YASSMIN MEDINA LAVER

Characterization and function of progesterone receptor membrane component 2 (PGRMC2) in the human endometrium

Thesis
2025

PhD program in Biomedicine and Biotechnology
International PhD thesis

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VNIVERSITAT DE VALÈNCIA

Valencia, 2025

Portada, contraportada y todos los detalles internos diseñados por mi pesadilla favorita:
mi hermano, Yoshua Medina.

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Characterization and function of progesterone receptor membrane component 2 (PGRMC2) in the human endometrium

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Bachelor's degree in Biology

Master's degree in Biotechnology of Assisted Human Reproduction

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Valencia, January 2025

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CERTIFICO QUE:

El trabajo de investigación titulado: "**Characterization and function of progesterone receptor membrane component 2 (PGRMC2) in the human endometrium**" ha sido realizado íntegramente por Yassmin Medina Laver bajo mi dirección. Dicha memoria está concluida y reúne todos los requisitos para su presentación y defensa como TESIS DOCTORAL ante un tribunal.

Y para que así conste a los efectos oportunos, firmo la presente certificación en Valencia a 05 de enero de 2025.

Fdo. Dr. Francisco Domínguez Hernández

VNIVERSITAT DE VALÈNCIA

Dr. José Bellver Pradas, Ginecólogo especialista con Doctorado en Reproducción Humana en el Instituto Valenciano de Infertilidad (IVIRMA Valencia), Catedrático en el Departamento de Pediatría, Obstetricia y Ginecología de la Facultad de Medicina de la Universidad de Valencia e Investigador en Fundación IVI.

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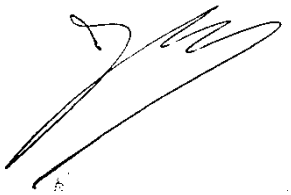


Fdo. Dr. José Bellver Pradas

A esos dos ojitos brillantes que siempre fueron calma y ahora viven en mi alma:

Coco.

ACKNOWLEDGMENTS

Desde antes de aprender a leer, algo en mi interior ya estaba conectado con este mundo que ahora me apasiona. Tenía alrededor de cuatro años cuando descubrí, en la biblioteca de mi barrio, en Adeje, un libro que ilustraba el desarrollo de un bebé desde sus primeras etapas en el útero hasta el nacimiento. No sé cuántas veces lo alquilé, pero sé que siempre era el mismo. Mi madre me preguntaba por qué no escogía otro, cuando había tantos por descubrir. Sin saberlo, aquel libro sembró en mí una semilla que, décadas después, germinaría en este trabajo.

Aunque no éramos una familia con muchos recursos, nunca me faltaron las ganas de aprender. Crecí en un entorno humilde, donde estudiar no solo era un privilegio, sino también un compromiso personal que asumí desde muy joven. No era algo que mis padres me pidieran; de hecho, nunca tuvieron que recordarme hacer las tareas. Estudiar era algo natural para mí. Mi padre, siempre realista respecto a la situación que nos rodeaba, solía decirme: "En esta casa no quiero vagos. Si no quieres estudiar, a los 16 te pongo de camarera en el hotel". Quizá por eso, desde adolescente, aprendí a compaginar mis estudios con trabajos ocasionales.

Decidí estudiar Biología, convencida de que allí encontraría mi futuro profesional. Sin embargo, al llegar al último año de la carrera, me vi inmersa en una crisis existencial. ¿Había desperdiciado años de esfuerzo en una disciplina que no terminaba de llenarme? Nacer y crecer en Tenerife añadía una dificultad extra, una isla donde el turismo domina la economía y representa la mayor fuente de empleo, era complicado imaginarse eligiendo algo tan distinto a lo que siempre había visto a mi alrededor. Fue entonces, por un giro inesperado del destino, cuando mis últimas prácticas universitarias me llevaron a una clínica de reproducción asistida. Allí, rodeada de microscopios y embriones, redescubrí aquella fascinación infantil por el desarrollo de la vida.

Esa revelación marcó un antes y un después. Busqué programas de especialización hasta que encontré el Máster en Biotecnología de la Reproducción Humana Asistida de la Universidad de Valencia y, aunque no conocía a nadie que lo hubiera cursado ni tenía referencias, decidí

intentarlo. Y, contra todo pronóstico, fui aceptada a la primera, y así comenzó mi carrera en este apasionante campo.

Al principio, Valencia fue un lugar solo para continuar mis estudios, pero con el tiempo me di cuenta de que se estaba convirtiendo en mucho más. Como dirían los valencianos, *de fora vindran i de casa ens trauran*, y me quedé. Aquí no solo encontré mi camino profesional, sino también una nueva familia valenciana que, como yo, venían de fuera. La ciudad me abrió sus brazos y me enseñó lo que significa sentirse parte de la "terreta", convirtiéndome en una simple chica canaria que encontró su lugar entre *valencians, pólvora, foc i passió*.

Este trabajo representa un homenaje a aquel sueño que comenzó con un libro prestado, una niña curiosa y una familia que siempre me enseñó a valorar el esfuerzo y compromiso. Desde aquellas primeras ilustraciones del embarazo hasta esta tesis, el camino ha sido largo, y, en ocasiones, arduo. Pero también ha estado lleno de momentos de aprendizaje, crecimiento personal y, sobre todo, de personas que me han acompañado a lo largo de esta etapa, y a quienes debo agradecer por su apoyo, orientación y paciencia. Espero ser concisa, que ya les aviso que nunca lo soy.

En primer lugar, quiero agradecer a Paco, mi director principal, quien me dio la oportunidad de embarcarme en esta aventura. Su capacidad para guiarme sin imponer esa figura de "jefe" hizo sentirme siempre a gusto, sin miedo a compartir mis dudas o errores, lo que me permitió aprender a mi propio ritmo. Agradezco también a Pepe, quien ha sido mi co-director, por su apoyo y por estar siempre dispuesto a ofrecerme su consejo.

I would also like to express my gratitude to Dr. Francesco DeMayo, for giving me the opportunity to learn in a different laboratory. I gained invaluable insights into the world of high-level research while working in your lab.

Agradezco a toda Fundación IVI y a los investigadores principales, Nicolás Garrido, Irene Cervelló, Sonia Herraiz, Patricia Díaz y Hortensia Ferrero, por su apoyo y orientación en todo momento.

Además, quiero agradecer al resto de jefes y compañeros de los otros laboratorios y centros en los que he tenido la suerte de formarme. Desde la clínica IVIRMA Bilbao, el Instituto

Universitario de Enfermedades Tropicales y Salud Pública de Canarias, el *National Institute of Environmental Health Sciences* (NIEHS) en Carolina del Norte, hasta la clínica IRMO en Tenerife, cada uno de estos lugares ha sido crucial para mi formación y crecimiento profesional.

I want to express my gratitude to the Ovumia clinics, where I currently work, for allowing me to work in my dream job as an embryologist. Thank you for the opportunity to grow both professionally and personally, for being part of such a wonderful team, and for supporting me every step of the way. Kiitos kaikille Ovumia Tampereella, että olette olleet osa tätä matkaa. Tack till hela Ovumia Stockholm för allt stöd och för att ni har gjort denna resa så minnesvärd.

Gracias a todo el personal de la clínica IVIRMA Valencia que ha contribuido al reclutamiento de donantes y la obtención de muestras, en especial a la Dra. Pilar Alamá. A los Servicios de Genómica y Proteómica de la Universidad de Valencia, especialmente a Vicente Sentandreu y Oreto Antúnez, por su apoyo y colaboración en los análisis realizados.

También me gustaría agradecer al resto de mi grupo de investigación. Rober, mi mini director de tesis, mi guía a escondidas ¿cómo agradecerte todo lo que has hecho por mí? Si pudiera encontrar las palabras justas para expresarlo, no serían suficientes. Desde el inicio de este viaje, cada vez que me sentía perdida, ahí estabas tú con tus consejos sabios, iluminando mi camino. No sé cómo has aguantado a este pollito desorientado, pero lo hiciste, y me guiaste con paciencia y con esa generosidad que te caracteriza. Me has enseñado a seguir creyendo en mí misma. Sin ti, este viaje hubiera sido imposible. Pedro, ¿cómo hablar de ti sin que se me queden cortas las palabras? Eres mucho más que el alma de la fiesta, eres casa, eres hogar. No solo has sido mi compañero de trabajo, has sido mi apoyo en los momentos más oscuros, mi persona favorita de Fundación. Esos momentos en lo que subía a la torre a visitarte, para simplemente charlar sin prisas, fueron mi salvavidas. Me has enseñado, sin darte cuenta, que la verdadera fuerza no está solo en el trabajo, sino en las personas que te acompañan en el viaje. Gracias por ser mi faro, mi hermano de otra madre. A Andrea, por tantos momentos y confidencias, tu forma de ver la investigación me anima a seguir creyendo en la ciencia. A Stefi, tu orientación fue clave desde el inicio de este viaje. Indra, fuiste calma en medio de la tormenta, siempre podía contar con tu apoyo. Gracias también a Robertito, que, aunque hayamos coincidido poco, ha sido un placer. Y, por último, pero no menos importante, a Ali.

Eres la mamá del laboratorio, la que hace que todo funcione, que todo sea más sencillo, eres los engranajes del grupo. Tu experiencia y tu apoyo han sido cruciales para que mi tesis tomara forma, y no sé qué habría hecho sin ti.

Quiero dedicar unas palabras especiales al despacho 1.17 y a las gallinas del corral que lo llenaron de vida. María Gil, perla del Túria, gracias por mostrarme la esencia de Valencia de una manera tan especial, quien me introdujo el verdadero espíritu de las Fallas desde dentro y quien me enseñó que la manera de echar el arroz a la paella es un arte. Anita, alcaldesa de lo que te propongas, siempre poniéndole ese toque rosa y adorable a cualquier situación. Rosa Pacheco, ambas *gymrats*, siempre me encantó tu forma de ser, tan cercana y adorable. Majo, *la millor professora de valencià. Sense tu, no hauria pogut anar omplint el meu diccionari valencià-castellà. Com diu el dit, "Gallina vella fa bon caldo", i tu fas que siga una veritat indiscutible per tot el que aportes.* María Gómez, esa chispa de alegría que siempre llevabas contigo. Gracias por enseñarme a valorar el significado de *tornarem*. Adolfo, el gallo del corrillo, siempre dispuesto a ayudar en lo que fuera. Eres el ejemplo perfecto de lo que significa estar ahí para lo que necesiten.

Agradecer también a otros compañeros de Fundación que marcaron de manera especial mi recorrido. Comenzando por ti, Emiliet, *vas ser el meu primer company de taula, les vivències que compartim junts donarien per escriure una novel·la. Sempre t'estaré eternament agraïda per la persona que ets, per la bondat que sempre has mostrat*. Noelia, la sevillana más guapa, mi revelación de Australia. La persona con la que puedo hablar eternamente, con la que siempre encuentro conexión en cada tema, por más estúpido que sea. Asunta, contigo me convertí en madre en Australia. Nunca unos rizos reflejaron tanta fuerza y personalidad como los tuyos. Alba, cariñito, echo de menos ese despiste tuyo, esa forma de no tener miedo de admitir tus despistes en el lab. Amparo, compañera de sofocos. Solo nosotras sabemos lo que se siente estar metidas en un horno de habitación y que nadie te entienda. Ana Corachán, tu apellido guanche siempre me hizo creer en ti. Tú en Chicago y yo en Carolina del Norte. Sara, nunca vi a alguien luchar tan fuerte por una ciencia bien hecha como tú. Siempre estás dispuesta a dar la cara por los demás, y no solo en lo profesional. Gracias por ser tan auténtica. A Luismi, por enseñarme a hacer las cosas bien hechas. Un verdadero guerrero de tormentas. A Irene, Lucía, Jessi, Anna Buigues, Diana, Clara, Juan Carlos, Patri Sebastián, Almu, Pablo, Rosa

Galán, María Marchante… sois tantos los que, en algún momento, habéis tenido unas buenas palabras, un gesto amable que hizo más llevadero todo. Gracias a todos por estar ahí.

Quiero hacer extensible este agradecimiento al resto de personas que han hecho posible nuestro trabajo en Fundación, como la UAGI al completo, Carmen, Elena, Alba, Davinia, Silvia, Marcos, etc.

MJ, you were not just my neighbor, you were my guardian angel during my time in North Carolina. So many nights spent with Chloe, sharing dinners and trying to solve the world. You two made that time so much easier, and I'll always be grateful for everything you did for me. You will always have a special place in my heart.

A toda mi familia valenciana porque, sinceramente, Valencia no tendría sentido sin ustedes. Y como no, empiezo contigo, María Cristina, son tantos los recuerdos, tantas las experiencias y tantas las confidencias que compartimos. Nuestra vida parecía sacada de una *sitcom*. Yassmin Medina y María Cristina Carbajo, 9 $\frac{3}{4}$. Con las cámaras en el piso nos hubiéramos hecho millonarias protagonizando la comedia más caótica. Sin duda, marcas una cicatriz con momentos que jamás voy a olvidar. Los conciertos de Manuel Carrasco en el 99, esos "Buenos días, qué bueno que a mi lado sigas" que nos acompañaron cada mañana. Sin ti, esta ciudad no sería lo mismo, y yo tampoco sería la misma. Gracias por ser ese pilar inquebrantable, por ser mi hermana, por estar siempre ahí en lo bueno y en lo malo, por siempre decir sí a todo, por los bailes, risas...y por los llantos y las noches sin dormir, solo nosotras sabemos por donde hemos pasado. Pepi, ¿cómo te agradezco todo lo que has hecho por mí? Gracias por ser la anfitriona más increíble, junto a Fran, por darme siempre ese cariño tan incondicional, por quererme de una manera tan intensa y sincera, me haces sentir especial. No sé qué haría sin ti, sin tu intensidad, sin tus abrazos y sin tu "azento". Eres familia. Fran, el anfitrión chipionero más auténtico que conozco. Antonio, mi arma, el más sevillano, el compañero de Euromillones. El amigo que siempre tiene la *playlist* perfecta, con "temasos" de San Benito (PRRR). Gracias por darme consejos sinceros, sé que vienen desde el mejor lugar. "Tamo'h bien, sobran los billetes de cien". Eva, definición de bondad, gracias por emitir esa luz. Eres maravillosa. Marina, compañera de festivales, mi pequeño saltamontes. Siempre con purpurina en el bolso y en la vida, lista para lo que venga. A Rosalba e Ismael, por ser el

ejemplo de fortaleza y dedicación. Alejandro M, gracias por tu dulce energía y por siempre aportar tu toque especial.

A lo largo de este camino, he tenido la suerte de encontrarme con personas que han marcado la diferencia. Gabi, la elegancia que te define sin esfuerzo. Gracias por haberte cruzado en mi camino durante el máster, sé que siempre estarás ahí por muchos kilómetros que nos separen. Maribel, por enseñarme que la vida se lleva mejor con carácter y determinación, *collons*!. Ana Conrado, mi salvación en Finlandia, el alma joven que me despierta. Tampere, nieve, munkit, Tappara, todo eso lleva tu nombre. Eres la hermana pequeña que nunca supe que necesitaba. *Yksi sisar on parempi kuin kymmenen ystävää*. A Ale, mi Ale, compañero de batallas crossfiteras, el que puede pasarse horas escuchando mis dramas sin cansarse, el que me ayuda aún sin pedírselo, el único sudor que estoy dispuesta a abrazar. Gracias por ser el que siempre está. A todo Boga Crossfit, en especial a Berni, Chris y Rama. Y a todos los demás, a Zaira, Carmen, Belén, Jesús, Amparo, a aquellos que compartieron conmigo las pequeñas cosas, las grandes risas y los abrazos.

A Ander, gracias por aguantarme y por creer en mí cuando yo dudaba, por apoyarme en los momentos más bajos y por sacarme una sonrisa cuando los ataques de ansiedad parecían invadirlo todo. Gracias por mostrarme la vida a ritmo de salsa caleña y por enseñarme a llevar las cosas con más calma. A Alejandra y Nando, gracias por tratarme siempre como una hija, por preocuparos por mí y por darme tanto apoyo.

A mis amigos de toda la vida. Almu, no tengo palabras suficientes para agradecerte todo lo que me has dado a lo largo de los años, desde aquella mesa al final de clase hasta hoy. Eres esa amiga con la que me siento en casa, como si tu familia fuera también la mía. Gracias por ser siempre mi refugio. Josito, gracias por estar siempre y por tu apoyo incondicional (aunque más bien siempre sea vacile). Sois la familia que se elige. Os quiero. A Ana, mi canaria australiana, compañera de universidad, profesión y tarimas.

A Javi. Mi tormento y mi salvación, mi calma y mi agitación. Con el que puedo pasar horas y horas hablando sin cansarnos (aunque a veces me saques de quicio con la “única verdad”). Gracias por darme un hueco tan especial en tu vida, y por darme la certeza de que siempre estarás en la mía. Por acompañarme en todas las etapas, especialmente en las más oscuras.

Gracias por enseñarme a no confiar tanto en la gente y por defenderme con espada si hace falta. Por no dejarme sola, por recorrer los más de 3000 kilómetros que separan Paiporta de Estocolmo más de 6 veces en un año para pasar frío. Por ser mi compañero Disney. Porque es "difísil". Por ser la persona que ha protagonizado mi vida desde 2022, por haber llegado de una forma tan inesperada. La línea 8 ha pasado a ser mi línea favorita. *Quin xiquet més templat acompanya la meua vida.*

Por supuesto, gracias a mi familia. Gracias a la familia Medina por enseñarme la palabra humildad. *Thanks to Laver's family, even though there's distance between us, I always feel you so close. Specially to my lovely grandma, Sandra, your warmth and love have a way of crossing any distance.*

A mi padre, José María, por enseñarme lo que significa trabajar duro desde joven para mantener a mi abuela y a mis tíos. Has sido la persona más luchadora que he conocido, tu fortaleza es algo que llevo conmigo todos los días. A mi hermano, Yoshua, gracias por ser el verdadero compañero de mi vida, con solo 10 meses de diferencia, como si de gemelos nos tratáramos. Mis únicas peleas físicas. Nuestro vínculo es irrompible, pase lo que pase, siempre a tu vera. A mi madre, Alexa. Aunque compartamos el mismo carácter, eres la calma de mi vida. Eres hogar. La que sabe cómo calmar mi ansiedad y la primera a la que quiero llamar cuando necesito consuelo. La que me ha cuidado incluso a la distancia. Gracias por darme la vida, por ser quien siempre sabe cómo hacer que todo se sienta mejor. Soy quien soy gracias a ti.

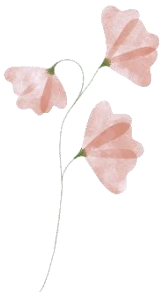
Por último, quiero dedicar unas palabras al amor más grande que he tenido, a Coco. Aunque no me puedas leer, gracias por enseñarme lo que es el amor infinito, por ser ese compañero incondicional que, con solo cuatro patitas, movió mi mundo de una forma que nunca imaginé. Espero que donde sea que estés, estés corriendo como un ruin entre jardines, tomando el sol, y recibiendo el amor que te mereces. Te amo y nunca te olvido.

Lo más difícil de haberme ido de España ha sido la distancia con todos los que habéis formado parte de mi camino. Porque cuando tomas la decisión de perseguir tus sueños, sabes que, a veces, ese sueño viene acompañado de sacrificios. Aunque físicamente sigo estando lejos, mi corazón nunca ha dejado de estar con ustedes. Está en cada rincón de cada recuerdo, en cada

conversación que compartimos, en cada momento vivido. Espero que, algún día, la vida me permita estar de nuevo cerca, que podamos compartir risas, abrazos, o simplemente estar, sin importar la distancia.

A cada uno de ustedes, gracias. Gracias por ser parte de mi viaje y por hacerme sentir siempre tan bienvenida.

Os quiero.



Yassmin.

Seijaku.

La presente tesis doctoral ha sido realizada en los laboratorios de Fundación IVI. El reclutamiento de las participantes y la recogida de las muestras ha sido realizado en la clínica de IVIRMA Valencia. Los análisis transcriptómicos y proteómicos han sido realizados en los servicios de genómica y proteómica, respectivamente, de la Universidad de Valencia.

Este trabajo ha sido posible gracias a la financiación por parte de la Generalitat Valenciana, tanto por la contratación de personal investigador en formación de carácter predoctoral (ACIF/2019/262) así como para la realización de estancias predoctorales fuera de la Comunidad Valenciana (BEFPI/2021/057); también apoyado por la financiación de Fundación IVI (1911-FIVI-103-FD).

This PhD thesis was carried out in the laboratories of IVI Foundation. The recruitment of the participants and the collection of the samples was carried out at the IVIRMA Valencia clinic. Transcriptomic and proteomic analyses were performed at the genomic and proteomic services, respectively, of the University of Valencia.

This work has been possible thanks to funding from the Generalitat Valenciana, both for the recruitment of predoctoral research trainees (ACIF/2019/262) as well as for predoctoral stays outside the Valencian Community (BEFPI/2021/057); also supported by funding from IVI Foundation (1911-FIVI-103-FD).

ABSTRACT

The endometrial response to progesterone (P4) is mediated by both classical P4 receptors (classical PGRs) and non-classical P4 receptors (non-classical PGRs). While significant research has been conducted on certain non-classical PGRs, such as P4 receptor membrane component 1 (PGRMC1), the role of P4 receptor membrane component 2 (PGRMC2), another non-classical PGR with structural similarity to PGRMC1, remains unexplored in humans, particularly in the context of P4 signalling and endometrium establishment for embryo implantation. Considering this, the main objective of this PhD thesis was to provide a detailed characterization of PGRMC2 expression across different phases of the human natural menstrual cycle and to explore its specific functional role in human endometrial stromal cells (hEnSCs) during decidualization, a process essential for successful embryo invasion and pregnancy establishment.

To achieve this, a comprehensive prospective study was designed, incorporating 77 endometrial biopsies from 66 oocyte donors. Total endometrial PGRMC2 expression was evaluated across different phases of the menstrual cycle using immunohistochemistry, RT-qPCR, and western blotting. *In vitro* decidualization of hEnSCs was induced by treatment with cyclic adenosine monophosphate (cAMP) and medroxyprogesterone acetate (MPA) during 4 days. In addition, PGRMC2's role during decidualization was further examined through transcriptomic (performing RNA-seq technique) and proteomic (using SWATH-MS procedure) analyses following siRNA-mediated *PGRMC2* silencing. Prolactin (PRL) secretion was measured not only as a marker of decidualization but also to evaluate whether *PGRMC2* silencing affected the decidualization process of hEnSCs, while a trophoblast outgrowth model, where decidualized hEnSCs (d-hEnSCs) with and without *PGRMC2* silencing were co-cultured with human trophoblastic spheroids (JEG-3), were carried out to assess PGRMC2's involvement in embryo invasion-related processes. Additionally, immunofluorescence analysis of Ki-67 expression was conducted to determine whether the transfection process affected hEnSCs proliferation during *in vitro* decidualization.

Results showed that *PGRMC2* gene expression fluctuates significantly throughout the human menstrual cycle, with its levels being significantly higher during the mid-secretory phase compared to the late secretory phase ($P < 0.05$), coinciding with the window of implantation,

where both glandular and stromal cells exhibited elevated PGRMC2 protein staining. d-hEnSCs revealed a marked increase in PGRMC2 expression at both the mRNA ($P < 0.0001$) and protein ($P < 0.001$) levels compared to non-decidualized hEnSCs (nd-hEnSCs), particularly in cell membranes and organelles. Interestingly, *PGRMC2*, along with its structurally similar counterpart *PGRMC1*, was found to be expressed at significantly higher levels than the classical *PGRs* during all phases of the menstrual cycle and during both d- and nd-hEnSCs ($P < 0.05$).

The silencing of *PGRMC2* in d-hEnSCs led to the identification of 4687 differentially expressed genes (DEGs) and 28 differentially expressed proteins (DEPs) in d-hEnSCs. Functional enrichment analysis of the DEGs revealed that the 2420 upregulated genes after *PGRMC2* silencing were primarily associated with processes such as endoplasmic reticulum function, vesicular transport, cell morphogenesis, angiogenesis, cell migration and cell adhesion. In contrast, the 2267 downregulated genes were linked to key biological pathways, including aerobic respiration and protein biosynthesis. At the proteomic level, enrichment analysis of the altered proteins highlighted their roles in aerobic respiration, cellular responses, metabolism, localization of endoplasmic reticulum proteins, and ribonucleoside biosynthesis.

As transfection processes can sometimes interfere with normal cellular behavior, Ki-67 immunofluorescence staining was performed to assess cell proliferation in d-hEnSCs after *PGRMC2* silencing. No statistically significant differences were found in the proliferation rates between negative control, scramble (Sc), and *PGRMC2*-silenced (i*PGRMC2*) d-hEnSCs, suggesting that *PGRMC2* silencing does not impair cell proliferation in this context.

Finally, PRL levels showed a significant increase in i*PGRMC2* d-hEnSCs compared to Sc d-hEnSCs ($P < 0.05$). This suggests that PGRMC2 absence may trigger a compensatory rise in PRL levels, potentially indicating impaired decidual function. Given this results along with transcriptomic findings showing that decidualization-related pathways were affected, we conducted an *in vitro* trophoblast outgrowth analysis where showed that *PGRMC2* silencing compromised the ability of d-hEnSCs to support trophoblast invasion ($P < 0.05$).

In summary, we conclude that PGRMC2 is a pivotal regulator of decidualization in hEnSCs, with broad implications in modulating endometrial receptivity and supporting embryo invasion. These findings provide valuable insights into the non-classical PGRs signalling

mechanisms in the human endometrium, with potential relevance for understanding fertility disorders and improving therapeutic approaches for implantation failure.

RESUMEN

I. INTRODUCCIÓN

1. El sistema reproductor femenino

1.1. Anatomía y fisiología del sistema reproductor femenino

El sistema reproductor femenino está formado por órganos internos, que incluyen los ovarios, las trompas de Falopio y el útero; y por órganos externos, compuestos por la vagina, la vulva, el perineo y la uretra. Los ovarios, además de albergar los ovocitos, producen las principales hormonas sexuales femeninas: estradiol o estrógeno (E2) y progesterona (P4). Las trompas de Falopio son responsables de transportar los ovocitos hacia la cavidad uterina, donde ocurre la fertilización y la formación del cigoto. Por su parte, el útero facilita la implantación, la formación de la placenta y el desarrollo embrionario hasta el parto.

Asimismo, la pared uterina consta de tres capas con funciones diferenciadas: el perimetrio, capa externa delgada; el miometrio, capa muscular que soporta el embarazo y genera las contracciones necesarias para el parto; y el endometrio, capa interna mucosa, cuya complejidad será abordada en la siguiente sección.

1.2. El endometrio humano

El endometrio humano es un tejido regulado hormonalmente que desempeña un papel esencial en la implantación del embrión y el mantenimiento del embarazo. Además, en ausencia de embarazo, este tejido es responsable del proceso de menstruación. Dado que el endometrio es fundamental para la implantación embrionaria, cualquier alteración puede comprometer directamente la fertilidad femenina.

1.2.1 Composición celular y organización del endometrio

El endometrio humano está formado por células epiteliales (hEnECs) y estromales (hEnSCs), sostenidas por una red vascular, células inmunitarias endometriales [células *natural killers* uterinas (uNK), macrófagos y linfocitos T] y un nicho endógeno de células madre (MSCs). El

compartimento epitelial está dividido en epitelio luminal y glándulas epiteliales, ambos formados por células columnares. Su principal función es regular y permitir la implantación del embrión. Por otra parte, el compartimento estromal contiene células tipo fibroblasto. La red vascular endometrial, que se origina en el miometrio, es esencial para la formación y el desarrollo de nuevos vasos sanguíneos (angiogénesis) que ocurre regularmente a lo largo del ciclo menstrual, facilitando la implantación embrionaria y la placentación si se alcanza la fecundación.

El endometrio se divide en dos capas: el estrato funcional (*stratum functionalis*), la cual es la porción que sufre los mayores cambios y se desprende durante la menstruación; y el estrato basal (*stratum basalis*), el cual permanece intacto, proporcionando los vasos sanguíneos y la regeneración necesaria para formar una nueva capa funcional en el siguiente ciclo menstrual.

1.2.2 Función del endometrio y el ciclo menstrual

En respuesta al E2 y la P4, el endometrio experimenta cambios fisiológicos que lo preparan para la implantación del embrión, lo que se conoce como la "ventana de implantación" (WOI). Esta ventana ocurre entre los días 19-22 del ciclo menstrual.

El ciclo menstrual se divide en: la fase proliferativa (días 0-14) y la fase secretora (días 15-28), con una duración promedio de 28 días. La fase proliferativa incluye la menstruación (días 0-4) y la ovulación (alrededor del día 14). La fase secretora comienza con la diferenciación del endometrio gracias al aumento de los niveles de P4. Si no ocurre la implantación embrionaria, el descenso de P4 induce la menstruación, iniciando un nuevo ciclo.

1.3. Mecanismos moleculares en la implantación y el desarrollo fetal temprano

Tras la fertilización, el cigoto experimenta divisiones mitóticas mientras es transportado por la trompa de Falopio hacia la cavidad uterina, convirtiéndose en blastocisto a los 5-6 días, el cual es capaz de implantar en el endometrio. Este blastocisto consta de dos componentes: la masa celular interna (ICM), que dará lugar al feto, y el trofoectodermo (TE), que formará la placenta.

La implantación embrionaria se puede dividir en tres etapas principales: aposición, adhesión e invasión, en las cuales intervienen diversas moléculas clave como citoquinas, factores de crecimiento, prostaglandinas, enzimas y moléculas de adhesión. El proceso de implantación es seguido por el desarrollo fetal temprano durante el primer trimestre.

1.4. Ómicas endometriales

El estudio del endometrio a nivel molecular, conocido como "ómicas del endometrio", se centra principalmente en dos áreas: la transcriptómica y la proteómica, que han avanzado considerablemente en los últimos años.

1.4.1 Transcriptómica endometrial

La transcriptómica estudia la expresión génica y su relación con procesos biológicos, como la receptividad endometrial y la implantación embrionaria. A través de estudios transcriptómicos, se han identificado perfiles de expresión génica que distinguen estados receptivos de no receptivos del endometrio. Este enfoque ha permitido identificar genes clave, como el factor inhibidor de leucemia (*LIF*), el cual facilita la interacción entre el embrión y hEnSCs, promoviendo la decidualización y regulando procesos como la adhesión y diferenciación celular. Sin embargo, la variabilidad experimental y las alteraciones en patologías como los fallos de implantación recurrentes (RIF) y endometriosis dificultan su aplicación clínica. La combinación de transcriptómica con otros enfoques "ómicos" podría mejorar el diagnóstico y tratamiento de problemas de fertilidad, optimizando los resultados clínicos.

1.4.2 Proteómica endometrial

La proteómica se centra en la identificación y cuantificación de proteínas, así como en el análisis de sus modificaciones post-traduccionales, siendo clave para caracterizar el ciclo endometrial e identificar biomarcadores de receptividad y patologías. Estudios iniciales revelaron 186 proteínas diferencialmente expresadas durante el ciclo menstrual, asociando proteínas del citoesqueleto a la fase proliferativa y metabólicas a la fase secretora. Avances como la espectrometría de masas han mejorado estos análisis, destacando proteínas como el componente 1 del receptor de membrana de la progesterona (PGRMC1), cuya reducción se

relaciona con receptividad, y la proteína S100A10, cuya sobreexpresión afecta la implantación. Aunque persisten desafíos por la variabilidad experimental, la integración con otras tecnologías "ómicas" ofrece una visión más completa de las patologías endometriales, como la endometriosis y el cáncer de endometrio.

2. El proceso de decidualización

La P4 induce la diferenciación del endometrio, haciendo que sea receptivo para la implantación embrionaria. Un evento dependiente de P4 esencial en este proceso es la decidualización, caracterizada por la diferenciación de las hEnSCs en células deciduales, caracterizado por un aumento en la secreción de prolactina (PRL) y de la proteína vinculada al factor de crecimiento similar a la insulina 1 (IGFBP1), marcadores de decidualización. La decidualización comienza aproximadamente 6 días después de la ovulación y se caracteriza por cambios morfológicos en las hEnSCs, en la secreción de las glándulas uterinas, un aumento en la presencia de uNK, y remodelación vascular para soportar el crecimiento embrionario.

El proceso de decidualización está sincronizado por la convergencia de las vías de señalización del adenosín monofosfato cíclico (AMPc) y la P4, siendo el AMPc intracelular crucial para lograr y mantener el estado decidualizado de las hEnSCs. Además, investigaciones recientes han señalado que la reprogramación metabólica, en particular en el metabolismo de aminoácidos y esfingolípidos, es crucial para este proceso, así como el estado de decidualización del endometrio, afectando la motilidad del trofoblasto. Por lo tanto, un fallo en la decidualización puede llevar a infertilidad, abortos recurrentes y trastornos uteroplacentarios, como la preeclampsia, vinculada a una decidualización defectuosa y alteraciones en la expresión de genes clave, como la anexina A2 (*ANXA2*). Además de la preeclampsia, también se asocia con endometriosis, infertilidad anovulatoria y pérdida recurrente del embarazo.

2.1 Modelos de la decidualización *in vitro*

Durante la decidualización, además de la secreción de PRL e IGFBP1, se sabe que dentro de las hEnSCs, los niveles de AMPc aumentan tras varios días de tratamiento con una forma sintética de la P4 (llamada progestina). Este proceso ha sido ampliamente estudiado en

cultivos celulares, donde se han desarrollado diversos protocolos para inducir la decidualización *in vitro*. Estos protocolos comúnmente incluyen tratamientos con E2 y P4, o progestinas, que inducen la expresión de AMPc intracelular, o directamente con un análogo de AMPc. La duración del tratamiento varía considerablemente entre los estudios, desde solo unas horas hasta más de 10 días.

En el contexto de la decidualización *in vitro*, la respuesta de las hEnSCs a estos compuestos está asociada con una modulación temporal del ciclo celular: un arresto inicial en la fase G0/G1 del ciclo celular, seguido por un arresto posterior en las fases G2/M. Es importante destacar que la cinética de señalización de AMPc y P4 varía entre ambas. Mientras que la señalización de AMPc muestra una respuesta decidual rápida e inmediata, induciendo un fenotipo decidual completo en los primeros tres días de tratamiento, la señalización de P4 genera un efecto más gradual pero persistente en el tiempo, requiriendo entre 14 y 23 días de tratamiento.

Recientemente, un estudio encontró que los cambios involucrados por tratamientos con AMPc (solo o combinado con progestinas) resultaron en aproximadamente el doble de genes diferencialmente expresados (DEGs) en comparación con los encontrados usando solo progestinas o combinado con E2. El uso de AMPc influyó significativamente en funciones celulares asociadas con la angiogénesis, la inflamación, la respuesta inmune y la implantación embrionaria, mientras que el uso de las progestinas afectó las vías de señalización de la insulina.

Como se puede comprobar, la selección de compuestos adecuados para los estudios de decidualización *in vitro* es crucial para replicar mejor las condiciones *in vivo* y mejorar nuestra comprensión de la biología endometrial y la salud reproductiva.

3. La hormona progesterona

La P4, producida por la placenta, ovarios y glándulas suprarrenales, regula procesos clave del ciclo menstrual, como la ovulación, la fertilización, la implantación, el desarrollo embrionario y la preparación para el parto, además de tener funciones no reproductivas en el sistema cardiovascular, nervioso central y mantenimiento óseo.

