured placental cells from preeclamptic women. *Cytokine* **2013**, *61*, 245–250.
93. Olmos-Ortiz, A.; Noyola-Martinez, N.; Barrera, D.; Zaga-Clavellina, V.; Avila, E.; Halhali, A.; Biruete, B.; Larrea, F.; Diaz, L. IL-10 inhibits while calcitriol reestablishes placental antimicrobial peptides gene expression. *J. Steroid Biochem. Mol. Biol.* **2014**, doi:10.1016/j.jsbmb.2014.07.012.
94. Halhali, A.; Acker, G.M.; Garabedian, M. 1,25-dihydroxyvitamin D3 induces *in vivo* the decidualization of rat endometrial cells. *J. Reprod. Fertil.* **1991**, *91*, 59–64.

95. Barrera, D.; Avila, E.; Hernandez, G.; Halhali, A.; Biruete, B.; Larrea, F.; Diaz, L. Estradiol and progesterone synthesis in human placenta is stimulated by calcitriol. *J. Steroid Biochem. Mol. Biol.* **2007**, *103*, 529–532.
96. Barrera, D.; Avila, E.; Hernandez, G.; Mendez, I.; Gonzalez, L.; Halhali, A.; Larrea, F.; Morales, A.; Diaz, L. Calcitriol affects hCG gene transcription in cultured human syncytiotrophoblasts. *Reprod. Biol. Endocrinol.* **2008**, *6*, 3.
97. Stephanou, A.; Ross, R.; Handwerger, S. Regulation of human placental lactogen expression by 1,25-dihydroxyvitamin D3. *Endocrinology* **1994**, *135*, 2651–2656.
98. Binkley, N.; Ramamurthy, R.; Krueger, D. Low vitamin D status: Definition, prevalence, consequences, and correction. *Endocrinol. Metab. Clin. North Am.* **2010**, *39*, 287–301.
99. Levine, B.S.; Song, M. Pharmacokinetics and efficacy of pulse oral *versus* intravenous calcitriol in hemodialysis patients. *J. Am. Soc. Nephrol.* **1996**, *7*, 488–496.
100. Batchelor, A.J.; Watson, G.; Compston, J.E. Changes in plasma half-life and clearance of 3h-25-hydroxyvitamin D3 in patients with intestinal malabsorption. *Gut* **1982**, *23*, 1068–1071.
101. Dawson-Hughes, B.; Heaney, R.P.; Holick, M.F.; Lips, P.; Meunier, P.J.; Vieth, R. Estimates of optimal vitamin D status. *Osteoporos. Int.* **2005**, *16*, 713–716.
102. Pepe, J.; Romagnoli, E.; Nofroni, I.; Pacitti, M.T.; de Geronimo, S.; Letizia, C.; Tonnarini, G.; Scarpiello, A.; D'Erasmo, E.; Minisola, S. Vitamin D status as the major factor determining the circulating levels of parathyroid hormone: A study in normal subjects. *Osteoporos. Int.* **2005**, *16*, 805–812.
103. Heaney, R.P. Functional indices of vitamin D status and ramifications of vitamin D deficiency. *Am. J. Clin. Nutr.* **2004**, *80*, 1706–1709.
104. Vigano, P.; Lattuada, D.; Mangioni, S.; Ermellino, L.; Vignali, M.; Caporizzo, E.; Panina-Bordignon, P.; Besozzi, M.; di Blasio, A.M. Cycling and early pregnant endometrium as a site of regulated expression of the vitamin D system. *J. Mol. Endocrinol.* **2006**, *36*, 415–424.
105. Barrera, D.; Noyola-Martinez, N.; Avila, E.; Halhali, A.; Larrea, F.; Diaz, L. Calcitriol inhibits interleukin-10 expression in cultured human trophoblasts under normal and inflammatory conditions. *Cytokine* **2012**, *57*, 316–321.
106. Avila, E.; Garcia-Becerra, R.; Rodriguez-Rasgado, J.A.; Diaz, L.; Ordaz-Rosado, D.; Zugel, U.; Steinmeyer, A.; Barrera, D.; Halhali, A.; Larrea, F.; *et al*. Calcitriol down-regulates human ether a go-go 1 potassium channel expression in cervical cancer cells. *Anticancer Res.* **2010**, *30*, 2667–2672.
107. Delvin, E.E.; Gagnon, L.; Arabian, A.; Gibb, W. Influence of calcitriol on p rolactin and prostaglandin production by human decidua. *Mol. Cell Endocrinol.* **1990**, *71*, 177–183.
108. Narahara, H.; Miyakawa, I.; Johnston, J.M. The inhibitory effect of 1,25-dihydroxyvitamin D3 on the secretion of platelet-activating factor acetylhydrolase by human decidual macrophages. *J. Clin. Endocrinol. Metab.* **1995**, *80*, 3121–3126.
109. Daftary, G.S.; Taylor, H.S. Endocrine regulation of HOX genes. *Endocr. Rev.* **2006**, *27*, 331–355.
110. Holick, M.F.; Binkley, N.C.; Bischoff-Ferrari, H.A.; Gordon, C.M.; Hanley, D.A.; Heaney, R.P.; Murad, M.H.; Weaver, C.M. Evaluation, treatment, and prevention of vitamin D deficiency: An Endocrine Society clinical practice guideline. *J. Clin. Endocrinol. Metab.* **2011**, *96*, 1911–1930.

111. Ross, A.C.; Manson, J.E.; Abrams, S.A.; Aloia, J.F.; Brannon, P.M.; Clinton, S.K.; Durazo-Arvizu, R.A.; Gallagher, J.C.; Gallo, R.L.; Jones, G.; *et al*. The 2011 Report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: What clinicians need to know. *J. Clin. Endocrinol. Metab.* **2011**, *96*, 53–58.
112. Aghajafari, F.; Nagulesapillai, T.; Ronksley, P.E.; Tough, S.C.; O'Beirne, M.; Rabi, D.M. Association between maternal serum 25-hydroxyvitamin D level and pregnancy and neonatal outcomes: Systematic review and meta-analysis of observational studies. *BMJ* **2013**, *346*, 1169.
113. Barrett, H.; McElduff, A. Vitamin D and pregnancy: An old problem revisited. *Best Pract. Res. Clin. Endocrinol. Metab.* **2010**, *24*, 527–529.
114. Bodnar, L.M.; Simhan, H.N. Vitamin D may be a link to black-white disparities in adverse birth outcomes. *Obstet. Gynecol. Surv.* **2010**, *65*, 273–284.
115. Christesen, H.T.; Falkenberg, T.; Lamont, R.F.; Jorgensen, J.S. The impact of vitamin D on pregnancy: A systematic review. *Acta Obstet. Gynecol. Scandinav.* **2012**, *91*, 1357–1367.
116. Nassar, N.; Halligan, G.H.; Roberts, C.L.; Morris, J.M.; Ashton, A.W. Systematic review of first-trimester vitamin D normative levels and outcomes of pregnancy. *Am. J. Obstet. Gynecol.* **2011**, *205*, 208.e201–208.e207.
117. Thorne-Lyman, A.; Fawzia, W.W. Vitamin D during pregnancy and maternal, neonatal and infant health outcomes: A systematic review and meta-analysis. *Paediatr. Perinat. Epidemiol.* **2012**, *26*, 75–90.
118. Urrutia, R.P.; Thorp, J.M. Vitamin D in pregnancy: Current concepts. *Curr. Opin. Obstet. Gynecol.* **2012**, *24*, 57–64.
119. Wei, S.Q.; Qi, H.P.; Luo, Z.C.; Fraser, W.D. Maternal vitamin D status and adverse pregnancy outcomes: A systematic review and meta-analysis. *J Matern. Fetal Neonatal Med.* **2013**, *26*, 889–899.
120. Report of the National high blood pressure Education Program Working Group on high blood pressure in pregnancy. *Am. J. Obstet. Gynecol.* **2000**, *183*, 1–22.
121. ACOG practice bulletin. Diagnosis and management of preeclampsia and eclampsia. Number 33, january 2002. American College of Obstetricians and Gynecologists. *Int. J. Gynaecol. Obstet.* **2002**, *77*, 67–75.
122. Baumwell, S.; Karumanchi, S.A. Pre-eclampsia: Clinical manifestations and molecular mechanisms. *Nephron Clin. Pract.* **2007**, *106*, c72–c81.
123. Palei, A.C.; Spradley, F.T.; Warrington, J.P.; George, E.M.; Granger, J.P. Pathophysiology of hypertension in pre-eclampsia: A lesson in integrative physiology. *Acta Physiol.* **2013**, *208*, 224–233.
124. Gernand, A.D.; Bodnar, L.M.; Klebanoff, M.A.; Parks, W.T.; Simhan, H.N. Maternal serum 25-hydroxyvitamin D and placental vascular pathology in a multicenter US cohort. *Am. J. Clin. Nutr.* **2013**, *98*, 383–388.
125. Halhali, A.; Bourges, H.; Carrillo, A.; Garabedian, M. Lower circulating insulin-like growth factor I and 1,25-dihydroxyvitamin D levels in preeclampsia. *Rev. Invest. Clin.* **1995**, *47*, 259–266.
126. Halhali, A.; Diaz, L.; Avila, E.; Ariza, A.C.; Garabedian, M.; Larrea, F. Decreased fractional urinary calcium excretion and serum 1,25-dihydroxyvitamin D and IGF-I levels in preeclampsia. *J. Steroid Biochem. Mol. Biol.* **2007**, *103*, 803–806.

127. Halhali, A.; Tovar, A.R.; Torres, N.; Bourges, H.; Garabedian, M.; Larrea, F. Preeclampsia is associated with low circulating levels of insulin-like growth factor I and 1,25-dihydroxyvitamin D in maternal and umbilical cord compartments. *J. Clin. Endocrinol. Metab.* **2000**, *85*, 1828–1833.
128. Diaz, E.; Halhali, A.; Luna, C.; Diaz, L.; Avila, E.; Larrea, F. Newborn birth weight correlates with placental zinc, umbilical insulin-like growth factor I, and leptin levels in preeclampsia. *Arch. Med. Res.* **2002**, *33*, 40–47.
129. Robinson, C.J.; Alanis, M.C.; Wagner, C.L.; Hollis, B.W.; Johnson, D.D. Plasma 25-hydroxyvitamin D levels in early-onset severe preeclampsia. *Am. J. Obstet. Gynecol.* **2010**, *203*, 366.e361–366.e366.
130. Xu, L.; Lee, M.; Jeyabalan, A.; Roberts, J.M. The relationship of hypovitaminosis D and IL-6 in preeclampsia. *Am. J. Obstet. Gynecol.* **2014**, *210*, 149.e141–149.e147.
131. Ullah, M.I.; Koch, C.A.; Tamanna, S.; Rouf, S.; Shamsuddin, L. Vitamin D deficiency and the risk of preeclampsia and eclampsia in Bangladesh. *Horm. Metab. Res.* **2013**, *45*, 682–687.
132. Wei, S.Q.; Audibert, F.; Luo, Z.C.; Nuyt, A.M.; Masse, B.; Julien, P.; Fraser, W.D. Maternal plasma 25-hydroxyvitamin D levels, angiogenic factors, and preeclampsia. *Am. J. Obstet. Gynecol.* **2013**, *208*, 390.e391–390.e396.
133. Baker, A.M.; Haeri, S.; Camargo, C.A., Jr.; Espinola, J.A.; Stuebe, A.M. A nested case-control study of midgestation vitamin D deficiency and risk of severe preeclampsia. *J. Clin. Endocrinol. Metab.* **2010**, *95*, 5105–5109.
134. Bodnar, L.M.; Catov, J.M.; Simhan, H.N.; Holick, M.F.; Powers, R.W.; Roberts, J.M. Maternal vitamin D deficiency increases the risk of preeclampsia. *J. Clin. Endocrinol. Metab.* **2007**, *92*, 3517–3522.
135. Abedi, P.; Mohaghegh, Z.; Afshary, P.; Latifi, M. The relationship of serum vitamin D with pre-eclampsia in the iranian women. *Matern. Child. Nutr.* **2014**, *10*, 206–212.
136. Diaz, L.; Arranz, C.; Avila, E.; Halhali, A.; Vilchis, F.; Larrea, F. Expression and activity of 25-hydroxyvitamin D-1 alpha-hydroxylase are restricted in cultures of human syncytiotrophoblast cells from preeclamptic pregnancies. *J. Clin. Endocrinol. Metab.* **2002**, *87*, 3876–3882.
137. Bodnar, L.M.; Simhan, H.N.; Catov, J.M.; Roberts, J.M.; Platt, R.W.; Diesel, J.C.; Klebanoff, M.A. Maternal vitamin D status and the risk of mild and severe preeclampsia. *Epidemiology* **2014**, *25*, 207–214.
138. Woodham, P.C.; Brittain, J.E.; Baker, A.M.; Leann Long, D.; Haeri, S.; Camargo, C.A.J.; Boggess, K.A.; Stuebe, A.M. Midgestation maternal serum 25-hydroxyvitamin D level and soluble fms-like tyrosine kinase 1/placental growth factor ratio as predictors of severe preeclampsia. *Hypertension* **2011**, *58*, 1120–1125.
139. Haugen, M.; Brantsæter, A.L.; Trogstad, L.; Alexander, J.; Roth, C.; Magnus, P.; Meltzera, H.M. Vitamin D supplementation and reduced risk of preeclampsia in nulliparous women. *Epidemiology* **2009**, *20*, 720–726.
140. Hyppönen, E.; Cavadino, A.; Williams, D.; Fraser, A.; Vereczkey, A.; Fraser, W.D.; Bánhidy, F.; Lawlor, D.; Czeizel, A.E. Vitamin D and pre-eclampsia: Original data, systematic review and meta-analysis. *Ann. Nutr. Metab.* **2013**, *63*, 331–340.

141. Tabesh, M.; Salehi-Abargouei, A.; Esmaillzadeh, A. Maternal vitamin D status and risk of pre-eclampsia: A systematic review and meta-analysis. *J. Clin. Endocrinol. Metab.* **2013**, *98*, 3165–3173.
142. Shand, A.W.; Nassar, N.; von Dadelszen, P.; Innis, S.M.; Green, T.J. Maternal vitamin D status in pregnancy and adverse pregnancy outcomes in a group at high risk for pre-eclampsia. *BJOG* **2010**, *117*, 1593–1598.
143. Powe, C.E.; Seely, E.W.; Rana, S.; Bhan, I.; Ecker, J.; Karumanchi, S.A.; Thadhani, R. First trimester vitamin D, vitamin D binding protein, and subsequent preeclampsia. *Hypertension* **2010**, *56*, 758–763.
144. Mashburn, J. Vaginal infections update. *J. Midwifery Womens Health* **2012**, *57*, 629–634.
145. Denney, J.M.; Culhane, J.F. Bacterial vaginosis: A problematic infection from both a perinatal and neonatal perspective. *Semin. Fetal Neonatal Med.* **2009**, *14*, 200–203.
146. Allsworth, J.E.; Peipert, J.F. Prevalence of bacterial vaginosis: 2001–2004 National Health and Nutrition Examination Survey data. *Obstet. Gynecol.* **2007**, *109*, 114–120.
147. Prietl, B.; Treiber, G.; Pieber, T.R.; Amrein, K. Vitamin D and immune function. *Nutrients* **2013**, *5*, 2502–2521.
148. Hewison, M. Vitamin D and immune function: An overview. *Proc. Nutr. Soc.* **2012**, *71*, 50–61.
149. Bodnar, L.M.; Krohn, M.A.; Simhan, H.N. Maternal vitamin D deficiency is associated with bacterial vaginosis in the first trimester of pregnancy. *J. Nutr.* **2009**, *139*, 1157–1161.
150. Dunlop, A.L.; Taylor, R.N.; Tangpricha, V.; Fortunato, S.; Menon, R. Maternal vitamin D, folate, and polyunsaturated fatty acid status and bacterial vaginosis during pregnancy. *Infect. Dis. Obstet. Gynecol.* **2011**, *2011*, 216217.
151. Hensel, K.J.; Randis, T.M.; Gelber, S.E.; Ratner, A.J. Pregnancy-specific association of vitamin D deficiency and bacterial vaginosis. *Am. J. Obstet. Gynecol.* **2011**, *204*, 41–49.
152. Rustveld, L.O.; Kelsey, S.F.; Sharma, R. Association between maternal infections and preeclampsia: A systematic review of epidemiologic studies. *Matern. Child. Health J.* **2008**, *12*, 223–242.
153. Hertting, O.; Holm, A.; Luthje, P.; Brauner, H.; Dyrdak, R.; Jonasson, A.F.; Wiklund, P.; Chromek, M.; Brauner, A. Vitamin D induction of the human antimicrobial peptide cathelicidin in the urinary bladder. *PLoS One* **2010**, *5*, 15580.
154. Boggess, K.A.; Espinola, J.A.; Moss, K.; Beck, J.; Offenbacher, S.; Camargo, C.A. Vitamin D status and periodontal disease among pregnant women. *J. Periodontol.* **2011**, *82*, 195–200.
155. Lowry, M.B.; Guo, C.; Borregaard, N.; Gombart, A.F. Regulation of the human cathelicidin antimicrobial peptide gene by 1alpha,25-dihydroxyvitamin D in primary immune cells. *J. Steroid Biochem. Mol. Biol.* **2014**, *143*, 183–191.
156. Mendez-Samperio, P. The human cathelicidin hCAP18/LL-37: A multifunctional peptide involved in mycobacterial infections. *Peptides* **2010**, *31*, 1791–1798.
157. Campbell, G.R.; Spector, S.A. Autophagy induction by vitamin D inhibits both mycobacterium tuberculosis and human immunodeficiency virus type 1. *Autophagy* **2012**, *8*, 1523–1525.
158. Campbell, G.R.; Spector, S.A. Vitamin D inhibits human immunodeficiency virus type 1 and mycobacterium tuberculosis infection in macrophages through the induction of autophagy. *PLoS Pathog.* **2012**, *8*, 1002689.

159. Wan, M.; van der Does, A.M.; Tang, X.; Lindbom, L.; Agerberth, B.; Haeggstrom, J.Z. Antimicrobial peptide LL-37 promotes bacterial phagocytosis by human macrophages. *J. Leukoc. Biol.* **2014**, *95*, 971–981.
160. Frew, L.; Stock, S.J. Antimicrobial peptides and pregnancy. *Reproduction* **2011**, *141*, 725–735.
161. Thota, C.; Farmer, T.; Garfield, R.E.; Menon, R.; Al-Hendy, A. Vitamin D elicits anti-inflammatory response, inhibits contractile-associated proteins, and modulates Toll-like receptors in human myometrial cells. *Reprod. Sci.* **2013**, *20*, 463–475.
162. Bodnar, L.M.; Rouse, D.J.; Momirova, V.; Peaceman, A.M.; Sciscione, A.; Spong, C.Y.; Varner, M.W.; Malone, F.D.; Iams, J.D.; Mercer, B.M.; *et al*. Maternal 25-hydroxyvitamin D and preterm birth in twin gestations. *Obstet. Gynecol.* **2013**, *122*, 91–98.
163. Thorp, J.M.; Camargo, C.A.; McGee, P.L.; Harper, M.; Klebanoff, M.A.; Sorokin, Y.; Varner, M.W.; Wapner, R.J.; Caritis, S.N.; Iams, J.D.; *et al*. Vitamin D status and recurrent preterm birth: A nested case-control study in high-risk women. *BJOG* **2012**, *119*, 1617–1623.
164. American Diabetes Association. Gestational diabetes mellitus. *Diabetes Care* **2003**, *26* (Suppl. 1), 103–105.
165. American Diabetes Association. Standards of medical care in diabetes--2012. *Diabetes Care* **2012**, *35* (Suppl. 1), 11–63.
166. Wang, Z.; Kanguru, L.; Hussein, J.; Fitzmaurice, A.; Ritchie, K. Incidence of adverse outcomes associated with gestational diabetes mellitus in low- and middle-income countries. *Int. J. Gynaecol. Obstet.* **2013**, *121*, 14–19.
167. Wendland, E.M.; Torloni, M.R.; Falavigna, M.; Trujillo, J.; Dode, M.A.; Campos, M.A.; Duncan, B.B.; Schmidt, M.I. Gestational diabetes and pregnancy outcomes—A systematic review of the world health organization (WHO) and the International Association of Diabetes in Pregnancy Study Groups (IADPSG) diagnostic criteria. *BMC Pregnancy Childbirth* **2012**, *12*, 23.
168. Norman, A.W.; Frankel, J.B.; Heldt, A.M.; Grodsky, G.M. Vitamin D deficiency inhibits pancreatic secretion of insulin. *Science* **1980**, *209*, 823–825.
169. Cho, G.J.; Hong, S.C.; Oh, M.J.; Kim, H.J. Vitamin D deficiency in gestational diabetes mellitus and the role of the placenta. *Am. J. Obstet. Gynecol.* **2013**, *209*, 560 e561–560 e568.
170. Clifton-Bligh, R.J.; McElduff, P.; McElduff, A. Maternal vitamin D deficiency, ethnicity and gestational diabetes. *Diabetes Med.* **2008**, *25*, 678–684.
171. Parlea, L.; Bromberg, I.L.; Feig, D.S.; Vieth, R.; Merman, E.; Lipscombe, L.L. Association between serum 25-hydroxyvitamin D in early pregnancy and risk of gestational diabetes mellitus. *Diabetes Med.* **2012**, *29*, 25–32.
172. Soheilykhah, S.; Mojibian, M.; Rashidi, M.; Rahimi-Saghand, S.; Jafari, F. Maternal vitamin D status in gestational diabetes mellitus. *Nutr. Clin. Pract.* **2010**, *25*, 524–527.
173. Zhang, C.; Qiu, C.; Hu, F.B.; David, R.M.; van Dam, R.M.; Bralley, A.; Williams, M.A. Maternal plasma 25-hydroxyvitamin D concentrations and the risk for gestational diabetes mellitus. *PLoS One* **2008**, *3*, 3753.
174. Zuhur, S.S.; Erol, R.S.; Kuzu, I.; Altuntas, Y. The relationship between low maternal serum 25-hydroxyvitamin D levels and gestational diabetes mellitus according to the severity of 25-hydroxyvitamin D deficiency. *Clinics (Sao Paulo)* **2013**, *68*, 658–664.