Durante el periodo periovulatorio, es esencial para procesos como la capacitación espermática, la hiperactivación, la quimiotaxis y la reacción acrosómica, los cuales son esenciales para una fertilización y desarrollo embrionario óptimos. Sin embargo, alteraciones en la señalización de ésta pueden causar complicaciones ginecológicas como miomas, sangrado menstrual anormal, endometriosis y adenomiosis, así como una relación con el cáncer de mama y de endometrio. Además, la actuación inadecuada de P4 se asocia con abortos espontáneos y partos prematuros, lo que refuerza su importancia para lograr un correcto embarazo.

3.1 Mecanismos de acción de los receptores de progesterona (PGRs)

Los efectos fisiológicos de la P4 son mediados por su unión a los receptores de P4 (PGRs) en las células diana. Los PGRs se clasifican en dos tipos: clásicos y no clásicos, cada uno con funciones y roles diferentes.

Los receptores clásicos se han identificado en numerosos órganos y tejidos, como los ovarios, el útero, las trompas de Falopio, la placenta, el cerebro, el páncreas, los huesos, las glándulas mamarias y el tracto urinario. Tras unirse a la P4, estos receptores se transforman en factores de transcripción que regulan la expresión de genes modulados por esta hormona, lo que genera respuestas celulares sostenidas que se manifiestan en el transcurso de varias horas.

Sin embargo, estudios en ratones *knockout* que carecen de PGRs clásicos han demostrado que las células aún pueden responder a la P4 a través de receptores de membrana no clásicos. Estos receptores facilitan efectos hormonales rápidos mediante la activación inmediata de mensajeros secundarios y vías de señalización. Las vías de señalización de los PGRs clásicos y no clásicos generan diversas respuestas celulares, cuyos efectos varían según el tipo de tejido y la etapa del ciclo menstrual.

3.1.1 PGRs clásicos

Los receptores clásicos de la P4 presentan dos isoformas principales: PGR-α (94 kDa) y PGR-β (120 kDa), transcritas a partir del mismo gen en el cromosoma 11 y diferenciándose por el uso de distintos promotores. Su transcripción depende principalmente del E2, que activa

elementos de respuesta en la región promotora, aunque también existen mecanismos independientes de E2.

Estructuralmente, los PGRs clásicos cuentan con un dominio de unión al ADN (DBD), ubicado en la región N-terminal con una función de activación (AF1), y un dominio inhibidor (ID). En su extremo C-terminal se encuentra el dominio de unión al ligando (LBD), con un segundo dominio de activación (AF2). En el caso de PGR-β incluye un dominio adicional (AF3) debido a una región extra en su extremo N-terminal. También se ha descrito una tercera isoforma, PGR-γ (60 kDa), localizada en la placenta humana, cuyo rol preciso es aún desconocido, aunque influye en las actividades transcripcionales de *PGR-α* y *PGR-β*.

En ausencia de su ligando, los PGRs clásicos permanecen en el citoplasma dentro de un complejo de proteínas chaperonas. Al unirse la P4 al dominio LBD, provoca cambios conformacionales que liberan al receptor del complejo chaperona, permitiendo su translocación al núcleo. Una vez en el núcleo, los PGRs se dimerizan y se unen a las secuencias específicas de respuesta hormonal (HRE) en los promotores de los genes diana, activando su transcripción.

Las isoformas de PGRs clásicos interactúan entre sí modulando su actividad. PGR-α inhibe a PGR-β mediante su ID, regulando así el impacto de la P4 en las células diana. En las células luteínicas, altos niveles de P4 inducen una mayor expresión de *PGR-α*, que a su vez reprime la transcripción de *PGR-β*. Por el contrario, niveles bajos de P4 reducen la expresión de *PGR-α*, incrementan la de *PGR-β* y, en consecuencia, potencian la acción de la P4.

3.1.2 PGRs no clásicos

Los PGRs no clásicos están localizados en la superficie celular, lo cual les permiten mediar respuestas rápidas tras la unión de la P4, activando segundos mensajeros, como la quinasa MAP (MAPK), y vías de señalización en cuestión de segundos. Estas respuestas inmediatas contrastan con las acciones más prolongadas mediadas por los PGRs clásicos.

Los receptores no clásicos se dividen en dos familias principales: los receptores de membrana de la P4 (mPRs) y los componentes de los receptores de membrana de la P4 (PGRMCs). La familia de los mPRs, también conocida como receptores progestina y AdipoQ

(PAQR), incluye las variantes mPRα, mPRβ, mPRγ, mPRɗ y mPRε (también denominadas PAQR7, PAQR8, PAQR5, PAQR6 y PAQR9, respectivamente). Por su parte, la familia PGRMCs incluye a PGRMC1, PGRMC2, neudecina y neuferricina. Recientemente, se han señalado interacciones entre PGRMC1 y mPRα, lo que sugiere un posible *crosstalk* entre ambas familias de receptores no clásicos.

A pesar de su menor afinidad de unión a P4 en comparación con los PGRs clásicos, los PGRs no clásicos están implicados en una variedad de procesos biológicos. Entre estos se incluyen la modulación del comportamiento sexual en ratones, la maduración meiótica en ovocitos de *Xenopus*, la reacción acrosómica en espermatozoides humanos y la regulación del flujo iónico en tejidos como neuronas murinas, miocitos vasculares y células epiteliales de rata. La relevancia de estos receptores radica en su capacidad de generar respuestas rápidas y diversificadas en tejidos específicos. Aunque su estudio aún está en desarrollo, los PGRs no clásicos representan un componente crucial en la señalización hormonal, con implicaciones en la reproducción y otros procesos biológicos esenciales.

4. Receptores clásicos y no clásicos de la progesterona en tejidos reproductivos

La acción de la P4 es mediada por los PGRs clásicos y no clásicos, que influyen en procesos como el desarrollo folicular y la ovulación. En el desarrollo folicular, los PGRs clásicos son fundamentales para la ovulación, pero no son esenciales en las etapas tempranas del ciclo folicular. En cambio, los PGRs no clásicos, como PGRMC1, son clave para el desarrollo de los folículos antrales y la reserva ovárica. También están involucrados en la maduración del ovocito y la competencia reproductiva, procesos fundamentales para el éxito reproductivo. Los PGRs orquestan una variedad de procesos reproductivos en el tracto femenino, abarcando desde la ovulación hasta la preparación del endometrio para la implantación embrionaria.

4.1 PGRs en el útero

Los PGRs afectan a diversos tipos celulares en el útero, como las hEnECs y las hEnSCs, así como a las células del miometrio. A través de su acción, los PGRs son esenciales en la

proliferación celular, la diferenciación y la remodelación del endometrio, preparando el tejido para la implantación embrionaria.

4.1.1 PGRs en el endometrio

Los PGRs median la acción de la P4 en el endometrio. De hecho, durante la fase folicular, el E2 induce la expresión de los PGRs clásicos, lo que facilita la preparación del endometrio para la posterior acción de la P4 en la fase lútea.

La P4, a través de los PGRs, actúa sobre diferentes capas celulares en el endometrio. En las hEnECs, los PGRs inician la receptividad endometrial mediante la activación de vías de señalización como WNT y la regulación de factores clave como SOX17 y HOXA10, esenciales para la diferenciación celular y la remodelación del endometrio. Además, la P4 modula la expresión de COX2, que está involucrada en la producción de prostaglandinas, esenciales para la decidualización y la receptividad del endometrio.

En las hEnSCs, los PGRs clásicos regulan la señalización a través de vías de señalización como la del *hedgehog*, que implican factores de transcripción como IHH y GLI1. Estas señales coordinan la interacción entre las hEnSCs y las hEnECs, promoviendo los cambios estructurales y bioquímicos necesarios para la implantación. Además, el factor de transcripción FOXO1, activado por vías como PI3K/AKT, juega un papel clave en la regulación de la decidualización, promoviendo la expresión de los principales marcadores de este proceso, PRL e IGFBP1.

Por otro lado, los PGRs no clásicos, como PGRMC1 y PGRMC2, actúan a través de cascadas de señalización rápidas, incluyendo las vías AMPc/PKA y MAPK, e interactúan con otros reguladores clave, como SERBP1 y NOTCH1. Estas vías no solo regulan la decidualización, sino que también modulan la expresión de genes relacionados con la remodelación de la matriz extracelular, la migración celular y la adhesión. Además, se ha demostrado que PGRMC1 influye en la expresión de los marcadores de decidualización PRL e IGFBP1, reforzando aún más su papel en la preparación del endometrio para la implantación.

Aunque los mecanismos exactos de estos receptores no clásicos aún no se han estudiado en profundidad, su contribución a la decidualización y la receptividad endometrial sugiere que son objetivos prometedores para futuras investigaciones.

Por otro lado, el equilibrio entre las isoformas clásicas de PGR-α y PGR-β también es crucial. En la fase proliferativa, el E2 promueve la expresión de ambos receptores en hEnECs y hEnSCs, pero al final de la fase secretora, los niveles de PGR-α disminuyen en hEnECs, mientras que los de PGR-β permanecen constantes, favoreciendo la secreción glandular. Por el contrario, las hEnSCs mantienen consistentemente la expresión de PGR-α, que es vital para la función uterina normal y la implantación embrionaria. Un exceso de PGR-α puede inducir hiperproliferación en el epitelio luminal y glandular, causando infertilidad, mientras que una sobreexpresión de ambas isoformas en células estromales inhibe la decidualización en hEnSCs. Una alteración en los niveles de PGRs clásicos se asocia con complicaciones, como preeclampsia, RIF y pérdidas gestacionales recurrentes.

Los PGRs no clásicos también deben de mantener un equilibrio en su expresión en el endometrio humano. Por ejemplo, los transcritos de PGRMC1, mPRγ y mPRε aumentan durante la fase proliferativa, mientras que los de mPRα y PGRMC2 se elevan en la fase secretora, coincidiendo con los picos de P4. Aunque el mPRβ es más abundante que el mPRα, su expresión permanece relativamente estable durante el ciclo menstrual, salvo por una disminución observada en mujeres con abortos espontáneos recurrentes. Del mismo modo, una alteración en la expresión de estos receptores afecta la fertilidad femenina. Pues la sobreexpresión de PGRMC1 durante la fase secretora inhibe la decidualización, mientras que su baja expresión puede comprometer la receptividad uterina. Además, se ha visto que la disfunción de los PGRs no clásicos en el endometrio se asocia con trastornos reproductivos como la endometriosis.

5. PGRMC2

PGRMC2 se encuentra en el cromosoma 4, es estructuralmente similar a PGRMC1 y se expresa en varios tejidos reproductivos, como el útero, los ovarios y la placenta. PGRMC2 cumple funciones específicas que incluyen la inhibición de la migración de células de cáncer de ovario SKOV-3, la regulación del metabolismo de esteroles, la participación en la proliferación celular mediante la activación de la vía ERK, y su asociación con el estado de reserva ovárica en mujeres jóvenes. Además, está involucrada en la regulación del ciclo celular y en la progresión de la endometriosis, donde inhibe la proliferación celular.

En modelos animales, la deficiencia de PGRMC2 ha demostrado consecuencias importantes, como la aparición prematura de senescencia uterina, diferente de la senescencia fisiológica que ocurre durante la decidualización, y fallos en la implantación y post-implantación embrionaria. Sin embargo, existe poca información sobre la expresión de PGRMC2 a lo largo del ciclo menstrual humano y su papel en la receptividad endometrial o en la decidualización. Por otro lado, PGRMC2 también ha sido identificado como un posible biomarcador en ciertos tipos de cáncer, como en cáncer de ovario y cáncer de endometrio.

El conocimiento limitado sobre PGRMC2 en los tejidos reproductivos resalta la necesidad de investigaciones más profundas. En este contexto, nuestro estudio busca cubrir esta laguna mediante la caracterización de la expresión de PGRMC2 en el endometrio humano a lo largo del ciclo menstrual y en hEnSCs sometidas a un modelo de decidualización *in vitro*. Además, evaluamos las implicaciones funcionales de PGRMC2 mediante ensayos de silenciamiento, integrando análisis transcriptómicos y proteómicos. También exploramos su papel en el proceso de invasión embrionaria utilizando un modelo in vitro; y finalmente, comparamos la expresión de *PGRMC2* con *PGRMC1* y los *PGRs* clásicos, proporcionando un análisis exhaustivo sobre su relevancia funcional en el endometrio.

II. HIPÓTESIS

Basado en la evidencia de que PGRMC2 está presente en diversos tejidos reproductivos y parece desempeñar un papel clave en el endometrio humano, nuestra hipótesis sugiere que PGRMC2 actúa como regulador en la decidualización de las hEnSCs, contribuyendo significativamente a la formación de un nicho óptimo para que ocurra una correcta implantación embrionaria. Además, planteamos que la presencia de PGRMC2 endometrial es indispensable en el proceso de invasión embrionaria, desempeñando así un papel crucial en las primeras etapas del embarazo.

III. OBJETIVOS

El objetivo principal de esta tesis fue caracterizar PGRMC2 a nivel endometrial a lo largo del ciclo menstrual en mujeres sanas y estudiar su papel durante la decidualización *in vitro* de las

células estromales endometriales y la implantación embrionaria. Los objetivos específicos constan de:

• Describir la expresión génica y la abundancia de proteína de PGRMC2 en el endometrio humano, localizándolo a lo largo del ciclo menstrual.

• Comparar la expresión de *PGRMC2* en el endometrio con la de *PGRMC1* y los *PGRs* clásicos.

• Estudiar el comportamiento y la localización celular de PGRMC2 durante el proceso de decidualización *in vitro* y compararlo con la expresión de *PGRMC1* y los *PGRs* clásicos.

• Evaluar las implicaciones funcionales de PGRMC2 mediante un ensayo de silenciamiento en células estromales endometriales humanas decidualizadas, integrando técnicas transcriptómicas y proteómicas.

• Corroborar si la invasión trofoblástica se ve afectada al silenciar *PGRMC2* en células estromales endometriales humanas decidualizadas utilizando un modelo *in vitro* de invasión embrionaria.

IV. DISEÑO EXPERIMENTAL

La tesis se divide en dos grupos de experimentos. En el primero se incluyen aquellos experimentos que tienen como objetivo la caracterización de PGRMC2 en el endometrio humano a lo largo del ciclo menstrual (experimentos *in vivo*). El segundo grupo, abarcan los estudios funcionales de PGRMC2 en células estromales endometriales (experimentos *in vitro* y funcionales).

V. MATERIAL Y MÉTODOS

1. Aprobación ética

El estudio, aprobado por el comité ético de IVIRMA Valencia (protocolo 1911-FIVI-103-FD), contó con participantes que dieron su consentimiento informado y cumplieron con las normativas de protección de datos.

2. Participantes

Entre 2020 y 2022, se reclutaron donantes de 18-35 años con IMC normal, ciclos regulares y fertilidad comprobada en las clínicas de IVIRMA Valencia. Se excluyeron casos con patologías uterinas, tratamientos hormonales recientes o factores que alteraran los resultados. Esta investigación fue financiada por el Instituto de Salud Carlos III (ISCIII) mediante las ayudas concedidas a F.D. (PI20/00405 y PI23/00860), cofinanciada por la Unión Europea, una beca predoctoral de la Generalitat Valenciana (ACIF/2019/262) y fondos internos de la Fundación IVI (1911-FIVI-103-FD).

3. Recolección de tejido endometrial

Se recolectaron 77 muestras de tejido endometrial de 66 donantes mediante biopsias con cánula Pipelle de Cornier®.

Las muestras endometriales del grupo 1 se obtuvieron a lo largo del ciclo menstrual natural y se clasificaron en cinco fases: proliferativa temprana (EP; *n* = 9; días 3-8), proliferativa tardía (LP; *n* = 8; días 9-14), secretora temprana (ES; *n* = 9; días 15-18), secretora media (MS; *n* = 8; días 19-22) y secretora tardía (LS; *n* = 7; días 23-28). Cada muestra se dividió para análisis de mRNA, proteínas e inmunohistoquímica, priorizando mRNA y proteínas en caso de que el tamaño fuera insuficiente.

Las muestras del grupo 2 de experimentos se recolectaron durante ciclos de estimulación ovárica controlada en el día de la extracción ovocitaria. Se utilizaron para análisis de mRNA (*n* = 15), proteínas (*n* = 15), localización celular y subcelular de PGRMC2 (*n* = 10 y *n* = 7, respectivamente), ensayos de invasión trofoblástica (*n* = 6), RNA-seq (*n* = 4) y SWATH-MS (*n* = 5). Algunas muestras permitieron usarse para múltiples análisis para maximizar los datos obtenidos.

4. Inmunohistoquímica de PGRMC2 en tejido endometrial humano

Para los análisis de inmunohistoquímica (*n* = 14), las muestras del grupo 1 se fijaron en paraformaldehído al 4%, se incluyeron en parafina, y se cortaron en secciones de 5 µm. Tras

la desparafinización, las secciones se incubaron con un anticuerpo policlonal anti-PGRMC2 (dilución 1:300) validado previamente. Los controles negativos se realizaron sin el anticuerpo primario y los positivos utilizaron tejido intestinal humano. La inmunorreactividad se visualizó con DAB y se contrastó con hematoxilina. Las secciones se montaron con *SafeMount* y se analizaron con un microscopio Nikon Eclipse 80i.

5. Aislamiento, cultivo celular y decidualización *in vitro* de hEnSCs

Se aislaron células estromales endometriales (hEnSCs) de las muestras del grupo 2 de experimentos tras digerir tejido con colagenasa IA. Las células estromales se separaron por sedimentación gravitacional y se cultivaron en placas de cultivo antes de ser inducidas a decidualización *in vitro* con bromo-AMPc y medroxiprogesterona (MPA) durante 4 días. Las células control no recibieron tratamiento hormonal.

El levantamiento de las células de la placa de cultivo para los análisis posteriores se realizó con TrypLE Select 1X. La decidualización se verificó mediante la medición de PRL en el medio celular (ELISA, $n = 15$) y la reorganización del citoesqueleto con tinción de F-actina ($n = 10$). Las imágenes de F-actina se obtuvieron con un microscopio fluorescente Zeiss AXIO.

6. Inmunofluorescencia de PGRMC2 en hEnSCs

Para analizar la localización de PGRMC2 en hEnSCs de muestras del grupo 2, las células decidualizadas (d-hEnSCs, $n = 10$) y no decidualizadas (nd-hEnSCs, $n = 10$) se fijaron, permeabilizaron y bloquearon para prevenir uniones inespecíficas de otras proteínas. Posteriormente, se incubaron con un anticuerpo primario anti-PGRMC2 (0.4 µg/mL) y un anticuerpo secundario fluorescente Alexa Fluor 488. Los núcleos se tiñeron con DAPI. Las imágenes se obtuvieron mediante un microscopio de fluorescencia Zeiss, lo que permitió observar y comparar la distribución de PGRMC2 en ambas condiciones celulares.

7. Extracción del proteoma subcelular en hEnSCs

Se empleó un kit comercial para fraccionar las proteínas citoplasmáticas, de membrana/orgánulos y nucleares de d-hEnSCs y nd-hEnSCs obtenidas de las muestras del

grupo 2 de experimentos (*n* = 7 por fracción subcelular). La pureza de las fracciones se verificó mediante western blot (WB) usando marcadores específicos: Hsp70 para el citoplasma, CD98 para membranas/orgánulos e histona H3 para el núcleo. La expresión de PGRMC2 en estas fracciones se cuantificó mediante WB, utilizando β-actina como control de carga, y los resultados se expresaron como abundancia relativa de proteínas (PGRMC2/β-actina) ± desviación estándar (SD).

8. Extracción de proteínas y WB

Se analizaron los niveles de PGRMC2 (≈28 kDa) en tejido endometrial a lo largo del ciclo menstrual (grupo 1, *n* = 35) y en d-hEnSCs (grupo 2, *n* = 15). Las proteínas se extrajeron con un tampón de lisis RIPA, seguido de centrifugación para recuperar el sobrenadante. La concentración de proteínas se determinó mediante el ensayo de Bradford.

Las proteínas (15 µg por muestra) se separaron por SDS-PAGE, se transfirieron a membranas PVDF y se detectaron con anticuerpos específicos contra PGRMC2, PGRMC1 y receptores clásicos de la P4 (PGR-α y PGR-β). La β-actina se usó como control de carga. Las bandas se visualizaron mediante quimioluminiscencia y se analizaron con ImageJ, expresando los resultados como abundancia relativa normalizada (media ± SD). Esto permitió comparar los niveles de PGRMC2 entre fases del ciclo menstrual y condiciones de decidualización *in vitro*, proporcionando información clave sobre su función en el endometrio.

9. Extracción de RNA, transcripción inversa y qPCR a tiempo real.

Se evaluó la expresión relativa de mRNA de *PGRMC2* en las distintas fases del ciclo menstrual (*n* = 38; EP, *n* = 8; LP, *n* = 8; ES, *n* = 8; MS, *n* = 7; LS, *n* = 7) y en hEnSCs (*n* = 15) bajo condiciones de decidualización *in vitro* mediante PCR cuantitativa a tiempo real (RT-qPCR), comparándola con *PGRMC1* y *PGRs* clásicos.

9.1 Extracción y cuantificación de RNA

El RNA se extrajo con Trizol (en el caso de los tejidos endometriales) o el kit de RNeasy Mini (en el caso de hEnSCs) y se cuantificó con un espectrofotómetro NanoDrop. Posteriormente, se sintetizó cDNA a partir de 0.5 µg de RNA por muestra.

9.2 RT-qPCR

Se utilizó el sistema StepOnePlus y el PowerUp SYBR Green Master Mix. Las reacciones se realizaron por duplicado con *primers* específicos para *PGRMC2*, *PGRMC1*, *PGRs* clásicos y otros genes para la validación de la transcriptómica.

9.3 Análisis de datos y expresión relativa génica

La expresión relativa se calculó mediante el método $2^{-\Delta\Delta CT}$, normalizando con *GAPDH* como control endógeno. Los resultados se expresaron como *fold change* (FC) y se compararon entre fases del ciclo y condiciones decidualizadas.

10. Silenciamiento de *PGRMC2* en hEnSCs

Para evaluar el papel de PGRMC2, se realizó un silenciamiento génico en hEnSCs mediante siRNA específico. Las células transfectadas con siRNA para *PGRMC2* (i*PGRMC2* d-hEnSCs) se compararon con controles internos (scramble; Sc d-hEnSCs) y células control negativo no transfectadas (control d-hEnSCs). La eficacia del silenciamiento se verificó mediante RT-qPCR y WB.

10.1 Inmunofluorescencia de Ki-67 después del silenciamiento de *PGRMC2* en hEnSCs

Se evaluó si la proliferación celular se veía afectada tras el silenciamiento de *PGRMC2* mediante tinción del marcador de proliferación Ki-67 (n = 3). Las células se fijaron, permeabilizaron y se tiñeron con anticuerpos específicos. A continuación, la proliferación se cuantificó como porcentaje de células Ki-67 positivas sobre el total (células marcadas con DAPI) ± SD.

10.2 Niveles de PRL después del silenciamiento de *PGRMC2* en hEnSCs

Se midieron los niveles de PRL en el medio de cultivo de las d-hEnSCs tras el silenciamiento de *PGRMC2* mediante la técnica de ELISA (*n* = 14), expresando los resultados como porcentaje relativo respecto al control.

10.3 Análisis transcriptómico mediante RNA-seq

Se comparó Sc d-hEnSCs e i*PGRMC2* d-hEnSCs mediante un conteo bruto obtenido de las bibliotecas de datos de RNA-seq. Posteriormente, la matriz fue procesada y sometida a un análisis estadístico exhaustivo.

10.3.1 Análisis de genes diferencialmente expresados (DEGs)

Se identificaron DEGs entre Sc d-hEnSCs e i*PGRMC2* d-hEnSCs mediante DESeq2, seleccionando DEGs con *P* valor ajustado < 0.01 y FC >2 o <0.5 (*n* = 4).

10.3.2 Análisis de enriquecimiento funcional

Los DEGs se analizaron con g:Profiler y Cytoscape, identificando procesos biológicos, componentes celulares y funciones moleculares afectados. Los resultados se representaron mediante mapas funcionales de enriquecimiento. Cytoscape utiliza los valores de *P* de g:Profiler para representar la significancia de cada nodo mediante su color, calculado con $-\log_{10}$(*P* valor)*sign(NES). El NES permite comparaciones estandarizadas entre conjuntos de genes, facilitando la interpretación visual del enriquecimiento.

10.3.3 Validación de RNA-seq

Los resultados de RNA-seq se validaron mediante RT-qPCR en genes seleccionados por su relevancia en la reproducción femenina (como *CXCL14* y *HGF*), normalizando los datos con *GAPDH* y expresándolos como FC ± SD (*n* = 4).

10.4 Análisis proteómico mediante SWATH-MS

Se realizó el análisis SWATH-MS en d-hEnSCs (*n* = 5) y se agruparon para construir una biblioteca espectral utilizando un sistema Ekspert nanoLC 425 y un espectrómetro de masas nanoESI qQTOF MS 6600. Los datos se procesaron con ProteinPilot v5.0 para identificar y cuantificar proteínas.

10.4.1 Análisis de expresión diferencial de proteínas (DEPs)

Se cargaron 20 µg de proteína total por muestra en geles 1D-SDS-PAGE para su una mejor concentración y purificación. Los archivos obtenidos fueron analizados con Peak View y Marker View (FDR < 1%), normalizando las áreas proteicas con respecto al total de áreas cuantificadas. Las proteínas con $P < 0.05$ se consideraron DEPs entre Sc d-hEnSCs e i*PGRMC2* d-hEnSCs.

10.4.2 Análisis de enriquecimiento funcional

Los DEPs comunes se analizaron mediante g:Profiler (FDR < 0.05) para identificar procesos biológicos, funciones moleculares y componentes celulares enriquecidos. Los resultados se visualizaron como mapas de enriquecimiento funcional mediante el plugin EnrichmentMap en Cytoscape, lo que facilitó la interpretación de relaciones biológicas complejas.

10.5 Invasión trofoblástica tras el silenciamiento de *PGRMC2* en hEnSCs

Se utilizó un modelo de co-cultivo con células d-hEnSCs (control, Sc e i*PGRMC2*; *n* = 6) y esferoides trofoblásticos derivados de la línea celular JEG-3. Los esferoides, marcados con *CellTracker* azul, se formaron en placas de baja adhesión durante 48 horas y luego se transfirieron a placas donde estaban cultivadas las d-hEnSCs.

La expansión del área trofoblástica (EA) se evaluó a las 24 y 48 horas mediante un microscopio fluorescente AXIO y se cuantificó con ImageJ. Los datos se normalizaron dividiendo el EA de cada esferoide por el EA promedio en d-hEnSCs control. Además, se verificó la decidualización midiendo PRL por ELISA para descartar que las variaciones en la invasión estuvieran influenciadas por diferencias en este proceso.

11. Análisis estadístico

El análisis estadístico y la generación de gráficos se realizaron con GraphPad Prism 8.3.0. La normalidad de los datos se evaluó mediante pruebas como Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk y Kolmogorov-Smirnov.

Se aplicó el test de *one-way* ANOVA para analizar la expresión génica de *PGRMC2*, *PGRMC1* y *PGR-t* a lo largo del ciclo menstrual y las diferencias de abundancia proteica de PGRMC2 entre control, Sc e i*PGRMC2* d-hEnSCs. El test de Kruskal-Wallis se utilizó para evaluar la expresión de *PGR-β* y *PGRMC2* en WB tanto en el ciclo menstrual como en el modelo de invasión trofoblástica entre control, Sc e i*PGRMC2* d-hEnSCs. El test de Wilcoxon para muestras pareadas analizó la expresión génica y proteica de PGRMC2 en hEnSCs. Las pruebas t-pareadas evaluaron la expresión de *PGRMC2* y genes validados por RNA-seq, así como los niveles de PRL entre nd- y d-hEnSCs. Además, se comparó la expresión de mRNA de *PGRMC2*, *PGRMC1*, *PGR-t* y *PGR-β* entre d-hEnSCs y nd-hEnSCs, así como diferencias en la expresión génica de *PGRMC2* entre control, Sc e i*PGRMC2* en d-hEnSCs, y el porcentaje de tinción de Ki-67 mediante el test de Friedman. Y, por último, se evaluó la abundancia de PGRMC2 en fracciones subcelulares mediante el test de *two-way* ANOVA.

En todas las pruebas, los resultados se normalizaron y compararon con la fase EP (en los análisis a lo largo del ciclo menstrual) o con un control endógeno según fuera apropiado, y se consideró significativo un $P < 0.05$.

VI. RESULTADOS

Para presentar los resultados de esta tesis, estos se dividen en las dos áreas de estudio mencionadas previamente: la caracterización de la expresión de PGRMC2 en el endometrio humano a lo largo del ciclo menstrual y el análisis funcional de PGRMC2 en el estroma endometrial humano bajo condiciones de decidualización *in vitro*.

1. Caracterización de la expresión de PGRMC2 en el endometrio humano durante el ciclo menstrual

1.1 Localización, expresión de mRNA y abundancia proteica de PGRMC2 a lo largo del ciclo menstrual

1.1.1 Expresión génica de PGRMC2 en tejido endometrial total

El análisis génico en muestras de tejido endometrial de donantes de ovocitos bajo condiciones hormonales naturales mostró una disminución significativa en los niveles de mRNA de *PGRMC2* durante la fase LS en comparación con la fase MS ($P < 0.05$).

1.1.2 Expresión proteica de PGRMC2 en tejido endometrial total

Mediante WB, aunque se detectó una banda de 28 kDa correspondiente a la proteína de PGRMC2 en todas las muestras endometriales analizadas, no se observaron diferencias significativas en la abundancia proteica entre las fases del ciclo menstrual.

1.1.3 Localización de PGRMC2 en tejido endometrial total

Las imágenes de inmunohistoquímica mostraron cambios dinámicos en la distribución de PGRMC2 a lo largo del ciclo menstrual, con mayor abundancia durante la fase MS, especialmente en las glándulas epiteliales endometriales y las hEnSCs, y disminuyendo en LS en todos los subtipos celulares del endometrio. Sin embargo, las células epiteliales luminales mantuvieron una expresión constante desde las fases proliferativas hasta la fase MS. Estos cambios en la tinción de PGRMC2 coinciden con los niveles de mRNA observados, confirmando una disminución significativa de PGRMC2 durante la fase LS en comparación con la fase MS.

1.2 Diferencias en los niveles de mRNA entre *PGRs* clásicos y no clásicos en el endometrio humano durante el ciclo menstrual

La comparación de estos receptores mostró que los niveles de mRNA de *PGRMC1* ($P < 0.05$) y los *PGRs* clásicos ($P < 0.05$) disminuyen significativamente durante la fase secretora en

comparación con la fase proliferativa. Por el contrario, *PGRMC2* no presentó una reducción significativa durante la fase MS.

Al comparar los niveles de mRNA de los receptores no clásicos y clásicos en cada fase, se observó que los no clásicos mostraron mayores niveles de expresión en todas las fases del ciclo ($P < 0.05$). *PGRMC1* destacó como el receptor más expresado en las fases proliferativas y en ES, mientras que *PGRMC2* predominó en la fase LS. Durante la fase MS, los receptores no clásicos *PGRMC1* y *PGRMC2* presentaron un aumento significativo en su expresión en comparación con los *PGRs* clásicos ($P < 0.05$).

2. Estudios funcionales de PGRMC2 en células estromales endometriales

2.1 Localización, análisis de mRNA y proteína de PGRMC2 en hEnSCs durante la decidualización in vitro

Dado los resultados obtenidos en los experimentos *in vivo*, se procedió a analizar la expresión de PGRMC2 en d-hEnSCs y nd-hEnSCs utilizando un modelo de decidualización *in vitro*. La efectividad del modelo se validó mediante la medición de niveles de PRL, que resultaron significativamente más altos en las d-hEnSCs ($P < 0.0001$), confirmando su correcta decidualización.

2.1.1 Expresión génica de *PGRMC2* en hEnSCs

Los niveles de mRNA de *PGRMC2* en d-hEnSCs mostraron un aumento significativo en comparación con las nd-hEnSCs ($P < 0.0001$). Este incremento refuerza los hallazgos de análisis previos sobre su papel en la decidualización.

2.1.2 Expresión proteica de PGRMC2 en hEnSCs

Dado que los niveles de mRNA no siempre reflejan directamente la cantidad de proteína, se evaluaron los niveles proteicos de PGRMC2 mediante WB. Los resultados confirmaron un aumento significativo de la proteína en d-hEnSCs frente a nd-hEnSCs ($P < 0.001$), consistente con los datos de expresión génica.

2.1.3 Localización de PGRMC2 en hEnSCs

La tinción por inmunofluorescencia permitió observar que PGRMC2 se localiza principalmente en las membranas de d-hEnSCs, mientras que en nd-hEnSCs se distribuye de forma más dispersa y menos definida. Para corroborar estos resultados, se fraccionaron citoplasma, membranas/orgánulos y núcleos de las nd-hEnSCs y d-hEnSCs y, a continuación, se analizaron mediante WB. Los resultados confirmaron una mayor abundancia de PGRMC2 en la fracción de membranas/orgánulos en comparación con otras fracciones subcelulares ($P < 0.05$).

2.2 Diferencias en los niveles de mRNA entre *PGRs* clásicos y no clásicos tras la decidualización *in vitro* en hEnSCs

En las nd-hEnSCs, los *PGRs* no clásicos mostraron niveles de mRNA significativamente mayores que los clásicos ($P < 0.001$). Además, tras la decidualización *in vitro*, la expresión de mRNA de los *PGRs* no clásicos también aumentó significativamente ($P < 0.05$), siendo *PGRMC2* el más expresado. Estos resultados reflejan patrones similares a los observados en el tejido endometrial total durante el ciclo menstrual natural.

2.3 Silenciamiento de *PGRMC2* en d-hEnSCs

2.3.1 Validación del silenciamiento de *PGRMC2*

Se evaluó la eficiencia del silenciamiento de *PGRMC2* en hEnSCs mediante RT-qPCR y WB y se observó una reducción del 80% en la expresión génica ($P < 0.01$) y una disminución del 60% en la abundancia proteica ($P < 0.001$) de *PGRMC2* en d-hEnSCs transfectadas con siRNA específico. Estos resultados confirmaron la eficacia del silenciamiento y garantizaron la fiabilidad de los experimentos posteriores.

2.3.2 Impacto del silenciamiento de *PGRMC2* en la proliferación celular en d-hEnSCs

Para asegurar que el método de transfección no afectaba la proliferación celular, se analizó la expresión del marcador de proliferación Ki-67 mediante inmunofluorescencia en d-hEnSCs control, Sc e i*PGRMC2*. No se encontraron diferencias significativas en la proporción de células

positivas para Ki-67 entre los grupos, confirmando que el proceso de transfección no alteró la proliferación celular.

2.3.3 Impacto del silenciamiento de *PGRMC2* en los niveles de PRL en d-hEnSCs

Se evaluó el efecto del silenciamiento de *PGRMC2* en la decidualización midiendo los niveles de PRL. Los niveles de PRL fueron significativamente más altos en las i*PGRMC2* d-hEnSCs en comparación con las Sc d-hEnSCs ($P < 0.05$).

2.3.4 Efectos transcriptómicos del silenciamiento de *PGRMC2* durante la decidualización

El análisis RNA-seq en i*PGRMC2* d-hEnSCs reveló 4687 DEGs (2420 sobreexpresados y 2267 infraexpresados, FDR < 0.05). El análisis de enriquecimiento funcional posterior demostró que los genes sobreexpresados están involucrados en procesos biológicos como la angiogénesis, migración celular, adhesión celular, función del retículo endoplásmico (ER), transporte vesicular y biosíntesis de lípidos, mientras que los infraexpresados se asociaron con respiración aeróbica y síntesis ribosomal. A nivel celular, los genes altamente expresados en i*PGRMC2* d-hEnSCs estuvieron vinculados con el núcleo, ER, aparato de Golgi y matriz extracelular, mientras que los menos expresados se asociaron con mitocondrias y ribosomas.

Posteriormente, se realizó una validación de los resultados de RNA-seq mediante RT-qPCR, confirmando el incremento en los niveles génicos de *TNC* ($P < 0.05$) y la infraexpresión de *PGRMC2* ($P < 0.0001$), *DEPP1* ($P < 0.05$) y *CXCL14* ($P < 0.05$) cuando se silenciaba *PGRMC2*. No se observaron diferencias significativas en *COL1A1* ni *HGF*.

2.3.5 Efectos proteómicos del silenciamiento de *PGRMC2* durante la decidualización

Dado que se observaron diferencias significativas en la expresión de mRNA entre las fases MS y LS del ciclo menstrual, pero no a nivel proteico en el análisis de WB, se exploraron los efectos proteómicos del silenciamiento de *PGRMC2* en d-hEnSCs mediante SWATH-MS. El análisis identificó 2070 proteínas en total, con 28 DEPs, de las cuales 4 estaban sobreexpresadas y 24 infraexpresadas ($P < 0.05$).

El análisis de enriquecimiento funcional de las DEPs reveló alteraciones en procesos biológicos clave como respiración celular, respuesta celular, metabolismo, localización de proteínas en el ER y biosíntesis de ribonucleósidos. En cuanto a componentes celulares, se asociaron principalmente con membranas de orgánulos, vesículas, matriz extracelular, ER y mitocondrias. A nivel funcional, se detectaron cambios significativos en la unión molecular, actividad transportadora y biosíntesis de esteroides.

2.3.6 Efectos del silenciamiento de *PGRMC2* en la invasión trofoblástica

A partir de los resultados transcriptómicos y proteómicos que evidenciaron alteraciones en procesos relacionados con migración celular, adhesión y remodelación de la matriz extracelular, se evaluó el impacto del silenciamiento de *PGRMC2* en la invasión trofoblástica mediante un modelo *in vitro*.

En el co-cultivo de i*PGRMC2* d-hEnSCs con esferoides trofoblásticos JEG-3, se observó una disminución significativa en la expansión de los esferoides en las i*PGRMC2* d-hEnSCs en comparación con los controles ($P < 0.001$) y Sc d-hEnSCs ($P < 0.01$). De hecho, las esferas en i*PGRMC2* d-hEnSCs mostraron una expansión similar entre las 24 y 48 horas.

VII. DISCUSIÓN

En el útero, tanto los PGRs clásicos como los no clásicos muestran fluctuaciones en su expresión a lo largo del ciclo menstrual, regulando eventos críticos para la receptividad endometrial y el desarrollo del embarazo. Aunque los receptores clásicos son fundamentales en la mayoría de los procesos reproductivos femeninos, las células, en ausencia de estos receptores, son capaces de responder a la P4, lo que sugiere un papel adicional de los receptores no clásicos, como PGRMC2.

Previamente, PGRMC2 se ha asociado con funciones como la inhibición del desarrollo de folículos antrales y la regulación de la expresión de factores angiogénicos y hormonales. Sin embargo, sus patrones de expresión y roles específicos en el endometrio humano, particularmente durante la decidualización, permanecen sin esclarecer. En este trabajo, se

examinó la expresión génica y proteica de PGRMC2 a lo largo del ciclo menstrual en tejido endometrial humano sano de mujeres donantes de ovocitos bajo condiciones normales.

Se observó que las glándulas epiteliales del endometrio presentaron la mayor expresión de PGRMC2 durante el ciclo, con una disminución significativa en la fase LS, lo que coincide con estudios previos en primates y pacientes con endometriosis. En las hEnSCs, la expresión de mRNA de *PGRMC2* osciló durante el ciclo, disminuyendo significativamente en la fase LS respecto a la MS. A diferencia de PGRMC1, cuya expresión mimetiza los patrones de expresión de los *PGRs* clásicos, *PGRMC2* mostró un aumento significativo en la fase MS, lo que refuerza su posible contribución al proceso de implantación. Sin embargo, no se observaron cambios a nivel proteico.

La discrepancia entre los niveles de mRNA y proteína de PGRMC2 a lo largo del ciclo menstrual indica que este receptor podría actuar a nivel genómico, modulando la expresión de genes sin necesariamente correlacionar con cambios en su propia abundancia proteica. Además, el análisis de WB mide los niveles totales de proteína, lo que podría no captar fluctuaciones transitorias o localizadas detectables mediante inmunohistoquímica, la cual mostró variaciones en la intensidad de la tinción de PGRMC2 entre las fases del ciclo menstrual.

Estudios previos han demostrado que PGRMC1 regula la receptividad endometrial y la decidualización, influyendo en procesos biológicos relacionados y en la producción de prostaglandinas. De la misma manera, en nuestro modelo *in vitro* de decidualización, la expresión génica y proteica de PGRMC2 aumentó significativamente en células decidualizadas, sugiriendo que su deficiencia podría contribuir a fallos reproductivos, como se ha documentado en modelos animales con anterioridad. Los análisis transcriptómicos y proteómicos respaldan esta hipótesis, mostrando que el silenciamiento de *PGRMC2* altera significativamente los niveles de expresión en d-hEnSCs. De hecho, el silenciamiento de *PGRMC2* desreguló genes clave asociados con la decidualización y la angiogénesis, como *ADAMTS-5*, *S100A10*, *LEPR* e *ITGB8*.

De hecho, los genes infraexpresados cuando se inhibe *PGRMC2* están involucrados en la respiración aeróbica, la producción de energía mitocondrial y la síntesis de proteínas,

reflejando defectos mitocondriales que podrían impactar en la fertilidad y la función endometrial. Además, nuestros resultados muestran que el silenciamiento de *PGRMC2* alteró significativamente las vías relacionadas con procesos oxidativos, como las proteínas MIRO1, QCR9, SDHB, CMC1 y DLST, indicando la ausencia de mecanismos compensatorios en la expresión génica para la reducción de proteínas mitocondriales.

A su vez, se observó una sobreexpresión de genes asociados al ER y al sistema de endomembranas, incluyendo el aparato de Golgi, la membrana plasmática y uniones celulares, lo que podría afectar la síntesis proteica. Genes relacionados con la homeostasis, como *ABCA1* y *CCR1*, también se vieron afectados, sugiriendo que PGRMC2 no solo regula funciones mitocondriales, sino también la estructura celular y la respuesta a señales celulares. Además, los genes sobreexpresados tras el silenciamiento de *PGRMC2* también están implicados en morfogénesis, angiogénesis, migración y adhesión celular, como ocurre con los *PGRs* clásicos cuando se silencian en hEnSCs, lo que sugiere que PGRMC2 y los PGRs clásicos actúan a la par en estos procesos.

A nivel proteómico, se detectó una sobreexpresión de proteínas localizadas en el ER en d-hEnSCs. Estos cambios corroboran el papel de PGRMC2 en la remodelación secretora que ocurre durante la decidualización.

En conjunto, la complementariedad entre PGRMC1 y PGRMC2 refuerza su papel en la remodelación citoplasmática que ocurre durante el proceso de decidualización. Asimismo, la implicación de PGRMC2 en el metabolismo lipídico y su biosíntesis sugiere que podría regular el ambiente lipídico del endometrio, esencial para la comunicación celular, así como los cambios estructurales y funcionales, cruciales para una correcta implantación embrionaria.

El silenciamiento de *PGRMC2* también reveló genes y proteínas relacionadas con sinapsis neuronales, lo que propone una función en procesos de comunicación celular y plasticidad en las hEnSCs, eventos cruciales para la decidualización y remodelación tisular. Este hallazgo inesperado apunta a una conexión entre vías de señalización neuronal y funciones endometriales, destacando la adaptabilidad de las hEnSCs ante señales celulares.

Además, el silenciamiento de *PGRMC2* alteró la expresión de biomarcadores de invasión embrionaria, como *S100A10*, *SERPINE1* y *CXCL12*, esenciales para la correcta invasión

trofoblástica. Nuestros hallazgos con el modelo de invasión *in vitro* evidencian que PGRMC2 regula la migración celular durante la invasión trofoblástica, mientras que el estrés elevado del ER asociado con la falta de PGRMC2 podría explicar los fallos post-implantacionales. Asimismo, el aumento de PRL en las i*PGRMC2* d-hEnSCs podría interpretarse como un mecanismo compensatorio, considerando que la PRL desempeña un papel clave al interactuar con citoquinas y factores de crecimiento que regulan procesos como la decidualización y la invasión trofoblástica. Sin embargo, la ausencia de PGRMC2 parece interrumpir estas conexiones, sugiriendo que, aunque el aumento de PRL intenta compensar esta deficiencia, no logra sostener eficazmente la decidualización ni la invasión trofoblástica.

Por último, la diferencia de expresión de los PGRs no clásicos frente a los clásicos en las fases menstruales y en las hEnSCs subraya la importancia de los PGRs no clásicos en las funciones reproductivas, destacando la importancia de la complementariedad entre la acción de PGRMC1 y PGRMC2.

Dado el tamaño limitado de las muestras en los análisis transcriptómicos, proteómicos, el modelo de invasión embrionaria y el análisis de Ki-67, las conclusiones de este estudio deben interpretarse con cautela. Mientras que el análisis transcriptómico mide los niveles de mRNA, estos no siempre se correlacionan con la expresión proteica debido a modificaciones post-transcripcionales y mecanismos reguladores. Por su parte, la proteómica presenta limitaciones, como la dificultad para detectar proteínas de baja abundancia, lo cual podría explicar el reducido número de DEPs identificadas. Además, la complejidad de las modificaciones post-traduccionales y la naturaleza dinámica del proteoma complican la interpretación de los datos. No obstante, la consistencia entre los hallazgos transcriptómicos y proteómicos respalda la relevancia biológica de los cambios observados.

El uso de una población primaria de hEnSCs diferenciadas *in vitro* no refleja completamente la heterogeneidad del endometrio *in vivo* ni las comunicaciones paracrinas entre sus distintos tipos celulares. Asimismo, los resultados obtenidos por WB sobre PGRMC2 deben ser considerados con precaución, ya que podrían no describir plenamente la compleja regulación funcional en el endometrio. Además, este estudio solo analizó PGRMC2 en las hEnSCs, quedando por investigar su papel en el compartimento epitelial.

A pesar de estas limitaciones, los resultados tienen importantes implicaciones en la reproducción asistida. La identificación de PGRMC2 como regulador clave en la decidualización y la invasión embrionaria abre nuevas oportunidades para desarrollar terapias dirigidas que mejoren los tratamientos de fertilidad, identificar nuevos biomarcadores en la fisiología endometrial y personalizar las intervenciones médicas. Asimismo, PGRMC2 podría convertirse en una diana terapéutica para tratar afecciones como el RIF y la endometriosis, mejorar la receptividad endometrial y las tasas de embarazo y recién nacido vivo en pacientes sometidas a ART.

Futuras investigaciones podrían enfocarse en ampliar el tamaño de la población de muestras para validar estos hallazgos y explorar las interacciones de PGRMC2 con otros componentes endometriales. Estudios *in vivo* en modelos animales podrían proporcionar información valiosa sobre su papel en la implantación y el mantenimiento del embarazo. Además, técnicas avanzadas como la secuenciación de RNA de célula única podrían identificar las funciones específicas de PGRMC2 en cada tipo celular del endometrio. También sería relevante investigar la regulación epigenética de PGRMC2, lo que podría revelar nuevas capas de complejidad en su función y control.

Este estudio representa la primera caracterización de la expresión de PGRMC2 a lo largo del ciclo menstrual humano y su rol en la decidualización *in vitro* de las hEnSCs. Proporciona un análisis comparativo entre los sistemas de PGRs clásicos y no clásicos de P4, junto con un enfoque transcriptómico y proteómico para evaluar las implicaciones funcionales del silenciamiento dirigido de *PGRMC2*. Sin embargo, se necesitan estudios adicionales para validar el mecanismo de acción de PGRMC2 y su relación con otros receptores durante la decidualización, con el fin de comprender plenamente su papel en la reproducción femenina.

VIII. CONCLUSIONES

Las principales conclusiones extraídas de esta tesis son:

1. La expresión génica de *PGRMC2* fluctúa en el endometrio humano a lo largo del ciclo menstrual, alcanzando un pico significativo durante la fase media secretora en comparación con la fase tardía secretora.

2. No se detectaron cambios en la abundancia de la proteína PGRMC2 a lo largo del ciclo menstrual humano, a pesar de las variaciones significativas en el mRNA. En contraste, el análisis de inmunohistoquímica reveló variaciones en la intensidad de PGRMC2, particularmente en las células glandulares y estromales.

3. Tras la decidualización *in vitro*, la expresión génica y la abundancia proteica de PGRMC2 aumentaron significativamente en las células estromales endometriales humanas, particularmente en las membranas celulares y los orgánulos.

4. La expresión de *PGRs* no clásicos fue significativamente mayor que la de los *PGRs* clásicos a lo largo del ciclo menstrual y en las células estromales endometriales humanas, tanto no decidualizadas como decidualizadas. Mientras que la expresión génica de *PGRMC1* y los *PGRs* clásicos disminuyó significativamente durante las fases secretoras, *PGRMC2* es el receptor más expresado en la fase tardía secretora.

5. El silenciamiento de *PGRMC2* en células estromales endometriales humanas decidualizadas resultó en cambios significativos a nivel transcriptómico y proteómico. Estos cambios afectaron a una variedad de procesos biológicos, como la angiogénesis, migración celular, adhesión celular y respuesta celular; así como a componentes celulares como el ER, vesículas, la matriz extracelular, los orgánulos, las membranas plasmáticas y los componentes mitocondriales.

6. El silenciamiento de *PGRMC2* perjudica la capacidad de las células estromales endometriales decidualizadas para facilitar la invasión trofoblástica, subrayando la importancia de PGRMC2 en la interacción entre las células estromales endometriales y el trofoblasto.

INDEX

ABBREVIATIONS

1D-SDS-PAGE	One-Dimensional Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
8-Br-cAMP	8-Bromo-cyclic adenosine monophosphate
ABCA1	ATP Binding Cassette Subfamily A Member 1
ADAMTS-5	A Disintegrin and Metalloproteinase with Thrombospondin Motifs 5
AF1	Activation Function 1
AF2	Activation Function 2
AF3	Activation Function 3
AKT	Protein Kinase B
AMH	Antimüllerian Hormone
ANXA2	Annexin A2
ART	Assisted Reproductive Technology
BMI	Body Mass Index
BMP2	Bone Morphogenetic Protein 2
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary DNA
cGMP	Cyclic Guanosine Monophosphate
CD98	L-type amino acid transporter 1
CMC1	Copper Metabolism Domain Containing Protein 1
COL1A1	Collagen Type I Alpha 1 Chain
COOH	Carboxyl Terminal Region
COUP-TFII	Chicken Ovalbumin Upstream Promoter Transcription Factor II
COX2	Cyclooxygenase 2
CRH	Corticotropin-Releasing Hormone
Cyt	Cytochrome
CXCL12	Chemokine (C-X-C Motif) Ligand 12
CXCL14	Chemokine (C-X-C Motif) Ligand 14

DAB	Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA-Binding Domain
db-cAMP	Dibutyryl Cyclic Adenosine Monophosphate
DEGs	Differentially Expressed Genes
DEPP1	DEPP Autophagy Regulator 1
DEPs	Differentially Expressed Proteins
DES	Diethylstilbestrol
DHT	Dihydrotestosterone
DLST	Dihydrolipoamide S-Succinyltransferase
DMEM	Dulbecco's Modified Eagle Medium
EA	Expanded Area
E2	Estradiol
E2R	Estradiol Receptors
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
EP	Early Proliferative Phase
ER	Endoplasmic Reticulum
ERE	Estrogen Response Elements
ERK1/2	Extracellular Signal-Regulated Kinases 1 and 2
ES	Early Secretory Phase
F-actin	Filamentous Actin
FBS	Fetal Bovine Serum
FDR	False Discovery Rate
FGF	Fibroblast Growth Factors
FOXO1	Forkhead Box Protein O1
FSH	Follicle-Stimulating Hormone
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
GLI1	GLI Family Zinc Finger 1
HAND2	Neural Crest Derivatives Expressed Transcript 2
HB-EGF	Heparin-Binding Epidermal Growth Factor
h	Hours
hCG	Human Chorionic Gonadotropin
hEnECs	Human Endometrial Epithelial Cells
hEnSCs	Human Endometrial Stromal Cells

HGF	Hepatocyte Growth Factor
HOXA10	Homeobox A10
HRP	Horseradish Peroxidase
IHH	Indian Hedgehog
GFBP1	Insulin-Like Growth Factor-Binding Protein 1
IGF-1	Insulin-Like Growth Factor 1
IGF1R	Insulin-Like Growth Factor 1 Receptor
IL-1β	Interleukin 1 Beta
IL-11	Interleukin 11
IUD	Intrauterine Device
LBD	Ligand-Binding Domain
LEPR	Leptin Receptor
LH	Luteinizing Hormone
LP	Late Proliferative Phase
LS	Late Secretory Phase
MAPK	MAP Kinase
MIRO1	Mitochondrial Rho GTPase 1
MMPs	Matrix Metalloproteinases
MPA	Medroxyprogesterone 17-Acetate
mPRα	Membrane Progesterone Receptor Alpha
mPRβ	Membrane Progesterone Receptor Beta
mPRγ	Membrane Progesterone Receptor Gamma
mPRδ	Membrane Progesterone Receptor Delta
mPRε	Membrane Progesterone Receptor Epsilon
MS	Mid-Secretory Phase
MSCs	Mesenchymal Stem Cells
MUC-1	Glycoprotein Mucin 1
NES	Normalized Enrichment Score
NFKβ	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NOTCH1	Neurogenic Locus Notch Homolog Protein 1
OCP	Oral Contraceptive Pills
ORA	Over-Representation Analysis

P4	Progesterone
PAI-1	Plasminogen Activator Inhibitor 1
PAQR	Progestin and AdipoQ Receptors
PBS	: Phosphate-Buffered Saline
PGE2	Prostaglandin E2
PI3K/AKT	Phosphatidylinositol 3-Kinase/Protein Kinase B Pathway
PKA	Protein Kinase A
PKG	Protein Kinase G
PLC	Phospholipase C
PGR	Progesterone Receptor
PGR-α	Progesterone Receptor Alpha Isoform
PGR-β	Progesterone Receptor Beta Isoform
PGR-γ	Progesterone Receptor Gamma Isoform
PGRMC1	Progesterone Receptor Membrane Component 1
PGRMC2	Progesterone Receptor Membrane Component 2
PRL	Prolactin
PTCH1	Patched 1 Protein
RIF	Recurrent Implantation Failure
RNA-seq	RNA Sequencing
RLN	Relaxin
RT	Room Temperature
RT-qPCR	Real Time Quantitative Polymerase Chain Reaction
Sc	Scrambled siRNA Control
SD	Standard Deviation
SDHB	Succinate Dehydrogenase Complex Subunit B
SERBP1	SERPINE1 mRNA Binding Protein 1
SERPINE1	Plasminogen Activator Inhibitor Type 1
SH2	Src Homology 2 Domain
SH3	Src Homology 3 Domain
siRNA	Small Interfering RNA
SMO	Smoothened Protein
SOX	SRY-Box Transcription Factor
SPP1	Secreted Phosphoprotein 1

SUMO	Small Ubiquitin-Like Modifier
SWATH-MS	Sequential Window Acquisition of All Theoretical Mass Spectra
TGF-β	Transforming Growth Factor Beta
TIMPs	Tissue Inhibitors of Metalloproteinases
TM	Transmembrane Domain
TNC	Tenascin C
TE	Trophoectoderm
uNK	Uterine Natural Killer Cells
uPA	Urokinase-Type Plasminogen Activator
VEGF-A	Vascular Endothelial Growth Factor-A
WB	Western Blot
WNT	Wingless-Related Integration Site

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I. INTRODUCTION

I. INTRODUCTION

1. THE FEMALE REPRODUCTIVE SYSTEM

1.1. ANATOMY AND PHYSIOLOGY OF THE FEMALE REPRODUCTIVE SYSTEM

The female reproductive system is composed of internal reproductive organs and external genitalia (Jones and Lopez, 2014; Roura *et al.*, 2014). The internal organs include the ovaries, fallopian tubes, and uterus, which play key roles in ovulation, embryo transport and implantation, pregnancy development and parturition. The external genitalia contain the vagina, vulva, perineum, and urethra.

The ovaries host the oocytes and produce and secrete female sex hormones [mainly estrogen or estradiol (E2) and progesterone (P4)]. The fallopian tubes transport the oocyte from the ovaries to the uterine cavity and it is divided into three zones: infundibulum, ampulla and isthmus. During copulation, sperm travel up from the vagina to the ampulla zone to fertilize the oocyte and following, the zygote would be transported to the uterine cavity. Lastly, the uterus harbours the embryo implantation, placenta formation and embryo fetal development until birth (Graziottin and Gambini, 2015). The anatomy of the human internal female reproductive organs is illustrated in Figure 1.

The uterine wall consists of three distinct layers: perimetrium, which is the thin outermost and serous layer covering the external surface, the myometrium, the thick middle muscular layer capable of supporting growing fetus(es) during pregnancy and producing very strong contractions required for birth; and finally, the endometrium, the internal, mucous layer and more complex layer of the uterus that will be explained in detail in the following section.

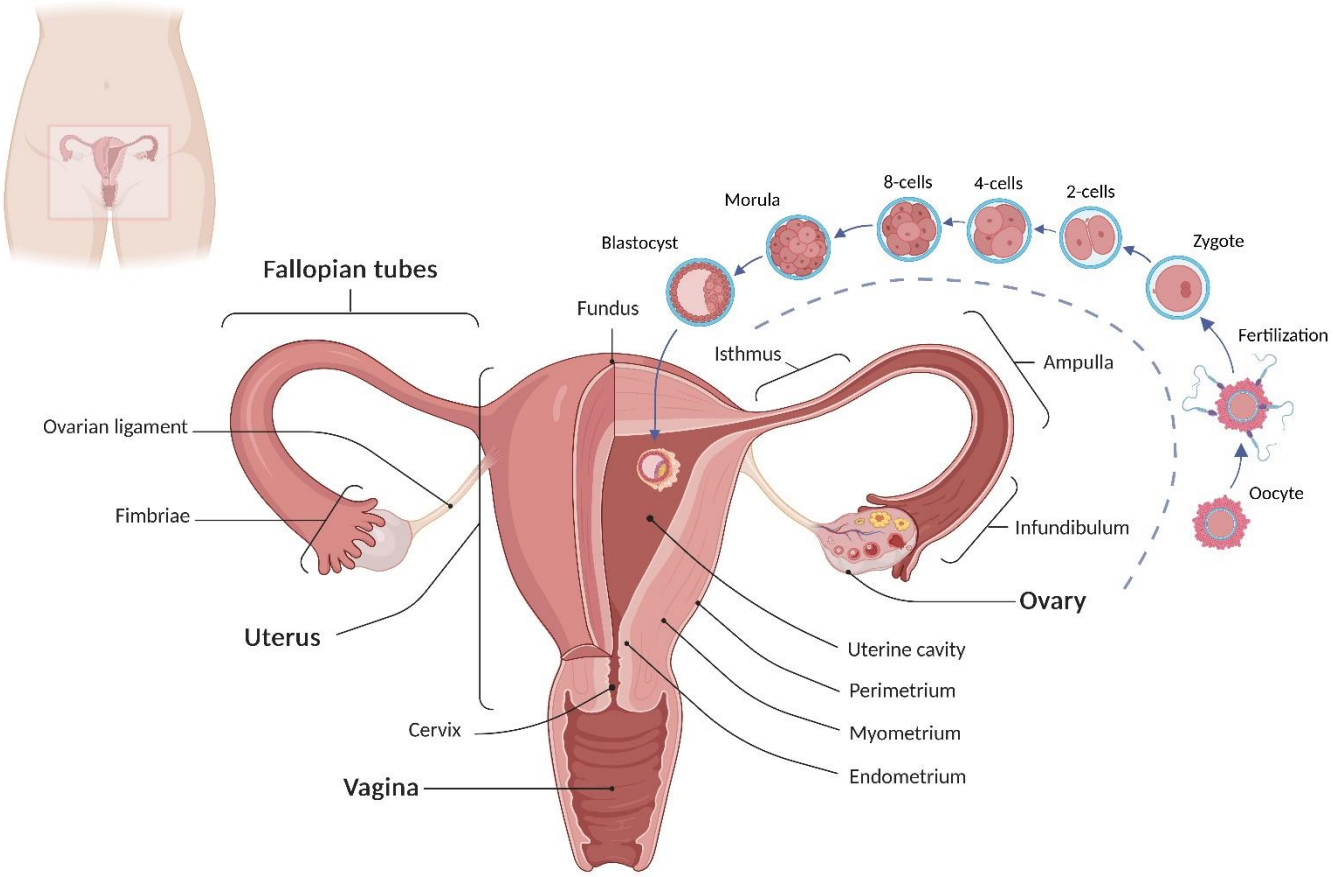

Figure 1. Anatomy of the human female internal organs of the reproductive system and the process of embryo formation and implantation. The primary internal organs of the human female reproductive system, illustrated in the figure, play crucial roles in gestation. Successful pregnancy requires several critical steps: the proper development and release of a mature oocyte from the ovaries, its fertilization by a sperm cell in the ampulla section of the fallopian tubes, an appropriate pre-implantation development as it travels through the fallopian tubes, and finally, blastocyst implantation in the uterine endometrium. *Created with BioRender.com (2024).*

1.2. THE HUMAN ENDOMETRIUM

The human endometrium is a hormonally-regulated tissue that plays a critical role in embryo implantation, pregnancy maintenance (Salazar and Calzada, 2007; Loutradis *et al.*, 2008; Morel *et al.*, 2016) and menstruate in the absence of pregnancy (Critchley *et al.*, 2020). Since the endometrium is essential for embryo implantation (Graziottin and Gambini, 2015), any disorder or complication in this mucous tissue can directly compromise female fertility.

1.2.1 The endometrium: cellular composition and organization

The endometrium is composed of epithelial and stromal compartments supported by an endogenous niche of stem cells, endometrial immune cells, and a vascular network (Figure 2A) (Carrascosa *et al.*, 2018).

The endometrial epithelial compartment consists of a monolayer of columnar polarized cells and can be divided into luminal epithelium and epithelial glands, both composed of endometrial epithelial cells (hEnECs). The primary function of this arrangement is to regulate and allow embryo implantation. Meanwhile the stromal compartment contains fibroblast cells known as endometrial stromal cells (hEnSCs) (Simón *et al.*, 2009) which play a pivotal role during endometrial establishment (Simón *et al.*, 2009; Cunningham *et al.*, 2022).

The endometrial immune cell population, located in the stroma, is composed of uterine natural killer cells (uNK), macrophages and T lymphocytes (Simón *et al.*, 2009). On the other hand, the endometrial vascular network initiates in the myometrium and leads to the endometrium. This vascular network is essential for the regularly-occurring intrinsic angiogenesis along the menstrual cycle (Adair and Montani, 2011): during menstruation where new blood vessels are regenerated from the existing ones, at the beginning of the cycle when the tissue grows and the arteries elongate, and during endometrial establishment phase when the spiral arteries sprout. Likewise, if fertilization occurs, angiogenesis takes place during embryo implantation and placentation (Cunningham *et al.*, 2022). A reservoir of mesenchymal stem cells (MSCs) is also found in this compartment (Figure 2A-B).

The endometrium can also be divided in two layers, the *stratum functionalis* (functional layer) and the *stratum basalis* (basal layer). The functional layer of the endometrium is the portion that undergoes the most changes and is shed during menstruation. Meanwhile, the underlying basal layers remain in place to act as an endometrial supply, ramifying the blood vessels from basal layer to functional layer and regenerating the new functional layer in the next menstrual cycle (Figure 2B).

1.2.2 The endometrial function and the menstrual cycle

The primary function of the endometrium is to provide a suitable environment for embryo implantation and the corresponding pregnancy. Accordingly, in response to the cyclic action of steroid hormones (such as E2 and P4), endometrial tissues undergo physiological changes as the functional layer thickens by 5-7 mm during each menstrual cycle to enhance receptivity to embryo implantation, a process commonly referred to as the "window of implantation" (WOI) (Graham and Clarke, 1997; Baerwald *et al.*, 2012; Ruiz-Alonso *et al.*, 2013; Yoshinaga, 2014). This short period normally occurs between days 19-22 of the menstrual cycle (Navot *et al.*, 1991; Domínguez *et al.*, 2003), and represents a complex and multifactorial process, involving molecular, cellular and tissue changes.

The human menstrual cycle consists mainly of two phases: proliferative phase (days 0-14) and secretory phase (days 15-28), with a usual total duration of 28 days (Figure 2), though regular menstrual cycles can span from 24 to 35 days. The menstruation (days 0-4) is integrated in the beginning of the proliferative phase. The proliferative phase can be divided into two phases: early proliferative (EP; days 0-8) and late proliferative (LP; days 9-14). In addition, the secretory phase also can be segregated in three phases: early secretory phase (ES; days 15-18), mid-secretory phase (MS; days 19-22) and late secretory (LS; 23-28) (Figure 2C). In response to follicle-stimulating hormone (FSH) produced by the pituitary gland during the proliferative phase, ovarian follicles grow (follicular phase) and in turn produce E2 that induces proliferation of the steroid-responsive endometrial cells, and ultimately thickens the endometrium for potential implantation. On day 14, a spike in luteinizing hormone (LH; also produced by the anterior pituitary), triggers ovulation of a mature oocyte from the ovary. The secretory phase in the human endometrium begins after ovulation and is marked by the ovulatory site transforming into a P4-secreting corpus luteum (Figure 2D-E). In turn, the increasing P4 during early secretory phase induces differentiation of the endometrium where mitotic activity of the endometrial cells occurs in the first three days after ovulation. Then stromal cells contribute to thickening the endometrium, resulting in remarkable changes that open the WOI (Figure 2C-D). With increasing P4 and decreasing E2 during the early secretory phase, the secretion of glycogen and mucus is also increased. In the mid-secretory phase, the spiral arteries become increasingly coiled and longer, contributing to decidualization (which

induces morphological change of the fibroblasts, described more in detail in the following section) and embryo receptivity during WOI. The secretory activity of endometrial glands reaches a maximum around six days after ovulation. If pregnancy is achieved, P4 continues to be secreted until the fetus can adequate levels to sustain the pregnancy. In addition to its effect on the endometrium, P4 decreases the frequency and intensity of uterine contractions, which helps prevent expulsion of the implanted embryo (Hawkins and Matzuk, 2008; Mihm *et al.*, 2011).

If embryo implantation does not occur, the ovary stops producing hormones during the late secretory phase (days 23-28) and the P4 withdrawal initiates the menstruation in the proliferative phase (days 0-4), where the arteries vasoconstrictors and the endometrial lining is broken down and shed (Figure 2C-D). Finally, a new increase in E2 levels, produced by another pool of growing ovarian follicles, begins a new cycle to proliferate again the endometrium forming a new functional layer in the following cycle (Critchley *et al.*, 2020) (Figure 2C-E).

Clinical evaluations of endometrial receptivity often rely on morphological measures, such as endometrial thickness and pattern, or on ultrasound-based measures of endometrial vascularization. However, these approaches do not provide a complete picture of endometrial status. "Omics" studies, including transcriptomics and proteomics, may help to better understand endometrial receptivity by providing a broader and more comprehensive view of the biological processes involved. Nevertheless, to fully harness the potential of "omics" technologies, it is essential to first explore and characterize the underlying molecular mechanisms that regulate implantation and early fetal development.

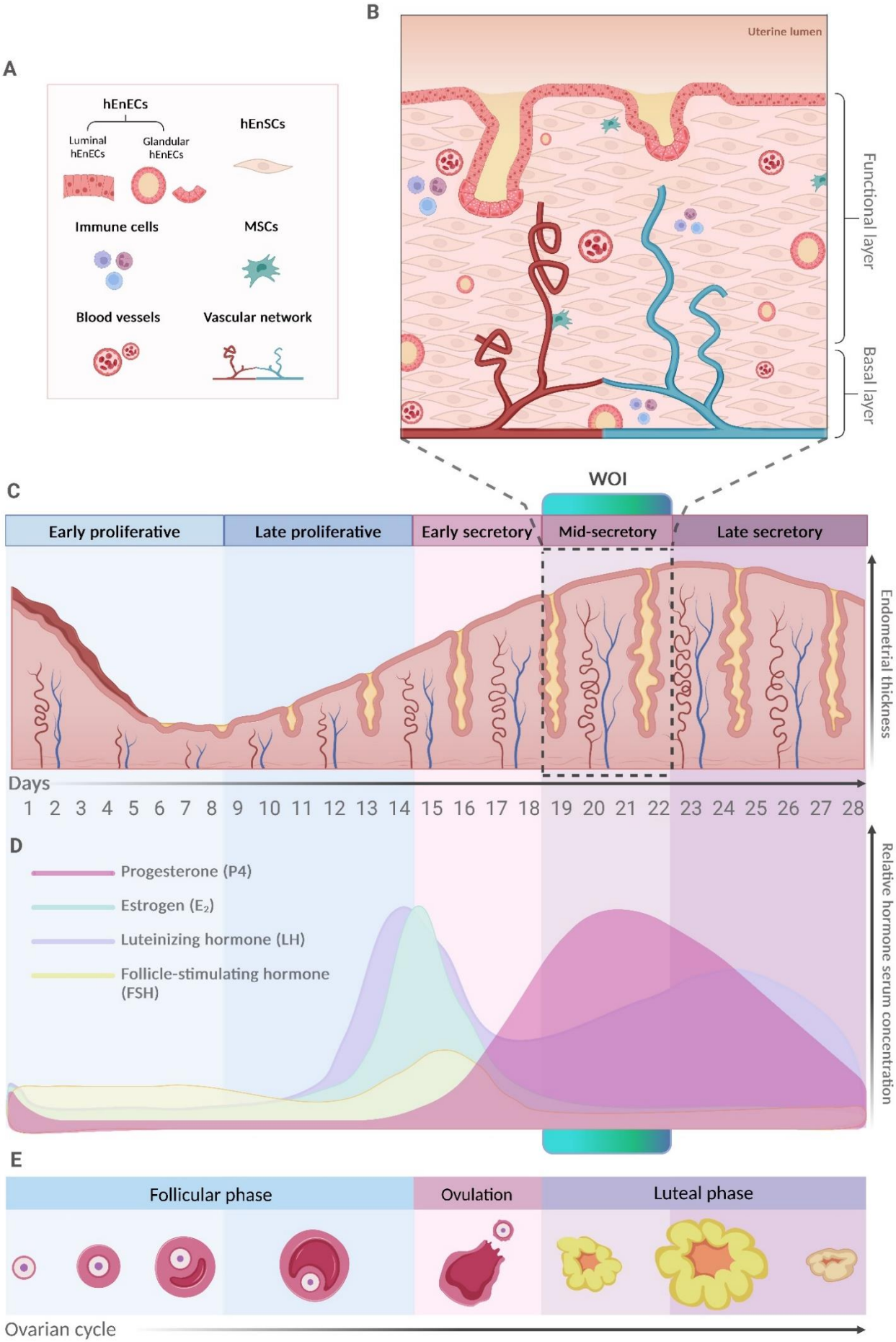
A
hEnECs
Luminal hEnECs
Glandular hEnECs
hEnSCs
Immune cells
MSCs
Blood vessels
Vascular network
B
Uterine lumen
Functional layer
Basal layer
C
WOI
Early proliferative
Late proliferative
Early secretory
Mid-secretory
Late secretory
Endometrial thickness
Days
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
D
Progesterone (P4)
Estrogen (E2)
Luteinizing hormone (LH)
Follicle-stimulating hormone (FSH)
Relative hormone serum concentration
E
Follicular phase
Ovulation
Luteal phase
Ovarian cycle

Figure 2. The human menstrual cycle. (A) Figure legend of the main cellular components in the human endometrium, including luminal and glandular human endometrial epithelial cells (hEnECs), human endometrial stromal cells (hEnSCs), immune cells, mesenchymal stem cells (MSCs), blood vessels, and the vascular network. These different cellular components appear in the detailed diagram of the endometrial layers during mid-secretory phase (B), highlighting the functional and basal layers. (C) Schematic representation of the endometrial growth throughout the menstrual cycle, accordingly divided into five endometrial phases (early proliferative, late proliferative, early secretory, mid-secretory and late secretory phases). The window of implantation (WOI) is indicated during days 19-22, showing the endometrial receptivity for embryo implantation. (D) The fluctuations in the relative serum concentrations of gonadotropic hormones [luteinizing hormone (LH), and follicle-stimulating hormone (FSH)], and ovarian hormones [progesterone (P4), and estrogen (E2)] during the menstrual cycle are also shown. These hormonal changes correlate with the different endometrial phases of the menstrual cycle. (E) The relative concentrations of the hormones correspond with the ovarian cycle stages: follicular phase, ovulation, and luteal phase. This panel illustrates the development of ovarian follicles in the folliculogenesis, the process of ovulation, and the formation of the corpus luteum. *Created with BioRender.com (2024).*

1.3. MOLECULAR MECHANISMS IN IMPLANTATION AND EARLY FETAL DEVELOPMENT

Successful embryo implantation requires synchronized communication between an embryo and a receptive endometrium during the WOI. This intricate process is governed by a diverse array of molecules, including cytokines, growth factors, prostaglandins, enzymes, and adhesion molecules (Ochoa-Bernal and Fazleabas, 2020).