175. Maghbooli, Z.; Hossein-Nezhad, A.; Karimi, F.; Shafaei, A.R.; Larijani, B. Correlation between vitamin D3 deficiency and insulin resistance in pregnancy. *Diabetes Metab. Res. Rev.* **2008**, *24*, 27–32.
176. Napartivaumnuay, N.; Niramitmahapanya, S.; Deerochanawong, C.; Suthornthepavarakul, T.; Sarinnapakorn, V.; Jaruyawongs, P. Maternal 25 hydroxyvitamin D level and its correlation in thai gestational diabetes patients. *J. Med. Assoc. Thail.* **2013**, *96* (Suppl. 3), 69–76.
177. Burris, H.H.; Rifas-Shiman, S.L.; Kleinman, K.; Litonjua, A.A.; Huh, S.Y.; Rich-Edwards, J.W.; Camargo, C.A., Jr.; Gillman, M.W. Vitamin D deficiency in pregnancy and gestational diabetes mellitus. *Am. J. Obstet. Gynecol.* **2012**, *207*, 182.e181–182.e188.
178. Wang, O.; Nie, M.; Hu, Y.Y.; Zhang, K.; Li, W.; Ping, F.; Liu, J.T.; Chen, L.M.; Xing, X.P. Association between vitamin D insufficiency and the risk for gestational diabetes mellitus in pregnant chinese women. *Biomed. Environ. Sci.* **2012**, *25*, 399–406.
179. Poel, Y.H.; Hummel, P.; Lips, P.; Stam, F.; van der Ploeg, T.; Simsek, S. Vitamin D and gestational diabetes: A systematic review and meta-analysis. *Eur. J. Intern. Med.* **2012**, *23*, 465–469.
180. Senti, J.; Thiele, D.K.; Anderson, C.M. Maternal vitamin D status as a critical determinant in gestational diabetes. *J. Obstet. Gynecol. Neonatal. Nurs.* **2012**, *41*, 328–338.
181. Lau, S.L.; Gunton, J.E.; Athayde, N.P.; Byth, K.; Cheung, N.W. Serum 25-hydroxyvitamin D and glycated haemoglobin levels in women with gestational diabetes mellitus. *Med. J. Aust.* **2011**, *194*, 334–337.
182. Makgoba, M.; Nelson, S.M.; Savvidou, M.; Messow, C.M.; Nicolaides, K.; Sattar, N. First-trimester circulating 25-hydroxyvitamin D levels and development of gestational diabetes mellitus. *Diabetes Care* **2011**, *34*, 1091–1093.
183. McLeod, D.S.; Warner, J.V.; Henman, M.; Cowley, D.; Gibbons, K.; McIntyre, H.D. Associations of serum vitamin D concentrations with obstetric glucose metabolism in a subset of the Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study cohort. *Diabetes Med.* **2012**, *29*, 199–204.
184. Walsh, J.M.; McGowan, C.A.; Kilbane, M.; McKenna, M.J.; McAuliffe, F.M. The relationship between maternal and fetal vitamin D, insulin resistance, and fetal growth. *Reprod. Sci.* **2013**, *20*, 536–541.
185. Jelsma, J.G.; van Poppel, M.N.; Galjaard, S.; Desoye, G.; Corcoy, R.; Devlieger, R.; van Assche, A.; Timmerman, D.; Jans, G.; Harreiter, J.; *et al*. Dali: Vitamin D and lifestyle intervention for gestational diabetes mellitus (GDM) prevention: An european multicentre, randomised trial-study protocol. *BMC Pregnancy Childbirth* **2013**, *13*, 142.
186. Asemi, Z.; Hashemi, T.; Karamali, M.; Samimi, M.; Esmaillzadeh, A. Effects of vitamin D supplementation on glucose metabolism, lipid concentrations, inflammation, and oxidative stress in gestational diabetes: A double-blind randomized controlled clinical trial. *Am. J. Clin. Nutr.* **2013**, *98*, 1425–1432.
187. Mozaffari-Khosravi, H.; Hosseinzadeh-Shamsi-Anar, M.; Salami, M.A.; Hadinedoushan, H.; Mozayan, M.R. Effects of a single post-partum injection of a high dose of vitamin D on glucose tolerance and insulin resistance in mothers with first-time gestational diabetes mellitus. *Diabetes Med.* **2012**, *29*, 36–42.

188. Baker, A.M.; Haeri, S.; Camargo, C.A., Jr.; Stuebe, A.M.; Boggess, K.A. First-trimester maternal vitamin D status and risk for gestational diabetes (GDM) a nested case-control study. *Diabetes Metab. Res. Rev.* **2012**, *28*, 164–168.
189. Savvidou, M.D.; Akolekar, R.; Samaha, R.B.; Masconi, A.P.; Nicolaides, K.H. Maternal serum 25-hydroxyvitamin D levels at 11(+0) -13(+6) weeks in pregnant women with diabetes mellitus and in those with macrosomic neonates. *BJOG* **2011**, *118*, 951–955.
190. Farrant, H.J.; Krishnaveni, G.V.; Hill, J.C.; Boucher, B.J.; Fisher, D.J.; Noonan, K.; Osmond, C.; Veena, S.R.; Fall, C.H. Vitamin D insufficiency is common in indian mothers but is not associated with gestational diabetes or variation in newborn size. *Eur. J. Clin. Nutr.* **2009**, *63*, 646–652.
191. Tomedi, L.E.; Simhan, H.N.; Bodnar, L.M. Early-pregnancy maternal vitamin D status and maternal hyperglycaemia. *Diabetes Med.* **2013**, *30*, 1033–1039.
192. Mirzaei, K.; Hossein-Nezhad, A.; Keshavarz, S.A.; Eshaghi, S.M.; Koohdani, F.; Saboor-Yaraghi, A.A.; Hosseini, S.; Tootee, A.; Djalali, M. Insulin resistance via modification of PGC1alpha function identifying a possible preventive role of vitamin D analogues in chronic inflammatory state of obesity. A double blind clinical trial study. *Minerva Med.* **2014**, *105*, 63–78.
193. Alkharfy, K.M.; Al-Daghri, N.M.; Yakout, S.M.; Hussain, T.; Mohammed, A.K.; Krishnaswamy, S. Influence of vitamin D treatment on t ranscriptional regulation of insulin-sensitive genes. *Metab. Syndr. Relat. Disord.* **2013**, *11*, 283–288.
194. Manna, P.; Jain, S.K. Vitamin D up-regulates glucose transporter 4 (GLUT4) translocation and glucose utilization mediated by cystathionine-gamma-lyase (CSE) activation and H2Ss formation in 3T3L1 adipocytes. *J. Biol. Chem.* **2012**, *287*, 42324–42332.
195. Meerza, D.; Naseem, I.; Ahmed, J. Effect of 1, 25(OH)(2) vitamin D(3) on glucose homeostasis and DNA damage in type 2 diabetic mice. *J. Diabetes Complicat.* **2012**, *26*, 363–368.
196. Zhang, J.; Merialdi, M.; Platt, L.D.; Kramer, M.S. Defining normal and abnormal fetal growth: Promises and challenges. *Am. J. Obstet. Gynecol.* **2010**, *202*, 522–528.
197. Mayer, C.; Joseph, K.S. Fetal growth: A review of terms, concepts and issues relevant to obstetrics. *Ultrasound Obstet. Gynecol.* **2013**, *41*, 136–145.
198. Burris, H.H.; Mitchell, A.A.; Werler, M.M. Periconceptional multivitamin use and infant birth weight disparities. *Ann. Epidemiol.* **2010**, *20*, 233–240.
199. Kovacs, C.S. Maternal vitamin D deficiency: Fetal and neonatal implications. *Semin. Fetal Neonatal Med.* **2013**, *18*, 129–135.
200. Brunvand, L.; Quigstad, E.; Urdal, P.; Haug, E. Vitamin D deficiency and fetal growth. *Early Hum. Dev.* **1996**, *45*, 27–33.
201. Gale, C.R.; Robinson, S.M.; Harvey, N.C.; Javaid, M.K.; Jiang, B.; Martyn, C.N.; Godfrey, K.M.; Cooper, C. Maternal vitamin D status during pregnancy and child outcomes. *Eur. J. Clin. Nutr.* **2008**, *62*, 68–77.
202. Agarwal, R.; Virmani, D.; Jaipal, M.L.; Gupta, S.; Gupta, N.; Sankar, M.J.; Bhatia, S.; Agarwal, A.; Devgan, V.; Deorari, A.; *et al*. Vitamin D status of low birth weight infants in Delhi: A comparative study. *J. Trop. Pediatr.* **2012**, *58*, 446–450.

203. Gernand, A.D.; Simhan, H.N.; Klebanoff, M.A.; Bodnar, L.M. Maternal serum 25-hydroxyvitamin D and measures of newborn and placental weight in a U.S. multicenter cohort study. *J. Clin. Endocrinol. Metab.* **2013**, *98*, 398–404.
204. Ertl, R.; Yu, C.K.; Samaha, R.; Akolekar, R.; Nicolaides, K.H. Maternal serum vitamin D at 11–13 weeks in pregnancies delivering small for gestational age neonates. *Fetal Diagn. Ther.* **2012**, *31*, 103–108.
205. Namgung, R.; Tsang, R.C.; Specker, B.L.; Sierra, R.I.; Ho, M.L. Reduced serum osteocalcin and 1,25-dihydroxyvitamin D concentrations and low bone mineral content in small for gestational age infants: Evidence of decreased bone formation rates. *J. Pediatr.* **1993**, *122*, 269–275.
206. Van den Berg, G.; van Eijsden, M.; Vrijkotte, T.G.; Gemke, R.J. Suboptimal maternal vitamin D status and low education level as determinants of small-for-gestational-age birth weight. *Eur. J. Nutr.* **2013**, *52*, 273–279.
207. Scholl, T.O.; Chen, X. Vitamin D intake during pregnancy: Association with maternal characteristics and infant birth weight. *Early Hum. Dev.* **2009**, *85*, 231–234.
208. Mannion, C.A.; Gray-Donald, K.; Koski, K.G. Association of low intake of milk and vitamin D during pregnancy with decreased birth weight. *CMAJ* **2006**, *174*, 1273–1277.
209. Bodnar, L.M.; Catov, J.M.; Zmuda, J.M.; Cooper, M.E.; Parrott, M.S.; Roberts, J.M.; Marazita, M.L.; Simhan, H.N. Maternal serum 25-hydroxyvitamin D concentrations are associated with small-for-gestational age births in white women. *J. Nutr.* **2010**, *140*, 999–1006.
210. Kalra, P.; Das, V.; Agarwal, A.; Kumar, M.; Ramesh, V.; Bhatia, E.; Gupta, S.; Singh, S.; Saxena, P.; Bhatia, V. Effect of vitamin D supplementation during pregnancy on neonatal mineral homeostasis and anthropometry of the newborn and infant. *Br. J. Nutr.* **2012**, *108*, 1052–1058.
211. Young, B.E.; McNanley, T.J.; Cooper, E.M.; McIntyre, A.W.; Witter, F.; Harris, Z.L.; O'Brien, K.O. Maternal vitamin D status and calcium intake interact to affect fetal skeletal growth in utero in pregnant adolescents. *Am. J. Clin. Nutr.* **2012**, *95*, 1103–1112.
212. Diogenes, M.E.; Bezerra, F.F.; Rezende, E.P.; Taveira, M.F.; Pinhal, I.; Donangelo, C.M. Effect of calcium plus vitamin D supplementation during pregnancy in brazilian adolescent mothers: A randomized, placebo-controlled trial. *Am. J. Clin. Nutr.* **2013**, *98*, 82–91.
213. Morley, R.; Carlin, J.B.; Pasco, J.A.; Wark, J.D.; Ponsonby, A.L. Maternal 25-hydroxyvitamin D concentration and offspring birth size: Effect modification by infant VDR genotype. *Eur. J. Clin. Nutr.* **2009**, *63*, 802–804.
214. Jones, K.S.; Assar, S.; Harnpanich, D.; Bouillon, R.; Lambrechts, D.; Prentice, A.; Schoenmakers, I. 25(OH)D2 half-life is shorter than 25(OH)D3 half-life and is influenced by DBP concentration and genotype. *J. Clin. Endocrinol. Metab.* **2014**, *99*, 3373–3381.
215. McGrath, J.J.; Saha, S.; Burne, T.H.; Eyles, D.W. A systematic review of the association between common single nucleotide polymorphisms and 25-hydroxyvitamin D concentrations. *J. Steroid Biochem. Mol. Biol.* **2010**, *121*, 471–477.
216. Wang, T.J.; Zhang, F.; Richards, J.B.; Kestenbaum, B.; van Meurs, J.B.; Berry, D.; Kiel, D.P.; Streeten, E.A.; Ohlsson, C.; Koller, D.L.; *et al*. Common genetic determinants of vitamin D insufficiency: A genome-wide association study. *Lancet* **2010**, *376*, 180–188.

217. Barry, E.L.; Rees, J.R.; Peacock, J.L.; Mott, L.A.; Amos, C.I.; Bostick, R.M.; Figueiredo, J.C.; Ahnen, D.J.; Bresalier, R.S.; Burke, C.A.; *et al*. Genetic variants in CYP2R1, CYP24A1, and VDR modify the efficacy of vitamin D3 supplementation for increasing serum 25-hydroxyvitamin D levels in a randomized controlled trial. *J. Clin. Endocrinol. Metab.* **2014**, *99*, 2133–2137.
218. Jones, K.S.; Assar, S.; Vanderschueren, D.; Bouillon, R.; Prentice, A.; Schoenmakers, I. Predictors of 25(OH)D half-life and plasma 25(OH)D concentration in the Gambia and the UK. *Osteoporos. Int.* **2014**, doi: 10.1007/s00198-014-2905-0.
219. Karras, S.N.; Anagnostis, P.; Annweiler, C.; Naughton, D.P.; Petroczi, A.; Bili, E.; Harizopoulou, V.; Tarlatzis, B.C.; Persinaki, A.; Papadopoulou, F.; *et al*. Maternal vitamin D status during pregnancy: The mediterranean reality. *Eur. J. Clin. Nutr.* **2014**, *68*, 864–869.
220. Wuertz, C.; Gilbert, P.; Baier, W.; Kunz, C. Cross-sectional study of factors that influence the 25-hydroxyvitamin D status in pregnant women and in cord blood in Germany. *Br. J. Nutr.* **2013**, *110*, 1895–1902.
221. Wortsman, J.; Matsuoka, L.Y.; Chen, T.C.; Lu, Z.; Holick, M.F. Decreased bioavailability of vitamin D in obesity. *Am. J. Clin. Nutr.* **2000**, *72*, 690–693.
222. Abboud, M.; Puglisi, D.A.; Davies, B.N.; Rybchyn, M.; Whitehead, N.P.; Brock, K.E.; Cole, L.; Gordon-Thomson, C.; Fraser, D.R.; Mason, R.S. Evidence for a specific uptake and retention mechanism for 25-hydroxyvitamin D (25OHD) in skeletal muscle cells. *Endocrinology* **2013**, *154*, 3022–3030.
223. Abboud, M.; Gordon-Thomson, C.; Hoy, A.J.; Balaban, S.; Rybchyn, M.S.; Cole, L.; Su, Y.; Brennan-Speranza, T.C.; Fraser, D.R.; Mason, R.S. Uptake of 25-hydroxyvitamin D by muscle and fat cells. *J. Steroid Biochem. Mol. Biol.* **2014**, *144*, 232–236.
224. Josefson, J.L.; Feinglass, J.; Rademaker, A.W.; Metzger, B.E.; Zeiss, D.M.; Price, H.E.; Langman, C.B. Maternal obesity and vitamin D sufficiency are associated with cord blood vitamin D insufficiency. *J. Clin. Endocrinol. Metab.* **2013**, *98*, 114–119.
225. Bodnar, L.M.; Catov, J.M.; Roberts, J.M.; Simhan, H.N. Prepregnancy obesity predicts poor vitamin D status in mothers and their neonates. *J. Nutr.* **2007**, *137*, 2437–2442.
226. Crozier, S.R.; Harvey, N.C.; Inskip, H.M.; Godfrey, K.M.; Cooper, C.; Robinson, S.M. Maternal vitamin D status in pregnancy is associated with adiposity in the offspring: Findings from the Southampton Women's Survey. *Am. J. Clin. Nutr.* **2012**, *96*, 57–63.
227. Morales, E.; Romieu, I.; Guerra, S.; Ballester, F.; Rebagliato, M.; Vioque, J.; Tardon, A.; Rodriguez Delhi, C.; Arranz, L.; Torrent, M.; *et al*. Maternal vitamin D status in pregnancy and risk of lower respiratory tract infections, wheezing, and asthma in offspring. *Epidemiology* **2012**, *23*, 64–71.
228. Hossain, N.; Kanani, F.H.; Ramzan, S.; Kausar, R.; Ayaz, S.; Khanani, R.; Pal, L. Obstetric and neonatal outcomes of maternal vitamin D supplementation: Results of an open-label, randomized controlled trial of antenatal vitamin D supplementation in pakistani women. *J. Clin. Endocrinol. Metab.* **2014**, *99*, 2448–2455.

229. Wagner, C.L.; McNeil, R.; Hamilton, S.A.; Winkler, J.; Rodriguez Cook, C.; Warner, G.; Bivens, B.; Davis, D.J.; Smith, P.G.; Murphy, M.; *et al*. A randomized trial of vitamin D supplementation in 2 c ommunity health center networks in South Carolina. *Am. J. Obstet. Gynecol.* **2013**, *208*, 137.e1–137.e13.
230. Sahu, M.; Das, V.; Aggarwal, A.; Rawat, V.; Saxena, P.; Bhatia, V. Vitamin D replacement in pregnant women in rural north india: A pilot study. *Eur. J. Clin. Nutr.* **2009**, *63*, 1157–1159.
231. Yu, C.K.; Sykes, L.; Sethi, M.; Teoh, T.G.; Robinson, S. Vitamin D deficiency and supplementation during pregnancy. *Clin. Endocrinol. (Oxf.)* **2009**, *70*, 685–690.
232. Grant, C.C.; Stewart, A.W.; Scragg, R.; Milne, T.; Rowden, J.; Ekeroma, A.; Wall, C.; Mitchell, E.A.; Crengle, S.; Trenholme, A.; *et al*. Vitamin D during pregnancy and infancy and infant serum 25-hydroxyvitamin D concentration. *Pediatrics* **2014**, *133*, 143–153.
233. Dawodu, A.; Saadi, H.F.; Bekdache, G.; Javed, Y.; Altaye, M.; Hollis, B.W. Randomized controlled trial (RCT) of vitamin D supplementation in pregnancy in a population with endemic vitamin D deficiency. *J. Clin. Endocrinol. Metab.* **2013**, *98*, 2337–2346.
234. Hollis, B.W.; Johnson, D.; Hulsey, T.C.; Ebeling, M.; Wagner, C.L. Vitamin D supplementation during pregnancy: Double-blind, randomized clinical trial of safety and effectiveness. *J. Bone Min. Res.* **2011**, *26*, 2341–2357.
235. Hashemipour, S.; Lalooha, F.; Zahir Mirdamadi, S.; Ziaee, A.; Dabaghi Ghaleh, T. Effect of vitamin D administration in vitamin D-deficient pregnant women on maternal and neonatal serum calcium and vitamin d concentrations: A randomised clinical trial. *Br. J. Nutr.* **2013**, *110*, 1611–1616.
236. Roth, D.E.; al Mahmud, A.; Raqib, R.; Akhtar, E.; Black, R.E.; Baqui, A.H. Pharmacokinetics of high-dose weekly oral vitamin D3 supplementation during the third trimester of pregnancy in Dhaka, Bangladesh. *Nutrients* **2013**, *5*, 788–810.
237. Shakiba, M.; Iranmanesh, M.R. Vitamin D requirement in pregnancy to prevent deficiency in neonates: A randomised trial. *Singap. Med. J.* **2013**, *54*, 285–288.
238. Hollis, B.W. Assessment and interpretation of circulating 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D in the clinical environment. *Endocrinol. Metab. Clin. North Am.* **2010**, *39*, 271–286.
239. Volmer, D.A.; Mendes, L.R.; Stokes, C.S. Analysis of vitamin D metabolic markers by m ass spectrometry: Current techniques, limitations of the "gold standard" method, and anticipated future directions. *Mass Spectrom. Rev.* **2015**, *34,* 2–23.
240. Zhang, S.W.; Jian, W.; Sullivan, S.; Sankaran, B.; Edom, R.W.; Weng, N.; Sharkey, D. Development and validation of an LC-MS/MS based method for quantification of 25 hydroxyvitamin D2 and 25 hydr oxyvitamin D3 in human serum and plasma. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2014**, *961*, 62–70.
241. Adaway, J.E.; Keevil, B.G.; Owen, L.J. Liquid chromatography tandem mass spectrometry in the clinical laboratory. *Ann. Clin. Biochem.* **2015**, *52*, 18–38.
242. Baecher, S.; Leinenbach, A.; Wright, J.A.; Pongratz, S.; Kobold, U.; Thiele, R. Simultaneous quantification of four vitamin D metabolites in human serum using high performance liquid chromatography tandem mass spectrometry for vitamin D profiling. *Clin. Biochem.* **2012**, *45*, 1491–1496.