After successful fertilization, the zygote undergoes mitotic divisions while migrating through the fallopian tube toward the uterine cavity (Figure 1). By day 5 or 6, it transforms into a blastocyst, comprising 32-256 cells. The blastocyst consists of two distinct cellular components. Firstly, the inner cell mass (ICM) which will eventually develop into the fetus. Meanwhile the outer cell layer, the trophoectoderm (TE), give rise to the placenta, providing nourishment and protection to the developing fetus. The blastocyst freely moves within the uterine cavity until reaching the upper posterior wall in the fundus of the uterus (Kim and Kim, 2017). This process involves a sequence of molecular events (Figure 3) (Massimiani *et al.*, 2019):

1. Apposition: Apposition marks the first interaction between the embryo and the receptive endometrium. Blastocysts exit their zona pellucida, align the ICM with the endometrium, and

bind leukemia-inhibitory factor (LIF) secreted by the receptive endometrium via LIF receptors on the blastocoels. LIF triggers the transition of luminal epithelium from proliferative to differentiated ones. LIF also drives stromal proliferation via the epidermal growth factor (EGF) pathway, involving heparin-binding EGF (HB-EGF). Trophoblasts release human chorionic gonadotropin (hCG) and interleukin 1 beta (IL-1β), initiating the extracellular signal-regulated protein kinases 1/2 (Erk1/2) pathway in endometrial epithelial cells. This cascade induces prostaglandin E2 (PGE2) production through cyclooxygenase 2 (COX2) protein in endometrial stromal cells, promoting decidualization and vascular permeability (Massimiani *et al.*, 2019).

2. Adhesion: Trophoblasts adhere to the endometrial epithelium through L-selectin, integrin αvβ3, and endothelial cadherin (E-cadherin) molecules. Glycoprotein mucin 1 (MUC-1) forms a protective barrier if the embryo encounters a glycocalyx, so a reduction in MUC-1 expression allows embryo adhesion. Other molecules, like L-type amino acid transporter 1 (CD98), neurogenic locus notch homolog protein 1 (NOTCH1) receptors, and ligands, contribute to adhesion. Activation of NOTCH1 signalling by hCG upregulates forkhead box protein O1 (FOXO1), vital for decidualization (Kajihara *et al.*, 2013; Massimiani *et al.*, 2019). Additionally, cytokines, including chemotactic chemokines [such as C-X-C motif ligand 1, 12, 13 and 14 (CXCL1, CXCL12, CXCL13 and CXCL14, respectively)], are produced by various cell types at the maternal-fetal interface. Those synthesized by the endometrial epithelium can be secreted either apically into the uterine lumen, influencing blastocyst development, migration, and attachment, or basally, impacting the transformation of the underlying stroma. Decidualized stromal cells (explained in detail in the following section), which become a significant part of the decidua during pregnancy, also generate cytokines that promote the decidualization process and chemokines that attract leukocytes, such as uterine natural killer cells and macrophages, as well as aid in trophoblast migration (Salamonsen *et al.*, 2007).

3. Invasion: Trophoblasts penetrate the decidualized stroma, differentiating into cytotrophoblasts and syncytiotrophoblasts. They remodel maternal spiral arteries, ensuring proper blood flow. Trophoblast invasion involves extracellular matrix remodeling through urokinase-type plasminogen activator (uPA) and matrix metalloproteinases (MMPs). MMP-2 and MMP-9 production is triggered by IL-1β, transforming growth factor beta (TGF-β), or hCG.

This invasion is tightly regulated by tissue inhibitors of MMPs (TIMPs) and plasminogen activator inhibitor 1 (PAI-1) downstream of TGF-β signalling (Massimiani *et al.*, 2019).

The implantation process is succeeded by early fetal development during the first trimester (12 weeks) (Farquharson *et al.*, 2005). Feto-maternal immune tolerance mechanisms maintain pregnancy, safeguarding the embryo from the maternal immune system while defending against potential pathogens. Decidual uNK cells, macrophages, T-cells, and dendritic cells constitute the feto-maternal interface, ensuring successful pregnancy (Wang and Li, 2020). Understanding these mechanisms may unveil origins of reproductive disorders.

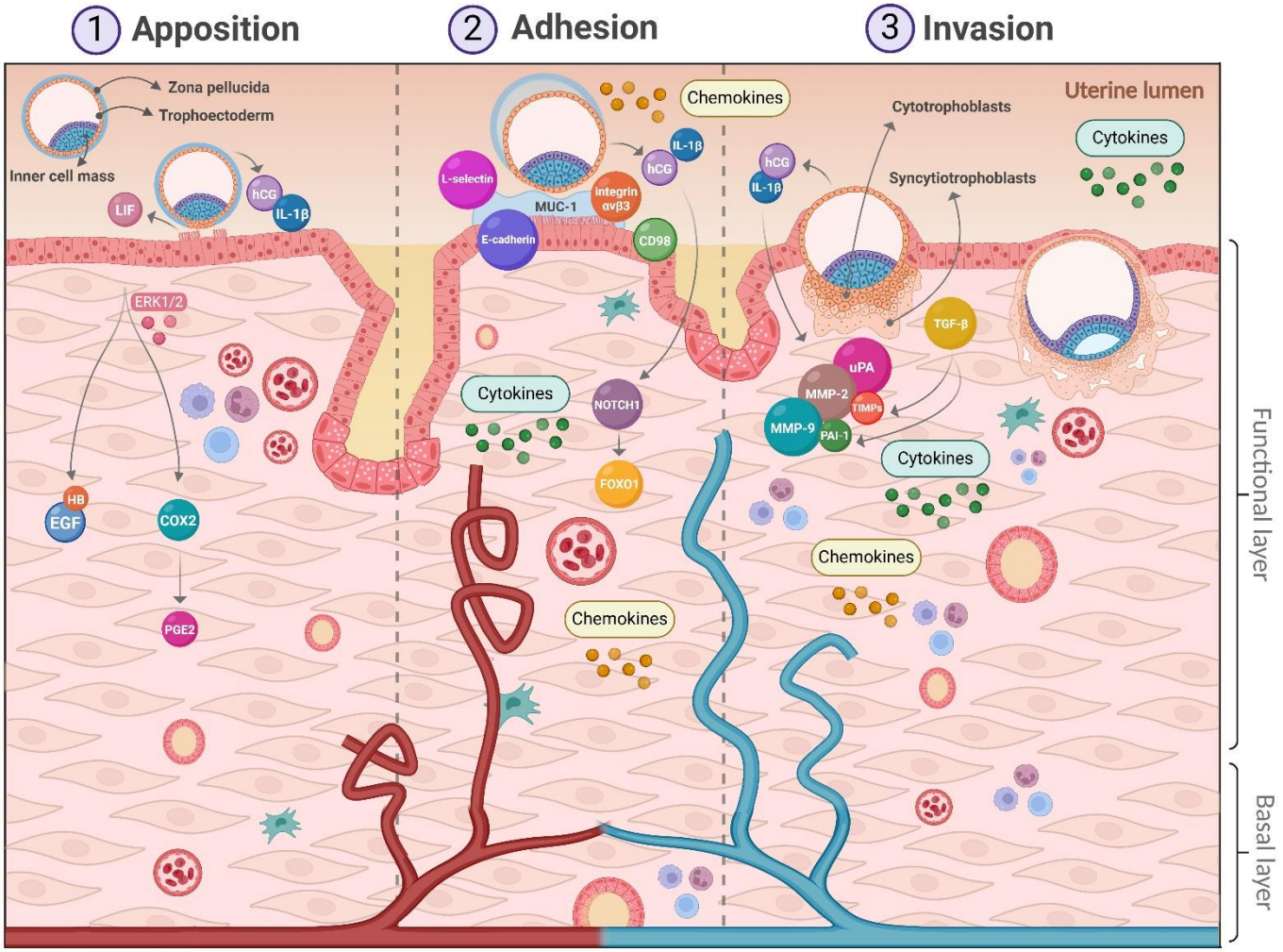

Figure 3. Molecular mechanisms in embryo implantation and early development. The primary molecular mechanisms involved in the various phases of human embryo implantation include: apposition (1), adhesion (2), invasion and early development (3). CD98, L-type amino acid transporter 1; COX2, cyclooxygenase 2; E-cadherin, endothelial cadherin; ERK1/2, extracellular signal-regulated protein kinases; FOXO1, forkhead box protein O1; HB-EGF, heparin-binding epidermal growth factor; hCG, human chorionic gonadotropin; IL-1β, interleukin 1 beta; LIF, leukemia inhibitory factor; MMP-2, matrix metalloproteinase 2; MMP-9, matrix metalloproteinase 9; MUC-

1, mucin 1; NOTCH1, neurogenic locus notch homolog protein 1; PAI-1, plasminogen activator inhibitor 1; PGE2, prostaglandin E2; TGF-β, transforming growth factor β; TIMPs, tissue inhibitors of metalloproteinases; uPA, urokinase-type plasminogen activator. *Created with BioRender.com (2024).*

1.4. ENDOMETRIAL OMICS

The study of the endometrium at the molecular level, often referred to as "endometrial omics," encompasses various approaches aimed at understanding the complex biological processes involved in endometrial function and pathology. This chapter delves into two primary areas of endometrial omics: transcriptomics and proteomics, which have both shown considerable advancements in recent years.

1.4.1 Endometrial transcriptomics

Transcriptomics attempts to analyze patterns of gene expression and to correlate them with their underlying biology. It also allows gene expression characterization at the mRNA level of a population, giving rise to a sample-specific molecular profile. This fact led to a new biomarker concept: transcriptomic signatures that define a biological process or disease, and thus represent new opportunities to characterize function or disease phenotypes (Supplitt *et al.*, 2021).

The study of endometrial transcriptomics has significantly advanced our understanding of endometrial receptivity and its role in successful embryo implantation. Endometrial transcriptomic analysis has been pivotal in identifying gene expression profiles that distinguish receptive from non-receptive endometrial states (Díaz-Gimeno *et al.*, 2014; Gómez *et al.*, 2015; Messaoudi *et al.*, 2019).

Over the past decade, the field of human endometrial transcriptomics has seen significant advancements, with numerous studies exploring various aspects of endometrial biology. Initially, research focused on understanding the transcriptomic expression during the menstrual cycle. Early studies by Horcajadas *et al*. (2004) and Giudice (2006) laid the groundwork by examining gene expression patterns throughout the menstrual cycle (Horcajadas *et al.*, 2004; Giudice, 2006). Critchley *et al*. (2006) and Horcajadas *et al*. (2007) further expanded on this by detailing the specific changes in gene expression during different

phases of the menstrual cycle (Critchley *et al.*, 2006; Horcajadas *et al.*, 2007). Aghajanova *et al*. (2008a, 2008b) later contributed to profiling menstrual cycle-associated gene expression in greater depth (Aghajanova *et al.*, 2008a, 2008b).

Building on these insights, several studies began to explore endometrial receptivity. Haouzi *et al*. (2012) and Ruiz-Alonso *et al*. (2012) investigated transcriptomic changes tied to endometrial receptivity, while Garrido-Gomez *et al*. (2013) offered detailed molecular insights into endometrial preparation for implantation (Haouzi *et al.*, 2012; Ruiz-Alonso *et al.*, 2012; Garrido-Gómez *et al.*, 2013). Martinez-Conejero *et al*. (2007) and Ruiz-Alonso *et al*. (2013) studied how assisted reproductive technology (ART) protocols influence gene expression (Martínez-Conejero *et al.*, 2007; Ruiz-Alonso *et al.*, 2013). These studies collectively highlighted the variability and complexity of endometrial receptivity, with Ruiz-Alonso *et al*. (2012) emphasizing that the WOI varies among women and is governed by unique gene expression signatures, a concept supported by Talbi *et al*. (2006) and Lessey and Young (2019) (Talbi *et al.*, 2006; Ruiz-Alonso *et al.*, 2012; Lessey and Young, 2019).

Simultaneously, transcriptomic research broadened its scope to include specific cellular compartments within the endometrium. Yanaihara *et al*. (2005), Evans *et al*. (2012), and Ulbrich *et al*. (2013) demonstrated distinct gene expression profiles for different cell types (Yanaihara *et al.*, 2005; Evans *et al.*, 2012; Ulbrich *et al.*, 2013). Recent studies have been focusing on the epigenetic regulation applying single-cell RNA sequencing to reveal dynamic changes in gene expression during the menstrual cycle, particularly in endometrial stem cells (Lee and Lee, 2023) and during the decidualization process (Liu *et al.*, 2020). These advances in transcriptomic technology and the identification of target molecules have enabled a deeper understanding of the crucial moment in the menstrual cycle, the WOI.

Despite the wealth of data generated, challenges remain. Variability in experimental designs, timing, sample collection, and data processing pipelines complicates cross-study comparisons, as highlighted previously by Horcajadas *et al*. (2007), Ruiz-Alonso *et al*. (2012), and Ulbrich *et al*. (2013), making it difficult to fully understand the complex nature of endometrial development. Comprehensive reviews like that of Altmäe *et al*. (2014) have identified key genes consistently associated with receptivity, however, they noted that the overlap of identified genes was relatively small, indicating the complexity and variability of the

endometrial transcriptome (Altmäe *et al.*, 2014). Wang and Yu (2018) provided an update on the progress of transcriptomic profiles of human endometrial receptivity emphasizing the potential of RNA sequencing to refine precision medicine approaches by detecting previously undiscovered genes (Wang and Yu, 2018). Recent advancements have enabled the identification of biomarkers, such as LIF (Aghajanova *et al.*, 2009), and the exploration of genes involved in adhesion, invasion, survival, differentiation, decidualization, and immune modulation (Messaoudi *et al.*, 2019; Zhang *et al.*, 2023), paving the way for more personalized ART strategies.

Parallel investigations addressed pathological conditions affecting the endometrium. Matsuzaki (2011) focused on transcriptomic profiles in endometriosis, while Doll *et al*. (2008) examined changes associated with endometrial cancer (Doll *et al.*, 2008; Matsuzaki, 2011). More recently, Miravet-Valenciano *et al*. (2017) identified differences in the mid-secretory endometrial transcriptome between women with and without endometriosis, providing insights into the molecular basis of this condition and its impact on receptivity (Miravet-Valenciano *et al.*, 2017). Similarly, Poli-Neto *et al*. (2020) investigated the transcriptomic signature of endometrial receptivity in women with endometriosis, suggesting that endometriosis significantly alters the gene expression profile of the endometrium (Poli-Neto *et al.*, 2020). Other studies identified biomarkers associated with recurrent implantation failure (RIF) (Basatvat *et al.*, 2021; Meltsov *et al.*, 2023; Lee *et al.*, 2024), while Chettiar *et al*. (2024) conducted a meta-analysis of endometrial transcriptome data, revealing novel molecular targets for RIF and providing further insights into the molecular basis of this condition (Chettiar *et al.*, 2024).

The identification of biomarkers for endometrial receptivity has significant clinical implications, particularly for ART. Biomarkers such as LIF have been studied extensively, although their clinical utility remains debated (Aghajanova *et al.*, 2009). High expression of LIF appears to be an indicator of a receptive endometrium in fertile women. However, studies have shown varying results concerning LIF concentrations in women with RIF, indicating the need for further research to clarify its role (Günther *et al.*, 2023). Recent studies, such as Meltsov *et al*., 2023, have highlighted the importance of molecular markers in assessing endometrial receptivity and their potential impact on ART outcomes (Meltsov *et al.*, 2023).

Despite the progress made, several challenges remain in the field of endometrial transcriptomics. One major challenge is the variability in gene expression profiles due to individual differences and the influence of external factors. Additionally, the functional relevance of many identified genes is still not fully understood.

In summary, research on endometrial transcriptomics has significantly advanced our understanding of the physiology of embryo implantation and enabled the identification of new tools for objectively evaluating endometrial receptivity. The ongoing advancements in RNA sequencing and data analysis techniques are expected to drive further discoveries, providing more precise and comprehensive profiling of the endometrial transcriptome. Endometrial transcriptomics has made substantial strides, offering valuable insights into the molecular mechanisms underlying endometrial function and pathology. Integrating transcriptomic data with other omics technologies promises to enhance the diagnosis, prognosis, and treatment of endometrial disorders. As research continues to progress, it is likely to contribute significantly to personalized medicine, ultimately improving patient outcomes and quality of life. However, further research is needed to understand transcriptomic changes in both physiological and pathological conditions, to gain deeper insights into endometrial receptivity and establish gene expression profiles.

1.4.2 Endometrial proteomics

Proteomics focuses on the identification and quantification of proteins, as well as the analysis of their post-translational modifications. It has been fundamental in characterizing the endometrial cycle and identifying biomarkers of endometrial receptivity and pathologies by detecting numerous proteins simultaneously. The field of reproductive medicine has adopted the use of proteomics for diagnostic markers, treatment targets, and post-translational modifications.

There have been several studies that have compared proteome results between endometrial tissues or uterine fluid from the proliferative and secretory phases, which may help us understand the molecular changes responsible for the transition from a non-receptive stage to a receptive stage, as well as the physiological development of the endometrium (Yang *et al.*, 2012; Evans *et al.*, 2019; Guo *et al.*, 2021).

In 1995, Byrjalsen *et al*. performed the first proteomic analysis of human endometrium in different stages, proliferative and secretory phase (Byrjalsen *et al.*, 1995). They identified 186 protein spots with altered expression throughout the menstrual cycle. Among these proteins, the authors found that cytoskeleton-related proteins were mostly synthesized in the proliferative phase, which may correlate with the high cell division rate of the proliferative endometrium. On the other hand, energy metabolism-related enzymes, annexin A4 precursor and cytokeratin 8, among other proteins, were found to be most highly expressed in the secretory phase, suggesting that endometrial cells undergo more functional changes during this period.

Other studies have attempted to investigate the shift of endometrial protein expression from the proliferative to secretory phase (Chen *et al.*, 2009; Parmar *et al.*, 2009; Rai *et al.*, 2010; Amjadi *et al.*, 2018; Meleady, 2018). The low reproducibility of the results in these studies may be due to several factors, such as the rapid changes that occur in the endometrium throughout the menstrual cycle, the differences in the time of endometrial biopsy in the studies, and the different criteria for determining differentially expressed proteins (DEPs). However, with the improvement of 2D technologies, two-dimensional differential in gel electrophoresis (2D DiGE) has emerged as a more accurate method.

Using MS technology, a study identified endometrial receptive biomarkers that were mainly involved in cell structure and motility, signal transduction, cell cycle, and immunity (Chen *et al.*, 2009). The study also found that multiple spots of the same identified protein suggest that post-transcriptional and post-translational modifications are important events in endometrial transition.

The study of the endometrial proteome has revealed biomarkers between pre-receptive and receptive endometrium. These differences in the proteins quantity have been found to be similar to those observed between proliferative and secretory endometrium. One notable protein, membrane-associated progesterone receptor component 1 (PGRMC1), showed decreased expression in the receptive phase (Chen *et al.*, 2009; Domínguez *et al.*, 2009; Li *et al.*, 2011a; Keator *et al.*, 2012; Salsano *et al.*, 2017). In addition, S100A10, member of the S100 family which is linked to reduced embryo implantation rates and poor pregnancy outcomes,

was found to be upregulated in the mid-secretory phase (Domínguez *et al.*, 2009; Liu *et al.*, 2012; Bissonnette *et al.*, 2016).

Despite two decades of research, there remains a lack of consensus on the molecular markers defining endometrial receptivity. However, recent studies have made significant strides in identifying key proteins involved in this process, which could improve fertility treatments and outcomes. Aplin *et al*. (2022) has provided new insights into the temporal expression patterns of proteins during the menstrual cycle, highlighting potential biomarkers that could be used to assess endometrial receptivity more accurately (Aplin and Stevens, 2022). Moreover, the integration of proteomics with other omics approaches, such as transcriptomics and metabolomics, has provided deeper insights into endometrial biology. For example, a study by Yang *et al*. (2024) utilized integrated transcriptomic and proteomic analyses to explore the role of ubiquitination in endometrial function and pathology. This multi-omics approach revealed novel regulatory mechanisms and potential therapeutic targets for endometrial disorders (Yang *et al.*, 2024).

By integrating data from different omics layers, researchers are gaining a more holistic view of the molecular events underlying not only endometrial receptivity but also diseases such as endometriosis and endometrial cancer (Aplin and Stevens, 2022; Romano *et al.*, 2023; Serambeque *et al.*, 2024).

The ongoing advancements in mass spectrometry and data analysis techniques are expected to drive further discoveries in this field, enabling more precise and comprehensive profiling of the endometrial proteome. Despite the new advances in these proteomics techniques, there is a need for further research to understand the proteomic changes in both physiological and pathological conditions to gain a deeper insight into endometrial receptivity and establish protein-protein interactions.

In conclusion, endometrial proteomics has made significant strides in recent years, providing valuable insights into the molecular mechanisms underlying endometrial function and pathology. The integration of proteomic data with other omics technologies holds great promise for improving the diagnosis, prognosis, and treatment of endometrial disorders. As research in this field continues to advance, it is expected to contribute significantly to the

development of personalized medicine approaches, ultimately improving patient outcomes and quality of life.

2. THE DECIDUALIZATION PROCESS

The P4 induces differentiation of the endometrium, resulting in critical changes that make the endometrium receptive and ready for the embryo to be implanted (Gellersen and Brosens, 2014; Okada *et al.*, 2018). One P4-dependent critical event is decidualization, which is characterized by profound morphological and biochemical differentiation of hEnSCs into decidual cells (Sakai *et al.*, 2003; Gellersen and Brosens, 2014), causing an increase of secreted prolactin (PRL) protein and insulin-like growth factor-binding protein 1 (IGFBP1), commonly used as markers of decidualization (Irwin *et al.*, 1994; Gellersen and Brosens, 2003).

Decidualization involves the differentiation of elongated fibroblast-like cells into rounded epithelioid-like cells (Pabona *et al.*, 2010; Ma *et al.*, 2011; Saleh *et al.*, 2011; Zhu *et al.*, 2014; Coulam, 2016). This change occurs in response to elevated P4 levels during the mid-secretory phase of the menstrual cycle (Gellersen and Brosens, 2014) (Figure 4). The process starts approximately 6 days after ovulation and is characterized by morphological changes in the hEnSCs, secretory transformation of the uterine glands, an influx of specialized uterine natural killer cells, and vascular remodelling to support the growing (Evans *et al.*, 2016; Schatz *et al.*, 2016; Okada *et al.*, 2018). The decidual cells provide nutrition for the implanting blastocyst and are characterized by enlarged and rounded nuclei, increased numbers of nucleoli, dense membrane-bound secretory granules, and expanded rough endoplasmic reticulum (ER) and Golgi complex (Gellersen and Brosens, 2014; Kajihara *et al.*, 2014).

The synchronization of the decidual response during the secretory phase of the menstrual cycle is intricate, relying on the convergence of the cyclic adenosine 3’:5’ monophosphate (cAMP) and P4 signalling pathways. While the significance of elevated P4 levels for decidualization is well-established, compelling evidence suggests that the initiation of this process hinges on elevated intracellular cAMP levels (Gellersen *et al.*, 1994; Brosens *et al.*, 1999a, 2002; Christian *et al.*, 2002b, 2002a; Mak *et al.*, 2002; Gellersen and Brosens, 2003;

Pohnke *et al.*, 2004). Importantly, this intracellular elevation of cAMP is paramount for achieving and maintaining the decidualized state of endometrial stromal cells (Telgmann *et al.*, 1997).

A crucial network for hEnSCs decidualization consists of proteins regulated by P4 and/or cAMP, such as homeobox A10 (HOXA10), FOXO1, signal transducers and activators of transcription, and heart and neural crest derivatives expressed transcript 2 (HAND2) (Huyen and Bany, 2011; Li *et al.*, 2011b; Cho *et al.*, 2013; Pawar *et al.*, 2014; Ujvari *et al.*, 2017) (Figure 4). On the other hand, cAMP also acts through others proteins network such as the small Ubiquitin-like modifier (SUMO) protein pathway within hEnSCs. It has been demonstrated that, in response to the surge of cAMP signalling, the SUMOylation of certain proteins, such as active isoforms of P4 receptors (PGRs), is essential for regulating the transcription of genes involved in the preparation and maintenance of the decidual environment (Jones *et al.*, 2006).

Recent advancements have further elucidated the complexity of decidualization. For instance, a 2024 study by Jia *et al*. revealed that metabolic reprogramming, particularly in amino acid and sphingolipid metabolism, is crucial during the decidualization process. This study utilized single-cell RNA sequencing to uncover the metabolic heterogeneity among decidual cells, highlighting the increased cellular activity and signalling propensity in highly metabolically active decidual cells. Additionally, the role of metabolic pathways, such as the pentose phosphate pathway, has been shown to be critical for decidualization, with inhibition of this pathway impairing the process both *in vitro* and *in vivo* (Jia *et al.*, 2024). In addition, a recent study explored the role of endometrial microvascular complexity and trophoblast outgrowth in gelatin methacryloyl hydrogels. This study demonstrated that the decidualization status of the endometrium modulates microvascular complexity and affects trophoblast motility, providing new insights into the dynamic interactions at the maternal-fetal interface (Zambuto *et al.*, 2024).

The transformation of hEnSCs into decidual cells is a significant event in the establishment of pregnancy as well as protecting the embryo from maternal immunological rejection and providing nutritional support prior to placental formation, regulating trophoblast invasion and placental formation. A failure in decidualization can lead to infertility, recurrent miscarriages,

and uteroplacental disorders. In fact, women experiencing recurrent miscarriages often show abnormal decidualization, leading to failed implantation or inadequate placentation.

Murata *et al*. (2022) developed a systematic repository of cell-cell communication for decidualization, revealing an aberrant ratio conversion of IGFBP1-expressing hEnSCs to insulin like growth factor 1 receptor (IGF1R)-expressing hEnSCs in patients with RIF (Murata *et al.*, 2022).

On the other hand, defective decidualization can lead to abnormal placentation, which has been associated with preeclampsia (Padilla, 2015). Preeclampsia is a pregnancy complication characterized by high blood pressure and damage to other organs, most commonly the liver and kidneys. Its etiology is multifactorial, but a significant relationship has been identified with defective decidualization of hEnSCs (Padilla, 2015). This is due to alterations in the expression of key genes involved in this process, such as annexin A2 (*ANXA2*), which is crucial for proper blastocyst implantation (Garrido-Gomez *et al.*, 2020). Additionally, these cells do not adequately secrete signalling molecules like PRL and IGFBP1, compromising the interaction between the embryo and the endometrium.

Besides its relationship with preeclampsia, defective decidualization has also been linked to other infertility issues. For instance, inadequate decidualization can contribute to anovulatory infertility, as stromal cells do not respond properly to the hormonal signals necessary for ovulation (Matsuyama *et al.*, 2024). Women with endometriosis also exhibit defective decidualization, suggesting that this alteration may be a contributing factor in their inability to conceive (Marquardt *et al.*, 2019). Defective decidualization has also been associated with recurrent pregnancy loss (Lucas *et al.*, 2020).

Overall, the intricate interplay between the cAMP and P4 pathways highlights the multifaceted nature of the decidualization process, where the orchestrated modulation of signalling cascades is central to the establishment and maintenance of the decidualized state within hEnSCs. Continued research and advancements in *in vitro* models of decidualization are essential for furthering our understanding of this critical process and developing treatments for conditions such as infertility and recurrent pregnancy loss.

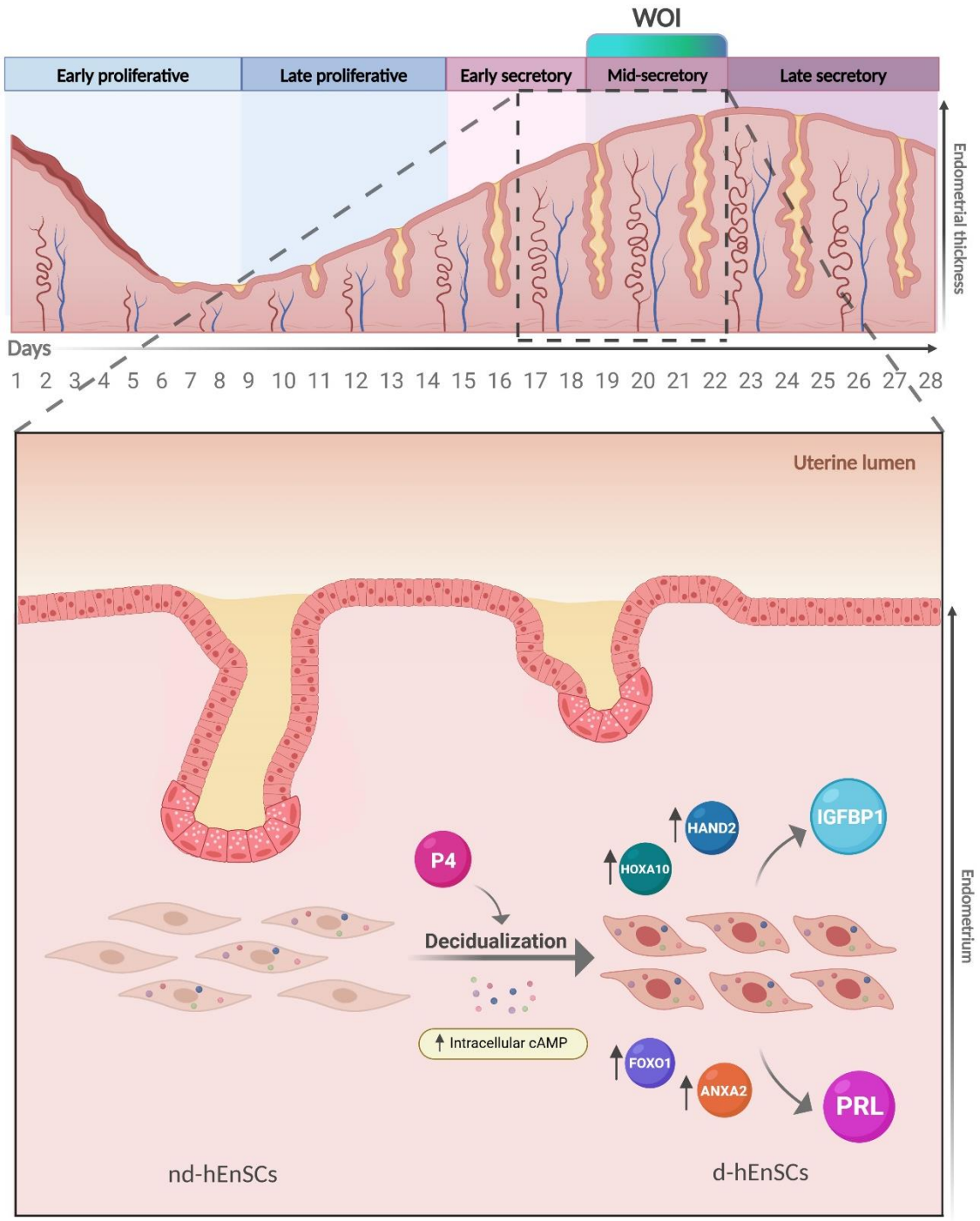

Figure 4. Molecular pathways involved in decidualization. The key molecules for decidualization are represented in this drawing. The stromal compartment in the human endometrium contains fibroblast-like cells, known as non-decidualized endometrial stromal cells (nd-hEnSCs). In response to elevated progesterone levels during the mid-secretory phase of the menstrual cycle, these cells are able to differentiate into rounded epithelioid-like cells, referred to as decidualized endometrial stromal cells (d-hEnSCs). This process causes an increase of secreted prolactin protein and insulin-like growth factor-binding protein 1, commonly used as markers of decidualization. ANXA2, annexin A2; cAMP, cyclic adenosine 3':5' monophosphate; FOXO1, forkhead box protein O1; HAND2, neural crest derivatives expressed transcript 2; HOXA10, homeobox A10; IGFBP1, insulin-like growth factor-binding protein 1; P4, progesterone; PRL, prolactin. *Created with BioRender.com (2024).*

2.1. *IN VITRO* MODELLING OF DECIDUALIZATION

During decidualization, in addition to the secretion of PRL and IGFBP1, the levels of cAMP inside the cells are known to increase after several days of synthetic form of P4 (progestin) treatment (Gellersen and Brosens, 2003). Various protocols have been developed to promote decidualization *in vitro* (Gellersen and Brosens, 2014; Doi-Tanaka *et al.*, 2024) (Table 1). These protocols typically involve the following treatments: E2 and P4 (or progestin), an analog that induces the expression of intracellular cAMP, a combination of a cAMP analog and P4 or progestin. The length of treatment differs greatly across studies, ranging from just a few hours to over 10 days.