243. Shah, I.; Petroczi, A.; Naughton, D.P. Method for simultaneous analysis of eight analogues of vitamin D using liquid chromatography tandem mass spectrometry. *Chem. Cent. J.* **2012**, *6*, 112.
244. Higashi, T.; Goto, A.; Morohashi, M.; Ogawa, S.; Komatsu, K.; Sugiura, T.; Fukuoka, T.; Mitamura, K. Development and validation of a method for determination of plasma 25-hydroxyvitamin D3 3-sulfate using liquid chromatography/tandem mass spectrometry. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2014**, *969*, 230–234.
245. Van den Ouweland, J.M.; Beijers, A.M.; van Daal, H. Overestimation of 25-hydroxyvitamin D3 by increased ionisation efficiency of 3-epi-25-hydroxyvitamin D3 in LC-MS/MS methods not separating both metabolites as determined by an LC-MS/MS method for separate quantification of 25-hydroxyvitamin D3, 3-epi-25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 in human serum. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2014**, *967*, 195–202.
246. Lee, S.; Oncescu, V.; Mancuso, M.; Mehta, S.; Erickson, D. A smartphone platform for the quantification of vitamin D levels. *Lab Chip* **2014**, *14*, 1437–1442.
247. Powe, C.E.; Evans, M.K.; Wenger, J.; Zonderman, A.B.; Berg, A.H.; Nalls, M.; Tamez, H.; Zhang, D.; Bhan, I.; Karumanchi, S.A.; *et al*. Vitamin D-binding protein and vitamin D status of black americans and white americans. *N. Engl. J. Med.* **2013**, *369*, 1991–2000.
248. Powe, C.E.; Karumanchi, S.A.; Thadhani, R. Vitamin D-binding protein and vitamin D in blacks and whites. *N. Engl. J. Med.* **2014**, *370*, 880–881.
249. Autier, P.; Boniol, M.; Pizot, C.; Mullie, P. Vitamin D status and ill health: A systematic review. *Lancet Diabet. Endocrinol.* **2014**, *2*, 76–89.
250. Bolland, M.J.; Grey, A.; Gamble, G.D.; Reid, I.R. The effect of vitamin D supplementation on skeletal, vascular, or cancer outcomes: A trial sequential meta-analysis. *Lancet Diabetes Endocrinol.* **2014**, *2*, 307–320.
251. Vitamin D: Chasing a myth? *Lancet Diabetes Endocrinol.* **2014**, *2*, 1; doi:10.1016/S2213-8587(13)70164-5.
252. Gillie, O. Controlled trials of vitamin D, causality and type 2 statistical error. *Public Health Nutr.* **2014**, doi:10.1017/S1368980014002304.

Journal of Steroid Biochemistry & Molecular Biology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

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

Chronic moderate ethanol intake differentially regulates vitamin D hydroxylases gene expression in kidneys and xenografted breast cancer cells in female mice

Janice García-Quiroz[a], Rocío García-Becerra[a], Galia Lara-Sotelo[a], Euclides Avila[a], Sofía López[a], Nancy Santos-Martínez[a], Ali Halhali[a], David Ordaz-Rosado[a], David Barrera[a], Andrea Olmos-Ortiz[a], María J. Ibarra-Sánchez[b], José Esparza-López[b], Fernando Larrea[a], Lorenza Díaz[a,*]

[a] Departamento de Biología de la Reproducción, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Avenida Vasco de Quiroga No. 15, Col. Belisario Domínguez Sección XVI, C.P. 14080, Ciudad de México, Mexico
[b] Unidad de Bioquímica, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Avenida Vasco de Quiroga No. 15, Col. Belisario Domínguez Sección XVI, C.P. 14080, Ciudad de México, Mexico

ARTICLE INFO

Article history:
Received 25 May 2016
Received in revised form 12 August 2016
Accepted 12 September 2016
Available online xxx

Keywords:
Alcohol
Vitamin D metabolism
Calcitriol
Breast cancer

ABSTRACT

Factors affecting vitamin D metabolism may preclude anti-carcinogenic effects of its active metabolite calcitriol. Chronic ethanol consumption is an etiological factor for breast cancer that affects vitamin D metabolism; however, the mechanisms underlying this causal association have not been fully clarified. Using a murine model, we examined the effects of chronic moderate ethanol intake on tumoral and renal *CYP27B1* and *CYP24A1* gene expression, the enzymes involved in calcitriol synthesis and inactivation, respectively. Ethanol (5% w/v) was administered to 25-hydroxyvitamin D_3-treated or control mice during one month. Afterwards, human breast cancer cells were xenografted and treatments continued another month. Ethanol intake decreased renal *Cyp27b1* while increased tumoral *CYP24A1* gene expression. Treatment with 25-hydroxyvitamin D_3 significantly stimulated *CYP27B1* in tumors of non-alcohol-drinking mice, while increased both renal and tumoral *CYP24A1*. Coadministration of ethanol and 25-hydroxyvitamin D_3 reduced in 60% renal 25-hydroxyvitamin D_3–dependent *Cyp24a1* upregulation ($P < 0.05$). We found 5 folds higher basal *Cyp27b1* than *Cyp24a1* gene expression in kidneys, whereas this relation was inverted in tumors, showing 5 folds more *CYP24A1* than *CYP27B1*. Tumor expression of the calcitriol target cathelicidin increased only in 25-hydroxyvitamin D_3-treated non-ethanol drinking animals ($P < 0.05$). Mean final body weight was higher in 25-hydroxyvitamin D_3 treated groups ($P < 0.001$). Overall, these results suggest that moderate ethanol intake decreases renal and tumoral 25-hydroxyvitamin D_3 bioconversion into calcitriol, while favors degradation of both vitamin D metabolites in breast cancer cells. The latter may partially explain why alcohol consumption is associated with vitamin D deficiency and increased breast cancer risk and progression.

1. Introduction

Breast cancer is the leading cause of cancer death among women worldwide and its incidence is still increasing. For this reason, identifying preventable risk factors is a priority for public health intervention. Amongst known risk factors, chronic ethanol intake stands as an important and modifiable etiological factor in the development and progression of breast cancer. In general, calculated percentage of alcohol-attributable breast cancer cases ranges between 5 and 9.4% [1–3]. In addition, for each additional 10 g alcohol (1 standard drink) per day, breast cancer risk is estimated to increase around 7% [4–6] and even moderate low levels of alcohol consumption (5.0–9.9 g/d) have been associated with a small rise in breast cancer risk [7]. Likewise, ethanol administration to female mice (10–15% ethanol in the drinking water) during 25 months induced spontaneous mammary tumors in almost half the number of ethanol-drinking mice, while no tumors were found in the control group [8]. A number of

* Corresponding author.
E-mail address: lorenzadiaz@gmail.com (L. Díaz).

http://dx.doi.org/10.1016/j.jsbmb.2016.09.011
0960-0760/

hypotheses have been raised in the literature to explain how alcohol increases breast cancer risk and tumor progression. For instance, ethanol impairs natural killer cells tumoricidal ability and activates estrogen related cell proliferation [9,10]. In addition, some alcohol metabolites such as acetaldehyde and oxygen reactive species are potentially carcinogenic [11]. In the present study, we aimed at identifying another potential mechanism that may help to explain the association between alcohol consumption and breast cancer. Given that high rates of incidence, relapse and mortality of breast cancer have been associated with vitamin D deficiency [12–14], we hypothesized that alcohol consumption could interfere with vitamin D metabolism in breast tumor cells, lowering production of the vitamin D active metabolite calcitriol and thus, preventing its local anti-carcinogenic effects. Indeed, vitamin D has become increasingly relevant in the oncological field due to the well-known calcitriol antineoplastic properties. Particularly in normal mammary epithelial cells, calcitriol mediates growth inhibition and terminal differentiation [15], while in malignant cells calcitriol interferes in almost every step of breast carcinogenesis and tumor progression by regulating the expression of genes involved in development and progression of breast cancer [16–18]. Endogenous calcitriol antitumorigenic effects take place in mammary epithelial cells given that all components of the vitamin D endocrine system are expressed in these cells. This includes the enzymes involved in vitamin D metabolism and the vitamin D receptor (VDR) [15]. Calcitriol is produced from 25-hydroxyvitamin D_3 ($25OHD_3$) after hydroxylation of its carbon-1 by the enzyme $25OHD_3$-1α-hydroxylase (CYP27B1). While the biological actions of calcitriol are mediated by the VDR, its bioavailability is decreased by the enzyme 1,25-dihydroxyvitamin D_3-24 hydroxylase (CYP24A1), which rapidly inactivates this metabolite. Of note, CYP24A1 may also hydroxylate $25OHD_3$, thus lowering availability of the substrate for CYP27B1 activity, and consequently, reducing calcitriol production [19,20]. Our hypothesis was conceived on the basis of previous studies showing that alcoholic subjects have reduced $25OHD_3$ and calcitriol serum concentrations and frequently suffer from alterations in calcium homeostasis [21,22]. Supporting the latter, animal models have shown that ethanol intake interferes with renal vitamin D metabolism [23–25]. However, to date there are no studies analyzing the regulation of *CYP27B1* and *CYP24A1* gene expression by moderate ethanol consumption in human breast cancer cells. Therefore, herein we investigated the latter in a murine model of chronic alcohol intake. Also, we studied the regulatory effect of ethanol consumption in intracrine vitamin D metabolism in xenografted breast cancer cells by analyzing calcitriol known transcriptional targets involved in angiogenesis and carcinogenesis. The results presented herein could provide partial mechanistic insights into causal relationships between chronic alcohol consumption and increased breast cancer risk, development and progression.

2. Material and methods

2.1. Reagents

Calcidiol (25-hydroxyvitamin D_3, $25OHD_3$) was purchased from Sigma-Aldrich (MO, USA) and was dissolved in dimethyl sulfoxide (DMSO). Ethanol was obtained from Herschi Trading (Mexico City, Mexico). Cell culture medium, fetal bovine serum (FBS), penicillin/streptomycin, and Trizol were obtained from Invitrogen Life Technologies (NY, USA). The oligonucleotides for real time polymerase chain reaction (qPCR) were from Integrated DNA Technologies (IO, USA). The reverse transcription (RT) system, TaqMan master reaction, TaqMan probes, and PCR microplates were from Roche Applied Science (IN, USA).

2.2. Characterization of cells by immunocytochemistry

The primary cell culture (MBCDF-D5 cells) was generated from a biopsy obtained from a female Mexican patient with invasive ductal breast carcinoma. The protocol was approved by the Human Research and Ethics Committee from the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán at Mexico City (Ref 1549, BQO- 008-06/9-1) and written informed consent was obtained from the patient. Cells were maintained in RPMI-1640 medium supplemented with 100 units/mL penicillin plus 100 μg/mL streptomycin and 5% heat-inactivated FBS in humidified atmosphere with 5% CO_2 at 37 °C.

Cultured cells were grown on glass coverslips and fixated in 96% ethanol. Antigen retrieval was achieved by autoclaving in immuno/DNA retriever with EDTA (BioSB, CA, USA) solution during 5 min. Slides were blocked with immunodetector peroxidase blocker (BioSB) and incubated with anti-estrogen receptor (ERα, 1:250, BioSB), anti-progesterone receptor (PR, 1:250, BioSB), anti-human epidermal growth factor receptor 1 (EGFR, 1:100, BioSB), anti-human epidermal growth factor receptor 2 (HER2, 1:100, Cell Signaling Technology, MA, USA), anti-VDR (1:100, Santa Cruz Biotechnology Inc, CA, USA), anti-vimentin (VIM, 1:100, Santa Cruz) and anti-cytokeratin 7 (CK-7, 1:300, BioSB) during 1 h or 2 h for the anti-ether à-go-go-1 potassium channel (KCNH1, 1:300, Novus Biologicals, CO, USA) antibody. After washing, the slides were sequentially incubated with immuno-Detector Biotin-Link and immunoDetector HRP label (BioSB) during 10 min each. Staining was completed with diaminobenzidine (DAB) and slides were counterstained with hematoxylin.

2.3. Therapeutic protocol and induction of tumors in athymic mice

Studies involving mice were performed according to the Official Mexican Norm 062- ZOO-1999. The study was approved by the Institutional Committee for the care and use of laboratory animals (protocol number BRE-1291-14/17-1, CINVA 1291) of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, where mice were housed in the animal facility. Athymic female BALB/c homozygous, inbred Crl:NU (NCr)-Foxn1nu nude mice (~6 weeks of age) were maintained in standard conditions. Sterilized water and feed (standard PMI 5053 feed) were given *ad libitum*. Animals were weighted and checked thrice per week in order to detect and alleviate any symptoms of pain. We carefully observed endpoints compatible with the scientific objectives of this work. Mice were randomly divided in 5 groups: 1) control, 2) ethanol (5% w/v, equivalent to approximately 12.5 g/day/kg in the drinking water), 3) vehicle (i.p. DMSO 17.5 μL/100 μL sterile saline solution), 4) $25OHD_3$ (i.p. 350 μg/kg/week in 100 μL sterile saline solution), and 5) combined ethanol + $25OHD_3$. Of note, for $25OHD_3$ dosing we administered the equivalent to 50 μg/kg/day all in one single shot per week, in order to reduce manipulation and discomfort of mice. Regarding alcohol administration, each mouse is estimated to drink 5–7 mL of water per day [26], which was taken into consideration to calculate the amount of ethanol added to the drinking water. As reference, a moderate ethanol consuming regimen in mice has been suggested to be around 5–10% ethanol in drinking water daily [27–29]; while in humans 10 g ethanol/day, which is equivalent to 3–6 drinks per week [7]. Supplemented water was changed every day. Mice were exposed to treatments during one month prior to subcutaneous breast cancer cell injection (2.0×10^6 cells in 0.1 mL of sterile saline solution into the upper part of the posterior limb of each mouse) and were maintained on alcohol and $25OHD_3$ treatments for another month after xenografting. Tumor dimensions were estimated three times per week with a caliper always by the same person. Tumor volume was calculated using the standard formula (length × width^2)/2,

Please cite this article in press as: J. García-Quiroz, et al., Chronic moderate ethanol intake differentially regulates vitamin D hydroxylases gene expression in kidneys and xenografted breast cancer cells in female mice, J. Steroid Biochem. Mol. Biol. (2016), http://dx.doi.org/10.1016/j.jsbmb.2016.09.011

where length is the largest dimension and width the smallest dimension perpendicular to the length. Relative tumor volume (RTV) was calculated for each tumor by dividing the tumor volume on the day of sacrifice by that on day 0 (which corresponded to the tumor volume at onset). Fold increase from initial volume was calculated subtracting 1 from RTV. Mice were euthanized 4 weeks after tumor cell injection and blood, tumors and kidneys were collected 14 h after the last 25OHD$_3$ dose.

2.4. Real time PCR (qPCR)

Total RNA was extracted by homogenizing tumor or renal tissue in the presence of Trizol reagent using the MagNA Lyser equipment (Roche) and 1.4-mm diameter ceramic beads. Two μg of total RNA were reverse-transcribed and resulting cDNAs were used for the qPCR. Amplifications were carried out in the LightCycler® 480 II from Roche, according to the following protocol: activation of Taq

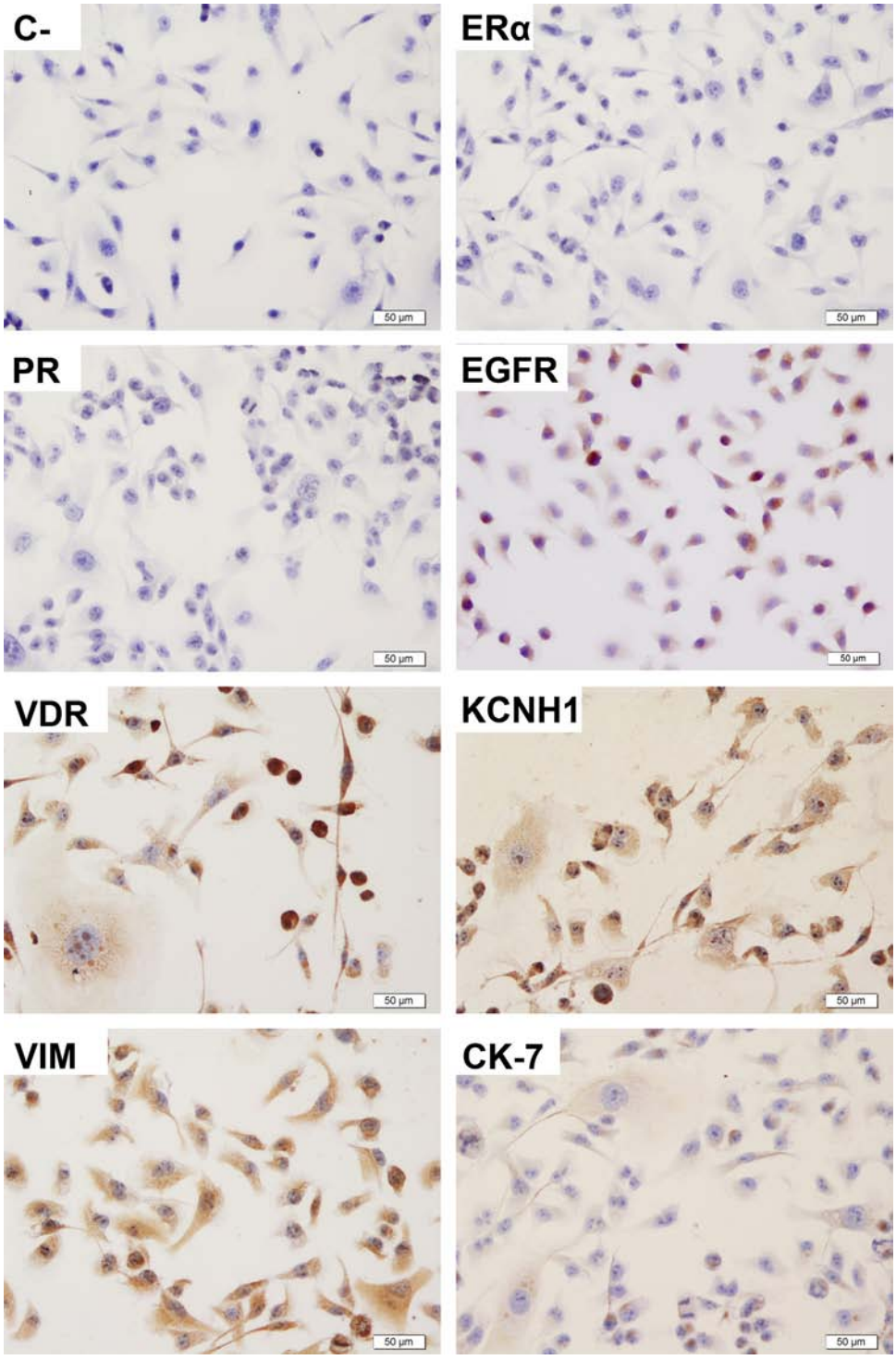

Fig. 1. Immunocharacterization of cultured MBCDF-D5 cells. MBCDF-D5 cells were negative for estrogen receptor-α (ERα) and progesterone receptor (PR), while were positive (brown staining) for epidermal growth factor receptor 1 (EGFR), vitamin D receptor (VDR), ether à-go-go-1 potassium channel (KCNH1), vimentin (VIM) and cytokeratin-7 (CK-7). Negative control (C-) was carried out in the absence of primary antibody. Representative images of cultured MBCDF-D5 cells are shown.

Please cite this article in press as: J. García-Quiroz, et al., Chronic moderate ethanol intake differentially regulates vitamin D hydroxylases gene expression in kidneys and xenografted breast cancer cells in female mice, J. Steroid Biochem. Mol. Biol. (2016), http://dx.doi.org/10.1016/j.jsbmb.2016.09.011

DNA polymerase and DNA denaturation at 95 °C for 10 min, followed by 45 amplification cycles consisting of 10 s at 95 °C, 30 s at 60 °C, and 1 s at 72 °C. The oligonucleotides pairs are shown in supplementary Table 1. In all cases gene expression was normalized against the housekeeping gene human β-actin (*ACTB*) or mouse β-actin (*Actb*), as corresponded.

2.5. Serum 25OHD$_3$ and calcium levels

Blood samples were obtained by cardiac puncture under anesthesia, causing exsanguination. Animal death was further guaranteed by cervical dislocation. The blood was centrifuged (3000 rpm/10 min) and serum samples of all mice in each experimental group were pooled and stored at −70 °C until analysis. Experiments were repeated at least 4 times. Serum calcium and 25OHD$_3$ concentrations were determined in the institutional clinical laboratories by colorimetric reaction between calcium and Arsenazo III (Beckman Coulter 2700, Beckman Coulter Inc., CA, USA) and by the Architect immunoassay (Abbott Diagnostics, Wiesbaden, Germany), respectively.

2.6. Western blot

A piece of the excised tumors was homogenized in RIPA buffer (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, 0.1% Nonidet P-40, 0.1% SDS, pH 7.4) in the presence of a protease inhibitor cocktail (Roche) using the MagNA Lyser equipment and ceramic beads. An equal amount of protein (55 micrograms) of tissue lysates was separated on 10% SDS-PAGE, transferred to nitrocellulose membranes and blocked with 5% non-fat milk in TBS-Tween. Membranes were incubated with rabbit anti-VDR (Santa Cruz Biotechnology CA, C-20 sc-1008, 1:200) overnight at 4 °C. As loading control, a monoclonal anti-GAPDH antibody was used (Millipore MA, MAB374, 1:8000). In the case of the cathelicidin antimicrobial peptide (CAMP), the proteins were separated using the Tricine-SDS-PAGE protocol containing 10% and 16% polyacrylamide. These membranes were incubated with mouse anti-CAMP (Santa Cruz Biotechnology CA, sc-166770, 1:200) and as loading control, a goat polyclonal anti-ACTB-horseradish peroxidase (HRP, I-19 sc-1616-HRP Santa Cruz Biotechnology CA, 1:200). For visualization of CAMP, VDR and GAPDH, membranes were incubated with goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, sc-2031, 1:2000) or goat anti-rabbit IgG-HRP (BioRad, CA, 1706515, 1:8000), as corresponded. Afterwards membranes were processed with the ECL + Plus Western blotting detection system (GE Healthcare, UK). Densitometric analysis of bands was performed using the Molecular Imager ChemiDoc XRS System and the Image Lab Software (Bio-Rad, CA, USA).

2.7. Statistical analysis

Statistical differences were determined by One-Way ANOVA followed by the Holm-Sidak test for pair-wise comparisons (SigmaStat, Jandel Scientific). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Cell characterization

MBCDF-D5 cells were negative for ERα and PR (Fig. 1). HER2 expression was not detected by immunocytochemistry but was found in low levels by Western blot (Esparza-López J. et al., unpublished data). In addition, cells moderately expressed CK-7 while presented high abundance of VIM, probably indicating epithelial-to-mesenchymal transition. Also, EGFR, the oncogenic protein KCNH1 and the VDR were highly expressed by these cells (Fig. 1). Overexpression of KCNH1 may confer a growth advantage to cancer cells while favors tumor progression [30]. Due to VDR endogenous expression, it is possible that these tumor-derived cells are able to respond to calcitriol stimuli. Functionality of VDR in cultured MBCDF-D5 cells was further corroborated by studying *CYP24A1* gene expression in the presence of 10 nM calcitriol. This resulted in significant *CYP24A1* gene expression stimulation compared to control cells (Supplementary Fig. 1).

3.2. In vivo *impact of chronic ethanol intake upon vitamin D-dependent effects*

3.2.1. *Chronic moderate ethanol intake differentially regulated* CYP27B1 *and* CYP24A1 *gene expression in mice kidneys and xenografted human breast cancer cells*

In the kidneys, chronic ethanol intake significantly decreased renal *Cyp27b1* ($P < 0.05$) while a non-significant increase of *Cyp24a1* gene expression was observed compared to controls. On the other hand, 25OHD$_3$ had no effect upon *Cyp27b1* but significantly stimulated *Cyp24a1* gene expression. Interestingly, coadministration of ethanol and 25OHD$_3$ diminished in 60% renal 25OHD$_3$–dependent *Cyp24a1* upregulation ($P < 0.05$, Fig. 2A and B).

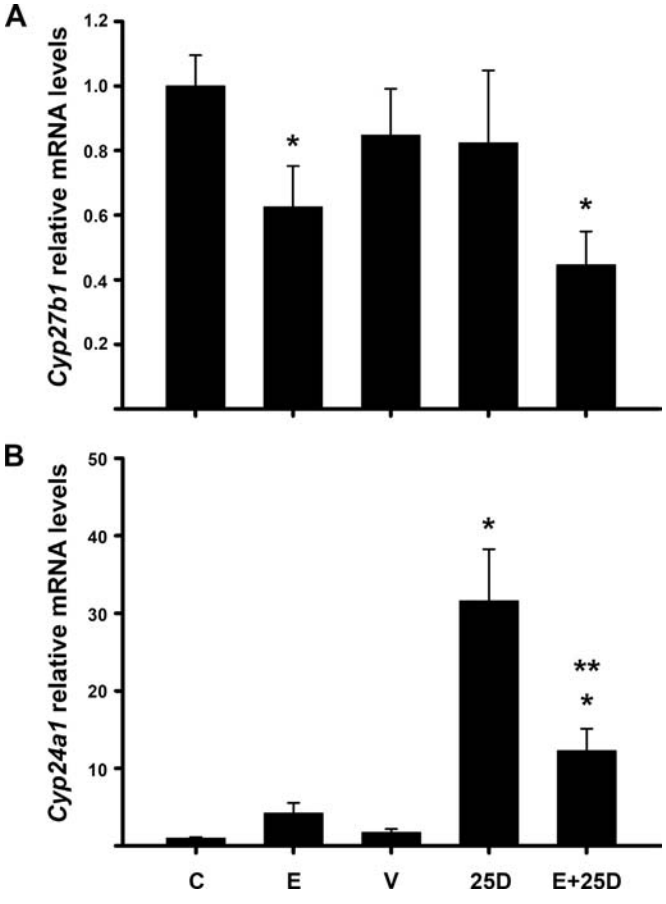

Fig. 2. Regulation of *Cyp27b1* and *Cyp24a1* gene expression in mice kidney. Mice were divided in the following groups: control (C), ethanol (E), vehicle (V), 25OHD$_3$ (25D), or combined E + 25D. A) The gene expression of *Cyp27b1* was significantly downregulated by E, while 25D had no effect upon this gene. However, the coadministration of E + 25D reduced *Cyp27b1* gene expression B) The gene expression of *Cyp24a1* was significantly upregulated by 25D, while its coadministration with E reduced this effect. Data are depicted as the mean ± SEM. Results were normalized against *Actb* mRNA expression; control values were set to one. N ≥ 12, * $P < 0.05$ *vs* C and V, ** $P < 0.05$ *vs* 25D.

Please cite this article in press as: J. García-Quiroz, et al., Chronic moderate ethanol intake differentially regulates vitamin D hydroxylases gene expression in kidneys and xenografted breast cancer cells in female mice, J. Steroid Biochem. Mol. Biol. (2016), http://dx.doi.org/10.1016/j.jsbmb.2016.09.011

In tumoral tissue, ethanol, 25OHD$_3$ and their combination increased tumoral *CYP24A1* in a similar manner, while *CYP27B1* gene expression was also stimulated by 25OHD$_3$ in non-ethanol-drinking mice (Fig. 3A and B). Since CYP27B1 and CYP24A1 ratio dictates whether 25OHD$_3$ will either be used for bioactive calcitriol production or will be inactivated, we evaluated the basal relative expression of the genes encoding for these two enzymes in tumors and kidneys. Of note, we found 5 folds higher basal *Cyp27b1* than *Cyp24a1* gene expression in kidneys, whereas this relation was inverted in MBCDF-D5 tumors, showing 5 folds more *CYP24A1* than *CYP27B1*. Renal tissue also showed significantly increased gene expression of both cytochromes when compared to xenografted breast cancer cells (Fig. 4).

3.2.2. Effects of endogenous calcitriol in the tumor mass

As a reflect of locally biosynthesized calcitriol, we studied known transcriptional targets of this secosteroid in order to get an insight into the effects of ethanol intake on 25OHD$_3$ metabolism and its consequences on tumor mass. For this, total RNA and protein were extracted from tumors. Selected genes were chosen based on their previously reported susceptibility to calcitriol regulation in breast cancer cells, and their involvement in pathways relevant for tumor progression. These genes were: *VEGFA*, *THBS1* and *KCNH1* [31–34]. The first two genes encode for vascular endothelial growth factor and thrombospondin-1, a proangiogenic and antiangiogenic factor, respectively; while the last one encodes for a voltage gated potassium channel which overexpression has been related to carcinogenesis, as previously mentioned. None of these genes were differentially expressed in tumors of xenotransplanted mice (data not shown). So, we decided to evaluate another calcitriol target with canonical vitamin D response elements. We chose cathelicidin (CAMP) due its high

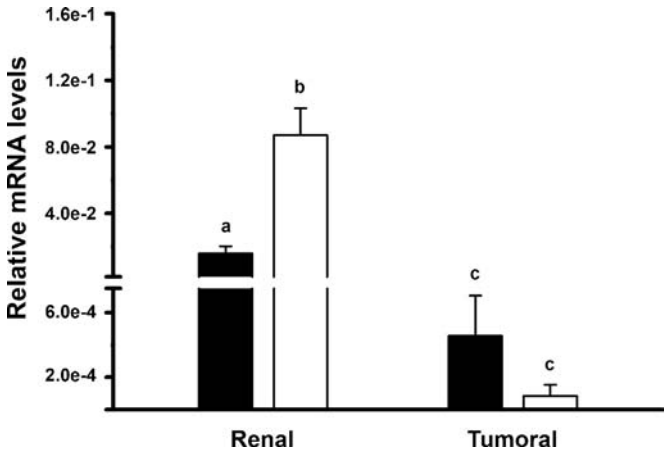

Fig. 4. Diferential vitamin D cytochromes gene expression in renal and tumoral tissue. We evaluated the relative expression of *CYP27B1* and *CYP24A1* at renal and tumoral level. In the mice kidneys, the basal expression of *Cyp27b1* was ~5 folds higher than that of *Cyp24a1*, while this relation was inverted in MBCDF-D5 tumors, showing 5 folds more *CYP24A1* than *CYP27B1* basal gene expression. Renal tissue also showed significantly more gene expression of both cytochromes compared to xenografted breast cancer cells. Data are depicted as the mean ± SEM. Results were normalized against actin mRNA expression. N ≥ 12, different letters indicate $P \leq 0.05$.

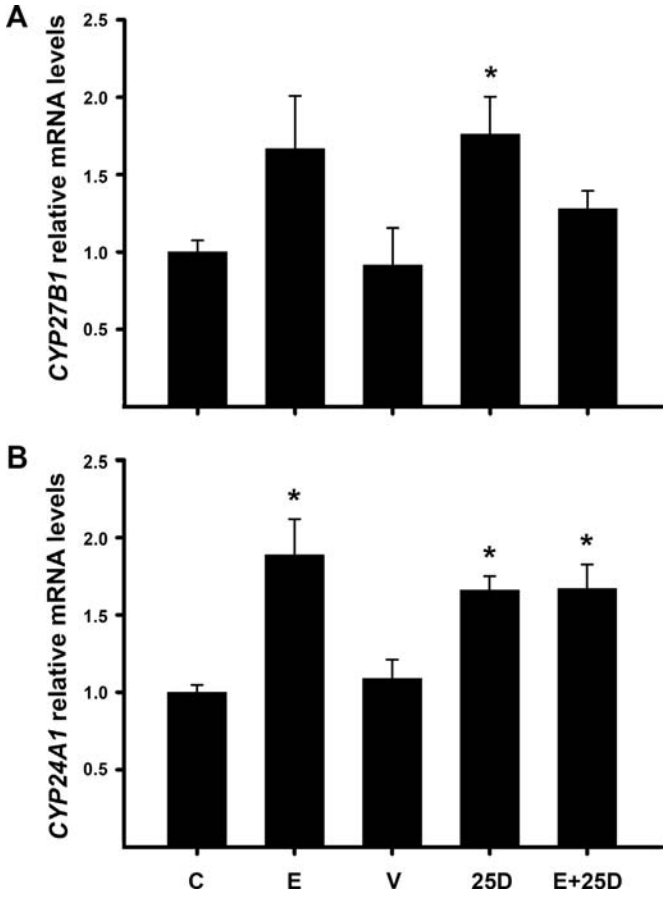

Fig. 3. Regulation of *CYP24A1* and *CYP27B1* gene expression in tumor tissue. Mice were divided in the following groups: control (C), ethanol (E), vehicle (V), 25OHD$_3$ (25D), or combined E + 25D. A) *CYP27B1* gene expression was stimulated in 25D-treated non-ethanol drinking mice. B) *CYP24A1* gene expression was significantly upregulated by E, 25D and the combined group (E + 25D). Data are depicted as the mean ± SEM. Results were normalized against *ACTB* mRNA expression; controls values were set to one. N ≥ 12, * $P < 0.05$ *vs* C.

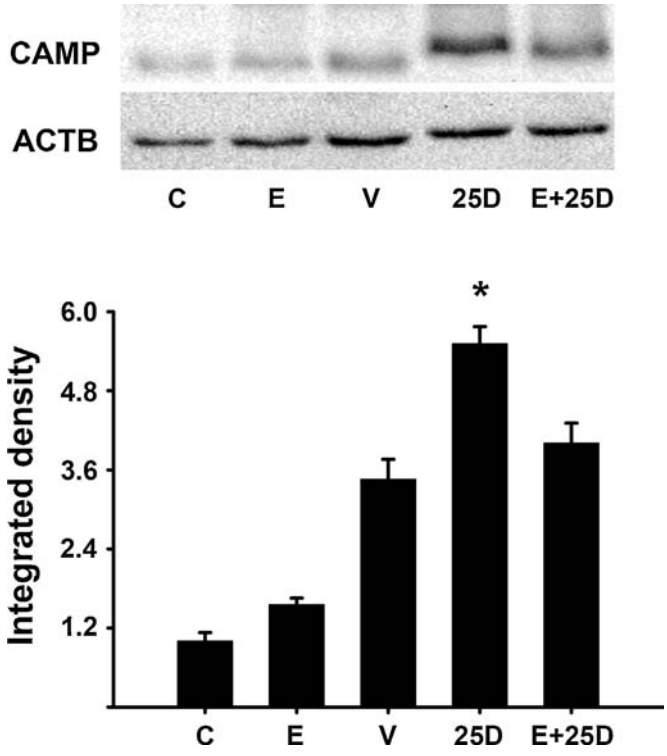

Fig. 5. Modulation of CAMP protein expression in xenografted tumors. Mice were divided in the following groups: control (C), ethanol (E), vehicle (V), 25OHD$_3$ (25D), or combined E + 25D. CAMP protein (25 kDa) was significantly more abundant in tumors from 25D treated mice, compared to controls (*$P < 0.05$). Data are depicted as the mean ± SEM. Actin (ACTB) protein (43 kDa) was used as a loading control. Control values were set to one. Representative images are shown. N ≥ 4 different tumors.

Please cite this article in press as: J. García-Quiroz, et al., Chronic moderate ethanol intake differentially regulates vitamin D hydroxylases gene expression in kidneys and xenografted breast cancer cells in female mice, J. Steroid Biochem. Mol. Biol. (2016), http://dx.doi.org/10.1016/j.jsbmb.2016.09.011

sensitivity to calcitriol in human cells [35]. As shown in Fig. 5 and as expected, Western blots of tumor homogenates showed that CAMP was significantly more abundant in samples from 25OHD$_3$-treated-mice than those from controls. Interestingly, when mice were concomitantly treated with ethanol and 25OHD$_3$, no statistical difference in CAMP expression was observed when compared to controls (Fig. 5). Two immunoreactive bands were detected: ~75 kDa and ~25 kDa. These bands might represent associations of uncleaved CAMP pro-protein with other proteins, since CAMP may form high molecular weight complexes [36,37] and different molecular weight CAMP bands have been described in breast tumors [38]. The relative abundance of these two CAMP variants was similar; therefore we only show results for the 25 kDa form (Fig. 5).

We also analyzed tumor VDR expression by Western blots. Regarding this, two immunoreactive bands were detected: a more abundant high molecular weight variant and the classic 50 kDa VDR band, which was weakly detected, as reported previously in breast cancer cells [39]. Optical density analysis showed a similar VDR protein abundance regardless the treatment (Supplementary Fig. 2).

3.2.3. *Effects of treatments upon 25OHD$_3$ and calcium serum concentrations*

Blood samples were drawn at the time of sacrifice, 14 h after the final 25OHD$_3$ weekly dose. As expected, in the case of 25OHD$_3$ mean serum levels were significantly higher in mice treated with this vitamin D metabolite compared to non-supplemented animals, reaching very high values in both ethanol-drinking and non-drinking groups (Table 1). On the other hand, ethanol intake increased 25OHD$_3$ levels compared to controls; however, this difference was not statistically different (Table 1). Regarding serum calcium levels, the administration of ethanol alone did not result in significant changes in calcemia compared to controls. However, in 25OHD$_3$-treated groups with or without ethanol drinking regimen, calcemia values were significantly higher when compared to the control group ($P < 0.05$), without reaching a hypercalcemic state (Table 1). In addition, serum calcium and 25OHD$_3$ levels were positively and significantly associated (R = 0.824, $P = 0.006$) in the 25OHD$_3$-treated groups (Fig. 6A), and without a significant association (R = 0.055, $P = 0.846$) in the non-25OHD$_3$ treated groups (Fig. 6B).

3.2.4. *Tumor volume*

Compared to the control group, neither alcohol consumption nor 25OHD$_3$ treatment significantly affected the establishment of MBCDF-D5 tumors after implantation. However, a suggestive delay on tumor onset was observed in the 25OHD$_3$-treated group *versus* all other groups, reaching significance when compared to ethanol-treated animals (Table 2). In general, increased tumor volume was observed in the ethanol-treated group; although this effect did not reach statistical significance (Fig. 7).

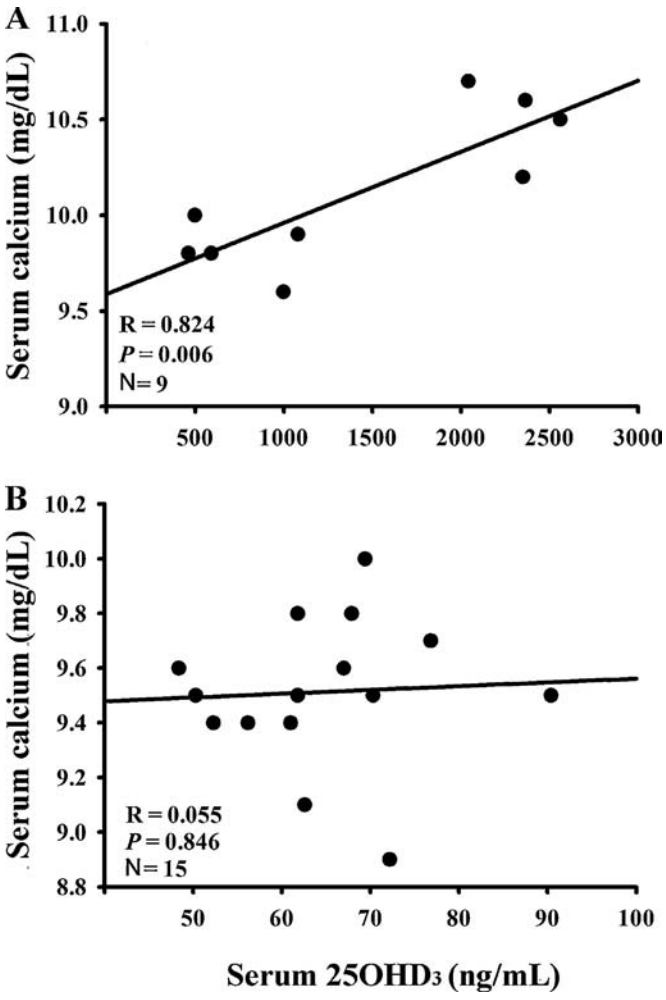

Fig. 6. Serum calcium and 25-hidroxy-vitaminD$_3$ association. A) 25OHD$_3$-treated groups. Serum calcium and 25OHD$_3$ were positively and significantly associated. B) Non-25OHD$_3$ treated groups. No association was found between the two parameters tested. The N represents pools of samples which include at least 3 mice in each pool from the same group.