Treatment	Duration	Check	Reference
Endometrial explants			
P4 + E2	6-10 days	PRL secretion	(Daly *et al.*, 1983)
P4	2-28 days	PRL secretion	(Maslar and Ansbacher, 1986)
Primary hEnSCs culture			
8-Br-cAMP	12-24 hours	PRL induction	(Telgmann *et al.*, 1997)
8-Br-cAMP	1-3 days	PRL, IGFBP1 mRNA	(Samalecos *et al.*, 2009)
8-Br-cAMP	2 days	Microarray analysis	(Popovici *et al.*, 2000)
8-Br-cAMP + E2	48 hours	Microarray analysis	(Tierney *et al.*, 2003)
CRH	8 days	PRL secretion	(Ferrari *et al.*, 1995)
db-cAMP	24-48 hours	PRL secretion	(Tang *et al.*, 1993)
FSH + LH or hCG	4-6 days	PRL secretion	(Tang and Gurpide, 1993)
MPA	10-20 days	PRL, IGFBP1 mRNA	(Tseng *et al.*, 1992)
MPA + 8-Br-cAMP	4-10 days	PRL secretion	(Brosens *et al.*, 1999a)
MPA + E2 + PGE2	10 days	PRL secretion	(Frank *et al.*, 1994)
MPA + IGF-1	28 days	PRL secretion	(Rosenberg *et al.*, 1991)
MPA + RLN	6 days	PRL secretion	(Telgmann *et al.*, 1997)
MPA + RLN + E2	6 days	PRL secretion	(Huang *et al.*, 1987)
MPA or P4	20 days	PRL secretion	(Zhu *et al.*, 1990)
P4 + Cortisone + 8-Br-cAMP	4 days	PRL, IGFBP1 mRNA	(Kuroda *et al.*, 2013)
P4 + DES + EGF	25 days	PRL secretion	(Irwin *et al.*, 1991)
P4 + DHT + 8-Br-cAMP	4-8 days	PRL, IGFBP1 secretion	(Cloke *et al.*, 2008)

P4 + E2	10-15 days	PRL secretion	(Irwin *et al.*, 1989)
P4 + E2	14 days	IGFBP1 secretion	(Hess *et al.*, 2007)
P4 + E2 + EGF	10 days	Microarray analysis	(Popovici *et al.*, 2000)
P4 + E2 + IL-11	6 days	PRL, IGFBP1 secretion	(Dimitriadis *et al.*, 2002)
PGE2 + Progestin	10 days	PRL secretion	(Frank *et al.*, 1994)
Decidual explants			
P4	3-10 days	PRL secretion	(Hamaguchi *et al.*, 1990)

Table 1. Overview of decidualization-inducing treatments for *in vitro* culture of human endometrial stromal cells (hEnSCs). 8-Br-cAMP, 8-Bromo-cyclic adenosine 3':5' monophosphate; CRH, corticotrophin-releasing hormone; DES, diethylstilbestrol; DHT, dihydrotestosterone; E2, estradiol; EGF, epidermal growth factor; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; IGF-1, insulin-like growth factor 1; IGFBP1, insulin-like growth factor-binding protein 1; IL-11, interleukin 11; LH, luteinizing hormone; MPA, medroxyprogesterone 17-acetate progestin; P4, progesterone; PGE2, prostaglandin E2; PRL, prolactin; RLN, relaxin. Adapted from (Gellersen and Brosens, 2014).

In human cell cultures, treatments with cAMP trigger decidualization and is associated also with the expression of PRL and IGFBP1 as decidual markers, and the presence of both cAMP and progestin increases this expression even further (Okada *et al.*, 2018). In primary culture, the extended exposure of hEnSCs to E2 and P4 has been demonstrated to elicit a significant increase in cAMP production. Interestingly, this induction of decidualization can be effectively impeded by the presence of a protein kinase A (PKA) inhibitor, underscoring the pivotal role of the cAMP pathway in the hEnSCs response to ovarian hormones, indicating the intimate interaction between cAMP and P4 signalling (Brar *et al.*, 1997; Yoshino *et al.*, 2003; Matsuoka *et al.*, 2010).

In the context of *in vitro* decidualization, the response of hEnSCs to cAMP analogs, E2, and medroxyprogesterone 17-acetate progestin (MPA) has been associated with a temporal modulation of the cell cycle. This progression involves initial cell cycle arrest in the G0/G1 phase, followed by subsequent arrest in the G2/M phases (Schmidt *et al.*, 2002; Cobrinik, 2005; Qian *et al.*, 2005; Takano *et al.*, 2007; Cloke *et al.*, 2008; Logan *et al.*, 2012). Notably, the signalling kinetics of cAMP and P4 differ in their respective contributions to decidualization initiation. Peak LH-triggered cAMP signalling exhibits a swifter and immediate decidualization

response, while P4 signalling elicits a more gradual yet persistent effect (Gellersen and Brosens, 2003; Tierney *et al.*, 2003).

cAMP induces a fully decidual phenotype within the first three days of treatment. In contrast, P4's response in the hEnSCs to perform the decidualization unfolds over a span of 14-23 days of treatment, culminating in a fully decidualized phenotype (Gellersen and Brosens, 2014).

A recent study explored the differential gene expression in hEnSCs induced by various stimuli, including cAMP, MPA and E2. The researchers aimed to understand how these different stimuli affect the transcriptome and cellular functions of hEnSCs. They found that stimuli involving cAMP (cAMP alone or combined with MPA) resulted in approximately twice as many differentially expressed genes (DEGs) compared to stimuli without cAMP (MPA alone or combined with E2). The cAMP-related stimuli significantly influenced cellular functions related to angiogenesis, inflammation, immune response, and embryo implantation, whereas MPA-related stimuli affected insulin signalling pathways. By comparing these *in vitro* findings with *in vivo* data, the study concluded that the combination of cAMP and MPA most closely mimics the natural decidualization process (Doi-Tanaka *et al.*, 2024). This research highlights the importance of selecting appropriate stimuli for *in vitro* decidualization studies to better replicate *in vivo* conditions and improve our understanding of endometrial biology and reproductive health.

3. PROGESTERONE HORMONE

P4, a vital steroid hormone, is synthesized not only by the placenta but also by the ovaries and adrenal glands. Its significance extends beyond its well-known role as the "pregnancy hormone," encompassing a wide range of physiological processes. While it is a key player in the intricate dynamics of female reproduction, P4 exerts its influence on multiple non-reproductive tissues as well, showcasing its versatile nature. In addition to its fundamental role in preparing the mammary gland for breastfeeding, P4 contributes to the overall health of the cardiovascular system, shapes the functions of the central nervous system, and actively

participates in maintaining bone health, highlighting its comprehensive impact on the body's overall well-being (Taraborrelli, 2015).

Within the female reproductive tract, P4 is involved in regulating a comprehensive sequence of essential events during the menstrual cycle, and its effects reflect through processes crucial to the establishment and preservation of pregnancy. These include ovulation, fertilization, implantation, embryonic development, breast development, and parturition (Graham and Clarke, 1997; Salazar and Calzada, 2007; Conneely *et al.*, 2008; Loutradis *et al.*, 2008; Szekeres-Bartho *et al.*, 2008; Yoshinaga, 2014; Morel *et al.*, 2016). During the periovulatory period, high P4 levels in the female reproductive tract play a pivotal role in processes such as sperm capacitation, hyperactivation, chemotaxis, and acrosome reaction. These processes are critical for optimal fertilization and subsequent embryo development (Tamburrino *et al.*, 2020).

Disruptions in P4 signalling can have far-reaching consequences, leading to a spectrum of gynecological complications. These complications span conditions like fibroids, abnormal menstrual bleeding, endometriosis, adenomyosis, and have even been implicated in breast and endometrial cancers. Additionally, inadequate P4 action has been linked to miscarriages and preterm labor, further underscoring its critical role in ensuring successful pregnancies (Wu *et al.*, 2006; Burney *et al.*, 2007; Ehn *et al.*, 2007; Ito *et al.*, 2007; Salazar and Calzada, 2007; Yin *et al.*, 2007; Szekeres-Bartho *et al.*, 2008; Branchini *et al.*, 2009; Kim *et al.*, 2013; Bunch *et al.*, 2014; Hirota, 2019a; Liao *et al.*, 2019). Addressing these challenges requires a deep understanding of the intricate mechanisms through which P4 exerts its effects, as this is essential for developing more effective therapeutic approaches, improving quality of life for individuals with these disorders, and increasing the chances of successful pregnancy and delivery.

3.1. PGRs MECHANISMS OF ACTION

The physiological effects of P4 are mediated by its binding to PGRs in target cells. PGRs are categorized into classical and non-classical types, each with distinct roles and functions (Kowalik *et al.*, 2013b). The classical PGRs have been identified across a range of organs and tissues, including the ovaries, uterus, fallopian tubes, placenta, testis, brain, pancreas, bone,

mammary gland, and urinary tract (Batra and Iosif, 1987; Iwai *et al.*, 1990; Doglioni *et al.*, 1990; Horie *et al.*, 1992; Amso *et al.*, 1994; Shanker and Rao, 1997; Bland, 2000; Conneely *et al.*, 2002; Thijssen, 2005; Abid *et al.*, 2008; Brinton *et al.*, 2008; Branchini *et al.*, 2009; Large and DeMayo, 2012; Plant, 2015; Wetendorf *et al.*, 2017; González-Orozco and Camacho-Arroyo, 2019; Barton *et al.*, 2020). Upon binding with P4, these receptors transform into transcription factors that govern the expression of P4-responsive genes. This intricate regulation triggers sustained cellular responses that manifest over a span of hours (Medina-Laver *et al.*, 2021).

In contrast, studies in knockout mice lacking these classical PGRs demonstrate that cells are still able to respond to P4 (Frye and Vongher, 1999) through a faster mechanism via non-classical membrane receptors (non-classical PGRs) (Kimura *et al.*, 2012). Non-classical PGRs facilitate rapid hormonal effects by initiating an array of secondary messengers and signalling pathways almost immediately upon P4 binding. Non-classical PGRs are important contributors in reproduction (Gellersen *et al.*, 2009; Medina-Laver *et al.*, 2021) regulating gene transcription (Karteris *et al.*, 2006; Salsano *et al.*, 2021), the acrosomal reaction in human sperm (Foresta *et al.*, 1993), sexual behavior in mice (Frye and Vongher, 1999; Frye *et al.*, 2006), and meiotic maturation in Xenopus oocytes (Finidori-Lepicard *et al.*, 1981).

The distinct signalling pathways of classical and non-classical PGRs result in diverse downstream responses, with the cellular effects of P4 varying according to tissue type and the stage of the menstrual cycle (Dias Da Silva *et al.*, 2024).

3.1.1 Classical PGRs

There are two main isoforms of classical PGR: PGR-α (94 kDa) and PGR-β (120 kDa). They are transcribed from the same gene, their distinction forged through the utilization of different promoters. In the human context, the *PGR* gene consists of an eight-exon sequence situated on chromosome 11 (11q22-q23) (Rousseau-Merck *et al.*, 1987; Misrahi *et al.*, 1993; Conneely and Lydon, 2000; Demayo and Lydon, 2020) (Figure 5), with its transcription primarily contingent upon E2. The engagement of E2 with E2 receptors (E2R) prompts the recognition of various E2 response elements (ERE) within the *PGRs* gene's promoter region, thereby inducing its expression. Yet, it's noteworthy that several non-E2-dependent mechanisms of *PGRs* expression regulation have also been described (Jacobsen and Horwitz, 2012).

Molecularly, PGR's structure comprises a DNA-binding domain (DBD) placed between an upstream N-terminal region containing an activation function (AF1) and an inhibitory domain (ID). Downstream, a ligand-binding domain (LBD) resides, housing another activation domain termed AF2 (Figure 5). PGR-β has an additional section located at the N-terminal end of the protein of approximately 164 extra amino acids in humans. Within this region, an extra activation domain, designated AF3, takes its place (Hill *et al.*, 2012). The panorama further broadens as various PGRs isoforms, stemming from the insertion of additional exons or via alternative splicing, make their appearance. Notably, these variants might not encode functional receptors; in contrast, they could impede or modulate the activities of PGR-α and PGR-β isoforms (Kowalik *et al.*, 2013b). Adding to the tapestry, a third isoform, PGR-γ (60 kDa), resides in the human placenta, its precise role still enigmatic. Nonetheless, it's known to form heterodimers with PGR-α and PGR-β, exerting influence over their transcriptional activities (Wei *et al.*, 1996).

Before engaging its ligand, unbound PGRs inhabit the cytoplasm, bound within a complex of chaperone proteins. Once P4 engages with the LBD, a cascade of conformational shifts transpires. PGR is emancipated from the embrace of chaperone proteins, enabling its journey into the nucleus. Inside the nucleus, PGR dimerizes, homing in on the hormone response element sequence (HRE) within the target gene's promoter.

Regardless of this heterogeneous tissue and isoform-specific gene expression regulation, PGRs isoforms also interact with one another by regulating their own activity. PGR-α assumes a role of inhibition over PGR-β's actions through its inhibitory domain (ID), effectively modulating the impact of P4 on its target cells (Pieber *et al.*, 2001).

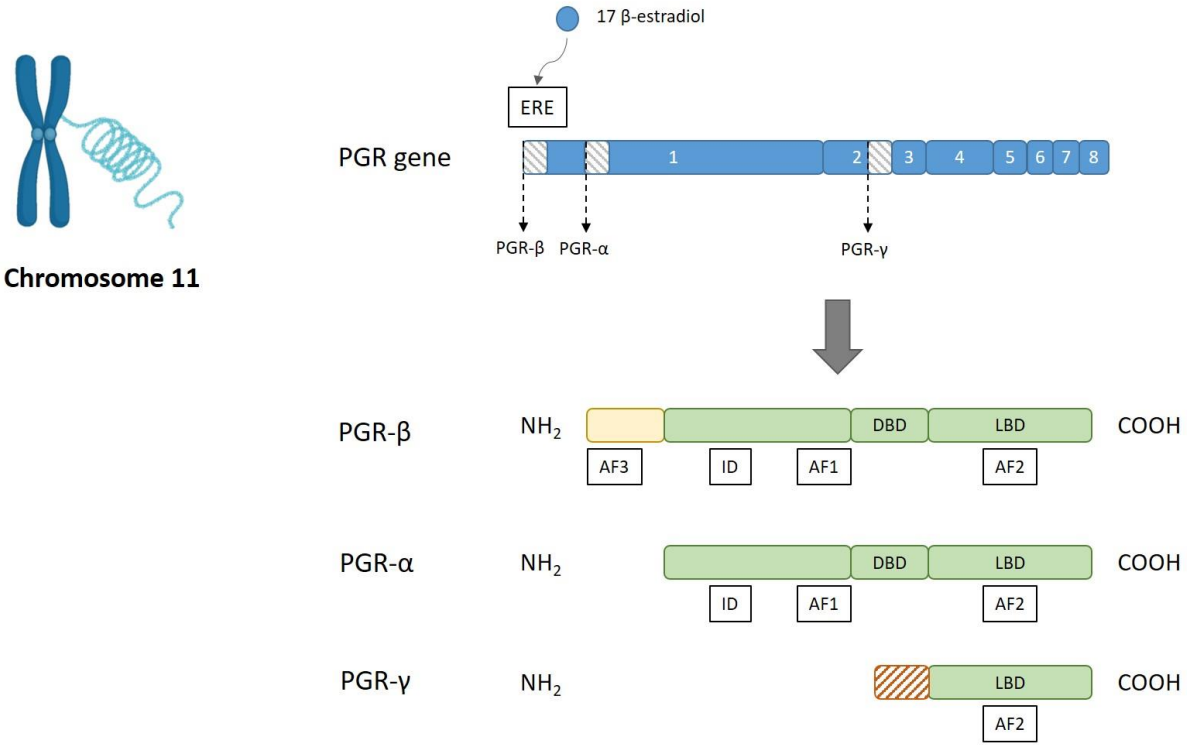

Figure 5. Schematic representation of the classical progesterone receptor (*PGR*) gene. *PGR* gene is located on chromosome 11 and in response to estrogen binding to ERE, the *PGR* gene produces different isoforms (*PGR-α*, *PGR-β*, and *PGR-γ*) through the activation of specific promoters. ERE, estrogen response elements; AF, activation function domain; ID, inhibitory domain; DBD, DNA-binding domain; LBD, ligand-binding domain; NH2, amino terminal region; COOH, carboxyl terminal region. Adapted from (Medina-Laver *et al.*, 2021).

This dynamic interrelationship is believed to underlie a compensatory mechanism that contributes to the meticulous regulation of P4's influence throughout the ovarian cycle. Within luteal cells, a finely tuned balance emerges: higher P4 levels induce a higher *PGR-α* expression. This augmented *PGR-α* presence, in turn, curbs *PGR-β* transcription, mitigating the effects of P4. Conversely, when P4 levels diminish within these cells, a converse scenario unfolds. Lower P4 levels in these cells may suppress *PGR-α* expression, increase *PGR-β* expression and subsequently enhance P4 action (Kowalik *et al.*, 2013b).

3.1.2 Non-classical PGRs

Non-classical progesterone receptors constitute a distinctive category of receptors located on the cell surface. These receptors are structurally related to G protein-coupled receptors and single transmembrane (TM) receptors, endowing them with the ability to mediate rapid responses upon binding to P4 (Gerdes *et al.*, 1998; Zhu *et al.*, 2003b) (Figure 6). In contrast to

classical P4 receptor-mediated actions, non-classical receptors trigger a diverse array of physiological effects by activating secondary messengers, such as MAP kinase (MAPK) and signal transduction pathways within seconds of P4 interaction (Migliaccio *et al.*, 1998; Saner *et al.*, 2003) (Figure 7).

This group of receptors can be divided into two primary families: the membrane progestin receptors (mPRs) family and the progesterone receptor membrane component (PGRMC) family (Pierson-Mullany and Lange, 2004; Tang *et al.*, 2005; Peluso, 2007; Thomas, 2008; Thomas and Pang, 2012) (Figure 6).

Members of the mPR family, which are proposed to have P4-binding activity, are also known as progestin and AdipoQ receptors (PAQR). They belong to the G protein-coupled receptor (GPCR) superfamily and include the membrane progesterone receptors mPRα, mPRβ, mPRγ, mPRđ, and mPRε (also referred to as PAQR7, PAQR8, PAQR5, PAQR6, and PAQR9, respectively). These receptors have been cloned and partially characterized in various mammalian species (Zhu *et al.*, 2003a). While *in vitro* studies have demonstrated biological functions for these receptors, the question of their functional identity as progesterone receptors remains a subject of inquiry due to their complex actions *in vitro* (Karteris *et al.*, 2006).

The second family of non-classical P4 receptors is the PGRMC family, which includes PGRMC1, PGRMC2 and the less studied neudecin, and also neuferricin. These non-classical PGRs share a similar non-covalent heme-binding domain related to cytochrome b5, a well-known functional interaction partner of microsomal cytochrome P450 (CYP) monooxygenase systems. PGRMC1 and PGRMC2 were initially recognized as heme-1 domain proteins (HPR6.6) and Dg6, respectively (Falkenstein *et al.*, 1996; Meyer *et al.*, 1996). Importantly, emerging research highlights potential interactions between PGRMC1 and mPRα, suggesting potential crosstalk among different families of non-classical progesterone receptors (Thomas *et al.*, 2014; Sueldo *et al.*, 2015). Nevertheless, some studies suggest that they are not even activated by P4 (Krietsch *et al.*, 2006).

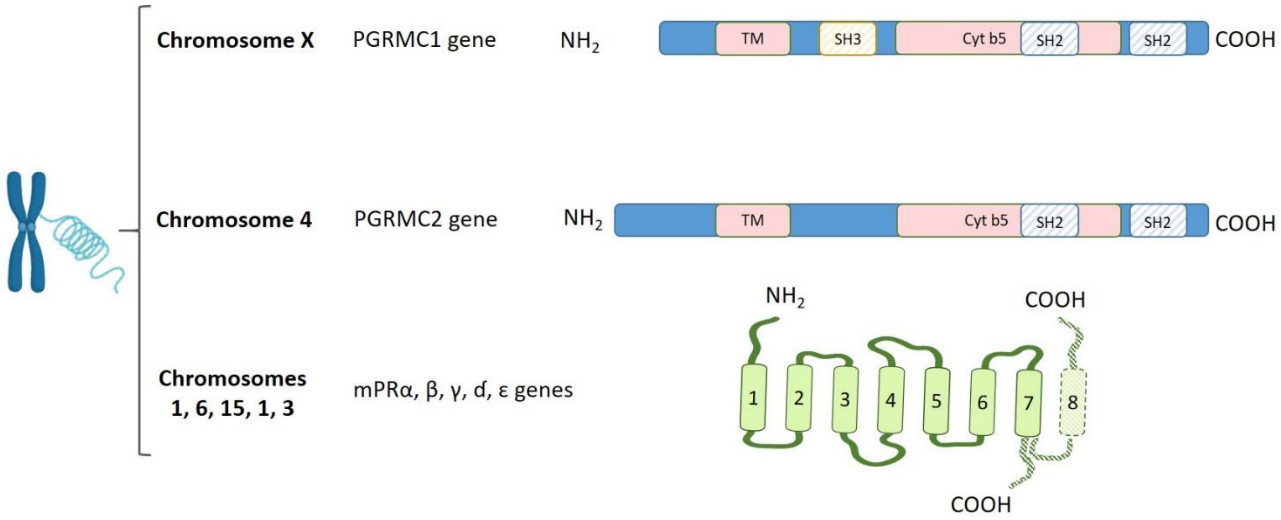

Figure 6. Schematic representation of the non-classical progesterone receptor (*PGRs*) genes. The figure illustrates the chromosomal localization of the non-classical *PGRs*. *PGRMC1* is located on the X chromosome, while *PGRMC2* resides on chromosome 4. Additionally, the genes encoding membrane progestin receptors (*mPRα*, *β*, *γ*, *δ*, and *ε*) are distributed across chromosomes 1, 6, 15, 1, and 3, respectively. Both *PGRMC1* and *PGRMC2* feature a single transmembrane domain (TM) at the N-terminal end, along with a cytochrome b5 (Cyt) domain. Analysis of Src homology 2 domain (SH2) and Src homology 3 domain (SH3) revealed specific binding sites that allow them to bind specifically to other proteins. The *mPRα*, *mPRβ*, and *mPRγ* receptors were initially proposed as traditional G protein-coupled membrane receptors, characterized by their typical 7-8 transmembrane (TM) domains, with the N-terminus positioned extracellularly. NH2, amino terminal region; COOH, carboxyl terminal region. Adapted from (Medina-Laver *et al.*, 2021).

Despite their relatively diminished P4 binding affinity in comparison to classical P4 receptors, non-classical PGRs have been associated with sexual behavior modulation in mice (Frye *et al.*, 2006), meiotic maturation in Xenopus oocytes (Finidori-Lepicard *et al.*, 1981), the acrosomal reaction in human sperm (Foresta *et al.*, 1993), as well as the regulation of ion efflux in murine neurons (Viéro *et al.*, 2006), vascular myocytes (Barbagallo *et al.*, 2001), and rat epithelial cells (Head *et al.*, 1999).

4. Classical and non-classical PGRs in reproductive tissues

P4 serves as a central orchestrator of essential processes in the female reproductive tract, extending its influence far beyond the uterus. The mechanisms through which P4 exerts its

effects involve both classical and non-classical PGRs. In the ovary, P4 action influences from follicular development to ovulation (Pelosi *et al.*, 2015). Classical PGRs expression in granulosa cells is triggered by the E2 surge, impacting ovulation (Iwai *et al.*, 1990). Furthermore, the corpus luteum's formation initiates P4 secretion, which interacts with both classical and non-classical PGRs across various cell types (Revelli *et al.*, 1996). Notably, PGRMC1 and PGRMC2 depletion in luteal cells disrupts growth factor expression, such as VEGF-a, Kit ligand and antimüllerian hormone (AMH) (Peluso *et al.*, 2018). This complex interplay extends to the hypothalamic-pituitary-gonadal axis, where P4's feedback action, mediated by classical and non-classical pathways, regulates gonad hormonal secretion (Scott *et al.*, 2002; Bashour and Wray, 2012).

Follicular development and recruitment also engage P4 receptors. While classical PGRs are indispensable for successful ovulation, they aren't vital for early follicular stages (Robker and Richards, 2000). In contrast, non-classical PGR, particularly PGRMC1, influence antral follicle development and ovarian reserve (Griffin *et al.*, 2014; Guo *et al.*, 2016; Will *et al.*, 2017). As oocyte competence acquisition is central to reproductive success, the involvement of PGRs, both classical and non-classical, is significant. Their roles in oocyte maturation, interaction with other receptors, and developmental competence are areas of active investigation (Robker and Richards, 2000; Thomas *et al.*, 2004; Deng *et al.*, 2009; Aparicio *et al.*, 2011; Lodde and Peluso, 2011; Jühlen *et al.*, 2016; Shi *et al.*, 2016; Terzaghi *et al.*, 2016; Roy *et al.*, 2017).

P4's action extends to the oviduct, where it regulates transport. The journey of cumulus-oocyte complexes and embryos is intricately regulated by steroid levels (Mahmood *et al.*, 1998). PGRs, both classical and non-classical, are expressed in the oviductal tissue of various species (Saint-Dizier *et al.*, 2012; Tahir *et al.*, 2013; Kowalik *et al.*, 2016; Hazano *et al.*, 2021). However, the specific contribution of PGRs in this context remains a subject of ongoing research.

In conclusion, P4 and its receptors navigate a complex network in the female reproductive tract. Their roles span from the ovaries, where P4 modulates follicular development and ovulation, to the oviduct, where it influences transport mechanisms. The interplay between classical and non-classical PGRs constructs reproductive processes, revealing the intricate nature of female reproduction's molecular synchronization.

4.1. PGRs IN THE UTERUS

In the human uterus, P4 action has been described following both classical (Conneely *et al.*, 2002) and non-classical (Gellersen *et al.*, 2009) signalling pathways. The expression of classical and non-classical PGRs in the uterus significantly oscillates throughout the menstrual cycle by regulating all the critical events that finally establish a receptive endometrial microenvironment and correct pregnancy (Salsano *et al.*, 2017, 2020; Medina-Laver *et al.*, 2021; Salsano *et al.*, 2021). During the follicular phase, extended exposure to estrogen induces the expression of classical PGRs in uterine cells. In the subsequent luteal phase, P4 exposure inhibits ER expression, which leads to estrogenic drive that, in turn, augments P4 responsiveness. Although the role of non-classical PGRs in the uterus is not well-known, its expression has been documented in human, rhesus monkey, mouse, zebrafish and bovine uterus (Mote *et al.*, 2000; Fernandes *et al.*, 2005; Zhang *et al.*, 2008; Luciano *et al.*, 2010; Keator *et al.*, 2012; Pru *et al.*, 2013; Salsano *et al.*, 2017; Kowalik *et al.*, 2019; Wu *et al.*, 2019a).

P4 exerts its effects in various uterine cell types, including hEnECs and hEnEScs, smooth muscle cells in the myometrium, and stromal fibroblasts in the cervix (Cowan *et al.*, 2004; Mesiano, 2007; Zhu *et al.*, 2014).

Understanding the interplay between classical and non-classical PGRs in the uterus is essential for comprehending the mechanisms underlying endometrial receptivity and successful pregnancy establishment. Certainly, we will focus on elucidating the roles of these receptors in the endometrium, as it is the primary reproductive tissue of this study, delving deeper into their intricate functions within this crucial reproductive context.

4.1.1 Role of PGRs in the endometrium

P4 plays a pivotal role in inhibiting cell proliferation in endometrial cell types, counteracting the stimulatory effects of E2 during the preovulatory phase. Conversely, P4 promotes structural and functional modifications, including decidualization that prepare the endometrium to receive and support embryo implantation (Jabbour *et al.*, 2006; Wetendorf and DeMayo, 2012).

Understanding the mechanisms of action of both classical and non-classical PGRs, as detailed previously, is crucial for obtaining a comprehensive view of their combined mechanisms in the human endometrium (Cope and Monsivais, 2022). Together, these receptors coordinate the response to P4 in the different cellular layers of the endometrium. Classical PGRs are primarily involved in transcriptional regulation, activating genes and signalling pathways necessary for cellular differentiation and structural remodeling within the endometrium (Dias Da Silva *et al.*, 2024). In hEnECs, classical PGRs initiate endometrial receptivity through pathways that involve essential proteins like the SRY-box transcription factor (SOX) family, which play a critical role in gene regulation during decidualization. SOX17, for example, modulates the wingless-related integration site (WNT) signalling pathway, important in endometrial cell differentiation and embryo implantation (Engert *et al.*, 2013; Kinnear *et al.*, 2019), while SOX4 can impact pathways related to cell cycle regulation and apoptosis (Chen *et al.*, 2016; Huang *et al.*, 2022; S *et al.*, 2024) (Figure 7).

Another transcription factor involved in the classical PGRs mechanism of action is GATA binding protein 2 (GATA2). This transcription factor enhances the expression of key genes involved in stromal cell proliferation and differentiation and collaborates with other transcription factors like HOXA10 to activate genes that support the structural and functional changes of the endometrial lining. HOXA10, in turns, promotes integrin αvβ3 expression creating a more receptive surface for embryo attachment (Zanatta *et al.*, 2010; Kohlmeier *et al.*, 2020; Keske *et al.*, 2024).

In contrast, within hEnSCs, classical PGRs regulate signalling pathways such as the Hedgehog pathway, involving transcription factors such as indian hedgehog (IHH), playing a vital role in the P4-regulated transformation of the endometrium. Once expressed, IHH acts as an intermediary in the communication between hEnECs and hEnSCs, coordinating the action of P4 in preparing the endometrial tissue. In response to IHH signalling, stromal cells activate smoothened (SMO) protein, trough patched 1 protein (PTCH1), which propagates the Hedgehog signal and induces the activation of GLI family of zinc finger transcription factors (such as GLI1). This signalling is critical for transforming the endometrium into a receptive environment, with adequate structural and biochemical changes to support implantation and early pregnancy (Wei *et al.*, 2010; Dias Da Silva *et al.*, 2024). Another Hedgehog target is

chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) protein which can directly influence the HAND2 transcription factor by promoting its expression during the decidualization process (Katayama *et al.*, 2006; Kurihara *et al.*, 2007; Oh *et al.*, 2023). COUP-TFII also interacts with bone morphogenetic protein 2 (BMP2) (Heng *et al.*, 2010), previously activated by the cAMP/PKA (Yin *et al.*, 2011) to enhance stromal differentiation WNT pathway and FOXO1 protein regulate stromal-epithelial interactions. FOXO1 also regulates the expression of the decidualization markers, PRL and IGFBP1 (Ujvari *et al.*, 2017). FOXO1 is activated by pathways like PI3K/AKT, which control its nuclear localization and activity, thereby enhancing its role in promoting decidualization (Yin *et al.*, 2011). Classical PGRs also acts directly through hEnSCs mediating the local production of COX2, via extracellular signal-regulated kinases 1 and 2 (ERK1/2) pathway, which derive to prostaglandins production, such as PGE2 as previously mentioned (Banu *et al.*, 2008; Kong *et al.*, 2019). PGE2 then modulates the activity of HAND2 that suppress the mitogenic activity of fibroblast growth factors (FGFs) (Figure 7) (Mazur *et al.*, 2015; Šućurović *et al.*, 2020).

Non-classical PGRs, meanwhile, act through rapid membrane-associated signalling cascades, to modulate gene expression indirectly (Dressing *et al.*, 2011). This includes cAMP/PKA and MAPK pathways, alongside additional regulators like cyclic guanosine monophosphate (cGMP), phospholipase C (PLC), and protein kinase G (PKG), which contribute to complex intracellular signalling (Migliaccio *et al.*, 1998; Pierson-Mullany and Lange, 2004; Mittelman-Smith *et al.*, 2017). These pathways can activate nuclear factor kappa-light-chain-enhancer of activated B cells (NFKβ), which regulates FOXO1 trough NOTCH1 pathway (King *et al.*, 2001; Su *et al.*, 2015; Shukla *et al.*, 2019). Finally, FOXO1 activation drives the expression of PRL and IGFBP1 in hEnSCs (Ujvari *et al.*, 2017; Jiang *et al.*, 2021). Non-classical PGRs signalling also intersects with other pathways, modulating factors such as SERPINE1 mRNA binding protein 1 (SERBP1), which helps to modulate gene expression involved in the structural and functional transformations of hEnSCs during decidualization (Salsano *et al.*, 2017) (Figure 7). However, the precise mechanisms of non-classical PGRs are still not fully understood.

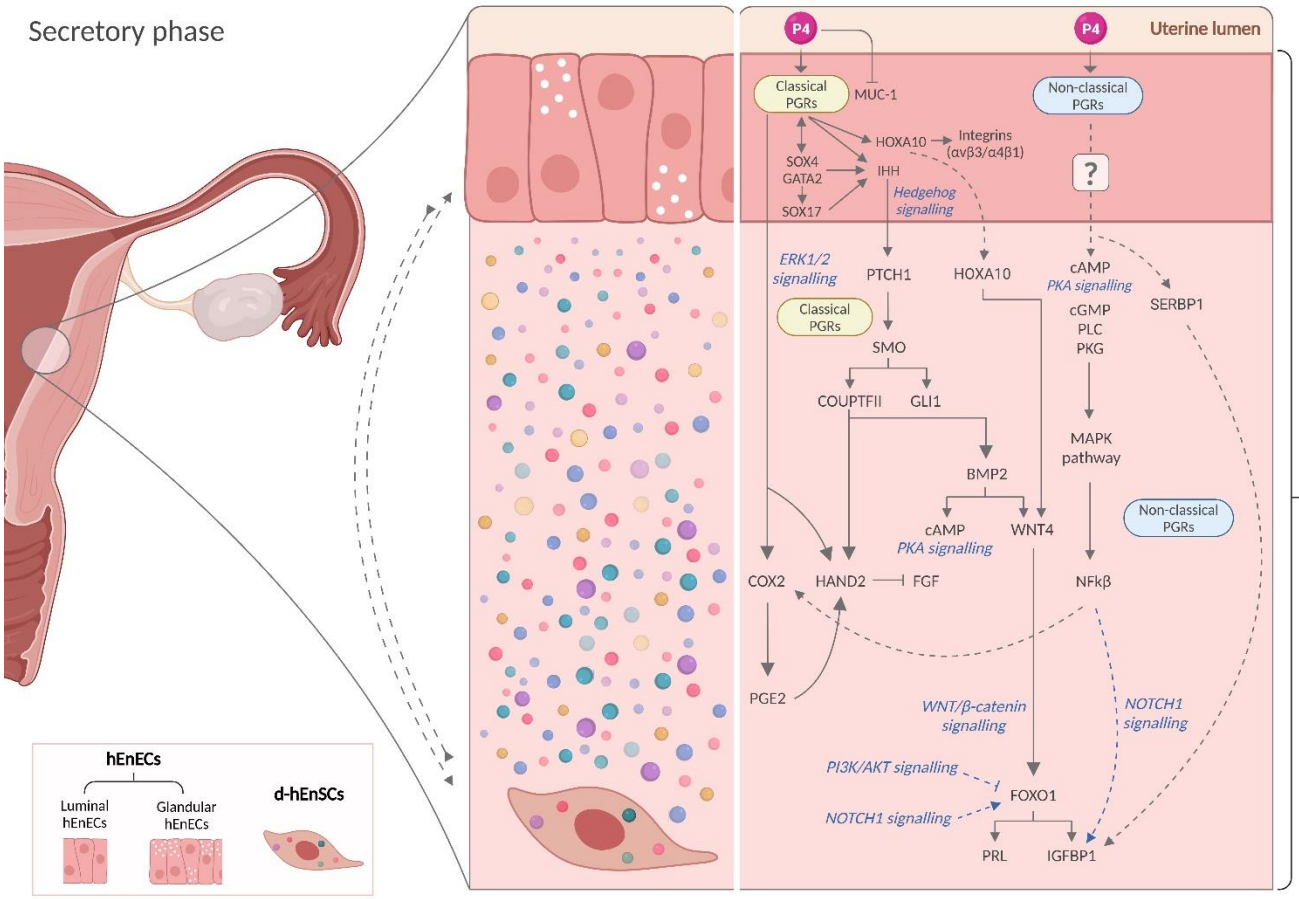

Figure 7. Classical and non-classical progesterone receptors (PGRs) signalling in response to progesterone during secretory phase of the human endometrium. Classical and non-classical PGRs mediate its action through the endometrial epithelial cells (hEnECs) or directly within the decidualized human endometrial stromal cells (d-hEnSCs). BMP2, bone morphogenetic protein 2; cAMP, cyclic adenosine 3':5' monophosphate; cGMP, cyclic guanosine monophosphate; COUPTFII, chicken ovalbumin upstream promoter transcription factor II; COX2, cyclooxygenase 2; FGF, fibroblast growth factors; FOXO1, forkhead box protein O1; GATA2, GATA binding protein 2; GLI1, GLI Family Zinc Finger 1; HAND2, neural crest derivatives expressed transcript 2; HOX10, homeobox A10; IHH, indian hedgehog; IGFBP1, insulin-like growth factor-binding protein 1; MAPK, MAP kinase; MUC-1, Glycoprotein mucin 1; NFKβ, nuclear factor kappa-light-chain-enhancer of activated B cells; NOTCH1, neurogenic locus notch homolog protein 1; P4, progesterone; PGE2, prostaglandin E2; PKA, protein kinase A; PKG, protein kinase G; PLC, phospholipase C; PRL, prolactin; PTCH1, patched 1; SERBP1, SERPINE1 mRNA binding protein 1; SMO, smoothened protein; SOX4, SRY-box transcription factor 4; SOX17, SRY-box transcription factor 17; WNT, wingless-related integration site. Adapted from (Dias Da Silva *et al.*, 2024). *Created with BioRender.com (2024).*

Alternatively, the balance between classical and non-classical PGRs isoforms also helps to maintain precise control over the reproductive hormonal effects in the endometrium (Patel *et al.*, 2015). During the menstrual cycle, the response to P4 varies due to cell-specific differences in *PGR-α* and *PGR-β* expression. In the proliferative phase, E2 drives *PGRs* expression in both stromal and epithelial cells rising their levels in tandem, but a decrease in

PGR-α occurs in epithelial cells by the end of the secretory phase, while *PGR-β* levels remain constant, aiding in glandular secretion. In contrast, stromal cells consistently express *PGR-α* (Mote *et al.*, 1999; Patel *et al.*, 2015). Although both isoforms are present in the endometrium, *PGR-α* appears vital for normal uterine function and implantation (Conneely *et al.*, 2002; Mulac-Jericevic *et al.*, 2003; Kaya *et al.*, 2015). Maintaining a balance between *PGR-α* and *PGR-β* expression is crucial for epithelial cell proliferation and the prevention of uterine disorders. Brosens *et al.* found that excessive expression of *PGR-α* and *PGR-β* in hEnSCs inhibits decidualization (Brosens *et al.*, 1999b). Moreover, overexpression of *PGR-α* leads to a hyperproliferative state in both the luminal and glandular endometrium, resulting in infertility (Fleisch *et al.*, 2009; Wetendorf *et al.*, 2017).

Classical PGRs dysfunction plays a critical role in early pregnancy complications, including conditions like preeclampsia. This disorder, marked by abnormal decidualization, reveals disrupted gene networks involving E2R and PGRs function, which may serve as indicators of a woman's risk for developing severe preeclampsia (Garrido-Gomez *et al.*, 2021). RIF has also been associated with decreased classical PGRs expression in the endometrium, as well as certain classical PGRs polymorphisms (Pisarska *et al.*, 2003; Rahnama *et al.*, 2019; Hosseinirad *et al.*, 2021). Recurrent pregnancy loss is another multifactorial condition which studies have linked with specific single nucleotide polymorphisms (SNPs) in the *PGRs* gene (Aruna *et al.*, 2010; Bahia *et al.*, 2018). These findings highlight that PGRs signalling and ratio of its isoforms, rather than P4 levels alone, could underlie many cases and open pathways for targeted therapies.

Non-classical PGRs have been observed to also be expressed in the endometrium of different mammals, as previously described. In most of them, these receptors also oscillate during the menstrual cycle to prepare the endometrium for embryo implantation. In humans, *PGRMCs*, *mPRα*, *mPRγ* and *mPRε* transcripts have been demonstrated to significantly vary according to the menstrual cycle phase (Fernandes *et al.*, 2005; Salsano *et al.*, 2017). In particular, transcripts of PGRMC1, mPRγ, and mPRε are upregulated in the proliferative phase and progressively decrease in the secretory phase, while mPRα and PGRMC2 mRNA are significantly overexpressed in the secretory phase, coinciding with the rise in P4 levels (Fernandes *et al.*, 2005; Salsano *et al.*, 2017; Wu *et al.*, 2019a). Although *mPRβ* is more

abundant than *mPRα* in the human endometrium, its expression remains relatively stable during the menstrual cycle (Fernandes *et al.*, 2005). Nonetheless, a reduction in endometrial *mPRβ* gene expression on days 10-14 of the human menstrual cycle has been observed in patients with a history of recurrent miscarriages (Lyzikova *et al.*, 2020).

PGRMC1 is involved in cell proliferation and is, thus, responsible for endometrium development in the first half of the menstrual cycle (Friel *et al.*, 2015). In fact, its ablation results in reduced fertility in female mice (Mccallum *et al.*, 2016). In the secretory phase, PGRMC1 stromal overexpression inhibits decidualization and creates an improper embryo implantation environment (Salsano *et al.*, 2017). In contrast, PGRMC1 downregulation in this phase may also impair uterine receptivity and blastocyst implantation (Wang *et al.*, 1998). Therefore, balanced PGRMC1 levels are necessary for proper endometrial receptivity. Garrido-Gómez *et al*. demonstrated that PGRMC1 behaves differently in human receptive endometrium vs. a non-receptive endometrium (Garrido-Gómez *et al.*, 2014). Salsano *et al*. detected PGRMC1 movement from the cytoplasm to the nucleus when decidualization process occurred in humans (Salsano *et al.*, 2017). This contrasts with the expression of the classical PGR, which is known to be absent in the endometrial stroma upon embryo implantation (Deryabin *et al.*, 2020). This interesting nuclear localization suggests a possible function for PGRMC1, regulating the expression of a specific set of distinct genes from the target genes of classical *PGRs* during decidualization. Alternatively, PGRMC1 stroma nuclear localization may lead to participation in cell cycle regulation when proliferative stromal cells are in transition to terminally differentiated decidual cells (Salsano *et al.*, 2020).

Endometrial PGRs dysregulation is associated with various reproductive disorders, such as endometriosis. This condition is characterized by abnormal endometrial tissue growth and morphology changes that mirror the eutopic endometrium's response to hormonal fluctuations (Bulun *et al.*, 2006). Therefore, its proliferation is induced by E2 exposure in the proliferative phase and is inhibited by P4 in the secretory phase. P4 resistance may cause this abnormal endometrial proliferation (Lyndrup *et al.*, 1987). Classical and non-classical PGRs expression has been reported in the ectopic endometrium of endometriosis patients and in endometrial hyperplasia, although studies yield mixed results (Nisolle *et al.*, 1994; Kunz *et al.*,

1996; Rocha-Junior *et al.*, 2019; Wu *et al.*, 2019a; Sletten *et al.*, 2020; Vázquez-Martínez *et al.*, 2020).

5. PGRMC2

PGRMC2 gene is located on chromosome 4 at the position 4q28.2 (Gerdes *et al.*, 1998). Moreover, PGRMC2 protein is structurally similar to PGRMC1 and it is expressed in various reproductive tissues, including the uterus, ovaries, and placenta (Gerdes *et al.*, 1998; Kimura *et al.*, 2012; Galmozzi *et al.*, 2019).

Despite its similarities to PGRMC1, PGRMC2 has distinct functions. Its multifaceted functions encompass the inhibition of the SKOV-3 ovarian cancer cells migration (Albrecht *et al.*, 2012), regulation of sterol metabolism (Kimura *et al.*, 2012; Wu *et al.*, 2019b), contribution to cell proliferation through ERK activation (Liu *et al.*, 2009), association with ovarian reserve status in young women (Skiadas *et al.*, 2012), participation in cell cycle regulation (Griffin *et al.*, 2014; Friel *et al.*, 2015; Peluso *et al.*, 2018, 2019; Li *et al.*, 2019; Wu *et al.*, 2019a), and is involved in the progression of endometriosis, inhibiting cell proliferation (Keator *et al.*, 2012; Bunch *et al.*, 2013). Despite its known functions, the role of PGRMC2 in the human endometrium, particularly in decidualization, remains elusive. Its importance is evident, in fact, PGRMC2 deficiency causes premature uterine senescence in PGRMC2 knockout mice [unlike normal senescence that occurs in the physiological decidualization process (Deryabin *et al.*, 2020; Lucas *et al.*, 2020)] and leads to post-implantation failure (Jiang *et al.*, 2002; Mccallum *et al.*, 2016; Clark *et al.*, 2017). A similar scenario has been observed in PGRMC1- and PGRMC2-knockout zebrafish (Keator *et al.*, 2012; Wu *et al.*, 2019a; Wu and Zhu, 2020). Additionally, there is limited knowledge regarding PGRMC2 expression across the human menstrual cycle and its function in human endometrial receptivity or decidualization process.

In addition to its role in reproductive tissues, PGRMC2 has been identified as a potential biomarker for certain gynecological cancers. Elevated levels of PGRMC2 have been detected in ovarian and endometrial cancers, indicating its potential involvement in tumor progression and chemoresistance (Peluso *et al.*, 2021). Targeting PGRMC2 in these cancers could offer new

therapeutic avenues for improving patient outcomes. Understanding how P4 signalling occurs in endometrial cells is important to guide the development of more effective therapeutic approaches to the numerous reproductive diseases caused by disrupted P4 signalling, resulting in subfertility or infertility (Szekeres-Bartho *et al.*, 2008; Bunch *et al.*, 2013; Kim *et al.*, 2013; Zhang and Murphy, 2014; Hirota, 2019b; Liao *et al.*, 2019).

Given the sparse literature available on this protein, particularly in reproductive tissues, our study aims to bridge this gap by investigating its role in endometrial function. In doing so, we characterized endometrial PGRMC2 expression throughout the human menstrual cycle and in hEnSCs subjected to an *in vitro* model of human endometrial decidualization. Additionally, we describe the functional implications of PGRMC2 with a silencing approach assay, integrating transcriptomic and proteomic techniques. To further understand its role, we evaluated the functional implication of PGRMC2 in the embryo invasion process using an outgrowth *in vitro* model. Finally, we also compared endometrial PGRMC2 expression with PGRMC1 and PGR, providing a comprehensive analysis of its functional relevance in the endometrium.

II. HYPOTHESIS

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Based on the evidence that PGRMC2, a multifunctional protein identified in various reproductive tissues, is likely to play a pivotal role in the human endometrium, we hypothesize that PGRMC2 serves as a critical regulator in orchestrating the intricate process of endometrial stromal cell transformation into decidual cells. This regulatory function is anticipated to contribute significantly to the establishment of an optimal microenvironment conducive to successful embryo implantation. Furthermore, we propose that PGRMC2 exerts an indispensable role in facilitating embryo invasion into the endometrial tissue, thereby playing a crucial role in the early stages of pregnancy establishment.

III. OBJECTIVES

III. OBJECTIVES

The main objective of this thesis dissertation was to characterize endometrial PGRMC2 along the human menstrual cycle in healthy women and to study the role of PGRMC2 during *in vitro* decidualization and embryo implantation.

Accordingly, the specific objectives were to:

- To describe the endometrial PGRMC2 gene expression and protein abundance and locate it along the human menstrual cycle.
- Compare the endometrial *PGRMC2* expression with *PGRMC1* and classical *PGRs* expression along the human menstrual cycle.
- To study PGRMC2 behaviour and cellular localization during the *in vitro* decidualization process and compare it with *PGRMC1* and classical *PGRs* expression.
- To evaluate the functional implications of PGRMC2 with a silencing approach assay in decidualized human endometrial stromal cells, integrating transcriptomic and proteomic techniques.
- To corroborate if trophoblast invasion is affected when *PGRMC2* is silenced in decidualized human endometrial stromal cells with an outgrowth *in vitro* model.

IV. EXPERIMENTAL DESIGN

IV. EXPERIMENTAL DESIGN

This thesis dissertation can be divided into two distinct groups of experiments (Figure 8).

Group 1: Characterization of PGRMC2 expression in the human endometrium across the menstrual cycle.

The first group focuses on characterizing the protein PGRMC2 in endometrial samples obtained from donors in the natural menstrual cycle (*in vivo* experiments). While the term "*in vivo* experiments" might suggest a clinical trial, in this context, it simply denotes the study of naturally occurring physiological processes. These samples were utilized to describe the expression patterns of PGRMC2 throughout the human natural menstrual cycle in the whole endometrium.

Group 2: Functional studies of PGRMC2 in endometrial stromal cells.

The second group of experiments aimed to elucidate the functional role of PGRMC2 in the decidualization process of endometrial stromal cells by studying the gene and protein expression of PGRMC2 in addition to conducting transcriptomic and proteomic analyses in decidualized endometrial stromal cells (*in vitro* and functional experiments, respectively). Furthermore, the role of PGRMC2 in trophoblast invasion into decidualized endometrial stromal was also investigated. Endometrial samples were obtained from oocyte donors undergoing controlled ovarian stimulation cycles as part of an egg donation program. Controlled ovarian stimulation involved the administration of estradiol valerate (6 mg/day; Meriestra, Novartis, Barcelona, Spain) from the first or second day of menstruation for a period of 10-15 days until the day of follicle pick-up. Endometrial samples were sometimes large enough to be used for multiple analyses to maximize the data obtained. These thorough analyses aimed to provide a detailed understanding of how PGRMC2 influences the cellular environment during the critical process of decidualization.

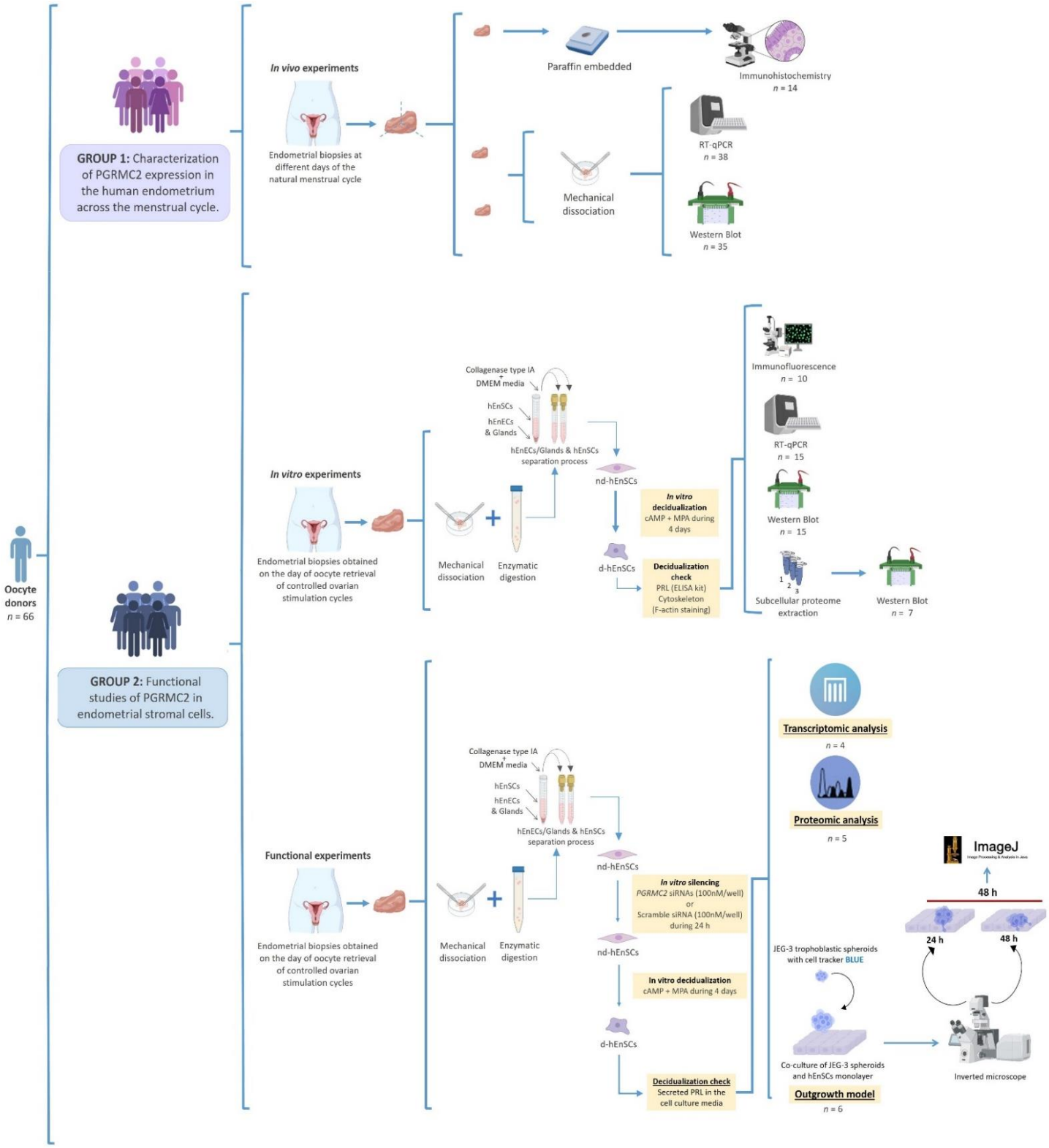


Figure 8. Study design. Endometrial biopsies were collected from 66 donors at different days of the natural menstrual cycle for the group 1 of experiments or undergoing oocyte donation cycles for the group 2 of experiments. For the group 1 of experiments, endometrial biopsies were cut into three sections. One of said sections was used to perform immunohistochemistry. The other two sections were used to analyze PGRMC2

expression by RT-qPCR and western blot. For the group 2 of experiments *(in vitro* experiments and functional experiments), hEnSCs were isolated from endometrial biopsies to analyze PGRMC2 localization, mRNA expression, and protein abundance during *in vitro* decidualization and to study transcriptomically and proteomically the differences in d-hEnSCs when *PGRMC2* was silenced. Finally, an outgrowth model using JEG-3 spheroids was carried out silencing *PGRMC2* in d-hEnSCs. d-hEnSCs, decidualized human endometrial stromal cells; DMEM, Dulbecco's modified Eagle medium; h, hours; hEnSCs, human endometrial stromal cells; hEnECs, human endometrial epithelial cells; MPA, medroxyprogesterone 17-acetate progestin; nd-hEnSCs, non-decidualized human endometrial stromal cells; PGRMC2, progesterone receptor membrane component 2; PRL, prolactin; RT-qPCR, quantitative reverse transcription polymerase chain reaction. *Created with BioRender.com (2024).*

Henceforth, we will delineate the following sections of the thesis by categorizing them into the two experimental groups conducted.

V. MATERIAL AND METHODS

V. MATERIAL AND METHODS

1. ETHICAL APPROVAL

The study was approved by the ethical committee for clinical research at the Instituto Universitario, Instituto Valenciano de Infertilidad (IU-IVIRMA Valencia), Spain (protocol number 1911-FIVI-103-FD). All participants provided written informed consent before undergoing surgical procedures and sample collection. Relevant clinical information of each participant (e.g., participants age, BMI, ovarian reserve, stage of the menstrual cycle, use of medication, uterine pathologies, allergies, diseases, drug or alcohol use, and reproductive outcomes) were asked or evaluated before the procedure and in accordance with the data protection law.

2. PARTICIPANTS

Donors were recruited between January 2020 and March 2022, in the IVI clinics located in Valencia, under the direction of the IVI Foundation (IIS La Fe, Valencia, Spain). This research was funded by Instituto de Salud Carlos III (ISCIII) granted to F.D. (PI20/00405 and PI23/00860), co-funded by the European Union, supported by a predoctoral research grant from Generalitat Valenciana (ACIF/2019/262) and through an internal funding by IVI Foundation (1911-FIVI-103-FD).

Donors seeking oocyte donation program in their natural menstrual cycle (group 1 of experiments) or under ovarian stimulation cycle (group 2 of experiments) meeting the following criteria were consulted by assisted reproductive practitioners for inclusion in the study through IVIRMA clinic gynaecology practices between 2020 and 2022.

- Inclusion criteria:

  - Aged 18-35 years old.
  - Body mass index (BMI) of 18.0-28.9 kg/m^2.

- Regular menstrual cycles (24-35 days).
- Normal ovarian reserve antral follicle count > 8.
- Proven fertility (>1 birth).

- Exclusion criteria:
  - Use of hormone treatment or oral contraceptive pills (OCP).
  - Ovarian stimulation (only for group 1).
  - Presence of intrauterine device (IUD).
  - Uterine pathologies (e.g., adenomyosis, endometriosis, endometritis, pelvic inflammatory disease).
  - Abnormal bleeding.
  - Irregular menstrual cycle: <24 or >35 days.
  - Any pregnancy in the 3 months prior to sample collection.
  - Special circumstances: stress, infections (cold, candidiasis, etc.), alcohol or drug use.

The participants were checked by the first day of the menstrual cycle and ultrasonographically evaluated of follicle growth to stablish the menstrual cycle stage and to exclude those with uterine pathologies.

3. ENDOMETRIAL TISSUE COLLECTION

A total of 77 endometrial tissue samples were obtained from 66 healthy fertile egg donors who fulfilled the criteria of the IVIRMA Valencia oocyte donation program (Figure 8). All of them were obtained from the uterine fundus with a cannula Pipelle de Cornier® (CCD Laboratories, France).

Endometrial tissue specimens used for the experiments in group 1 were collected at different days of the natural menstrual cycle and were distributed into five groups: early proliferative phase (EP; *n* = 9, days 3-8), late proliferative phase (LP; *n* = 8, days 9-14), early secretory phase (ES; *n* = 9, days 15-18), mid-secretory phase (MS; *n* = 8, days 19-22), and late secretory phase (LS; *n* = 7, days 23-28). Each biopsy was divided into three pieces for three

different experiments: immunohistochemistry (*n* = 14), mRNA quantification (*n* = 38), and protein quantification (*n* = 35). In cases where the biopsy size did not allow for three pieces, it was prioritized for mRNA and protein analysis.

The experiments belong to group 2, endometrial tissue specimens were obtained on the day of oocyte retrieval of controlled ovarian stimulation cycles of donors. A total of 15 samples (*n* = 15) were used for mRNA quantification, *n* = 15 for protein abundance quantification, *n* = 10 for PGRMC2 cellular localization by immunofluorescence and *n* = 7 for PGRMC2 subcellular localization, *n* = 6 for trophoblast invasion assays, *n* = 4 for RNA sequencing (RNA-seq) analysis and *n* = 5 for Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH-MS) analysis. Some larger endometrial samples were used for multiple analyses to maximize the data obtained. These analyses focused on comparing the expression, functionality, and molecular profiling of PGRMC2 in endometrial stromal cells that were decidualized *in vitro versus* those that were not.

4. PGRMC2 IMMUNOHISTOCHEMISTRY IN HUMAN ENDOMETRIAL TISSUE

For immunohistochemical analysis (*n* = 14), endometrial tissues classified in group 1 of experiments were immediately fixed in 4% (vol/vol) paraformaldehyde, embedded in paraffin, cut in 6 mm sections (5 µm thickness), and placed on microscope slides (Thermo Scientific, USA). After deparaffinization using xylene and rehydration with decreasing concentrations of ethanol and distilled water, sections were rinsed with 1× phosphate-buffered saline (1× PBS) and treated with a heat-induced antigen retrieval solution with citrate buffer at pH 6.0 as previously described (Salsano *et al.*, 2017).

The prepared slides were incubated overnight at 4°C within a humidified chamber, utilizing a rabbit polyclonal anti-human PGRMC2 antibody (PA5-59465, Invitrogen, USA). This specific antibody has been extensively validated in previous research studies, including those by Richardson *et al*. and Lozovyy *et al*. (Richardson *et al.*, 2020; Lozovyy *et al.*, 2021). The antibody

was diluted at a ratio of 1:300 in a solution containing 3% bovine serum albumin (BSA; Sigma-Aldrich, Spain) to ensure optimal binding conditions.

To ensure the accuracy and validity of the immunohistochemical staining, we included both negative and positive controls in our experiments. The negative controls consisted of slides incubated with 1× PBS and 3% BSA but without the primary antibody, thus confirming the specificity of the antibody staining. The positive controls involved the use of human intestine tissue (TTR008-25EA, Sigma-Aldrich, Spain), known to express the target protein.

Following the primary antibody incubation, secondary antibody was applied using the peroxidase/diaminobenzidine (DAB) kit from DAKO (Agilent Technologies, USA). Immunoreactivity was then visualized using DAB as the chromogen, producing a brown color at the site of the antigen-antibody reaction. After this, the slides were counterstained with hematoxylin, which provided a blue-purple contrast to the DAB staining, and were thoroughly washed with distilled water to remove excess reagents.

Finally, the slides were mounted with SafeMount mounting medium (Sigma-Aldrich, Spain). Slides without antibodies (negative controls) were analyzed by hematoxylin–eosin staining to check the overall condition and integrity of the biopsies. All prepared slides were examined under a Nikon Eclipse 80i microscope, which is equipped with a Nikon digital sight DS-L1 camera.

5. hEnSCs ISOLATION, CELL CULTURE, AND *IN VITRO* DECIDUALIZATION

To conduct the analyses for experimental group 2, endometrial samples were used for isolating human endometrial stromal cells (hEnSCs). The samples were first minced into smaller pieces (≤ 1 mm^3) with the assistance of blades, and then subjected to an overnight digestion at 4°C using a 1:10 dilution of collagenase type IA in phenol red-free Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, Spain). This process facilitated the breakdown of the extracellular matrix, allowing for the isolation of hEnSCs via gravity

sedimentation as previously outlined by Dominguez *et al*. (Dominguez *et al.*, 2010). This process entails utilizing the natural tendency of different components to separate based on their density when allowed to stand undisturbed for several minutes. The heavier, undigested tissue fragments, which include extracellular matrix residues and larger cell clumps such as those found in endometrial epithelial cells, gradually settle to the bottom of the tube due to gravity. Meanwhile, the lighter stromal cells, which are now free-floating as single cells or small clusters due to the enzymatic digestion, remain suspended in the supernatant. This supernatant, which contains the desired stromal cells, can then be carefully collected from the top, ensuring minimal contamination from the pellet that has settled at the bottom. This method effectively isolates the stromal cells, providing a relatively pure population for subsequent experiments.

Following isolation, primary hEnSCs were resuspended and cultured in phenol red-free DMEM/nutrient mixture F12 medium (DMEM/F12; Gibco Thermo Scientific, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS; Gibco Thermo Scientific, USA) and 0.2% antibiotics and antifungals (80 µg/mL gentamicin and 0.4 µg/mL amphotericin B), plated in 24-well plates and incubated 24 hours at 37°C with 5% CO_2 before being induced with *in vitro* decidualization treatment. At 70%–80% confluency, the medium was replaced with DMEM/F12 containing 2% FBS and 0.2% antibiotics and antifungals. They were induced through treatment with a stable cAMP analog, 0.5 mM bromo-cAMP (Sigma-Aldrich, Spain), and 1 µM MPA (Sigma-Aldrich, Spain) over 4 days. Bromo-cAMP and medroxyprogesterone are commonly used compounds to mimic the hormonal conditions of decidualization *in vitro*, inducing changes in gene expression and cellular morphology characteristic of decidualized cells (Salker *et al.*, 2010; Barragan *et al.*, 2016). Control hEnSCs were cultured simultaneously without hormone treatment, serving as non-decidualized hEnSCs (nd-hEnSCs).

To detach the cells from the wells, an incubation with TrypLE Select 1X (12563029, Gibco, Thermo Fisher Scientific, USA) for 5 minutes at 37 °C was performed. TrypLE Select is a recombinant enzyme-based solution designed to gently break down the extracellular matrix and the cell-cell adhesion proteins. This process facilitates cell detachment while preserving cell viability. Compared to the traditional use of trypsin, TrypLE Select offers several advantages. While trypsin, a protease derived from porcine sources, is widely used for cell

detachment, its enzymatic activity can sometimes compromise cell surface proteins or viability if not carefully timed. In contrast, TrypLE Select is animal-origin-free and exhibits a more controlled and milder enzymatic activity, making it especially suitable for sensitive cell types or experiments requiring prolonged handling.

After the enzymatic digestion, the action of TrypLE Select was neutralized by the addition of fresh growth medium, which dilutes and inactivates the enzyme, ensuring that cells maintain their structural and functional integrity. Additionally, mechanical scraping with the pipette tip along the bottom of the well was performed to dislodge any remaining adherent cells.

After neutralization of the enzymatic action, the cell suspension was purified by centrifugation for 5 min at 2,000 rpm. Finally, the supernatant was discarded. It's important to note that cell detachment was performed only for cells intended for downstream analyses such as RT-qPCR, western blotting (WB), transcriptomics, proteomics, etc., and not for assays like immunofluorescence or trophoblast invasion studies, where cells remain in culture.

Decidualization was checked by measuring secreted human PRL (*n* = 15) in the conditioned medium, collected before detaching them, by enzyme-linked immunosorbent assay (ELISA; Novus Biologicals, USA) and by assessing cytoskeletal reorganization via F-actin staining (*n* = 10) in both d-hEnSCs and nd-hEnSCs. The ELISA had a detection limit of 2 ng/mL PRL, and colorimetric values were quantified against a standard curve. Results are expressed as µlU/mL PRL [mean ± standard deviation (SD)] (Figure 11).

For F-actin staining, cells were fixed with 4% paraformaldehyde for 15 min, washed and incubated with 1:1,000 Rhodamine Phalloidin (ab235138, Abcam, UK) at room temperature (RT). The structure of the cytoskeleton was visualized and photographed using a fluorescence inverted AXIO microscope (Zeiss, Germany).

6. PGRMC2 IMMUNOFLUORESCENCE IN hENSCS

To assess the localization of PGRMC2 in hEnSCs extracted from group 2 of endometrial samples, following *in vitro* decidualization treatment, both nd-hEnSCs (*n* = 10) and d-hEnSCs (*n* = 10), cells were grown to 70-80% of confluence in 24-well plates and then fixed with 4%

paraformaldehyde for 15 minutes, washed thoroughly and permeabilized using 0.2% Triton-X 100 to allow antibody access to intracellular proteins. Subsequently, the cells were blocked with 5% BSA to prevent non-specific binding and incubated overnight at 4°C with 0.4 µg/mL of rabbit polyclonal anti-human PGRMC2 antibody (PA5-59465, Invitrogen, USA) diluted in 3% BSA.

Following primary antibody incubation, the cells were exposed to a secondary antibody, Alexa Fluor 488 (Santa Cruz Biotechnology, Germany), at a dilution of 1:1,000 in 3% BSA for 1 hour at RT in the dark to prevent photobleaching. To visualize nuclei, the cells were mounting with 6-diamidino-2-phenylindole (DAPI; P36931, Thermo Scientific, USA).

Each experiment was conducted in duplicate to ensure reproducibility and reliability of the results. The cytoskeletal structure and PGRMC2 localization were visualized using a fluorescence inverted AXIO microscope (Zeiss, Germany). This setup enabled the detailed observation and photographing of the cells, providing valuable insights into the role and distribution of PGRMC2 in decidualized *versus* non-decidualized hEnSCs.

7. SUBCELLULAR PROTEOME EXTRACTION IN hENSCS

To investigate the subcellular localization of PGRMC2, we employed a ProteoExtract Subcellular Proteome Extraction Kit (539790, Calbiochem, Millipore, France) to fractionate the cytoplasmic, membrane/organelle, and nuclear proteins from nd-hEnSCs and d-hEnSCs from *in vitro* cultures of stromal cells from group 2 of endometrial samples. The procedure followed the manufacturer's guidelines (n = 7 per subcellular fraction).

The purity of each subcellular fraction was verified using WB. For the cytosolic fraction, we utilized a mouse monoclonal anti-human Hsp70 antibody (1:1,000 dilution; sc373867, Santa Cruz Biotechnology, Germany) (Mayer and Bukau, 2005). Hsp70 was selected as a marker for the cytosolic fraction due to its predominant localization in the cytosol and its crucial role as a chaperone protein involved in protein folding and stress response within this cellular compartment. This choice ensures that the cytosolic fraction is enriched with proteins

primarily localized in the cytosol, thus minimizing contamination from other cellular compartments.

For the membrane/organelle fraction, a rabbit polyclonal anti-human CD98 antibody (1:1,000 dilution; sc9160, Santa Cruz Biotechnology, Germany) was used (Fenczik *et al.*, 1997). CD98 serves as an essential marker for the membrane/organelle fraction owing to its function as a transmembrane protein involved in amino acid transport across the cell membrane. By utilizing CD98 as a marker, we confirm the enrichment of proteins associated with the cell membrane or subcellular organelles within this fraction, ensuring minimal contamination from cytosolic or other compartmental proteins.

The nuclear fraction was assessed with a rabbit polyclonal anti-human histone H3 antibody (1:1,000 dilution; PA5-16183, Invitrogen, USA) (Bourbousse *et al.*, 2012). Histone H3, being a core histone protein, is integral to chromatin structure and primarily localized within the cell nucleus. Utilizing histone H3 as a marker confirms the enrichment of nuclear proteins within this fraction, thereby minimizing contamination from cytosolic, membrane, or organelle proteins.

PGRMC2 expression in the various subcellular fractions was determined by WB, using β-actin as a loading control (1:1,000 dilution; sc47778, Santa Cruz Biotechnology, Germany). Protein quantification was performed using ImageJ software, and the abundance of each protein was normalized to β-actin levels. The results were presented as protein relative abundance (PGRMC2/β-actin) ± SD.

8. PROTEIN EXTRACTION AND WB

To investigate the protein abundance of PGRMC2 (≈ 28kDa) in the endometrium throughout different phases of the menstrual cycle (group 1 of samples) and in hEnSCs decidualized *in vitro* (group 2 of samples), we performed WB analysis. Protein was extracted from total endometrial samples (total n = 35; EP, n = 7; LP, n = 8; ES, n = 6; MS, n = 8; LS, n = 6) and from hEnSCs (n = 15), respectively.