3.3. In vivo *effects of treatments upon body weight and animal wellbeing*

Initial body weights mean from all groups was 17.94 ± 0.161 g. Final body weights mean in each group were: control 20.11 g ± 1.40 g, ethanol 20.74 g ± 1.57 g, vehicle 21.05 g ± 0.19 g, 25OHD$_3$ 22.07 g ± 0.96 g and 25OHD$_3$ plus ethanol 21.74 g ± 1.63 g. Only the groups supplemented with 25OHD$_3$ had higher mean body weight compared to the control group at the end of experiments ($P < 0.001$). In general, we did not find signs of health deterioration associated to treatment (*e.g.* dehydration, altered behavior, loss of

Table 1
Serum levels of calcium and 25OHD$_3$.

Parameters	C	E	V	25D	25D + E
Calcium (mg/dL)	9.3 ± 0.3	9.6 ± 0.1	9.7 ± 0.3	10.2 ± 0.6*	10.1 ± 0.3*
25OHD$_3$ (ng/mL)	59 ± 9	71 ± 15	63 ± 5	1499 ± 840**	1390 ± 1005**

Data are presented as mean ± standard deviation. Mice were divided in the following groups: control (C), ethanol (E), vehicle (V), 25OHD$_3$ (25D), or combined E + 25D. N ≥ 4 different pools, each pool included at least 3 mice from the same experimental group.
* $P < 0.05$ *vs* C and E.
** $P < 0.05$ *vs* C, E and V.

Table 2
Tumor onset.

Treatment	Media ± SD
C	17.7 ± 4.79
E	13.9 ± 7.24
V	17.54 ± 4.7
25D	20.63 ± 6.25*
E + 25D	18.75 ± 5.74

Data are presented as mean ± S.D of time elapsed between xenograft and tumor onset (days). Mice were divided in the following groups: control (C), ethanol (E), vehicle (V), 25OHD$_3$ (25D), or combined E + 25D. N ≥ 13.
* $P = 0.01$ *vs* E.

Please cite this article in press as: J. García-Quiroz, et al., Chronic moderate ethanol intake differentially regulates vitamin D hydroxylases gene expression in kidneys and xenografted breast cancer cells in female mice, J. Steroid Biochem. Mol. Biol. (2016), http://dx.doi.org/10.1016/j.jsbmb.2016.09.011

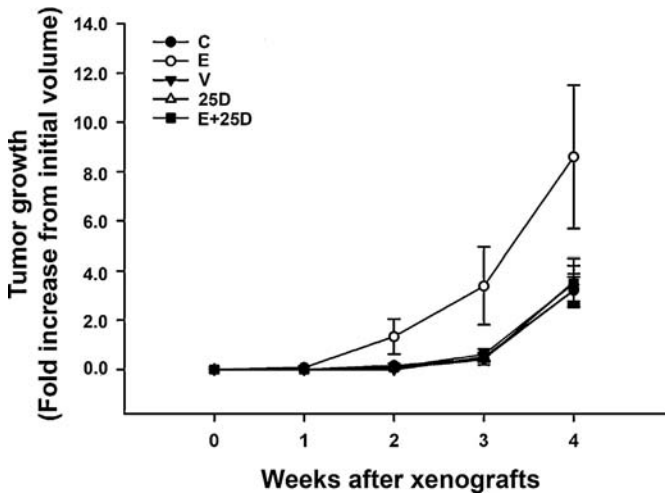

Fig. 7. Tumor growth depicted as fold increase from initial volume by weekly measures. Human breast cancer cells were injected on day 0 and afterwards tumor dimensions were measured. Mice were divided in the following groups: control (C), ethanol (E), vehicle (V), 25OHD$_3$ (25D), or combined E + 25D. The graphic depicts weekly measures (fold increase from initial volume) as the mean ± SEM.

appetite, weight loss), suggesting that the tested doses of ethanol and 25OHD$_3$ were well tolerated.

4. Discussion

In the current study, we examined the possibility that chronic moderate ethanol consumption might affect gene expression of enzymes involved in vitamin D metabolism in breast cancer cells, and consequently, impact on 25OHD$_3$-dependent expression of some calcitriol targets with relevant roles in breast cancer genesis and progression. We chose 25OHD$_3$ instead of cholecalciferol to bypass hepatic hydroxylation, given that liver functions may be compromised by alcohol intake; hence, 25OHD$_3$ bioavailability would not be a limiting factor. Under these conditions, we found that moderate chronic ethanol intake significantly increased *CYP24A1* but did not modify *CYP27B1* gene expression in tumor cells. However, ethanol drinking did significantly decrease renal *Cyp27b1*. These results suggest that the ethanol consumption regimen tested herein decreased 25OHD$_3$ bioconversion into calcitriol in the kidney, while increased breast cancer cells ability to degrade these two vitamin D metabolites. Our results are in line with those from a previous study performed in female rats, where ethanol drinking increased *Cyp24a1* while decreased *Cyp27b1* expression in the kidneys, resulting in reduced serum calcitriol levels [23]. Thus, we tested the impact of ethanol consumption on biological markers of calcitriol activity (*i.e.* gene expression and tumor volume) in xenografted breast tumors. Under the conditions used in this study, 25OHD$_3$ administration did not significantly delay tumor onset nor decreased tumor growth. This could be explained either by the cell phenotype (MBCDF-D5 cells are highly undifferentiated) or by the low intratumoral calcitriol concentration due to high degradation. The latter is possible given the high tumoral *CYP24A1*/*CYP27B1* ratio observed herein, which, on the other hand, might also explain why we did not find a significant modulation of *KCNH1*, *VEGFA* or *THSB1* gene expression by 25OHD$_3$ treatment. In contrast, in kidneys there were 5 folds higher basal *Cyp27b1* than *Cyp24a1*, suggesting that compared to kidneys, MBCDF-D5 tumors are very inefficient in producing calcitriol. Nevertheless, the results on tumoral CAMP protein and *CYP24A1* gene expression strongly suggest that local conversion of 25OHD$_3$ to calcitriol actually occurred in our xenografted breast cancer cells both in the presence and absence of systemic ethanol. CAMP is an antimicrobial peptide that in neoplastic cells has shown cytotoxic, antiproliferative and pro-apoptotic effects [40–42]. Transcription of *CAMP* is highly stimulated by calcitriol due to the vitamin D response element located in its promoter [35]. Interestingly, even though in both 25OHD$_3$-treated groups CAMP protein expression augmented compared to controls, the magnitude of the response was higher and significant only in mice non-consuming alcohol. This observation further supported that in the tumor mass calcitriol production and bioavailability were restricted by alcohol intake, consistently with our results showing increased tumoral *CYP24A1* in ethanol drinking mice and decreased *CYP27B1* in tumors from mice treated with the combined regimen compared to those treated only with 25OHD$_3$. Likewise, our results also suggest that a similar process is taking place at the renal level, given that co-administration of ethanol and 25OHD$_3$ reduced in 60% 25OHD$_3$–dependent *Cyp24a1* upregulation in this tissue, probably reflecting less bioactive calcitriol and thus, less expression of this canonical calcitriol-responsive gene. Elsewhere, ethanol consumption has shown to stimulate the production of oxidative stress in kidneys, resulting in increased renal CYP24A1 gene transcription as a result of mitogen-activated protein kinases signaling pathway activation. This specific mechanism of action of ethanol remains to be studied in our breast tumor-murine model [23]. On the other hand, given that calcitriol is a known negative regulator of renal *CYP27B1* [43], we expected to find its gene expression reduced by 25OHD$_3$ treatment in both kidneys and tumors. However; this was not the case. In fact, renal *Cyp27b1* gene expression was only reduced when mice were exposed to chronic ethanol intake, suggesting that alcohol may decrease systemic calcitriol concentration as a result of reduced renal *CYP27B1* expression; hence, lowering vitamin D anticancer effects in the whole body. Instead, in xenografted breast tumor cells 25OHD$_3$ significantly increased *CYP27B1* gene expression. This outcome agrees with previous studies showing that calcitriol and cholecalciferol augmented *CYP27B1* gene expression in breast cancer cells both *in vivo* and *in vitro* [44,45], and with reports showing breast tumor spilling of calcitriol into the circulation in cholecalciferol-supplemented mice [45]. This is explainable on the basis that extrarenal regulation of *CYP27B1* by calcitriol is a cell-type-selective event [46,47], and implies that bioconversion of 25OHD$_3$ into calcitriol in the mammary gland depends more on vitamin D nutritional status. Importantly, our results on tumor *CYP27B1* showed that alcohol intake may diminish 25OHD$_3$-dependent calcitriol production, preventing in this manner its beneficial antineoplastic effects in breast cancer cells. The latter highlights the importance of vitamin D sufficiency and the perils of alcohol consumption in women with risk of breast cancer or currently under therapy for this pathology.

In the present study, ethanol administration *per se* was not associated with significant changes in serum calcium or 25OHD$_3$. However, a non-significant increase in 25OHD$_3$ values was observed in ethanol consuming mice not-treated with this vitamin D metabolite compared to controls, which has also been observed in rat and human models of alcoholism [23,24,48]. In this regard, it has been suggested that higher serum 25OHD$_3$ levels in alcohol users may reflect disturbed vitamin D metabolism as a result of reduced bioconversion into calcitriol, rather than vitamin D sufficiency [48].

The highest values of calcemia were observed in the 25OHD$_3$-treated mice, in accordance with the stimulatory effect of calcitriol upon active intestinal calcium absorption and with previous studies showing that 25OHD$_3$ is more potent and effective than cholecalciferol in rising 25OHD$_3$ serum levels [49]. Herein, even though very high 25OHD$_3$ serum levels were reached in the groups treated with this metabolite, hypercalcemia was not detected in any mice and the clinical status of these animals was similar to

Please cite this article in press as: J. García-Quiroz, et al., Chronic moderate ethanol intake differentially regulates vitamin D hydroxylases gene expression in kidneys and xenografted breast cancer cells in female mice, J. Steroid Biochem. Mol. Biol. (2016), http://dx.doi.org/10.1016/j.jsbmb.2016.09.011

those observed in the other groups studied. This observation may suggest a differential vitamin D pharmacotoxicity between mice and humans. Most probably, vitamin D toxicity was avoided in $25OHD_3$–treated groups by the classical feedback autoregulatory loop involving increased *CYP24A1* expression, as seen in our renal and tumor data. Also, it is possible that urinary calcium excretion was higher in $25OHD_3$-treated groups. On the other hand, the positive association between serum calcium and $25OHD_3$ levels observed only in $25OHD_3$-treated mice corroborates that nutritional vitamin D status is one of the determinants of calcemia.

Whereas total body fat has been inversely associated with $25OHD_3$ levels in humans [50], in the murine model used herein we found a significantly higher mean final body weight in mice that received $25OHD_3$ compared to controls. This might be explained considering the fact that both VDR and its ligand positively and significantly impact on lipid accumulation, as seen in the VDR-KO-associated lean phenotype [51]. Alternatively, since mice were still developing at the beginning of the study, it is possible that $25OHD_3$ supplementation increased body weights as a result of augmented bone mineral content and decreased bone resorption, as reported in young girls [52]. These assumptions need to be further investigated. In line with this, vitamin D supplementation is known to protect against bone loss associated with chronic alcohol administration in female mice [53]. Nonetheless, in our study alcohol intake did not result in weight loss, must probably due to the moderate dose of alcohol used in this study.

In summary, our study indicates that chronic moderate alcohol intake rises renal and tumoral *CYP24A1* while lowers renal *Cyp27b1* gene expression. In addition, $25OHD_3$ supplementation significantly stimulated tumoral *CYP27B1* gene expression only in non-alcohol-drinking mice. These results suggest that even a low ethanol intake habit can actually decrease renal and tumoral $25OHD_3$ bioconversion into calcitriol and stimulate its degradation in breast tumors. The latter may partially explain why alcohol consumption is associated with vitamin D deficiency and breast cancer risk and progression.

Acknowledgments

We acknowledge with thank to Consejo Nacional de Ciencia y Tecnología (CONACyT), México, who supported this study with grant numbers 153862, 241034 and MOD-ORD-18-2014, PCI-006-06-14 to LD, EA and JGQ, respectively. The funder had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2016.09.011.

References

[1] M. Schutze, H. Boeing, T. Pischon, J. Rehm, T. Kehoe, G. Gmel, A. Olsen, A.M. Tjonneland, C.C. Dahm, K. Overvad, F. Clavel-Chapelon, M.C. Boutron-Ruault, A. Trichopoulou, V. Benetou, D. Zylis, R. Kaaks, S. Rohrmann, D. Palli, F. Berrino, R. Tumino, P. Vineis, L. Rodriguez, A. Agudo, M.J. Sanchez, M. Dorronsoro, M.D. Chirlaque, A. Barricarte, P.H. Peeters, C.H. van Gils, K.T. Khaw, N. Wareham, N.E. Allen, T.J. Key, P. Boffetta, N. Slimani, M. Jenab, D. Romaguera, P.A. Wark, E. Riboli, M.M. Bergmann, Alcohol attributable burden of incidence of cancer in eight European countries based on results from prospective cohort study, BMJ 342 (2011) d1584.
[2] C. Scoccianti, B. Lauby-Secretan, P.Y. Bello, V. Chajes, I. Romieu, Female breast cancer and alcohol consumption: a review of the literature, Am. J. Prev. Med. 46 (2014) S16–25.
[3] P. Boffetta, M. Tubiana, C. Hill, M. Boniol, A. Aurengo, R. Masse, A.J. Valleron, R. Monier, G. de The, P. Boyle, P. Autier, The causes of cancer in France, Ann. Oncol. 20 (2009) 550–555.
[4] N.E. Allen, V. Beral, D. Casabonne, S.W. Kan, G.K. Reeves, A. Brown, J. Green, Moderate alcohol intake and cancer incidence in women, J. Natl. Cancer Inst. 101 (2009) 296–305.
[5] R.C. Ellison, Y. Zhang, C.E. McLennan, K.J. Rothman, Exploring the relation of alcohol consumption to risk of breast cancer, Am. J. Epidemiol. 154 (2001) 740–747.
[6] L.S. Morch, D. Johansen, L.C. Thygesen, A. Tjonneland, E. Lokkegaard, C. Stahlberg, M. Gronbaek, Alcohol drinking, consumption patterns and breast cancer among Danish nurses: a cohort study, Eur. J. Public Health 17 (2007) 624–629.
[7] W.Y. Chen, B. Rosner, S.E. Hankinson, G.A. Colditz, W.C. Willett, Moderate alcohol consumption during adult life, drinking patterns, and breast cancer risk, JAMA 306 (2011) 1884–1890.
[8] T. Watabiki, Y. Okii, T. Tokiyasu, S. Yoshimura, M. Yoshida, A. Akane, N. Shikata, A. Tsubura, Long-term ethanol consumption in ICR mice causes mammary tumor in females and liver fibrosis in males, Alcohol Clin. Exp. Res. 24 (2000) 117S–122S.
[9] R. Yirmiya, S. Ben-Eliyahu, R.P. Gale, Y. Shavit, J.C. Liebeskind, A.N. Taylor, Ethanol increases tumor progression in rats: possible involvement of natural killer cells, Brain Behav. Immun. 6 (1992) 74–86.
[10] P.J. Brooks, S. Zakhari, Moderate alcohol consumption and breast cancer in women: from epidemiology to mechanisms and interventions, Alcohol. Clin. Exp. Res. 37 (2013) 23–30.
[11] G. Millonig, Y. Wang, N. Homann, F. Bernhardt, H. Qin, S. Mueller, H. Bartsch, H. K. Seitz, Ethanol-mediated carcinogenesis in the human esophagus implicates CYP2E1 induction and the generation of carcinogenic DNA-lesions, Int. J. Cancer 128 (2011) 533–540.
[12] A.A. Rose, C. Elser, M. Ennis, P.J. Goodwin, Blood levels of vitamin D and early stage breast cancer prognosis: a systematic review and meta-analysis, Breast Cancer Res. Treat. 141 (2013) 331–339.
[13] C.F. Garland, E.D. Gorham, S.B. Mohr, W.B. Grant, E.L. Giovannucci, M. Lipkin, H. Newmark, M.F. Holick, F.C. Garland, Vitamin D and prevention of breast cancer: pooled analysis, J. Steroid Biochem. Mol. Biol. 103 (2007) 708–711.
[14] T. Shao, P. Klein, M.L. Grossbard, Vitamin D and breast cancer, Oncologist 17 (2012) 36–45.
[15] J. Welsh, Vitamin D metabolism in mammary gland and breast cancer, Mol. Cell Endocrinol. 347 (2011) 55–60.
[16] L. Lowe, C.M. Hansen, S. Senaratne, K.W. Colston, Mechanisms implicated in the growth regulatory effects of vitamin D compounds in breast cancer cells, Recent Results Cancer Res. 164 (2003) 99–110.
[17] J.C. Fleet, Molecular actions of vitamin D contributing to cancer prevention, Mol. Aspects Med. 29 (2008) 388–396.
[18] J. Welsh, Cellular and molecular effects of vitamin D on carcinogenesis, Arch. Biochem. Biophys. 523 (2012) 107–114.
[19] N. Lopes, B. Sousa, D. Martins, M. Gomes, D. Vieira, L.A. Veronese, F. Milanezi, J. Paredes, J.L. Costa, F. Schmitt, Alterations in vitamin D signalling and metabolic pathways in breast cancer progression: a study of VDR CYP27B1 and CYP24A1 expression in benign and malignant breast lesions, BMC Cancer 10 (2010) 483.
[20] K. Townsend, C.M. Banwell, M. Guy, K.W. Colston, J.L. Mansi, P.M. Stewart, M.J. Campbell, M. Hewison, Autocrine metabolism of vitamin D in normal and malignant breast tissue, Clin. Cancer Res. 11 (2005) 3579–3586.
[21] J. Alvisa-Negrin, E. Gonzalez-Reimers, F. Santolaria-Fernandez, E. Garcia-Valdecasas-Campelo, M.R. Valls, R. Pelazas-Gonzalez, M.C. Duran-Castellon, M. de Los Angeles Gomez-Rodriguez, Osteopenia in alcoholics: effect of alcohol abstinence, Alcohol Alcohol 44 (2009) 468–475.
[22] K. Laitinen, M. Valimaki, C. Lamberg-Allardt, L. Kivisaari, M. Lalla, M. Karkkainen, R. Ylikahri, Deranged vitamin D metabolism but normal bone mineral density in Finnish noncirrhotic male alcoholics, Alcohol Clin. Exp. Res. 14 (1990) 551–556.
[23] K. Shankar, X. Liu, R. Singhal, J.R. Chen, S. Nagarajan, T.M. Badger, M.J. Ronis, Chronic ethanol consumption leads to disruption of vitamin D3 homeostasis associated with induction of renal 1,25 dihydroxyvitamin D3-24-hydroxylase (CYP24A1), Endocrinology 149 (2008) 1748–1756.
[24] R.T. Turner, R.C. Aloia, L.D. Segel, K.S. Hannon, N.H. Bell, Chronic alcohol treatment results in disturbed vitamin D metabolism and skeletal abnormalities in rats, Alcohol Clin. Exp. Res. 12 (1988) 159–162.
[25] M. Gascon-Barre, Interrelationships between vitamin D3 and 25-hydroxyvitamin D3 during chronic ethanol administration in the rat, Metabolism 31 (1982) 67–72.
[26] R. Jacoby, J. Fox, Biology and disease of mice, in: L.C.A.J. Fox, F.M. Loew, F.W. Quimby (Eds.), Laboratory Animal Medicine, Academic Press, Inc, San Diego, CA, 1984, pp. 31–89.
[27] E. Aberg, C.P. Hofstetter, L. Olson, S. Brene, Moderate ethanol consumption increases hippocampal cell proliferation and neurogenesis in the adult mouse, Int. J. Neuropsychopharmacol. 8 (2005) 557–567.
[28] E. Cebral, A. Motta, M. Boquet, M.A. de Gimeno, Effects of low chronic ethanol exposure on prostaglandin E synthesis by preimplantation mouse embryos, Prostaglandins Leukot. Essent. Fatty Acids 58 (1998) 249–255.
[29] B.A. Vorderstrasse, T. Wang, A.K. Myers, K.L. Wyrick, G.G. Meadows, Alcohol consumption suppresses mammary tumor metastasis in a syngeneic tumor transplantation model, Breast Cancer Res. Treat. 136 (2012) 729–737.
[30] L.A. Pardo, D. del Camino, A. Sanchez, F. Alves, A. Bruggemann, S. Beckh, W. Stuhmer, Oncogenic potential of EAG K(+) channels, EMBO J. 18 (1999) 5540–5547.
[31] J. Garcia-Quiroz, M. Rivas-Suarez, R. Garcia-Becerra, D. Barrera, I. Martinez-Reza, D. Ordaz-Rosado, N. Santos-Martinez, O. Villanueva, C.L. Santos-Cuevas,

Please cite this article in press as: J. García-Quiroz, et al., Chronic moderate ethanol intake differentially regulates vitamin D hydroxylases gene expression in kidneys and xenografted breast cancer cells in female mice, J. Steroid Biochem. Mol. Biol. (2016), http://dx.doi.org/10.1016/j.jsbmb.2016.09.011

E. Avila, A. Gamboa-Dominguez, A. Halhali, F. Larrea, L. Diaz, Calcitriol reduces thrombospondin-1 and increases vascular endothelial growth factor in breast cancer cells: implications for tumor angiogenesis, J. Steroid Biochem. Mol. Biol. 144 (Pt A) (2014) 215–222.
[32] J. Garcia-Quiroz, R. Garcia-Becerra, N. Santos-Martinez, D. Barrera, D. Ordaz-Rosado, E. Avila, A. Halhali, O. Villanueva, M.J. Ibarra-Sanchez, J. Esparza-Lopez, A. Gamboa-Dominguez, J. Camacho, F. Larrea, L. Diaz, In vivo dual targeting of the oncogenic Ether-a-go-go-1 potassium channel by calcitriol and astemizole results in enhanced antineoplastic effects in breast tumors, BMC Cancer 14 (2014) 745.
[33] R. Garcia-Becerra, L. Diaz, J. Camacho, D. Barrera, D. Ordaz-Rosado, A. Morales, C.S. Ortiz, E. Avila, E. Bargallo, M. Arrecillas, A. Halhali, F. Larrea, Calcitriol inhibits ether-a go–go potassium channel expression and cell proliferation in human breast cancer cells, Exp. Cell Res. 316 (2010) 433–442.
[34] V. Cazares-Ordonez, R.J. Gonzalez-Duarte, L. Diaz, M. Ishizawa, S. Uno, V. Ortiz, M.L. Ordonez-Sanchez, M. Makishima, F. Larrea, E. Avila, A cis-acting element in the promoter of human ether a go–go 1 potassium channel gene mediates repression by calcitriol in human cervical cancer cells, Biochem. Cell Biol. 93 (2015) 94–101.
[35] T.T. Wang, F.P. Nestel, V. Bourdeau, Y. Nagai, Q. Wang, J. Liao, L. Tavera-Mendoza, R. Lin, J.W. Hanrahan, S. Mader, J.H. White, Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression, J. Immunol. 173 (2004) 2909–2912.
[36] O. Sorensen, J.B. Cowland, J. Askaa, N. Borregaard, An ELISA for hCAP-18, the cathelicidin present in human neutrophils and plasma, J. Immunol. Methods 206 (1997) 53–59.
[37] Y. Wang, B. Agerberth, A. Lothgren, A. Almstedt, J. Johansson, Apolipoprotein A-I binds and inhibits the human antibacterial/cytotoxic peptide LL-37, J. Biol. Chem. 273 (1998) 33115–33118.
[38] J.D. Heilborn, M.F. Nilsson, C.I. Jimenez, B. Sandstedt, N. Borregaard, E. Tham, O. E. Sorensen, G. Weber, M. Stahle, Antimicrobial protein hCAP18/LL-37 is highly expressed in breast cancer and is a putative growth factor for epithelial cells, Int. J. Cancer 114 (2005) 713–719.
[39] J. Garcia-Quiroz, R. Garcia-Becerra, D. Barrera, N. Santos, E. Avila, D. Ordaz-Rosado, M. Rivas-Suarez, A. Halhali, P. Rodriguez, A. Gamboa-Dominguez, H. Medina-Franco, J. Camacho, F. Larrea, L. Diaz, Astemizole synergizes calcitriol antiproliferative activity by inhibiting CYP24A1 and upregulating VDR: a novel approach for breast cancer therapy, PLoS One 7 (2012) e45063.
[40] K. Okumura, A. Itoh, E. Isogai, K. Hirose, Y. Hosokawa, Y. Abiko, T. Shibata, M. Hirata, H. Isogai, C-terminal domain of human CAP18 antimicrobial peptide induces apoptosis in oral squamous cell carcinoma SAS-H1 cells, Cancer Lett. 212 (2004) 185–194.
[41] D. Winder, W.H. Gunzburg, V. Erfle, B. Salmons, Expression of antimicrobial peptides has an antitumour effect in human cells, Biochem. Biophys. Res. Commun. 242 (1998) 608–612.
[42] H. Bruns, M. Buttner, M. Fabri, D. Mougiakakos, J.T. Bittenbring, M.H. Hoffmann, F. Beier, S. Pasemann, R. Jitschin, A.D. Hofmann, F. Neumann, C. Daniel, A. Maurberger, B. Kempkes, K. Amann, A. Mackensen, A. Gerbitz, Vitamin D-dependent induction of cathelicidin in human macrophages results in cytotoxicity against high-grade B cell lymphoma, Sci. Trans. Med. 7 (2015) 282ra247.
[43] H.L. Henry, Regulation of vitamin D metabolism, Best Pract. Res. Clin. Endocrinol. Metab. 25 (2011) 531–541.
[44] L.L. Ooi, Y. Zheng, H. Zhou, T. Trivedi, A.D. Conigrave, M.J. Seibel, C.R. Dunstan, Vitamin D deficiency promotes growth of MCF-7 human breast cancer in a rodent model of osteosclerotic bone metastasis, Bone 47 (2010) 795–803.
[45] S. Swami, A.V. Krishnan, J.Y. Wang, K. Jensen, R. Horst, M.A. Albertelli, D. Feldman, Dietary vitamin D(3) and 1,25-dihydroxyvitamin D(3) (calcitriol) exhibit equivalent anticancer activity in mouse xenograft models of breast and prostate cancer, Endocrinology 153 (2012) 2576–2587.
[46] M.V. Young, G.G. Schwartz, L. Wang, D.P. Jamieson, L.W. Whitlatch, J.N. Flanagan, B.L. Lokeshwar, M.F. Holick, T.C. Chen, The prostate 25-hydroxyvitamin D-1 alpha-hydroxylase is not influenced by parathyroid hormone and calcium: implications for prostate cancer chemoprevention by vitamin D, Carcinogenesis 25 (2004) 967–971.
[47] M.M. Turunen, T.W. Dunlop, C. Carlberg, S. Vaisanen, Selective use of multiple vitamin D response elements underlies the 1 alpha,25-dihydroxyvitamin D3-mediated negative regulation of the human CYP27B1 gene, Nucleic Acids Res. 35 (2007) 2734–2747.
[48] K. Lee, Sex-specific relationships between alcohol consumption and vitamin D levels: the Korea national health and nutrition examination survey 2009, Nutr. Res. Pract. 6 (2012) 86–90.
[49] H.A. Bischoff-Ferrari, B. Dawson-Hughes, E. Stocklin, E. Sidelnikov, W.C. Willett, J.O. Edel, H.B. Stahelin, S. Wolfram, A. Jetter, J. Schwager, J. Henschkowski, A. von Eckardstein, A. Egli, Oral supplementation with 25(OH) D3 versus vitamin D3: effects on 25(OH)D levels, lower extremity function, blood pressure, and markers of innate immunity, J. Bone Miner. Res. 27 (2012) 160–169.
[50] M.B. Snijder, R.M. van Dam, M. Visser, D.J. Deeg, J.M. Dekker, L.M. Bouter, J.C. Seidell, P. Lips, Adiposity in relation to vitamin D status and parathyroid hormone levels: a population-based study in older men and women, J. Clin. Endocrinol. Metab. 90 (2005) 4119–4123.
[51] C.J. Narvaez, D. Matthews, E. Broun, M. Chan, J. Welsh, Lean phenotype and resistance to diet-induced obesity in vitamin D receptor knockout mice correlates with induction of uncoupling protein-1 in white adipose tissue, Endocrinology 150 (2009) 651–661.
[52] H.T. Viljakainen, A.M. Natri, M. Karkkainen, M.M. Huttunen, A. Palssa, J. Jakobsen, K.D. Cashman, C. Molgaard, C. Lamberg-Allardt, A positive dose-response effect of vitamin D supplementation on site-specific bone mineral augmentation in adolescent girls: a double-blinded randomized placebo-controlled 1-year intervention, J. Bone Miner. Res. 21 (2006) 836–844.
[53] K.E. Mercer, R.A. Wynne, O.P. Lazarenko, C.K. Lumpkin, W.R. Hogue, L.J. Suva, J. R. Chen, A.Z. Mason, T.M. Badger, M.J. Ronis, Vitamin D supplementation protects against bone loss associated with chronic alcohol administration in female mice, J. Pharmacol. Exp. Ther. 343 (2012) 401–412.

Please cite this article in press as: J. García-Quiroz, et al., Chronic moderate ethanol intake differentially regulates vitamin D hydroxylases gene expression in kidneys and xenografted breast cancer cells in female mice, J. Steroid Biochem. Mol. Biol. (2016), http://dx.doi.org/10.1016/j.jsbmb.2016.09.011

Journal of Steroid Biochemistry & Molecular Biology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

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

A time-course regulatory and kinetic expression study of steroid metabolizing enzymes by calcitriol in primary cultured human placental cells

Nancy Noyola-Martínez[a], Ali Halhali[a], Verónica Zaga-Clavellina[b], Andrea Olmos-Ortiz[a], Fernando Larrea[a], David Barrera[a,*]

[a] Departamento de Biología de la Reproducción, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Av. Vasco de Quiroga No. 15, Col. Belisario Domínguez, Sección XVI, México, D.F. 14080, Mexico
[b] Departamento de Inmunobioquímica, Instituto Nacional de Perinatología Isidro Espinosa de los Reyes, Montes Urales No. 800, Miguel Hidalgo 11000, México, D.F., Mexico

ARTICLE INFO

Article history:
Received 29 June 2016
Received in revised form 16 November 2016
Accepted 17 November 2016
Available online xxx

Keywords:
Trophoblast
Calcitriol
Cytochromes
Hydroxysteroid dehydrogenases
Placenta

ABSTRACT

1,25-dihydroxivitamin D_3 (calcitriol), is a secoesteroid involved in several placental functions. In particular, we and others showed that calcitriol regulates peptides, proteins, cytokines and hormones production in human trophoblastic cells. On the other hand, calcitriol modifies the activity and expression of some steroidogenic enzymes, a process that is considered tissue-specific. However, the effects of calcitriol on the expression of enzymes involved in the synthesis of sex steroids in placental tissue have not yet been entirely studied. The aim of the present study was to investigate the effects of calcitriol upon gene expression of several steroid enzymes such as cytochrome P450scc (*CYP11A1*), type 1 3β-hydroxysteroid dehydrogenase(*3β-HSDI*), *17β-HSD3*, 17α-hydroxylase/17,20 lyase (*CYP17A1*) and aromatase (*CYP19A1*) in primary cultures of human placental cells. Cell cultures were performed using placentas obtained immediately after delivery by caesarean section from normotensive healthy women and calcitriol effects were evaluated, at level of transcription, by qPCR. The results showed that: 1) from basal expression values of the five genes studied, 3β-HSDI was the most expressed gene ($P < 0.05$); 2) basal expression of all enzymes was significantly higher in cultured syncytiotrophoblast than in cytotrophoblasts ($P < 0.05$); 3) the presence of calcitriol in cultured trophoblast cells generally resulted in a stimulatory effect of *CYP11A1*, *CYP19A1* and *17β-HSD3* gene expression at 3 h of treatment whereas *3β-HSDI* was induced at 6 h ($P < 0.05$). However, a time-dependent variable was also observed; 4) protein expression of CYP11A1 and 3β-HSDI were not modified significantly by calcitriol, however that of CYP19A1 was regulated in similar fashion as gene expression. In conclusion, calcitriol affected in a time-dependent manner the expression of steroids metabolizing enzymes in human placental cell cultures.

1. Introduction

Calcitriol, is a secosteroid with both calcemic and non-calcemic biological activities [1]. Similar to steroids hormones, calcitriol is metabolized by human placenta, a tissue that is also considered a target for its hormonal actions. Indeed, this hormone regulates the expression or production of calbindins, cytokines, antimicrobial peptides as well as human chorionic gonadotropin (hCG), progesterone (P_4) and estradiol (E_2) in enriched trophoblastic cell preparations (ETC) [2–5]. In addition, it is well known that calcitriol differentially regulates CYP27B1 and CYP24A1, the two cytochromes involved in calcitriol synthesis and metabolism [6]. Likewise, its role in the regulation of other steroidogenic enzymes in a tissue-specific manner has been also reported [7].

Steroid hormones are synthetized from a common precursor called cholesterol and the steroidogenic enzymes involved are members of both the cytochrome (CYP) P450 superfamily and hydroxysteroid dehydrogenases (HSD) [8,9]. During pregnancy, the fluctuations of maternal steroids depend mainly of the placenta, and the major pathways involved in their synthesis have been well established [8]. In human placenta, after the conversion of

* Corresponding author at: Departamento de Biología de la Reproducción, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga No. 15, Tlalpan 14000, México, D.F, Mexico.
E-mail addresses: barrera1912@gmail.com, vidadav@hotmail.com (D. Barrera).

http://dx.doi.org/10.1016/j.jsbmb.2016.11.015
0960-0760/

cholesterol to pregnenolone by cytochrome P450 cholesterol side chain cleavage (P450scc; CYP11A1), pregnenolone is bioconverted mainly to P_4 by means of the type 1 3β-hydroxysteroid dehydrogenase (3β-HSDI). On the other hand, it well known that androgens from fetal tissues are necessary to produce the corresponding estrogens in the placenta means aromatase enzyme (CYP19A1). However, it has been showed *in vitro* the placental capability to express and synthetized androgens *de novo* by 17α-hydroxylase/17,20 lyase (CYP17A1) presence, a topic that is still discussed controversially [10,11].

Steroidogenic enzymes regulation is very complex [8,12], and the identification of factors regulating their expression has long been sought. At this respect, it has been demonstrated that some enzymes are target of the biological effects of calcitriol. In fact, it has been showed that calcitriol increased the 3β-HSDI activity in granulosa cells [13] and induces *3β-HSDI* and *CYP11A1* transcription in human glioma GI-1 cells [14]. In addition, it has been demonstrated that calcitriol up regulates *17β-HSD* type 2, type 4 and type 5 gene transcription in human prostate cancer lines cells, and both *CYP11A1* and *CYP17A1* in human adrenocortical NCI-H295R cells [15–17]. On the other hand, it has been shown that calcitriol stimulates aromatase activity in diverse cell types such as prostate, osteoblasts, human choriocarcinoma, purified immature rat Sertoli and rat granulosa cells [18–21]. Interestingly, Kinuta et al., [22] have showed that the activity and gene expression of *Cyp19A1* are decreased in the ovary, testis and epididymis of VDR null mutant mice, which indicates that calcitriol is an additional stimulator of aromatase. However, the effects of calcitriol on aromatase gene expression and enzyme activity are controversial, since is mainly inhibitory in breast cancer cells and human macrophages [7,23]. These findings indicate the differential tissue-specific effects of calcitriol.

We have previously shown that calcitriol induce P_4 and E_2 secretion in ETC [2]; however, its effects on the enzymes involved in this process have not been studied. Therefore, the aim of this study was to investigate the effects of calcitriol on expression of steroidogenic enzymes in primary cultures of human placental cells.

2. Materials and methods

2.1. Reagents

Culture media, fetal bovine serum (FBS), Trizol and all oligonucleotides for real time polymerase chain reaction (PCR) were by Invitrogen (CA, USA). Light Cycler® 480 probes master kit, TaqMan Master reaction, TaqMan probes, 96 well PCR microplates, the reverse transcription (RT) system and Proteases inhibitor cocktail were from Roche (Roche Applied Science and Roche Diagnostics, IN, USA). Calcitriol (1α,25-dihydroxycholecalciferol) was kindly donated from Hoffmann-La Roche Ltd (Basel, Switzerland). Deoxyribonuclease I and trypsin enzymes from bovine pancreas used for cell cultures were from Sigma-Aldrich (MO, USA). Percoll was from GE Healthcare (Uppsala, Sweden). CYP11A1 and CYP19A1 antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA) whereas CYP17A1 and 3β-HSDI antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and GAPDH antibody was from Millipore (Temecula, CA, USA).

2.2. Trophoblast cell culture

This protocol was approved by the Human Research Ethics Committee from the Instituto Nacional de Ciencias Médicas y Nutrición, Salvador Zubirán (No. BRE-535-12/15-1) and the Internal Review Board of the Instituto Nacional de Perinatología "Isidro Espinosa de los Reyes" in Mexico City (No. 212250-21131). Written informed consent was obtained from all participants. All pregnant women were from an urban area of Mexico City, 18–39 years old, previously normotensive, with no history of diabetes mellitus or thyroid, liver, renal disease. Term placentae (39–41 weeks of gestation) were acquired following caesarean section. All women had uncomplicated pregnancies, without evidence of active labor, cervical dilation or loss of the mucus plug. In addition, none had any clinical or microbiological signs of chorioamnionitis or lower genital tract infection; twin pregnancy was excluded from this study.

Placental cotyledons were dissected free of decidua and fetal membranes. The ETC were cultured as previously described [6,19]. Briefly: Villous tissue was enzymatically dispersed and cells were separated on density percoll gradients. Before plating, the viability of the percoll-enriched cells was estimated by dye exclusion (0.4% trypan-blue). Cells were plated at a density of 3×10^6 cells in flasks of 25 cm^2 with 4 mL each of supplemented medium [(DMEM HG) 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 mg/ml Fungizone], containing 10% heat-inactivated-FBS. Incubations were performed in humidified 5% CO_2-95% air at 37 °C. In order to remove all non-adherent cells, after 3 h to incubation, culture medium was aspirated and trophoblasts cells were washing with Hank's Balanced Salt Solution (HBSS) 1X. Treatments with calcitriol (1 or 100 nM) or its vehicle (ethanol 0.1%) were added in supplemented medium F-12 with penicillin and streptomycin. Afterward, RNA was extracted from cells for gene expression studies. Expression of human chorionic gonadotropin (hCGβ5) was used as control of the cell culture system and CYP24A1 gene as control of calcitriol function.

2.3. Calcitriol effects on CYP11A1, CYP17A1, CYP19A1, 3β-HSDI and 17β-HSD3 gene expression

Calcitriol effects upon gene expression were studied by extracting total RNA from treated cells using Trizol reagent [24]. In all cases, the amount and quality of RNA were estimated spectrophotometrically at 260/280 nm and a constant amount of RNA (2 μg) was reverse transcribed using a RT assay. Primers and probes for PCR amplifications are shown in Table 1. Identical PCR conditions were performed for all genes and in all cases normalized against glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) used as housekeeping gene.

Real time PCR amplifications were carried on a LightCycler® 480 II (Roche), as described below: The specific probe for each gene was added to a reaction mixture with 5.2 μL of PCR grade water, 0.1 μL of primer corresponding to the gene of interest [20 μM] and 3.5 μL of 2X enzyme. The mixture was homogenized and placed in plates of 96 wells to PCR. In each well 9 μL of the mixture and 1 μL cDNA are placed. The plate was then sealed and centrifuged at 1000 rpm for 30 s. Finally, the plate was placed in the thermocycler LightCycler480 II, where occurred activation of Taq DNA polymerase and DNA denaturation at 95 °C for 10 min, proceeded by 45 amplification cycles of 10 s at 95 °C, 30 s at 60 °C, and 1 s at 72 °C.

2.4. Calcitriol effects upon CYP11A1, CYP17A1, CYP19A1 and 3β-HSDI protein expression

In order to evaluate the calcitriol effects upon CYP11A1, CYP17A1, CYP19A1 *and* 3β-HSDI protein expression these were studied by Western blot. Briefly: Syncytiotrophoblast were incubated in the absence or presence of calcitriol 100 nM during 6 and 24 h. Afterwards cells were pelleted and lysed with RIPA buffer (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mMNaCl, 1% Nonidet P-40, 0.1% SDS, pH 7.4) in presence of a proteases inhibitor cocktail. Protein content

Please cite this article in press as: N. Noyola-Martínez, et al., A time-course regulatory and kinetic expression study of steroid metabolizing enzymes by calcitriol in primary cultured human placental cells, J. Steroid Biochem. Mol. Biol. (2016), http://dx.doi.org/10.1016/j.jsbmb.2016.11.015

Table 1
Oligonucleotides and probes used for real time PCR analysis.