The biopsies were first washed with 1× PBS to remove blood and other contaminants and then minced into smaller pieces (≤ 1 mm^3) using scalpels at 4°C. Proteins were extracted using RIPA lysis buffer. This buffer contained 1% Triton X-100, 1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 1× protease inhibitor (Roche complete 7×), and 1× phosphatase inhibitor mixture (Sigma-Aldrich, Spain) to prevent protein degradation and dephosphorylation during the extraction process. The tissue samples were incubated with the lysis buffer on ice for 20–30 minutes, allowing the buffer to solubilize cellular membranes and release proteins. Following lysis, the samples were centrifuged at 12,000 rpm for 20 minutes at 4°C to separate the cellular fragments from the protein-containing supernatant. This supernatant was carefully collected. Protein concentration in the supernatant was determined using the Bradford protein assay, a colorimetric assay that measures protein concentration based on the binding of Coomassie Brilliant Blue dye to proteins, following the manufacturer's protocol.

15 µg of protein per sample was denatured by heating at 95°C for 5 minutes. The denatured proteins were then separated by SDS-PAGE using 8% - 12% polyacrylamide gels, which resolve proteins based on their molecular weight. The separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, USA), a semi-dry transfer method that facilitates efficient protein transfer.

PGRMC2 protein expression was determined and compared to the expression of PGRMC1 (goat monoclonal anti-human PGRMC1, 0.33 µg/mL; ab48012, Abcam, UK) and classical PGRs (PGR-α and PGR-β isoforms; mouse monoclonal anti-human PGR, 1:500 dilution; MA1-410, Invitrogen, USA). β-Actin (mouse monoclonal anti-human β-actin; 1:2,000 dilution; Santa Cruz Biotechnology, Germany) was used as a loading control. The membranes were incubated with the primary antibodies overnight at 4°C, followed by incubation with corresponding secondary antibodies conjugated to horseradish peroxidase (HRP).

The protein bands were visualized using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, USA), a chemiluminescent substrate that emits light upon reaction with HRP. The emitted light was detected and quantified using an Amersham Imager

680 system. The PVDF membranes were scanned using a Fujifilm LAS-300, ensuring high-resolution imaging of the protein bands.

For quantification, the intensity of the protein bands was analyzed using ImageJ software. The protein abundance was normalized to β-actin band intensity to account for any variations in protein loading (protein relative abundance). The results were expressed as mean ± SD for protein relative abundance in total endometrial samples (group 1 of samples) and as fold change for group 2 of samples, providing a comparative measure of protein levels.

By performing these analyses, we were able to determine the variations in PGRMC2 protein levels throughout the menstrual cycle and under *in vitro* decidualization conditions, offering insights into the role of PGRMC2 in endometrial function.

9. RNA EXTRACTION, REVERSE TRANSCRIPTION, AND REAL-TIME qPCR

To determine the relative expression of *PGRMC2* mRNA in different phases of the menstrual cycle and in hEnSCs following *in vitro* decidualization, real time qPCR (RT-qPCR) was conducted, comparing it with the expression of *PGRMC1* and classical *PGRs*. The analyses comprised total endometrial samples (total *n* = 38; EP, *n* = 8; LP, *n* = 8; ES, *n* = 8; MS, *n* = 7; LS, *n* = 7) and *in vitro* decidualized hEnSCs (*n* = 15).

9.1 RNA EXTRACTION AND QUANTIFICATION

For RNA isolation from total endometrial samples and hEnSCs, Trizol and an RNeasy Mini kit (Qiagen, USA) were utilized, respectively. The RNA concentration was measured using a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA), ensuring precise assessment of RNA purity and quantity. Subsequently, cDNA synthesis was performed using the PrimeScript RT reagent kit (Takara, Japan) from 0.5 µg of RNA per sample.

9.2 REAL-TIME qPCR

The StepOnePlus qPCR System (Applied Biosystems, Spain) with PowerUp SYBR Green Master Mix (Applied Biosystems, Spain) was employed for RT-qPCR. Table 2 provides the list

of primers used for *PGRMC2*, *PGRMC1*, total PGR (*PGR-t*), *PGR-β* isoform (*PGR-β*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), hepatocyte growth factor (*HGF*), *CXCL14*, tenascin C (*TNC*), DEPP autophagy regulator 1 (*DEPP1*) and collagen type I alpha 1 chain (*COL1A1*). Reactions were conducted in duplicate using 96-well plates to ensure the accuracy and reproducibility of the results.

Gene	Forward primer (5'–3')	Reverse primer (5'–3')
PGRMC2	TCGAGAATGGGAAATGCAG	TTGTGATCCTTGGTATCTTCTTCA
PGRMC1	GGAAGAGATGCATCCAGGG	TGAGTACACAGTGGGCTCCT
PGR-t	GTGGGAGCTGTAAGGTCTTCTTTAA	AACGATGCAGTCATTTCTTCCA
PGR-β	TCGGACACCTTGCCTGAAGT	CTGTCCTTTTCTGGGGGACT
GAPDH	AGATCAAGAAGGTGGTGAAG	TTGTCATACCAGGAAATGAGC
HGF	CATGACCTGCAATGGGGAGA	CCTGTAGCCTTCTCCTTGACC
CXCL14	AAGGGACCCAAGATCCGCTA	GACACGCTCTTGGTGGTGAT
TNC	GTCAAGCAACCCAGCCAAAG	TCTGTCTGGGAAACACGTC
DEPP1	GACATCACCGCACGTTTCAG	ACTCCCCAAAAAGCCAGTCC
COL1A1	TGACGAGACCAAGAACTGCC	GCACCATCATTTCCACGAGC

Table 2. Primers used for RT-qPCR. PGRMC2, progesterone receptor membrane component 2; PGRMC1, progesterone receptor membrane component 1; PGR-t, total PGR; PGR-β, PGR-β isoform; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HGF, hepatocyte growth factor; CXCL14, C-X-C motif chemokine ligand 14; TNC, tenascin C; DEPP1, DEPP autophagy regulator 1; COL1A1, collagen type I alpha 1 chain.

9.3 DATA ANALYSIS AND RELATIVE GENE EXPRESSION

The $2^{-\Delta\Delta CT}$ method was utilized to calculate relative mRNA expression, normalizing values to *GAPDH* expression as an endogenous control. Results were expressed as fold change mean ± SD in gene expression during the menstrual cycle and in *in vitro* d-hEnSCs. Analysis of *PGRMC2* gene expression between different phases of the menstrual cycle was performed comparing the ΔC_T of each sample with the ΔC_T mean of EP-phase samples.

To compare classical and non-classical *PGRs* mRNA expression across the menstrual cycle stages and in hEnSCs, the inverse of ΔC_T ($1/\Delta C_T$) of each sample was compared between genes in each phase or decidual condition, respectively.

10. *PGRMC2* SILENCING IN hENSCs

To further investigate the role of PGRMC2 in hEnSCs, we performed gene silencing experiments. One day before transfection, cells were plated in 24-well plates according to previously described methods. Small interfering RNA (siRNA) is a synthetic RNA duplex designed to specifically target and degrade mRNA molecules, thereby silencing gene expression. A pool of three different siRNAs specific to *PGRMC2* (Origene, USA; Table 3) was utilized to knockdown the *PGRMC2* gene in decidualized hEnSCs (i*PGRMC2* d-hEnSCs). As an internal control, Trilencer-27 Universal Scrambled Negative Control siRNA Duplex (Origene, USA) was used at a concentration of 100 nM per well (Sc d-hEnSCs). This internal control serves to ensure that any observed effects are due to the specific knockdown of *PGRMC2* and not off-target effects or the transfection process itself. Trilencer-27 Universal Scrambled Negative Control siRNA Duplex contains a sequence that does not correspond to any known gene, thus ensuring non-specific effects of the siRNA molecule on gene expression are not observed.

Name	Duplex Sequences
PGRMC2 siRNA A	rArGrArUrArCrCrArArGrGrArUrCrArCrArArUrArArArCAG
PGRMC2 siRNA B	rArGrUrArArArArGrArArUrArUrUrGrCrArCrUrGrArUrAGA
PGRMC2 siRNA C	rUrUrGrArArCrUrUrUrGrUrArArArCrArArCrCrArArArGTC

Table 3. siRNAs sequences used for progesterone receptor membrane component 2 (*PGRMC2*) silencing. PGRMC2, progesterone receptor membrane component 2.

Additionally, non-decidualized hEnSCs and decidualized hEnSCs without transfection served as negative controls (control nd-hEnSCs and control d-hEnSCs, respectively).

The siRNAs pool targeting *PGRMC2* (100 nM per well) was transfected into cells using Lipofectamine 3000 Transfection Reagent (Invitrogen, USA) at a dose of 1.5 µl per well and incubated for 24 hours. Transfection agents, like Lipofectamine 3000, facilitate the delivery of siRNA into cells by forming complexes with the RNA molecules, thereby enhancing cellular uptake. Following transfection, *in vitro* decidualization was induced over a period of 4 days. The efficiency of *PGRMC2* silencing was assessed by RT-qPCR and WB.

10.1 Ki-67 immunofluorescence after *PGRMC2* silencing in d-hEnSCs

To evaluate if *PGRMC2* silencing was affecting d-hEnSCs proliferation, an extensive analysis of Ki-67 expression via immunofluorescence was conducted. This analysis was conducted to ensure that the siRNAs transfection itself did not adversely affect cell proliferation, as transfection processes can sometimes negatively influence cellular behavior (Birmingham *et al.*, 2006; Jackson and Linsley, 2010). The experimental groups included control d-hEnSCs (*n* = 3), Sc d-hEnSCs (*n* = 3), and i*PGRMC2* d-hEnSCs (*n* = 3).

This different treatment conditions cells cultured in 24-well plates were fixed with 4% paraformaldehyde for 15 minutes and subsequently washed with PBS. Permeabilization was achieved using 0.2% Triton-X 100 to allow intracellular antibody access. Following permeabilization, cells were blocked with 5% BSA to prevent non-specific binding. Subsequently, cells were incubated with a rabbit polyclonal anti-human Ki-67 primary antibody (ab15580, Abcam, UK) at a concentration of 0.5 µg/mL in 3% BSA at 4°C overnight. Cells were exposed to a secondary Alexa Fluor 488 antibody (Santa Cruz Biotechnology, Germany) at a dilution of 1:1,000 in 3% BSA for 1 hour at RT in the dark and mounted with DAPI. The percentage of proliferative cells (Ki-67 positive) was observed using a fluorescent inverted AXIO microscope (Zeiss, Germany). Image analysis was performed using ImageProPlus software to quantify the percentage of Ki-67 positive cells relative to the total number of cells (DAPI). Results are expressed as the percentage (%) of proliferative cells (Ki-67) / total cells (DAPI) ± SD.

10.2 PRL levels after *PGRMC2* silencing in d-hEnSCs

To evaluate the functional effect in the decidualization process of *PGRMC2* silencing in d-hEnSCs cultured *in vitro*, the decidualization process was checked by measuring the PRL levels secreted in the cell culture media using an ELISA kit according to the previously mentioned guidelines (*n* = 14).

The expression of *PGRMC2* in these cells was silenced following the protocol described in previous section. *PGRMC2* silencing was performed using specific siRNAs for *PGRMC2* and a

scramble control. PRL levels were referenced to the Sc decidual condition (Sc d-hEnSCs; 100 %) and results were expressed as percentage of PRL levels (%) (Figure 16).

10.3 TRANSCRIPTOMIC ANALYSIS BY RNA-seq

The raw count matrix derived from RNA-seq data libraries comparing Sc d-hEnSCs and i*PGRMC2* d-hEnSCs, hEnSCs extracted from group 2 of samples, was processed. A thorough statistical analysis was conducted (n = 4). Initially, the normalization of read counts was achieved by dividing the raw read counts by the scaling factor associated with the respective sample, ensuring accurate comparisons across different samples. This step is crucial as it accounts for variations in sequencing depth and RNA composition between samples.

10.3.1 Differentially gene expression analysis (DEGs)

DEGs between Sc d-hEnSCs and i*PGRMC2* d-hEnSCs were identified using the DESeq2 package (DESeq2 v1.34.0). DESeq2 is a widely-used statistical tool for analyzing count data from RNA-seq experiments. It models the counts using a negative binomial distribution and includes normalization to account for sequencing depth and other systematic biases. Genes were considered relevant when adjusted $P < 0.01$ and fold change was greater than 2 or less than 0.5.

10.3.2 Functional enrichment analysis

For downstream analysis, common DEGs with a false discovery rate (FDR)-adjusted *P*-value < 0.05 were selected for functional enrichment analysis. This analysis was conducted using g:Profiler, a tool that identifies enriched biological processes, molecular functions, and cellular components based on the list of DEGs. For this identification g:Profiler performs an over-representation analysis (ORA), where a list of genes of interest (e.g. DEGs for a condition) is compared to a list of background genes (e.g. the list of all genes evaluated in the assay) to distinguish the biological processes, molecular functions, and cellular components significantly associated with the DEGs. The parameters were set to g_SCS<0.05 and analyzed with g:Profiler (with a term size up to 2,000 genes). The resulting functional enrichment maps of biological processes and cellular components affected of DEGs were generated using the EnrichmentMap plugin in Cytoscape 3.10.2 software. The EnrichmentMap and AutoAnnotate

plugins cluster related biological terms, making it easier to interpret large sets of enrichment results (Reimand *et al.*, 2019).

Cytoscape uses the *P* values provided by g:Profiler to determine the significance of each node and represents this through node color, calculated as $-\log_{10}$(*P* value)*sign(NES), where NES (Normalized Enrichment Score) stands for normalized enrichment score. The use of $-\log_{10}$(*P* value) instead of the raw *P* value enhances visual interpretation, as it inversely scales significance: a higher value indicates a stronger enrichment. Additionally, NES is preferred for its standardized measure across gene sets, allowing for meaningful comparisons of enrichment levels after adjusting for gene set size.

10.3.3 RNA-seq validation

To validate the RNA-seq findings, RNA expression levels of selected genes were further analyzed in d-hEnSCs from the same endometrial samples used in the initial RNA-seq experiments (*n* = 4). The genes selected for validation included *CXCL14*, *DEPP1*, *PGRMC2*, *TNC*, *HGF*, and *COL1A1*. These genes were chosen not only for their significant differential expression observed in the RNA-seq analysis but also due to their known implications in female reproduction (Vollmer, 1994; Watanabe *et al.*, 2005; Nishiura *et al.*, 2005; Haibin *et al.*, 2009; Kuang *et al.*, 2009; Mokhtar *et al.*, 2010; Chen *et al.*, 2011; Stepanjuk *et al.*, 2019; Kim *et al.*, 2019; Albayrak *et al.*, 2021; Peng *et al.*, 2021; Poh *et al.*, 2021; Zhu *et al.*, 2021; Kawamura *et al.*, 2021; Liu *et al.*, 2021; Lin *et al.*, 2023).

Validation of RNA expression levels was performed using RT-qPCR, adhering to the previously described methodology for RNA extraction, reverse transcription, and RT-qPCR. The expression levels of the selected genes were normalized to the housekeeping gene *GAPDH*, ensuring consistency and reliability in the comparative analysis. Primers used for the validation experiments are listed in Table 2. The results of the RT-qPCR validation were expressed as fold change mean ± SD.

10.4 Proteomic analysis by SWATH-MS

The SWATH-MS procedure was carried out following the methodology described previously by Perez-Patiño *et al*. (Perez-Patiño *et al.*, 2016). In summary, all samples (*n* = 5), endometrial

stromal cells (control d-hEnSCs and i*PGRMC2* d-hEnSCs) extracted from group 2 samples, were pooled to construct the spectral library using a 12% precast gel (Bio-Rad Laboratories, USA) and an Ekspert nanoLC 425 nanoflow system (Eksigent Technologies, ABSCIEX) coupled to a nanoESI qQTOF MS (6600 plus TripleTOF, ABSCIEX) with which the resulting peptides were analyzed. Data from the mass spectrometer were processed using ProteinPilot v5.0 software, which generates a peak list directly from the 6600 TripleTOF wiff files, facilitating the identification and quantification of proteins.

10.4.1 Differentially protein expression analysis (DEPs)

For the individual SWATH analysis, 20 µg of total protein extracted from each sample was loaded onto a 1D-SDS-PAGE gel. This step is crucial for cleaning and concentrating the samples, ensuring that the proteins are adequately separated before mass spectrometry analysis. The wiff files obtained from the SWATH experiment were analyzed using Peak View 2.2 and Marker View software (Sciex), (FDR < 1%). Protein areas were normalized by the total sum of the areas of all quantified proteins, providing a relative quantification metric. Proteins with a P value < 0.05 were described as DEPs between negative control d-hEnSCs and i*PGRMC2* d-hEnSCs.

10.4.2 Functional enrichment analysis

Common DEPs were subjected to downstream functional enrichment analysis using g:Profiler, with a Benjamini-Hochberg FDR threshold of less than 0.05 (with a term size up to 10,000 proteins). This analysis helps in identifying significantly enriched biological processes, molecular functions, and cellular components associated with the DEPs. For this identification g:Profiler performs an ORA, where a list of proteins of interest (e.g. DEPs for a condition) is compared to a list of background proteins (e.g. the list of all proteins present in the proteomic library) to distinguish the biological processes, molecular functions, and cellular components significantly associated with the DEPs. The functional enrichment maps of DEPs related to biological processes and cellular components were generated using the EnrichmentMap plugin in Cytoscape 3.10.2 software. The use of EnrichmentMap and AutoAnnotate plugins allows for the visualization of complex data sets by clustering related biological terms, making

it easier to interpret the functional relationships and biological implications of the DEPs (Reimand *et al.*, 2019).

To assess the significance of each node, Cytoscape utilizes the *P* values obtained from g:Profiler, visually represented by node color and calculated as $-\log_{10}(P \text{ value})*\text{sign(NES)}$. This approach improves visual clarity by using $-\log_{10}(P \text{ value})$ instead of the raw *P* value, as it inversely adjusts significance: a greater value reflects a higher level of enrichment. NES provides a normalized measure across protein sets, enabling precise comparisons of enrichment levels by accounting for variations in protein set size.

10.5 TROPHOBLAST INVASION AFTER *PGRMC2* SILENCING IN d-hENSCS

To investigate trophoblast invasion, an outgrowth model was employed, involving co-culture of control, Sc, and i*PGRMC2* d-hEnSCs (*n* = 6) with human trophoblastic spheroids derived from the JEG-3 cell line (cat. HTB-36, ATCC, USA). The JEG-3 cell line, derived from human choriocarcinoma, is characterized by its ability to form syncytiotrophoblast and cytotrophoblast, indicative of trophoblastic properties crucial for studies related to embryonic implantation and placental development during pregnancy (Leibovitz *et al.*, 1976). Trophoblast cells were labelled with blue CellTracker fluorescent probes (1:1,000; Invitrogen, USA) and cultured for 48 hours in 6-well low-attachment plates to facilitate spheroid formation. Then, 48h-spheroids were transferred to 24-well plates containing the corresponding *in vitro* d-hEnSCs from group 2 samples, with 3-4 spheroids per well. Each experiment was performed in duplicate to ensure reproducibility.

The trophoblast expanded area (EA) was assessed using a fluorescent inverted AXIO microscope (Zeiss, Germany) at 24 and 48 hours, and the area was quantified using ImageJ software. Data is represented as normalized EA, being EA = 48h area - 24h area and normalizing by dividing the corresponding EA of each spheroid by the mean EA of spheroids cultured in control d-hEnSCs. This normalization method accounts for variations in spheroid expansion dynamics and enables comparison across different experimental conditions.

All decidualization procedures were verified by measuring secreted PRL using by ELISA, ensuring that the observed effects on trophoblast invasion were not influenced by variations in the decidualization process.

11. STATISTICAL ANALYSIS

GraphPad Prism 8.3.0 (San Diego, California) was used for statistical analyses and graphics generation. Normality and logarithmic tests (Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov test) were performed to analyze the distribution of our results.

Gene expression levels of *PGRMC2*, *PGRMC1*, and total PGR (*PGR-t*) throughout the menstrual cycle, the differences of gene expression between genes in EP, LP, ES, MS and LS phases and comparisons of PGRMC2 protein abundance between control, Sc and i*PGRMC2* d-hEnSCs were assessed by a one-way analysis of variance (ANOVA) test.

Furthermore, to explore variations in *PGR-β* mRNA expression and PGRMC2 protein expression via WB, both across the menstrual cycle, and for outgrowth model analysis between control, Sc and i*PGRMC2* d-hEnSCs, a Kruskal-Wallis test was employed. Wilcoxon test for paired samples was utilized to analyze PGRMC2 gene expression and protein abundance in hEnSCs.

Paired t-tests were conducted to evaluate the expression of *PGRMC2* and other genes used to validate RNA-seq results, as well as PRL levels in checking decidualization between nd- and d-hEnSCs, and between Sc d-hEnSCs and i*PGRMC2* d-hEnSCs. The Friedman test was applied to compare mRNA expression levels of *PGRMC2*, *PGRMC1*, *PGR-t*, and *PGR-β* in d-hEnSCs and nd-hEnSCs, as well as to assess significant differences in *PGRMC2* gene expression between control, Sc, and i*PGRMC2* d-hEnSCs, and to compare the percentage of Ki-67 protein staining between control, Sc and i*PGRMC2* d-hEnSCs. Moreover, the two-way ANOVA test was performed to assess PGRMC2 protein abundance in all subcellular fractions.

All analyses were meticulously conducted, with results normalized and compared to those of the EP phase (for comparisons of the same gene across the menstrual cycle) or endogenous control expression as appropriate. A significance level of $P < 0.05$ was considered statistically significant in all cases.

VI. RESULTS

VI. RESULTS

To present the findings of this thesis, the results will be divided into the two areas of study mentioned previously (explained in detail in previous sections): the first group focuses on the characterization of endometrial PGRMC2 expression along the natural human menstrual cycle, and the second group on the functional analysis of PGRMC2 in human endometrial stroma under *in vitro* decidualization.

1. CHARACTERIZATION OF PGRMC2 EXPRESSION IN THE HUMAN ENDOMETRIUM ACROSS THE MENSTRUAL CYCLE

1.1 PGRMC2 LOCALIZATION, mRNA EXPRESSION, AND PROTEIN ABUNDANCE ACROSS THE MENSTRUAL CYCLE

1.1.1 *PGRMC2* gene expression in total endometrial tissue

To study the expression pattern of *PGRMC2* across the different phases of the menstrual cycle, we performed RT-qPCR analysis on endometrial tissue samples from oocyte donors without ovarian stimulation. This approach allowed us to assess mRNA levels under natural hormonal conditions, avoiding alterations caused by external factors. The results revealed a significant decline in *PGRMC2* mRNA expression during the LS phase compared to the MS phase ($P < 0.05$; Figure 9I).

1.1.2 PGRMC2 protein expression in total endometrial tissue

While mRNA expression provides valuable insights into gene activity, it does not always directly correlate with protein levels due to post-transcriptional regulation, variations in protein stability, or differences in translational efficiency. Therefore, to complement the gene expression analysis, we analyzed PGRMC2 protein abundance across the menstrual cycle in order to understand its functional presence and potential activity in the tissue.

Through WB analysis, we identified a 28 kDa band corresponding to PGRMC2 in all endometrial samples examined. Intriguingly, we did not discern significant differences in PGRMC2 protein levels across the phases of the menstrual cycle (Figure 9J) as observed at the mRNA level.

1.1.3 PGRMC2 localization in total endometrial tissue

To map the localization and abundance of PGRMC2 within the endometrium, we performed immunohistochemistry technique on total human endometrial tissues from different menstrual phases. While previous analyses at the mRNA and protein levels provided valuable insights, examining protein localization is crucial, as discrepancies between mRNA and protein expression can arise from factors like translational regulation, protein stability, and cellular compartmentalization.

Imaging revealed dynamic changes in PGRMC2 distribution and quantity across the different menstrual phases (Figure 9A-H). Notably, PGRMC2 staining was more intense during the MS phase, particularly within endometrial epithelial glands and hEnSCs. This heightened staining contrasted sharply with the LS phase, where PGRMC2 protein expression exhibited a notable decrease across all cellular subtypes within the endometrium. Interestingly, luminal epithelial cells maintained a consistent expression of PGRMC2 protein from the proliferative phases through the MS phase.

This reduction in PGRMC2 staining is consistent with the observed changes in mRNA levels. Subsequent RT-qPCR analysis corroborated our immunochemistry findings, demonstrating a significant decline in PGRMC2 during the LS phase compared to the MS phase.

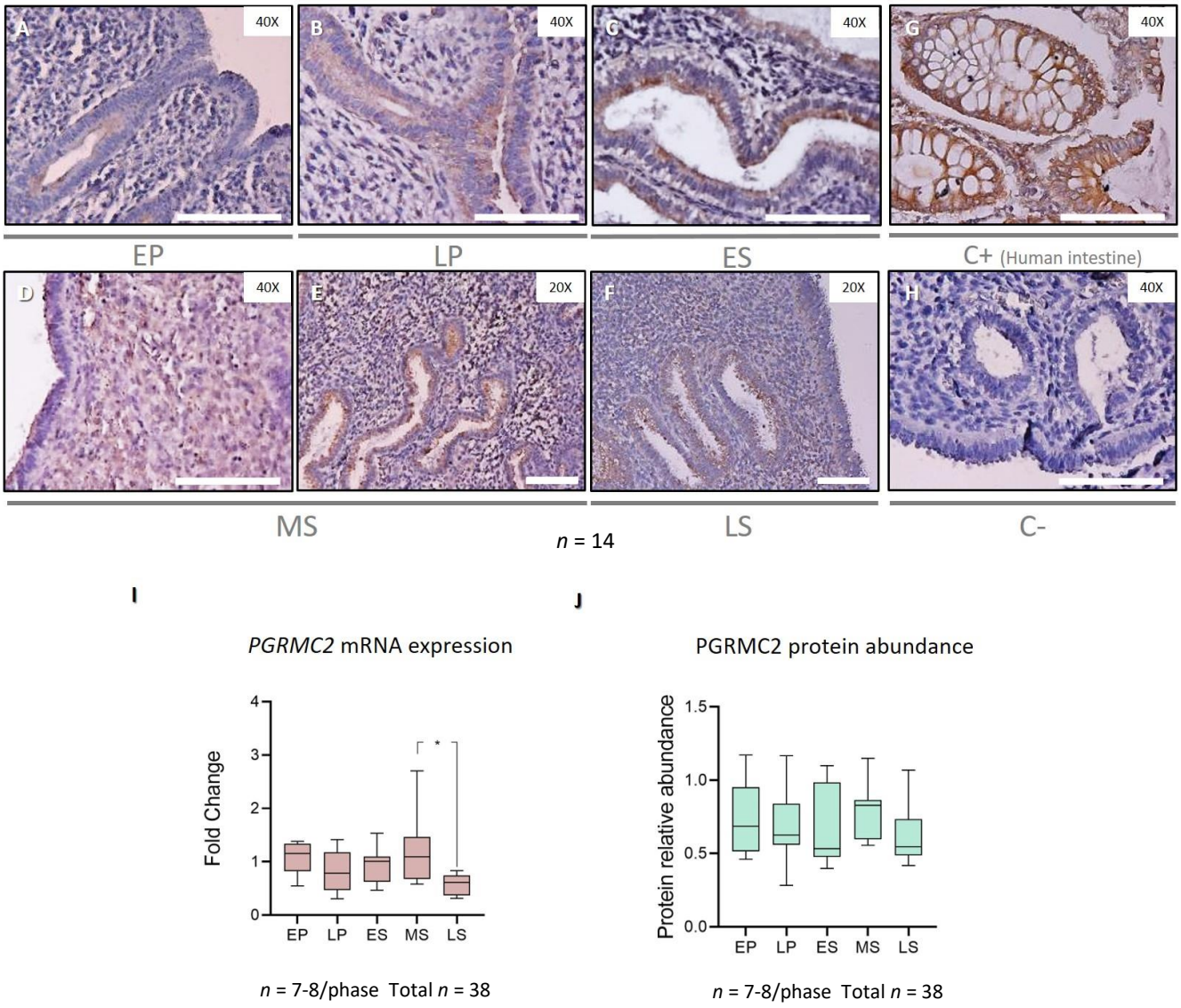


Figure 9. Progesterone receptor membrane component 2 (PGRMC2) localization, mRNA expression, and protein abundance in the human endometrium across the menstrual cycle. (A–F) Immunostaining of PGRMC2 in human endometrium at different menstrual cycle stages. Positive control tissue (human intestine) probed with anti-PGRMC2 (G) and a negative control (H) are also shown. Images were acquired at ×20 and ×40 magnifications. Scale bars: 100µm. A total of *n* = 14 human endometrial tissue samples were used. PGRMC2 mRNA and protein abundance were quantified by RT-qPCR (I; *n* = 38) and western blot (J; *n* = 35), respectively, in human endometrium throughout the menstrual cycle. Box and whiskers plots show PGRMC2 (I) gene expression, represented as mRNA fold change, and (J) protein abundance, represented as protein relative abundance, both in the different endometrial phases. In the plots, central bar denotes the median value, the box contains the 25th to 75th percentiles, and the whiskers mark the 5th and 95th percentiles; $^{*}P < 0.05$; EP, early proliferative; LP, late proliferative; ES, early secretory; MS, mid-secretory; LS, late secretory; C+, positive control; C–, negative control.

1.2 DIFFERENCES BETWEEN CLASSICAL AND NON-CLASSICAL *PGR*S mRNA LEVELS IN THE HUMAN ENDOMETRIUM ACROSS THE MENSTRUAL CYCLE

To gain a deeper understanding of the potential role of PGRMC2 in the human endometrium, we extended our analysis to include both classical and non-classical PGRs across the menstrual cycle. Given that PGRMC2 is a non-classical receptor, it was crucial to compare its expression patterns with PGRMC1 and the classical PGRs, to explore potential differences in how these receptors are regulated and function within the endometrium.

By examining both receptor types, our findings revealed that the expression levels of *PGRMC1* ($P < 0.05$) and the classical *PGRs* ($P < 0.05$) were significantly reduced during the secretory phases compared to the proliferative phases (Figure 10A-C). This pattern contrasted with the expression of *PGRMC2*, which did not exhibit a significant decrease during the MS phase (Figure 9I).

To quantify the presence of each receptor in each menstrual phase, we compared the mRNA levels of the genes encoding the non-classical and classical *PGRs* (Figure 10D-H). Non-classical *PGRs* consistently showed greater expression levels in all phases ($P < 0.05$). *PGRMC1* emerged as the most expressed receptor during the proliferative phases and ES. Conversely, *PGRMC2* was the most expressed receptor during the LS phase. In particular, during the MS phase, the expression of non-classical *PGRMC1* and *PGRMC2* significantly increased compared to the classical *PGRs* in endometrial tissue ($P < 0.05$; Figure 10G).

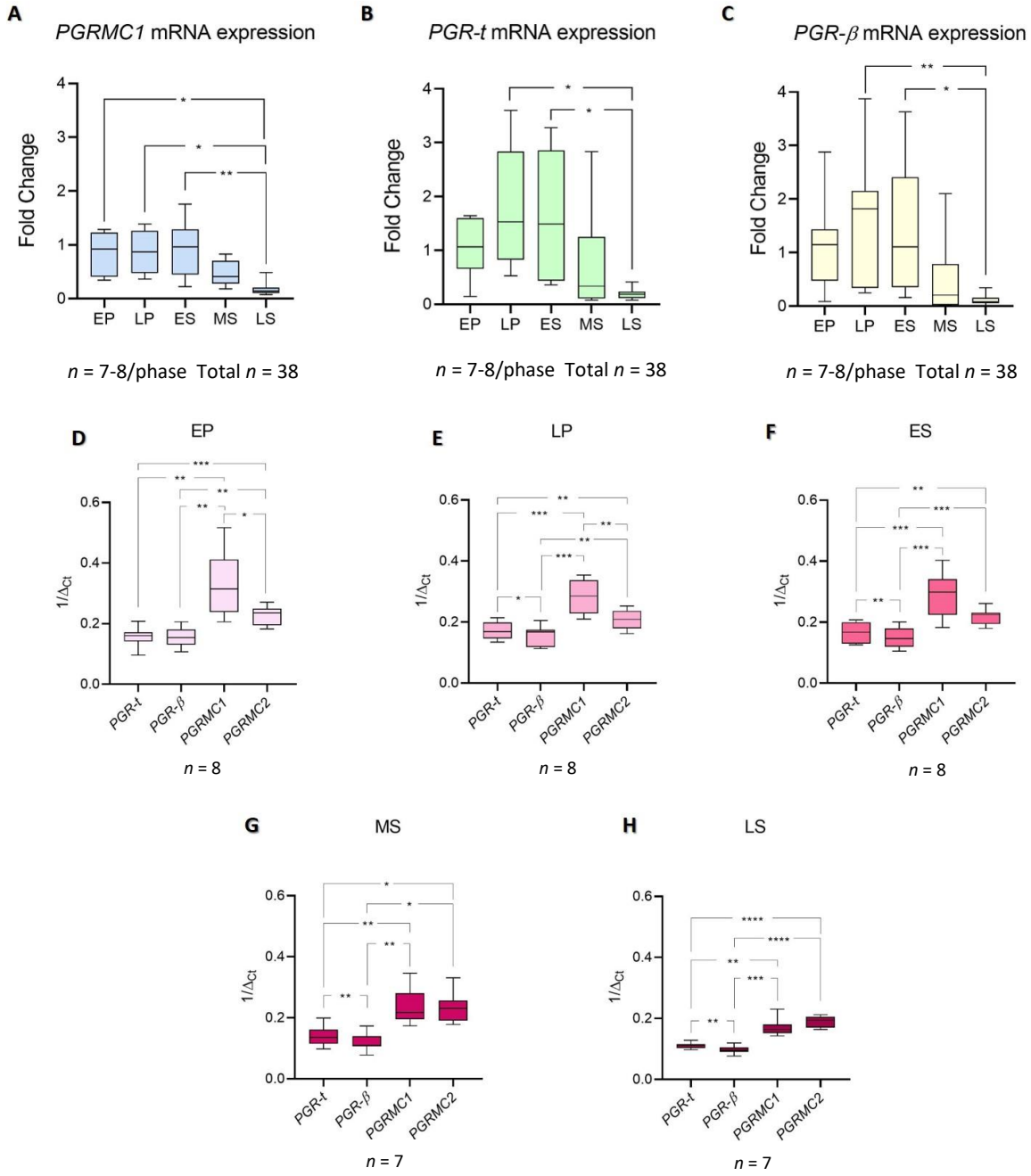


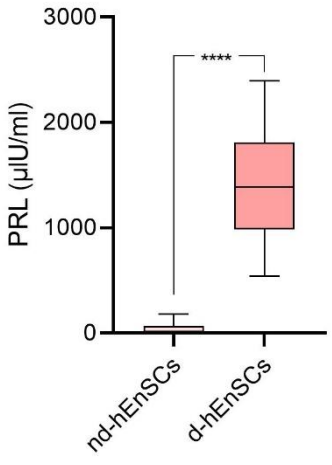
Figure 10. Differences in mRNA expression between classical and non-classical progesterone receptors (*PGRs*) across the human menstrual cycle. (A–C) Box and whiskers show the fold change expression of progesterone receptor membrane component 1 (*PGRMC1*), total progesterone receptors (*PGR-t*), and beta progesterone receptor (*PGR-β*) mRNA across the human menstrual cycle. A total of *n* = 38 human endometrial tissue samples were used. (D–H) Box and whiskers plots show the comparison of classical and non-classical *PGRs* mRNA expression in the human endometrium during each phase of the menstrual cycle. A total of *n* = 8 and *n* = 7 human endometrial tissue samples were used. mRNA gene expression was represented as fold change (A–C) and inverse of ΔCt (1/ΔCt) (D–H). In the plots, central bar denotes the median value, the box contains the 25th to 75th percentiles, and the whiskers mark the 5th and 95th percentiles; **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001 EP, early proliferative; LP, late proliferative; ES, early secretory; MS, mid-secretory; LS, late secretory.