Gen	Upper primer	Lower primer	Amplicon (nt)	[a]Probe	Accession Number
CYP11A1	aggaggggtggacacgac	ttgcgtgccatctcataca	60	59	M14565.1
CYP17A1	gcatcatagacaacctgagcaa	gggttttgttggggaaaatc	75	64	NM_000102.3
CYP19A1	gaattcatgcgagtctggatct	tcattatgtggaacatacttgagga	76	55	NM_000103.2
3β-HSDI	cggaccagaattgagagagg	gaatggctcatccagaattc	88	11	M35493.1
17β-HSD3	aacttgcaggcttagaaattgg	ggtgcgttcaggaaatgg	85	7	NM_000197.1
CYP24A1	catcatggccatcaaaacaa	gcagctcgactggagtgac	65	88	NM_000782.3
hCGβ5	gctcaccccagcatcctat	cagcagcaacagcagcag	131	70	NM_000737.2
GAPDH	agccacatcgctcagacac	gcccaatacgaccaaatcc	66	60	NM_002046.3

[a] From the universal probe library (Roche).

was determined in the cell lysates and equal amounts (30 μg protein) were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes and blocked overnight in TBST (10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) containing 5% skim milk. Then membranes were washed and incubated overnight at room temperature in the presence of anti-CYP11A1 (50 kDa) or anti-CYP19A1 (51 kDa) diluted 1:1000, or anti-CYP17A1 (55 kDa) or anti-3β-HSD (42 kDa) diluted 1:100 and 1:200 respectively, in TBST. After washing membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:8000), followed by chemiluminescent detection with Clarity Western ECL substrate (Bio-Rad laboratories, USA) according to the manufacturer's instructions. Visualization was achieved by Molecular Imager ChemiDoc XRS System and the densitometry was performed by Imagen Lab Software (Bio-Rad, Hercules, California, USA). The membranes was washed and incubated with Restore™ Western Blot Stripping Buffer (Thermo Fisher Scientific Inc. Rockford, IL, USA) to follow use with further antibody. For normalization, blots were incubated with anti-GAPDH (32 kDa, 1:8000) and processed as described above.

2.5. Statistical analysis

The results were described as the mean ± standard deviation (S. D.). Statistical differences for dose-response assays were determined by one-way ANOVA followed by appropriate post hoc tests (Holm-Sidak method for pair-wise comparisons), using a specialized software package (SigmaPlot 11.0, Jandel Scientific). For each experiment the final result was calculated considering the mean of triplicate from at least three separated cell cultures. Differences were considered statistically significant at $P < 0.05$.

3. Results

Trophoblast cells were seeded (3×10^6 cells per flask) and during three days of culture a progressive increase of *hCGβ5* gene expression was observed and used as a biological marker of placental differentiation from cytotrophoblasts to syncytiotrophoblast. Likewise, gene expression level of *CYP24A1* in ETC was used as a control of calcitriol bioactivity, as previously described [25].

3.1. Comparison of basal gene expression profiles of CYP11A1, CYP17A1, CYP19A1, 3β-HSDI and 17β-HSD3 enzymes in ETC

Fig. 1, shows the basal gene expression of each one of the steroidogenic enzymes studied. As depicted,the *3β-HSDI* transcript was the one with the highest ($P < 0.05$) expression level when compared with the other transcripts. In general, the expression profile level of these genes was as follows: *3β-HSDI* > *CYP19A1* ≥ *CYP11A1* > *17β-HSD3* ≥ *CYP17A1*.

3.2. Basal gene expression profiles during cell differentiation

Gene expression of steroidogenic enzymes was also studied during the differentiation process of trophoblast cells in culture. As shown in Fig. 2, 3 of the 5 enzymes showed a significantly higher expression on days 2 and 3 of cultures ($P < 0.05$ *vs* day 1). Gene expression of *CYP11A1* and *CYP17A1* reached a maximum and significant higher value at 72 h of plating ($P < 0.05$; Fig. 2).

3.3. Calcitriol effects upon gene expression of steroidogenic enzymes during 3, 6, 12 and 24 h of treatment in syncytiotrophoblast

Considering previous results from our laboratory, we decided to study the effects of calcitriol on gene expression of all enzymes at two different concentrations (1 and 100 nM) and for various times of incubation (3, 6, 12 and 24 h) as shown in Fig. 3. As depicted, after 3 h of treatment, 100 nM of calcitriol upregulated *CYP11A1* and *CYP19A1* gene expression whereas *CYP17A1* was down regulated after 6 h (Fig. 3A–C). Meanwhile,the relative gene expression of *3β-HSDI* was significantly increased around 6 h and *17β-HSD3* mRNA shown a biphasic effect between 3 and 6 h. On contrary, between 12 and 24 h of calcitriol treatment, *CYP11A1* and *CYP19A1* transcripts were decreased and *CYP17A1* gene transcription was increased whereas that the expression both *3β-* and *17β-HSDs* was stabilized since no further effects of calcitriol were observed after 12 h (Fig. 3D, E).

3.4. Calcitriol effects upon CYP11A1, CYP17A1, CYP19A1 and 3β-HSDI protein expression after 6 and 24 h of treatment in syncytiotrophoblasts

Calcitriol effects upon protein abundance of CYP11A1, CYP19A1 *and* 3β-HSDI are shown in Fig. 4. Protein expression of CYP11A1 and 3β-HSDI was not affected by treatment at 6 and 24 h (Fig. 4A

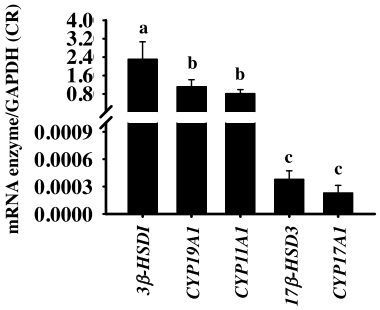

Fig. 1. Placental basal gene expression of steroidogenic enzymes. The data are expressed as concentration ratio (CR). Bars represent the data of at least 4 separated cell cultures. Different letters indicate significant differences at the $P < 0.05$.

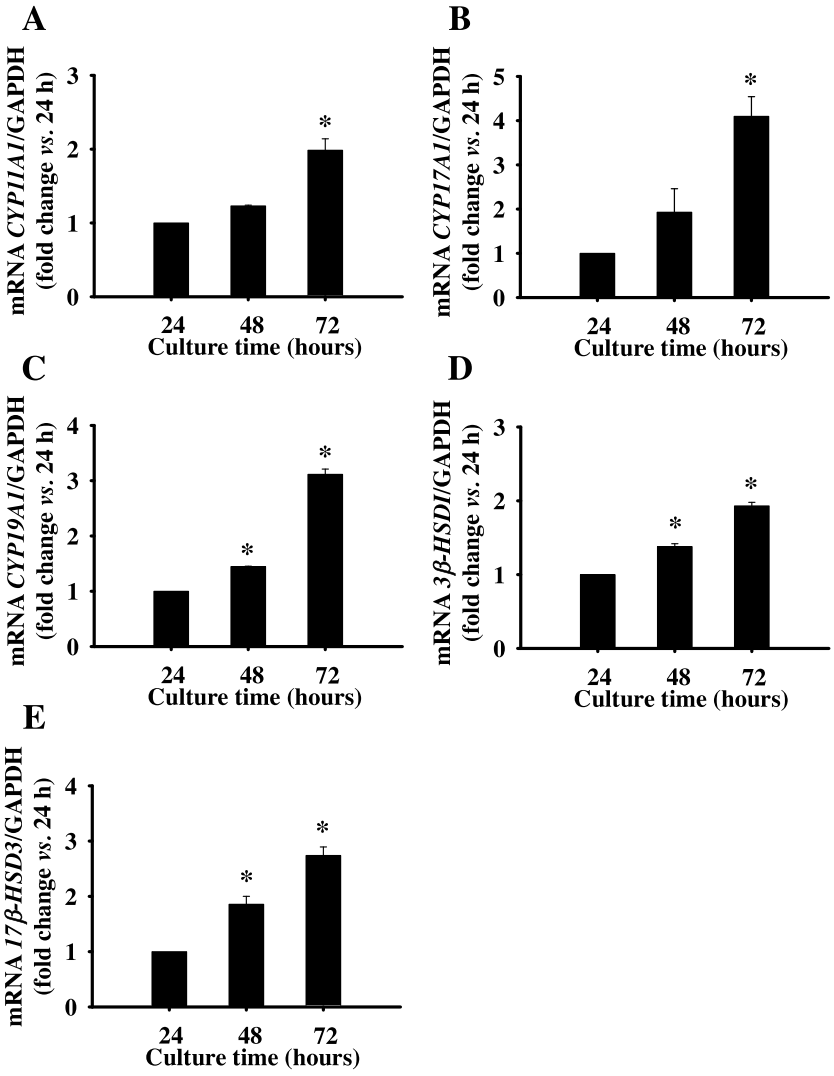

Fig. 2. Placental gene expression profiles of the indicated steroidogenic enzymes during differentiation process of trophoblast cell cultures. The data are expressed as fold changes as compared to 24 h of culture which value was arbitrarily set to 1. Bars represent the data of at least 3 separated cell cultures. *$P < 0.05$.

and C). However, the abundance of CYP19A1 was increased at 6 h and decreased at 24 h of incubation which was in accordance with gene expression of this enzyme at the same times of treatment (Fig. 4B). Regarding CYP17A1, its protein expression was not detected using the methodology used in this study (data no shown).

3.5. Calcitriol effect upon gene expression of enzymes during each day of differentiation

Fig. 5 shows the role of the cell differentiation process on the calcitriol effects on gene expression of the enzymes studied. As depicted, calcitriol at two doses (1 nM and 100 nM) was added at 3, 24 and 48 h of culture times that corresponded to cytotrophoblasts transition into syncytiotrophoblast. At each time, calcitriol treatment was incubated for 24 h and gene expression of each enzyme was analyzed. As shown in Fig. 5, calcitriol at 1 nM and 100 nM concentrations decreased *CYP11A1* in each stage of cell differentiation. Similarly, *CYP19A1* was inhibited but only in the syncytiotrophoblast while *CYP17A1* transcription was upregulated also in this last phenotype cell. Regarding the dehydrogenases, their expression was not significantly affected at any dose of calcitriol and the day of cell culture differentiation studied.

4. Discussion

Human pregnancy is a physiological condition associated with several changes in the concentrations of maternal and fetal-placental hormones, cytokines and peptides, among others, that ensure and guarantee the maintenance of gestation. Specifically, steroidogenic enzymes in placental tissue plays a pivotal role to produce and increase in steroid hormones levels, which assure uterine quiescence, immunological tolerance and adaptation during this period [26–29]. Therefore, the regulation of these factors, particularly in placenta deserves to be further studied.

It has been shown that syncytiotrophoblast cells are the main steroidogenic cell type in placenta [30–32]. This is in line with our results, where this structure had significantly higher enzymatic expression than that observed in cytotrophoblasts. In this study, the basal level of gene expression of the cytochromes being investigated was as follows: *CYP19A1* ≥ *CYP11A1* > *CYP17A1*, these results are in accordance with those reported by Pezzi et al. [11]. Regarding the dehydrogenases, the expression of *3β-HSDI* was significantly higher than that of *17β-HSD3* but in contrast to Pezzi et al.*3β-HSDI* was also more expressed than the three other cytochromes evaluated. The discrepancy may be due to differences in gestational age and preparation of the biological samples.

Please cite this article in press as: N. Noyola-Martínez, et al., A time-course regulatory and kinetic expression study of steroid metabolizing enzymes by calcitriol in primary cultured human placental cells, J. Steroid Biochem. Mol. Biol. (2016), http://dx.doi.org/10.1016/j.jsbmb.2016.11.015

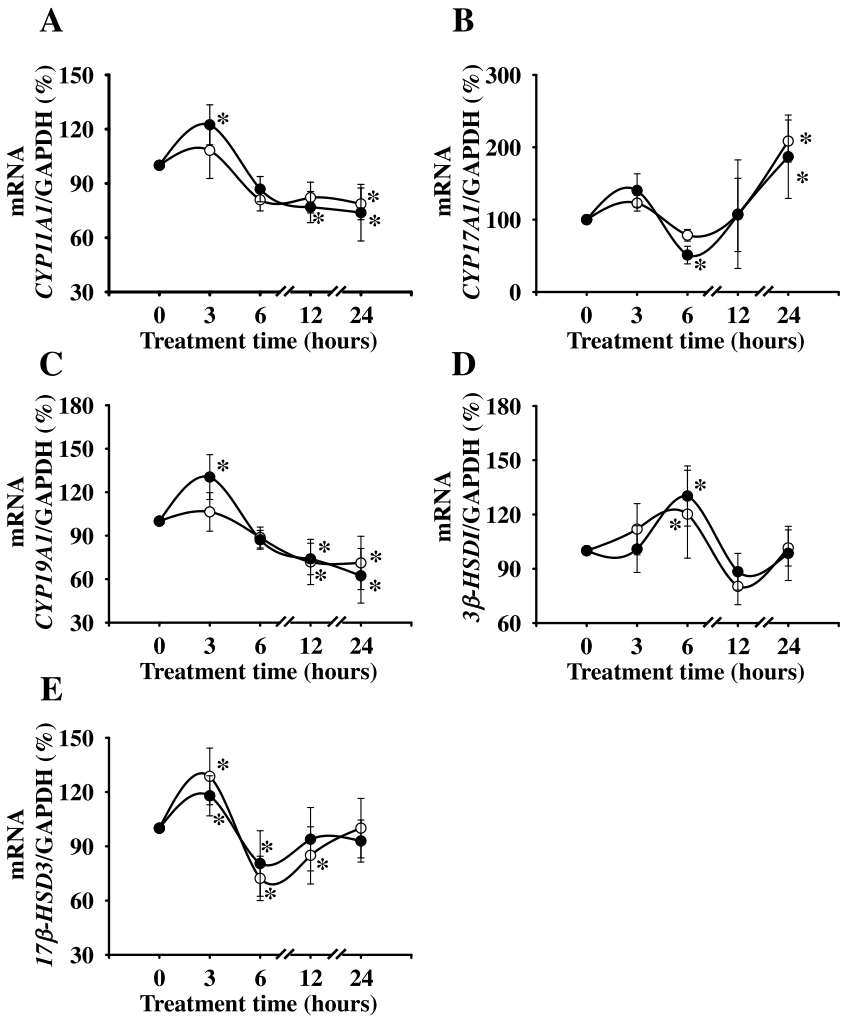

Fig. 3. Time-dependent effect of calcitriol treatment upon gene expression of the indicated steroidogenic enzymes in syncytiotrophoblast cells. Calcitriol 1 nM (open circles) and 100 nM (closed circles). The data are expressed as percent changes as compared to 0 h of treatment which value was arbitrarily set to 100. Bars represent the data of at least 3 separated cell cultures. *$P < 0.05$.

Indeed, we used cultured ETC obtained from term placentas while Pezzi et al. performed their study in placental homogenates obtained at 15–19 weeks of gestation after abortion induction. On the other hand, it has been demonstrated that placenta expresses several *17β-HSD* isozymes [32–34], but not the *17β-HSD3*. Interestingly, in our ETC culture model, we demonstrated that *17β-HSD3* is not only expressed but its expression increased along with the differentiation process. Overall, the results in the present study demonstrated that *CYP11A1*, *CYP19A1* and *3β-HSDI* are highly expressed in human placenta to provide steroids hormones such as P_4 and E_2 which are essential for the maintenance and adequate outcome of pregnancy [27,28].

These results demonstrated the hormonal phenotype of our culture and validated its use for the purposes of this study. The aim of this study was to investigate the role of calcitriol upon gene expression regulation of key steroidogenic enzymes involved in the synthesis of some steroids produced during normal pregnancy. It has previously shown the role of calcitriol in steroidogenesis; therefore, we thought of importance to evaluate if this hormone might be also involved in gene expression regulation of steroidogenic enzymes throughout different stages of placenta cell differentiation in culture and times of incubation.

The results in this study demonstrated that calcitriol upregulates *CYP11A1*, *CYP19A1* and *3β-HSDI* gene expression. In this regard, these results agreed with those previously reported by our laboratory showing the ability of calcitriol to stimulate in a dose-dependent manner both P_4 and E_2 secretion in ETC [2]. However, this effect was time-dependent according to the enzyme been studied. Similar results have been observed in other cell types from different sources, including established cell lines from brain and adrenocortical cells [14,17]. Interestingly enough was the observation of the down regulatory effect of calcitriol on *CYP11A1* and *CYP19A1* gene expression at 24 h of treatment. This inhibitory effect of calcitriol upon *CYP11A1* expression has been previously observed in mice IL-4-activated $CD8^+$ T cells by Schedel et al. [35]. These authors reported seven potential VDR-binding sites with repressor transcriptional VDR-dependent activity. Whether a similar promoter behavior is responsible of the inhibitory effects of calcitriol on *CYP11A1* and *CYP19A1* in this study deserves to be further investigated. Although the *CYP11A1* gene expression was altered, the protein abundance of this enzyme was not modified. This result may indicate that some translational mechanisms could be differentially regulated. Similarly, it has been reported by Beaudoin et al. [31] that P_4 and E_2 induce both *3β-HSDI* and

Please cite this article in press as: N. Noyola-Martínez, et al., A time-course regulatory and kinetic expression study of steroid metabolizing enzymes by calcitriol in primary cultured human placental cells, J. Steroid Biochem. Mol. Biol. (2016), http://dx.doi.org/10.1016/j.jsbmb.2016.11.015

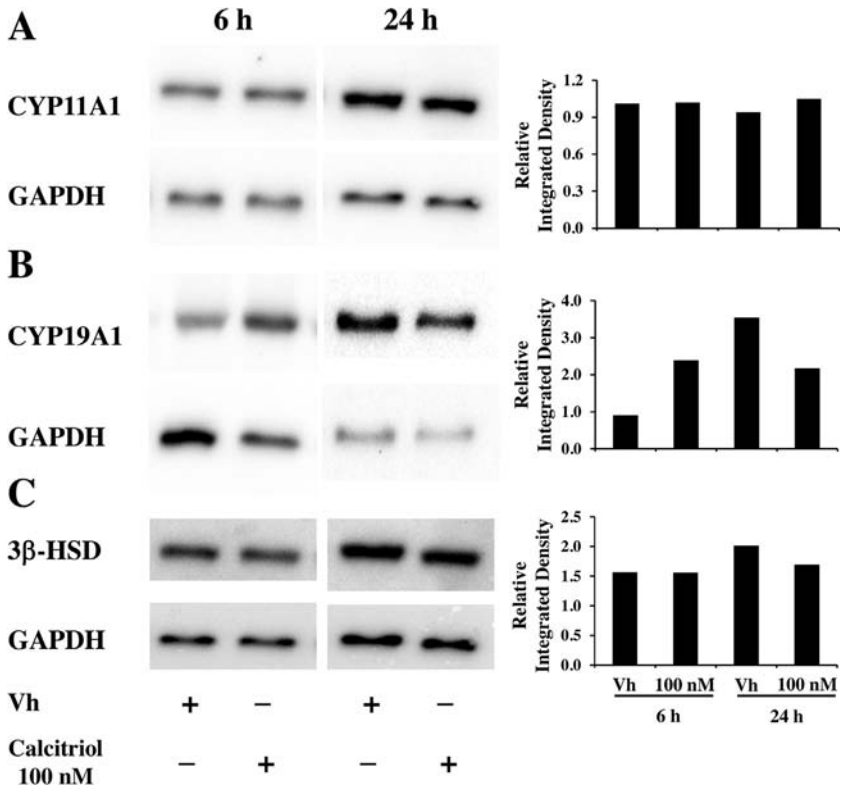

Fig. 4. Calcitriol effects upon syncytiotrophoblasts protein expression of the indicated steroidogenic enzymes at 6 and 24 h of treatment. Figures are representative of three independent experiments. Figures showed the abundance of proteins (left panel) and their respective relative integrated density (right panel).

CYP11A1 gene transcription but not the protein abundance of 3β-HSDI. Therefore, we suggest that after the induction of these steroid hormones by calcitriol [2], protein expression of these key enzymes could be preserved and the enzymatic activity affected positively.

In addition to its importance in steroid hormones biosynthesis, Slominski et al. [36] have demonstrated the participation of CYP11A1 in novels activities since this enzyme also metabolizes 7-dehydrocholesterol (7DHC), ergosterol, lumisterol 3 and hydroxylates the side chain of vitamin D_3 and D_2 [37–40]. In fact, it has been shown that this enzyme is able to convert vitamin D_3 to 20-hydroxyvitamin D_3 and 20,22-dihydroxyvitamin D_3 in female rat adrenal glands and human term placenta as well as vitamin D_2 form into 20-hydroxyvitamin D_2, 20,22-dihydroxyvitamin D_2 and 1,20-dihydroxyvitamin D_2 in several cells types such as epidermal keratinocytes, Caco-2 colon cells, adrenal glands and also in human placenta [36,37,41,42].

Regarding CYP19A1, its gene expression was inhibited after 24 h of treatment with calcitriol which is in accordance with others studies [7,18,23,43]. Interestingly, in the present study protein abundance of this enzyme was modified by calcitriol in similar fashion as its gene expression. In *in vivo* conditions, we cannot discard a modification of this effect of calcitriol upon this enzyme by the presence of others hormones, local environment factors, as well as others cells types found in human placenta. This possibility is not unlikely since both calcitriol and estrogen circulating levels increase during healthy pregnancy [44,45].

Additionally, we identified *CYP17A1* gene transcripts in both cyto- and syncytio-trophoblasts as previously reported [10,46,47]. However, the placental *CYP17A1* and *17β-HSD3* gene expression are very low in comparison with the others enzymes, which suggest minor production of androgens that could be biotransformed immediately to estrogens by CYP19A1 [10]. Indeed, calcitriol did induce *CYP17A1* mRNA after 24 h of treatment. This result is similar to the stimulatory effect previously described of calcitriol upon *CYP17A1* expression in adrenal cells; however those authors found that androgen production was decreased [17]. It is well known that *CYP17A1* mediates two different activities, 17α-hydroxylase activity that is regulated by *CYP17A1* gene expression and the 17,20-lyase activity that is regulated by post-transcriptional mechanisms [48]. Therefore, increased gene expression of *CYP17A1* observed in the present study by calcitriol could not result in more androgens synthesis but to be related also to production of 17α-hydroxypregnenolone and/or 17α-hydroxyprogesterone that are formed as intermediates to glucocorticoids [8,49–52], which merits further investigation.

Identifying the factors and molecules that regulate the steroideogenesis in the placental tissue is of importance, since pregnancy is dependent of steroids produced by this organ. In fact, several adverse pregnancy outcomes are associated with hormonal unbalance such as miscarried, preeclampsia and preterm delivery. In particular, it has been demonstrated in preeclamptic women that the androgens and estrogens production is abnormal as compared with normotensive pregnancy [53,54]. In addition, in this pathology the vitamin D status has been found deficient [55] and it has been demonstrated that the supplementation with vitamin D diminishes the risk of preeclampsia development.

In summary, we demonstrated that calcitriol is a placental regulator of expression of the enzymes involved in the sex steroids production in ETC and we suggest that continuous and increased production of calcitriol during pregnancy could preserve the initial regulation of the expression of these enzymes and that vitamin D supplementation could offer an alternative therapeutic action for patients with hormonal imbalance.

Please cite this article in press as: N. Noyola-Martínez, et al., A time-course regulatory and kinetic expression study of steroid metabolizing enzymes by calcitriol in primary cultured human placental cells, J. Steroid Biochem. Mol. Biol. (2016), http://dx.doi.org/10.1016/j.jsbmb.2016.11.015

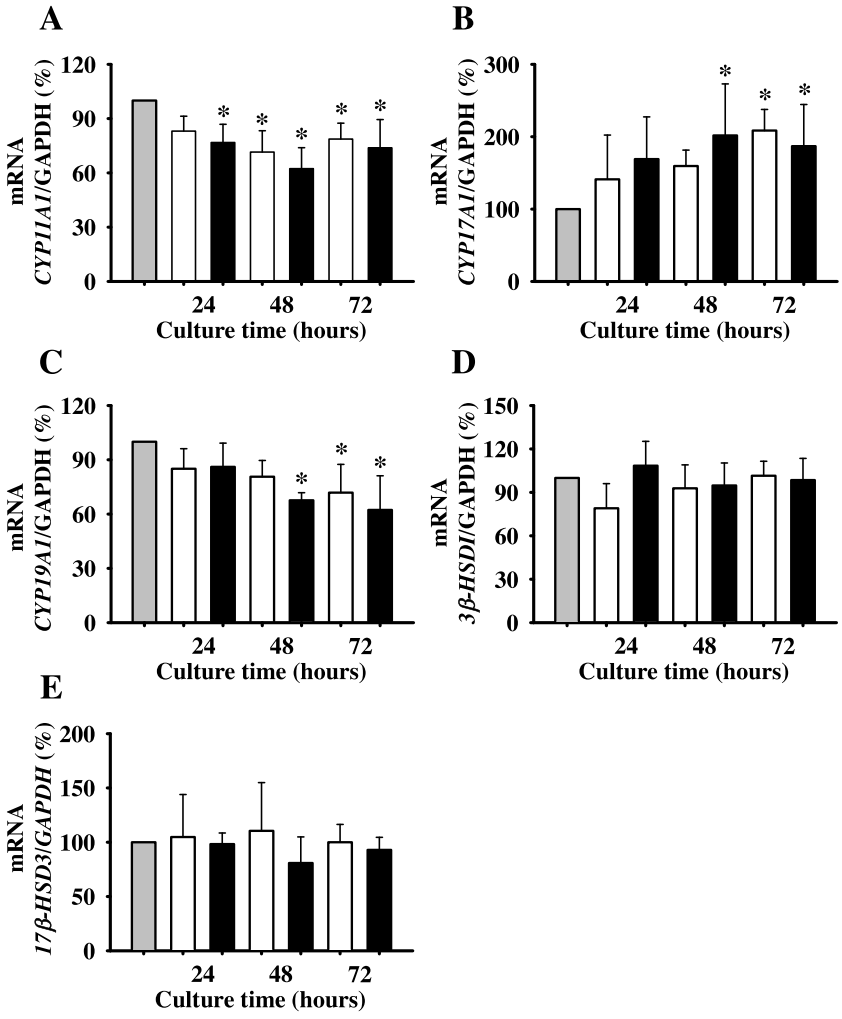

Fig. 5. Time-dependent effect of calcitriol treatment upon gene expression of the indicated steroidogenic enzymes during trophoblast cells differentiation. Calcitriol 1 nM (white bars) and 100 nM (black bars). Results are expressed as percent change of cells treated *vs* Vh (grey bars), which was assigned a value of 100%. $^{*}P < 0.05$ *vs* Vh. Bars represent the data of at least 3 separated cell cultures.

Acknowledgments

This article is part of the doctoral thesis of Nancy Noyola Martínez who is a Ph.D. student from Programa de Doctorado en Ciencias Biomédicas, Facultad de Medicina, Universidad Nacional Autónoma de México (UNAM, México), under a fellowship number 241393 from Consejo Nacional de Ciencia y Tecnología (CONACyT, México). We very much appreciate Isela Martínez-Reza, Mitzi García-Olivares and Mariana Segovía-Mendoza for their help with the collection of placental samples and technical assistance. This work was supported by a grant (154215) from CONACyT, México.

References

[1] A.S. Dusso, A.J. Brown, E. Slatopolsky, Vitamin D, Am. J. Physiol. Renal Physiol. 289 (1) (2005) F8–28.
[2] D. Barrera, E. Avila, G. Hernandez, A. Halhali, B. Biruete, F. Larrea, L. Diaz, Estradiol and progesterone synthesis in human placenta is stimulated by calcitriol, J. Steroid Biochem. Mol. Biol. 103 (3–5) (2007) 529–532.
[3] D. Barrera, E. Avila, G. Hernandez, I. Mendez, L. Gonzalez, A. Halhali, F. Larrea, A. Morales, L. Diaz, Calcitriol affects hCG gene transcription in cultured human syncytiotrophoblasts, Reprod. Biol. Endocrinol. 6 (2008) 3.
[4] D. Barrera, N. Noyola-Martinez, E. Avila, A. Halhali, F. Larrea, L. Diaz, Calcitriol inhibits interleukin-10 expression in cultured human trophoblasts under normal and inflammatory conditions, Cytokine 57 (3) (2012) 316–321.
[5] L. Diaz, N. Noyola-Martinez, D. Barrera, G. Hernandez, E. Avila, A. Halhali, F. Larrea, Calcitriol inhibits TNF-alpha-induced inflammatory cytokines in human trophoblasts, J. Reprod. Immunol. 81 (1) (2009) 17–24.
[6] E. Avila, L. Diaz, D. Barrera, A. Halhali, I. Mendez, L. Gonzalez, U. Zuegel, A. Steinmeyer, F. Larrea, Regulation of Vitamin D hydroxylases gene expression by 1,25-dihydroxyvitamin D3 and cyclic AMP in cultured human syncytiotrophoblasts, J. Steroid Biochem. Mol. Biol. 103 (1) (2007) 90–96.
[7] A.V. Krishnan, S. Swami, L. Peng, J. Wang, J. Moreno, D. Feldman, Tissue-selective regulation of aromatase expression by calcitriol: implications for breast cancer therapy, Endocrinology 151 (1) (2010) 32–42.
[8] A.H. Payne, D.B. Hales, Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones, Endocr. Rev. 25 (6) (2004) 947–970.
[9] W.L. Miller, R.J. Auchus, The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders, Endocr. Rev. 32 (1) (2011) 81–151.
[10] J.C. Escobar, S.S. Patel, V.E. Beshay, T. Suzuki, B.R. Carr, The human placenta expresses CYP17 and generates androgens de novo, J. Clin. Endocrinol. Metab. 96 (5) (2011) 1385–1392.
[11] V. Pezzi, J.M. Mathis, W.E. Rainey, B.R. Carr, Profiling transcript levels for steroidogenic enzymes in fetal tissues, J. Steroid Biochem. Mol. Biol. 87 (2–3) (2003) 181–189.
[12] J. Simard, M.L. Ricketts, S. Gingras, P. Soucy, F.A. Feltus, M.H. Melner, Molecular biology of the 3beta-hydroxysteroid dehydrogenase/delta5-delta4 isomerase gene family, Endocr. Rev. 26 (4) (2005) 525–582.
[13] Z. Merhi, A. Doswell, K. Krebs, M. Cipolla, Vitamin D alters genes involved in follicular development and steroidogenesis in human cumulus granulosa cells, J. Clin. Endocrinol. Metab. 99 (6) (2014) E1137–1145.
[14] T. Yagishita, A. Kushida, H. Tamura, Vitamin D(3) enhances ATRA-mediated neurosteroid biosynthesis in human glioma GI-1 cells, J. Biochem. 152 (3) (2012) 285–292.
[15] J.H. Wang, P. Tuohimaa, Regulation of cholesterol 25-hydroxylase expression by vitamin D3 metabolites in human prostate stromal cells, Biochem. Biophys. Res. Commun. 345 (2) (2006) 720–725.
[16] J.H. Wang, P. Tuohimaa, Regulation of 17beta-hydroxysteroid dehydrogenase type 2, type 4 and type 5 by calcitriol, LXR agonist and 5alpha-

dihydrotestosterone in human prostate cancer cells, J. Steroid Biochem. Mol. Biol. 107 (1–2) (2007) 100–105.
[17] J. Lundqvist, M. Norlin, K. Wikvall, 1alpha, 25-Dihydroxyvitamin D3 affects hormone production and expression of steroidogenic enzymes in human adrenocortical NCI-H295R cells, Biochim. Biophys. Acta 1801 (9) (2010) 1056–1062.
[18] C.T. Lee, J.Y. Wang, K.Y. Chou, M.I. Hsu, 1,25-Dihydroxyvitamin D3 increases testosterone-induced 17beta-estradiol secretion and reverses testosterone-reduced connexin 43 in rat granulosa cells, Reprod. Biol. Endocrinol. 12 (2014) 90.
[19] L. Zanatta, H. Bouraima-Lelong, C. Delalande, F.R. Silva, S. Carreau, Regulation of aromatase expression by 1alpha, 25(OH)2 vitamin D3 in rat testicular cells, Reprod, Fertil. Dev. 23 (5) (2011) 725–735.
[20] T. Sun, Y. Zhao, D.J. Mangelsdorf, E.R. Simpson, Characterization of a region upstream of exon I.1 of the human CYP19 (aromatase) gene that mediates regulation by retinoids in human choriocarcinoma cells, Endocrinology 139 (4) (1998) 1684–1691.
[21] Y.R. Lou, T. Murtola, P. Tuohimaa, Regulation of aromatase and 5alpha-reductase by 25-hydroxyvitamin D(3), 1alpha, 25-dihydroxyvitamin D(3), dexamethasone and progesterone in prostate cancer cells, J. Steroid Biochem. Mol. Biol. 94 (1–3) (2005) 151–157.
[22] K. Kinuta, H. Tanaka, T. Moriwake, K. Aya, S. Kato, Y. Seino, Vitamin D is an important factor in estrogen biosynthesis of both female and male gonads, Endocrinology 141 (4) (2000) 1317–1324.
[23] B. Villaggio, S. Soldano, M. Cutolo, 1,25-dihydroxyvitamin D3 downregulates aromatase expression and inflammatory cytokines in human macrophages, Clin. Exp. Rheumatol. 30 (6) (2012) 934–938.
[24] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem. 162 (1) (1987) 156–159.
[25] N. Noyola-Martinez, L. Diaz, E. Avila, A. Halhali, F. Larrea, D. Barrera, Calcitriol downregulates TNF-alpha and IL-6 expression in cultured placental cells from preeclamptic women, Cytokine 61 (1) (2013) 245–250.
[26] E.D. Albrecht, G.J. Pepe, Placental steroid hormone biosynthesis in primate pregnancy, Endocr. Rev. 11 (1) (1990) 124–150.
[27] J.S. Babischkin, R.W. Grimes, G.J. Pepe, E.D. Albrecht, Estrogen stimulation of P450 cholesterol side-chain cleavage activity in cultures of human placental syncytiotrophoblasts, Biol. Reprod. 56 (1) (1997) 272–278.
[28] G.J. Pepe, E.D. Albrecht, Actions of placental and fetal adrenal steroid hormones in primate pregnancy, Endocr. Rev. 16 (5) (1995) 608–648.
[29] M. Perusquia, J. Jasso-Kamel, Influence of 5alpha- and 5beta-reduced progestins on the contractility of isolated human myometrium at term, Life Sci. 68 (26) (2001) 2933–2944.
[30] F. Martinez, S. Olvera-Sanchez, M. Esparza-Perusquia, E. Gomez-Chang, O. Flores-Herrera, Multiple functions of syncytiotrophoblast mitochondria, Steroids 103 (2015) 11–22.
[31] C. Beaudoin, C.H. Blomquist, M. Bonenfant, Y. Tremblay, Expression of the genes for 3 beta-hydroxysteroid dehydrogenase type 1 and cytochrome P450scc during syncytium formation by human placental cytotrophoblast cells in culture and the regulation by progesterone and estradiol, J. Endocrinol. 154 (3) (1997) 379–387.
[32] C. Beaudoin, C.H. Blomquist, Y. Tremblay, Gene expression of 17 beta-hydroxysteroid dehydrogenase type 2 isozyme in primary cultures of human trophoblasts predicts different mechanisms regulating type 1 and type 2 enzymes, Endocrinology 136 (9) (1995) 3807–3814.
[33] N. Sakurai, Y. Miki, T. Suzuki, K. Watanabe, T. Narita, K. Ando, T.M. Yung, D. Aoki, H. Sasano, H. Handa, Systemic distribution and tissue localizations of human 17beta-hydroxysteroid dehydrogenase type 12, J. Steroid Biochem. Mol. Biol. 99 (4-5) (2006) 174–181.
[34] Y. Li, V. Isomaa, A. Pulkka, R. Herva, H. Peltoketo, P. Vihko, Expression of 3beta-hydroxysteroid dehydrogenase type 1, P450 aromatase, and 17beta-hydroxysteroid dehydrogenase types 1, 2, 5 and 7 mRNAs in human early and mid-gestation placentas, Placenta 26 (5) (2005) 387–392.
[35] M. Schedel, Y. Jia, S. Michel, K. Takeda, J. Domenico, A. Joetham, F. Ning, M. Strand, J. Han, M. Wang, J.J. Lucas, C. Vogelberg, M. Kabesch, B.P. O'Connor, E.W. Gelfand, 25D3 prevents CD8(+)Tc2 skewing and asthma development through VDR binding changes to the Cyp11a1 promoter, Nat. Commun. 7 (10213) (2016) 1–11.
[36] A.T. Slominski, W. Li, T.K. Kim, I. Semak, J. Wang, J.K. Zjawiony, R.C. Tuckey, Novel activities of CYP11A1 and their potential physiological significance, J. Steroid Biochem. Mol. Biol. 151 (2014) 25–37.
[37] O. Guryev, R.A. Carvalho, S. Usanov, A. Gilep, R.W. Estabrook, A pathway for the metabolism of vitamin D3: unique hydroxylated metabolites formed during catalysis with cytochrome P450scc (CYP11A1), Proc. Natl. Acad. Sci. U. S. A. 100 (25) (2003) 14754–14759.
[38] A. Slominski, I. Semak, J. Zjawiony, J. Wortsman, W. Li, A. Szczesniewski, R.C. Tuckey, The cytochrome P450scc system opens an alternate pathway of vitamin D3 metabolism, FEBS J. 272 (16) (2005) 4080–4090.
[39] M.N. Nguyen, A. Slominski, W. Li, Y.R. Ng, R.C. Tuckey, Metabolism of vitamin d2 to 17,20,24-trihydroxyvitamin d2 by cytochrome p450scc (CYP11A1), Drug Metab. Dispos. 37 (4) (2009) 761–767.
[40] A.T. Slominski, T.K. Kim, H.Z. Shehabi, I. Semak, E.K. Tang, M.N. Nguyen, H.A. Benson, E. Korik, Z. Janjetovic, J. Chen, C.R. Yates, A. Postlethwaite, W. Li, R.C. Tuckey, In vivo evidence for a novel pathway of vitamin D(3) metabolism initiated by P450scc and modified by CYP27B1, FASEB J. 26 (9) (2012) 3901–3915.
[41] A.T. Slominski, T.K. Kim, J. Chen, M.N. Nguyen, W. Li, C.R. Yates, T. Sweatman, Z. Janjetovic, R.C. Tuckey, Cytochrome P450scc-dependent metabolism of 7-dehydrocholesterol in placenta and epidermal keratinocytes, Int. J. Biochem. Cell Biol. 44 (11) (2012) 2003–2018.
[42] A.T. Slominski, T.K. Kim, H.Z. Shehabi, E.K. Tang, H.A. Benson, I. Semak, Z. Lin, C. R. Yates, J. Wang, W. Li, R.C. Tuckey, In vivo production of novel vitamin D2 hydroxy-derivatives by human placentas, epidermal keratinocytes, Caco-2 colon cells and the adrenal gland, Mol. Cell. Endocrinol. 383 (1–2) (2014) 181–192.
[43] T. Hayashi, N. Harada, Post-translational dual regulation of cytochrome P450 aromatase at the catalytic and protein levels by phosphorylation/dephosphorylation, FEBS J. 281 (21) (2014) 4830–4840.
[44] C.S. Kovacs, The role of vitamin D in pregnancy and lactation: insights from animal models and clinical studies, Annu. Rev. Nutr. 32 (2012) 97–123.
[45] E.A. Kuijper, J.C. Ket, M.R. Caanen, C.B. Lambalk, Reproductive hormone concentrations in pregnancy and neonates: a systematic review, Reprod. Biomed. Online. 27 (1) (2013) 33–63.
[46] J.C. Escobar, B.R. Carr, The protein kinase a pathway regulates CYP17 expression and androgen production in the human placenta, J. Clin. Endocrinol. Metab. 96 (9) (2011) 2869–2873.
[47] A. Loganath, K.L. Peh, P.C. Wong, Evidence for the biosynthesis of DHEA from cholesterol by first-trimester human placental tissue: source of androgens, Horm. Metab. Res. 34 (3) (2002) 116–120.
[48] P.J. Meis, 17 hydroxyprogesterone for the prevention of preterm delivery, Obstet. Gynecol. 105 (5 Pt 1) (2005) 1128–1135.
[49] W.L. Miller, R.J. Auchus, D.H. Geller, The regulation of 17,20 lyase activity, Steroids 62 (1) (1997) 133–142.
[50] C.E. Fluck, W.L. Miller, R.J. Auchus, The 17, 20-lyase activity of cytochrome p450c17 from human fetal testis favors the delta5 steroidogenic pathway, J. Clin. Endocrinol. Metab. 88 (8) (2003) 3762–3766.
[51] T.Y. Hsu, K.C. Lan, C.C. Tsai, C.Y. Ou, B.H. Cheng, M.Y. Tsai, H.Y. Kang, Y.H. Tung, Y. H. Wong, K.E. Huang, Expression of androgen receptor in human placentas from normal and preeclamptic pregnancies, Taiwan J, Obstet. Gynecol. 48 (3) (2009) 262–267.
[52] K. Sathishkumar, M. Balakrishnan, V. Chinnathambi, M. Chauhan, G.D. Hankins, C. Yallampalli, Fetal sex-related dysregulation in testosterone production and their receptor expression in the human placenta with preeclampsia, J. Perinatol. 32 (5) (2012) 328–335.
[53] M.T. Acromite, C.S. Mantzoros, R.E. Leach, J. Hurwitz, L.G. Dorey, Androgens in preeclampsia, Am. J. Obstet. Gynecol. 180 (1 Pt 1) (1999) 60–63.
[54] A. Hertig, P. Liere, N. Chabbert-Buffet, J. Fort, A. Pianos, B. Eychenne, A. Cambourg, M. Schumacher, N. Berkane, G. Lefevre, S. Uzan, E. Rondeau, P. Rozenberg, M.E. Rafestin-Oblin, Steroid profiling in preeclamptic women: evidence for aromatase deficiency, Am. J. Obstet. Gynecol. 203 (5) (2010) e471–479 (477).
[55] A. Olmos-Ortiz, E. Avila, M. Durand-Carbajal, L. Diaz, Regulation of calcitriol biosynthesis and activity: focus on gestational vitamin D deficiency and adverse pregnancy outcomes, Nutrients 7 (1) (2015) 443–480.

Please cite this article in press as: N. Noyola-Martínez, et al., A time-course regulatory and kinetic expression study of steroid metabolizing enzymes by calcitriol in primary cultured human placental cells, J. Steroid Biochem. Mol. Biol. (2016), http://dx.doi.org/10.1016/j.jsbmb.2016.11.015