2. FUNCTIONAL STUDIES OF PGRMC2 IN ENDOMETRIAL STROMAL CELLS

2.1 PGRMC2 LOCALIZATION, mRNA AND PROTEIN ANALYSIS IN hENSCs DURING *IN VITRO* DECIDUALIZATION

Given the previous mRNA analysis showing a significant decline in *PGRMC2* expression during the LS phase compared to the MS phase, and the observation of increased PGRMC2 staining in the stromal compartment during the MS phase in the previous immunohistochemical studies, we decided to further investigate its role in the decidualization process, as the observed changes in PGRMC2 levels coincide with the onset of decidualization. Furthermore, considering prior studies where another non-classical PGR, PGRMC1, plays an important role in decidualization (Salsano *et al.*, 2017, 2020, 2021), we aimed to determine whether PGRMC2 might also influence this critical process, either in a similar or distinct manner.

These analyses focused on its behavior during *in vitro* decidualization to determine whether similar patterns were evident in our *in vitro* model of decidualization. In this context, we examined the expression of PGRMC2 in both decidualized and non-decidualized hEnSCs. Our *in vitro* model of decidualization was validated by measuring PRL levels confirming the effectiveness of the model, as PRL levels were significantly higher in the d-hEnSCs ($P < 0.0001$; Figure 11).



n = 15

Figure 11. Prolactin (PRL) secreted during *in vitro* decidualization in human endometrial stromal cells (hEnSCs). Box and whiskers plots show the secreted PRL levels, measured by ELISA, in non-decidualized human endometrial stromal cells (nd-hEnSCs) and decidualized human endometrial stromal cells (d-hEnSCs). In the plot, central bar denotes the median value, the box contains the 25th to 75th percentiles, and the whiskers mark the 5th and 95th percentiles. PRL is expressed in µIU/mL. A total of n = 15 decidual human endometrial stromal cells (d-hEnSCs) were used. ****$P < 0.0001$.

2.1.1 *PGRMC2* gene expression in hEnSCs

To better understand the role of PGRMC2 during *in vitro* decidualization, we first quantified the mRNA expression levels of *PGRMC2* in d-hEnSCs and nd-hEnSCs. The results from RT-qPCR analysis demonstrated a significant increase in *PGRMC2* gene expression in d-hEnSCs compared to nd-hEnSCs ($P < 0.0001$; Figure 12A). This increase was particularly relevant, as it aligns with previous analyses.

2.1.2 PGRMC2 protein expression in hEnSCs

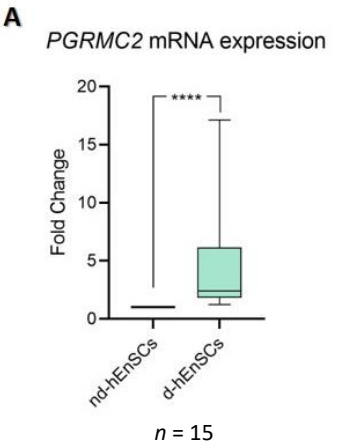
Given that mRNA expression does not always correlate directly with protein levels due to post-transcriptional regulation, examining protein expression provides crucial insights into the functional state of the cells. Therefore, to complement the gene expression data, we assessed PGRMC2 protein levels using WB analysis. Consistent with the mRNA results, PGRMC2 protein levels were significantly elevated in d-hEnSCs compared to nd-hEnSCs ($P < 0.001$; Figure 12B-C).

2.1.3 PGRMC2 localization in hEnSCs

To determine if the localization of PGRMC2 changes during decidualization, we performed immunofluorescence staining on primary hEnSCs that were induced to *in vitro* decidualization. Immunofluorescence allowed us to visualize the spatial distribution of PGRMC2 within the hEnSCs, providing valuable information on how the localization of this receptor might be regulated during decidualization.

The staining revealed that PGRMC2 was primarily localized to the membranes of d-hEnSCs (Figure 12E-G). In contrast, PGRMC2 was less concentrated and harder to pinpoint within specific cellular compartments in nd-hEnSCs (Figure 12D-F).

To verify the immunofluorescence results and to provide further insights, cytoplasmic, membrane/organelles, and nuclear fractions were isolated from both nd-hEnSCs and d-hEnSCs. WB analysis was conducted on these fractions to determine the presence of PGRMC2 in specific subcellular compartments. The results confirmed the presence of PGRMC2 in hEnSCs, with a significantly higher abundance of PGRMC2 in the membranes/organelles fraction compared to other subcellular fractions ($P < 0.05$; Figure 12H-I).



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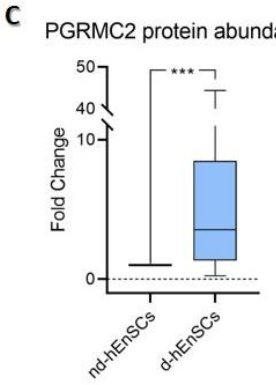
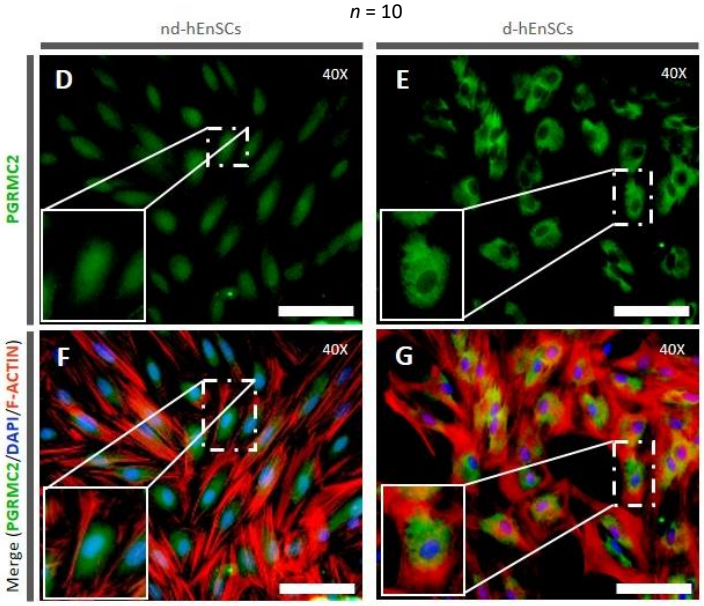
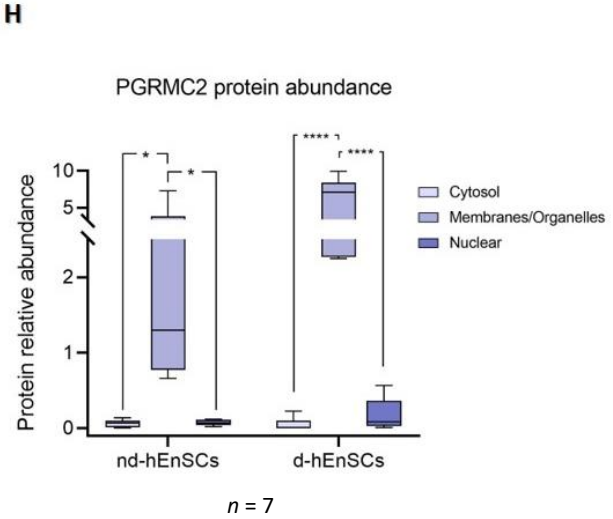


n = 15

n = 10





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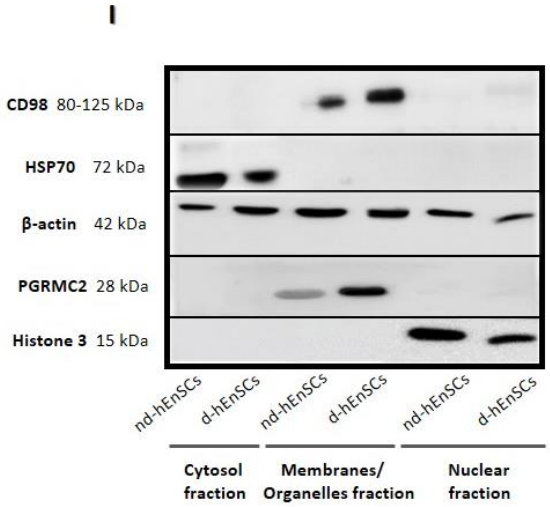

Figure 12. Progesterone receptor membrane component 2 (PGRMC2) mRNA expression, protein abundance, and subcellular localization in human endometrial stromal cells (hEnSCs) during *in vitro* decidualization. (A–C) Box and whiskers show the expression of PGRMC2 in non-decidualized human endometrial stromal cells (nd-hEnSCs) and decidualized human endometrial stromal cells (d-hEnSCs) by quantitative reverse transcription polymerase chain reaction (RT-qPCR) (A) and western blot (B and C); a total of *n* = 6 decidual human endometrial stromal cells (d-hEnSCs) were used. (D–G) PGRMC2 (green) localization in nd-hEnSCs and d-hEnSCs by immunofluorescence; F-actin, rhodamine phalloidin (red); nuclei, DAPI (blue). Merged images are presented in F and G. Images were acquired at ×40 magnifications. Scale bars: 100 μm. A total of *n* = 10 decidual human endometrial stromal cells (d-hEnSCs) were used. (H, I) Box and whiskers plots show the subcellular protein abundance of PGRMC2 in nd-hEnSCs and d-hEnSCs. In the plots, central bar denotes the median value, the box contains the 25th to 75th percentiles, and the whiskers mark the 5th and 95th percentiles. A total of *n* = 7 decidual human endometrial stromal cells (d-hEnSCs) were used. Specific markers for membrane/organelles (CD98), cytosol (HSP70), and nuclear fraction (His3) were used. Gene expression and protein abundance are represented as fold change during *in vitro* decidualization, while subcellular protein abundance is represented as protein relative abundance; **P* < 0.05; ****P* < 0.001; *****P* < 0.0001.

2.2 Differences between classical and non-classical *PGRs* mRNA levels after *in vitro* decidualization in hEnSCs

To confirm that the *in vitro* behavior of non-classical and classical *PGRs* corresponds to the patterns observed in endometrial tissue, we compared the expression of genes encoding *PGRs* in hEnSCs following decidualization in cell culture (Figure 13). This analysis aimed to evaluate how *PGRMC2* behaves relative to other *PGRs* in an *in vitro* decidualization model, and to compare the results with those observed in endometrial tissue during the menstrual cycle. By performing this comparison, we sought to determine whether the expression patterns of *PGRMC2* during decidualization mirrored the dynamics seen *in vivo* across the different phases of the menstrual cycle.

Firstly, we analyzed *PGRMC2*, *PGRMC1* and classical *PGRs* expression in non-decidualized cells to establish baseline expression levels of the receptor types in hEnSCs before the induction of decidualization. In nd-hEnSCs non-classical *PGRs* expression levels were significantly higher than classical *PGRs* (*P* < 0.001; Figure 13A).

Subsequently, we analyzed the same set of genes after inducing *in vitro* decidualization. A significant increase in the mRNA expression of non-classical *PGRs* was observed (*P* < 0.05).

Among the non-classical *PGRs*, *PGRMC2* gene expression was the highest, indicating a pronounced upregulation in response to decidualization stimuli (Figure 13B). These results align with the endometrial tissue observations, where non-classical *PGRs*, particularly PGRMC2, were altered in the decidualization process.

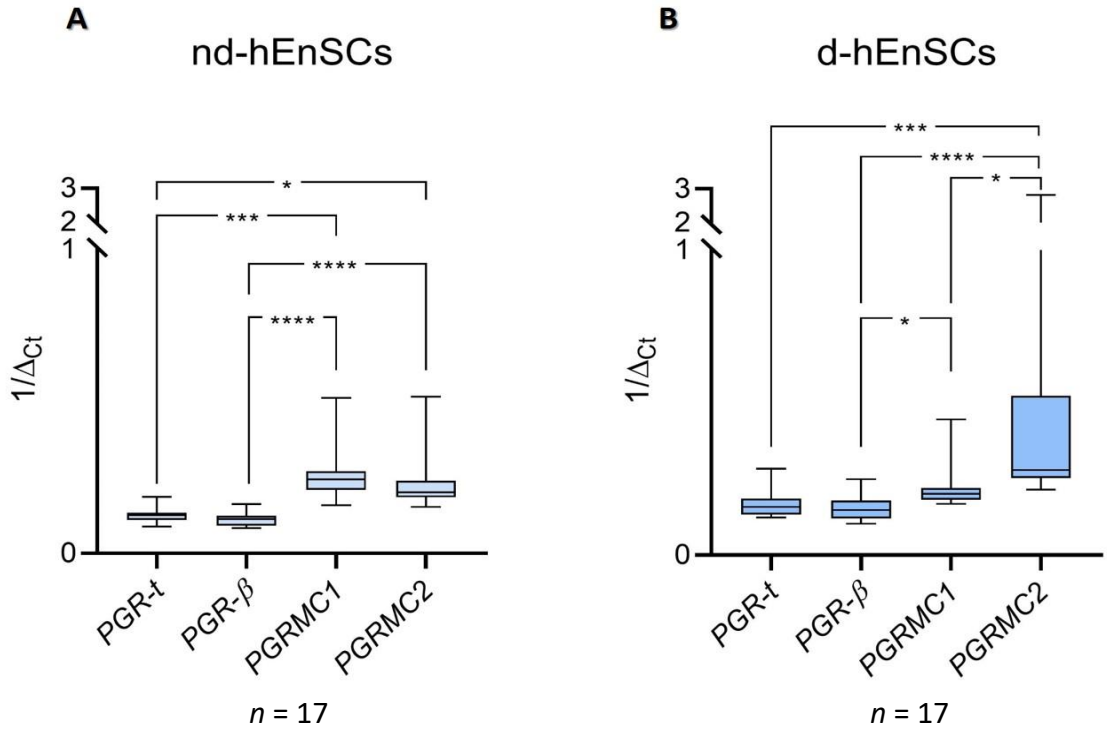

Figure 13. Differences in mRNA expression between classical and non-classical progesterone receptors (*PGRs*) in human endometrial stromal cells (hEnSCs) during *in vitro* decidualization. (A and B) Box and whiskers plots illustrate the fold change differences of total progesterone receptors (*PGR-t*), beta progesterone receptor (*PGR-β*), progesterone receptor membrane component 1 (*PGRMC1*), and progesterone receptor membrane component 2 (*PGRMC2*) mRNA expression in non-decidualized human endometrial stromal cells (nd-hEnSCs) (A) and decidualized human endometrial stromal cells (d-hEnSCs) (B). Gene expression is represented as inverse of ΔCt (1/ΔCt). In the plots, central bar denotes the median value, the box contains the 25th to 75th percentiles, and the whiskers mark the 5th and 95th percentiles. A total of *n* = 17 human endometrial stromal cells (hEnSCs) were used; **P* < 0.05; ****P* < 0.001; *****P* < 0.0001.

2.3 *PGRMC2* SILENCING IN d-hENSCS

2.3.1 Validation of *PGRMC2* silencing

The efficiency and effectiveness of our *PGRMC2* silencing method that we used in hEnSCs during *in vitro* decidualization were evaluated to ensure the reliability of subsequent

experiments through two analyses. In the first part, RT-qPCR analysis demonstrated a significant reduction in *PGRMC2* gene expression, showing an approximately 80% decrease in d-hEnSCs transfected with *PGRMC2* siRNAs compared to negative control d-hEnSCs ($P < 0.01$; Figure 14). Secondly, WB analysis validated the silencing efficiency at the protein level, revealing an approximate 60% reduction in PGRMC2 protein abundance in d-hEnSCs transfected with *PGRMC2* siRNAs compared to negative control d-hEnSCs ($P < 0.001$; Figure 14B). These results were crucial for verifying the efficacy of *PGRMC2* silencing confirming the successful of the technique, ensuring the reliability of subsequent experiments.

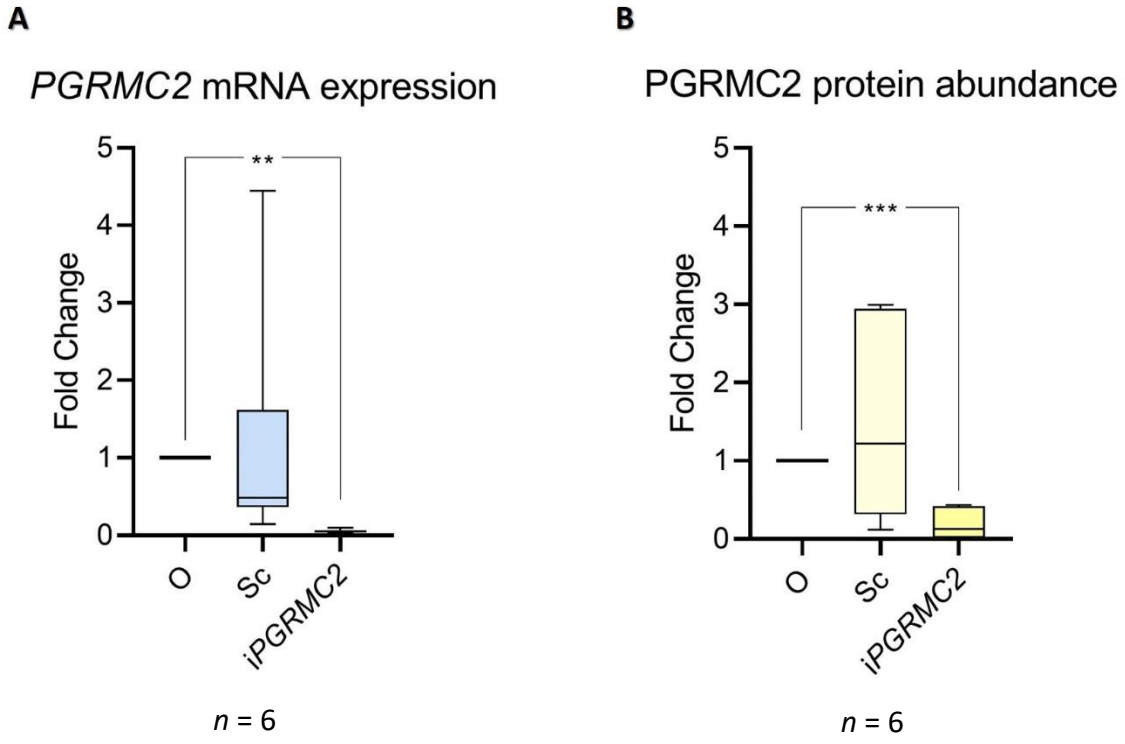

Figure 14. Progesterone receptor membrane component 2 (*PGRMC2*) levels after decidualized human endometrial stromal cells (d-hEnSCs) were transfected with *PGRMC2* siRNAs. (A) Box and whiskers plot show the fold change differences of *PGRMC2* mRNA levels between negative control (O), scramble control (Sc) and inhibited *PGRMC2* (i*PGRMC2*) decidualized endometrial stromal cells. (B) Box and whiskers plot show the fold change differences of PGRMC2 protein abundance between negative control (O), scramble control (Sc) and inhibited *PGRMC2* (i*PGRMC2*) decidual endometrial stromal cells. The central bars denote the median value, the box represents the 25th to 75th percentiles, and the whiskers indicate the 5th and 95th percentiles. A total of *n* = 6 decidual human endometrial stromal cells (d-hEnSCs) were used; $^{**}P < 0.01$; $^{***}P < 0.001$.

2.3.2 *PGRMC2* silencing impact in cell proliferation in d-hEnSCs

Due to the sensitivity of primary hEnSCs to experimental manipulations, it was crucial to ensure that the siRNA transfection method used for *PGRMC2* silencing did not interfere with the normal proliferation of the cells during the *in vitro* decidualization process. Transfection techniques, particularly those involving siRNAs, can sometimes induce unintended cellular stress or toxicity, potentially altering proliferation rates and confounding the interpretation of downstream analyses.

Potential effects were ruled out prior to conducting the transcriptomic and proteomic experiments by measuring the proliferation marker Ki-67 through immunofluorescence staining. This approach enabled us to quantify the proportion of Ki-67 positive cells and directly compare proliferation rates across three groups: control d-hEnSCs, Sc d-hEnSCs, and i*PGRMC2* d-hEnSCs. Fluorescence microscopy quantification was used to determine the number of Ki-67 positive cells.

Our analysis revealed no statistically significant differences in the number of Ki-67 positive cells among control d-hEnSCs, Sc, and i*PGRMC2* d-hEnSCs groups (Figure 15), confirming that the transfection method did not affect cell proliferation. By establishing consistent Ki-67 expression across the groups, we ensured that any effects observed in the subsequent transcriptomic and proteomic analyses could be attributed to *PGRMC2* silencing, rather than potential artifacts from the transfection process. This validation was essential for confirming the reliability and specificity of our experimental approach, providing a solid foundation for the interpretation of downstream findings.

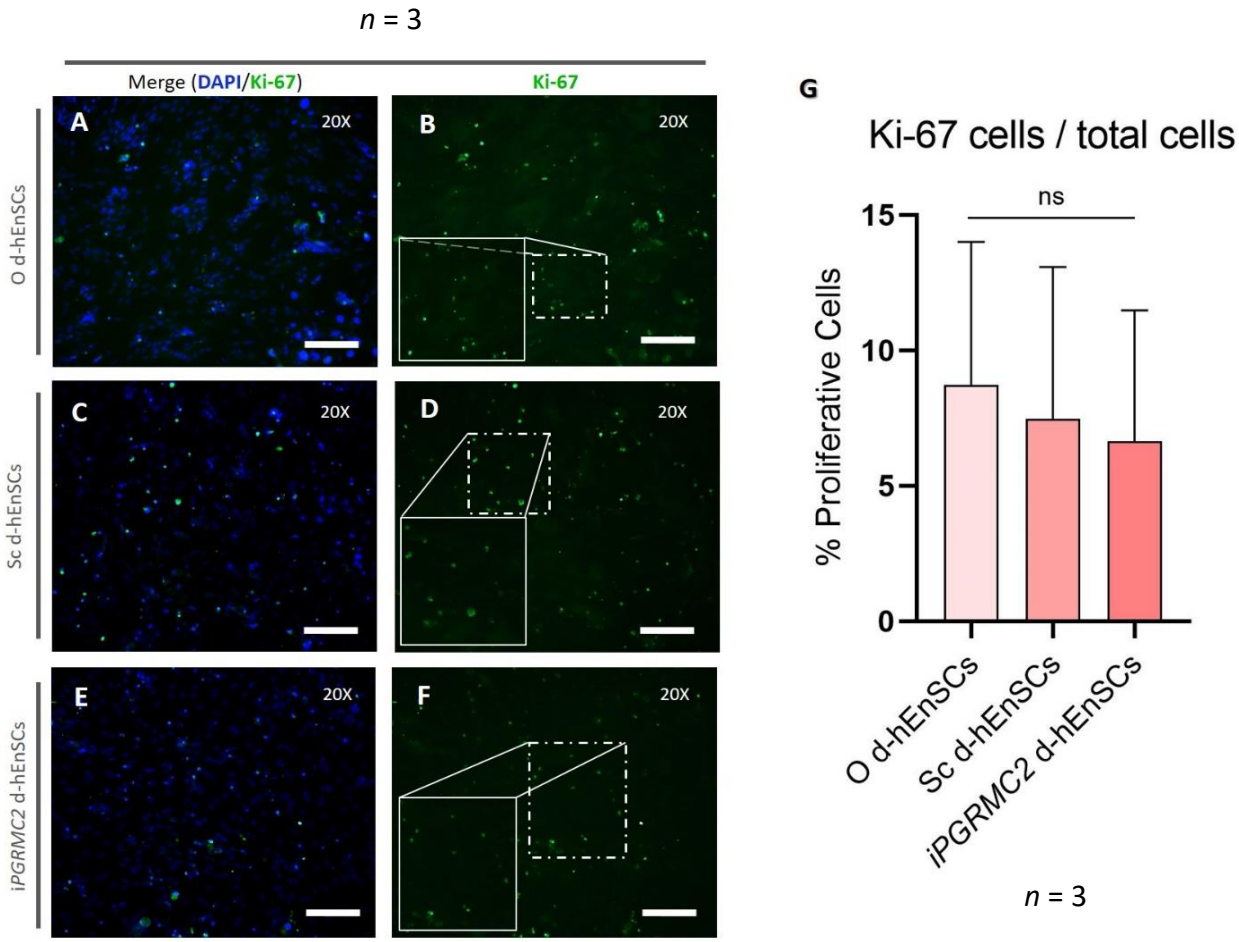

Figure 15. Immunofluorescence of proliferation marker Ki-67 in decidualized human endometrial stromal cells (d-hEnSCs) after progesterone receptor membrane component 2 (*PGRMC2*) silencing. Photographs show negative control (O d-hEnSCs; A-B), scramble control (Sc d-hEnSCs; C-D) and silenced *PGRMC2* (i*PGRMC*2; E-F) d-hEnSCs stained for Ki-67 protein (green) and DAPI (blue). Merged images are presented in A, C, E. Images were acquired at ×20 magnification. Scale bars: 100 µm. (G) Bars plot illustrates the differences between percentage of Ki-67 cells / total cells in d-hEnSCs after *PGRMC2* silencing. Data are represented as normalized percentage (%) of proliferative cells ± SD, where the bars contains the mean and the central bars denote the SD. A total of *n* = 3 d-hEnSCs were used; ns: not significant.

2.3.3 *PGRMC2* silencing impact in PRL levels in d-hEnSCs

Once we confirmed the reliability of our silencing method, the next aim was to evaluate the impact of *PGRMC2* silencing on the decidualization process in hEnSCs. A direct and effective way to evaluate this was by measuring the levels of PRL in the cell culture media, as PRL is a well-established marker of decidualization in hEnSCs. We chose to measure PRL secretion

specifically in the Sc d-hEnSCs and i*PGRMC2* d-hEnSCs groups because the Sc group reflects the same experimental conditions as the i*PGRMC2* group, except for the silencing of *PGRMC2*, and thus allows for a direct comparison between both groups.

A statistically significant difference in PRL secretion was found between Sc d-hEnSCs and i*PGRMC2* d-hEnSCs ($P < 0.05$). Specifically, the PRL levels were significantly higher in the i*PGRMC2* d-hEnSCs compared to the Sc d-hEnSCs (Figure 16).

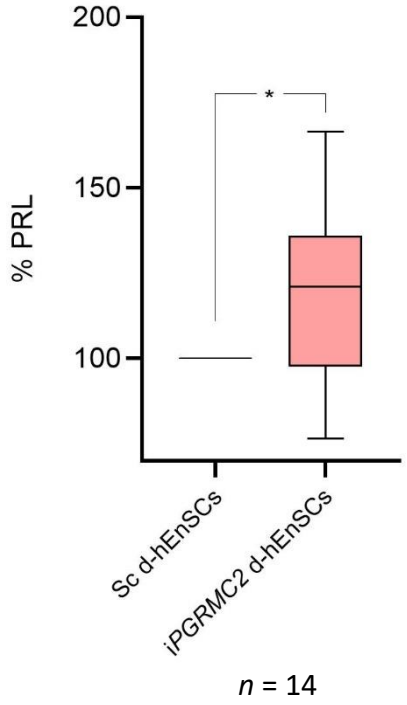

Figure 16. Prolactin (PRL) secreted in decidualized human endometrial stromal cells (d-hEnSCs) after progesterone receptor membrane component 2 (*PGRMC2*) silencing. Box and whiskers plot depict the secreted PRL levels, measured by ELISA, in scramble control (Sc d-hEnSCs) and inhibited *PGRMC2* (i*PGRMC*2) d-hEnSCs. In the plot, central bars denote the median value, the box represents the 25th to 75th percentiles, and the whiskers indicate the 5th and 95th percentiles. PRL is expressed in percentage of PRL levels (%). A total of $n = 14$ decidual human endometrial stromal cells (d-hEnSCs) were used. *$P < 0.05$.

2.3.4 Transcriptomic effects of *PGRMC2* silencing during decidualization

Building on our previous findings showing that *PGRMC2* silencing can impact the decidualization process, we performed *PGRMC2* silencing in hEnSCs and compared i*PGRMC2* d-hEnSCs with Sc d-hEnSCs using RNA-seq analysis. This approach allowed us to examine the

global gene expression changes associated with *PGRMC2* silencing and better understand how *PGRMC2* influences the hEnSCs transcriptomic landscape during decidualization.

RNA-seq analysis revealed 4687 DEGs between Sc d-hEnSCs and i*PGRMC2* d-hEnSCs (n = 4), with 2420 genes being upregulated and 2267 downregulated (FDR-adjusted $P < 0.05$) (Figure 17A & Supplementary Table S1). The biological relevance of these DEGs was explored through functional enrichment analysis. The results indicated that genes overexpressed in i*PGRMC2* d-hEnSCs were involved in various biological processes including angiogenesis, cell migration, cell adhesion, ER function, vesicular transport, lipid biosynthesis, cell signalling, phosphorylation, and morphogenesis. Conversely, genes that were under-expressed in i*PGRMC2* d-hEnSCs were primarily related to aerobic respiration, RNA translation, and ribosome biosynthesis (Figure 17B & Supplementary Table S2).

Regarding cellular components, the genes most highly expressed in i*PGRMC2* d-hEnSCs were associated with the nucleus, ER, Golgi apparatus, cell vesicles and vacuoles, microtubule organizing center, cell junctions and transport, neuron synapse, and the extracellular matrix. In contrast, the least expressed genes were linked to the mitochondria, ribosomes, and the catalytic complex, and organelles (Figure 18; Supplementary Table S2).

Concerning molecular functions, the highly expressed genes in i*PGRMC2* d-hEnSCs predominantly exhibited kinase activity, substrate binding, and interactions with extracellular matrix constituents. On the other hand, the least expressed genes were primarily involved in oxidoreductase activity, proton transport, and constituted structural components of ribosomes (Supplementary Table S2).

A

	Analysis type	Total DEGs	Upregulated	Downregulated
i*PGRMC2* d-hEnSCs *vs* Sc d-hEnSCs	Transcriptomic (n = 4)	4687	2420	2267

B

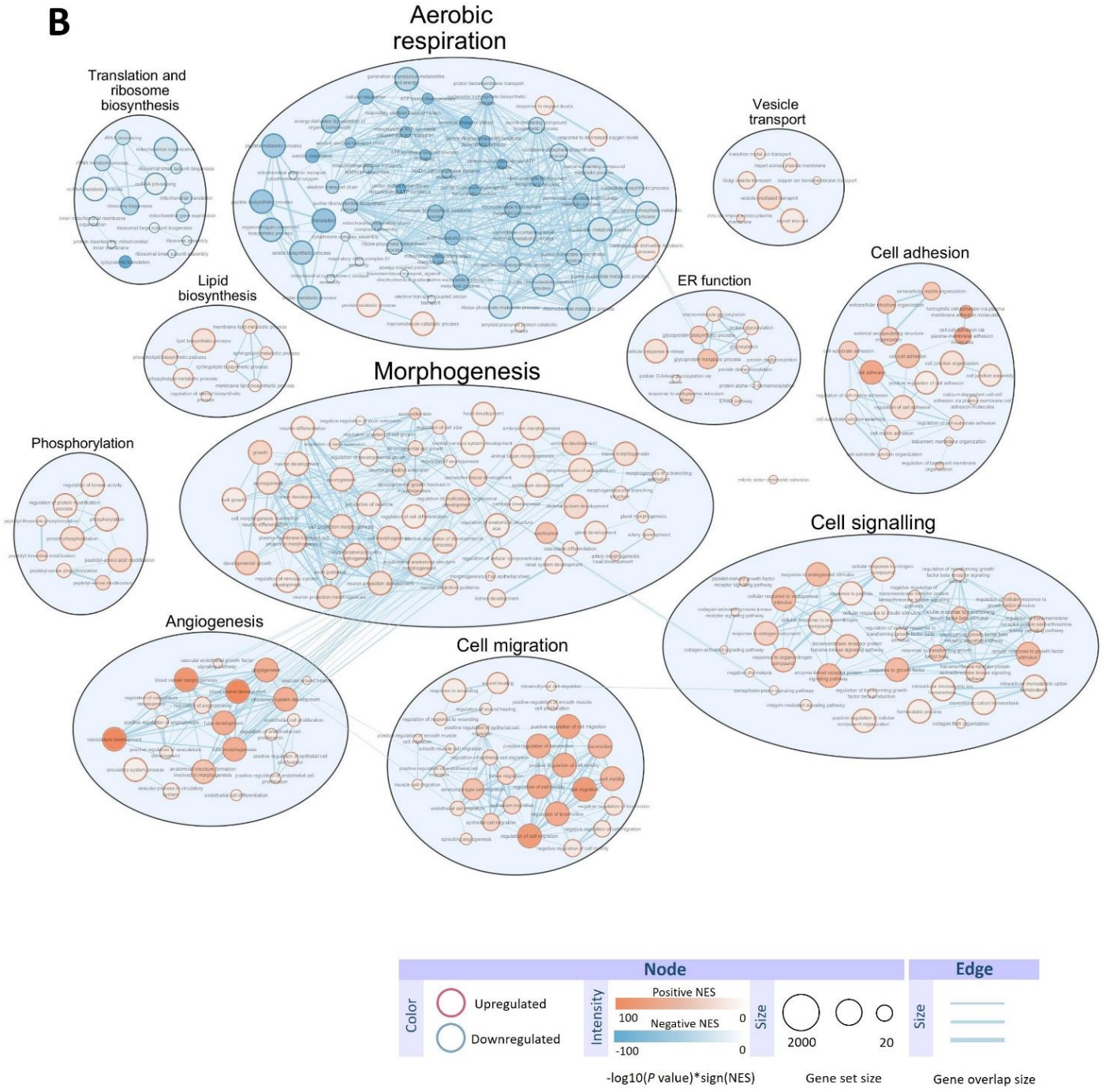

Figure 17. Transcriptomic analysis of decidualized human endometrial stromal cells (d-hEnSCs) when progesterone receptor membrane component 2 (*PGRMC2*) was silenced. (A) Differentially expressed genes (DEGs; false discovery rate (FDR)-adjusted $P < 0.05$) (total, upregulated and downregulated) in pairwise comparison of Scramble control d-hEnSCs (Sc d-hEnSCs) and d-hEnSCs with *PGRMC2* silenced (i*PGRMC2* d-

hEnSCs) (*n* = 4). (B) Transcriptomic enrichment map for gene ontology biological processes analyzed by g:Profiler in pairwise comparison of Sc d-hEnSCs and i*PGRMC2* d-hEnSCs. Node size represents the number of genes associated with a specific term. Node color represents the -$\log_{10}$(*P* value)*sign(NES). ER, Endoplasmic Reticulum; NES, Normalized Enrichment Score. Enrichment map was created with Cytoscape 3.10.2.

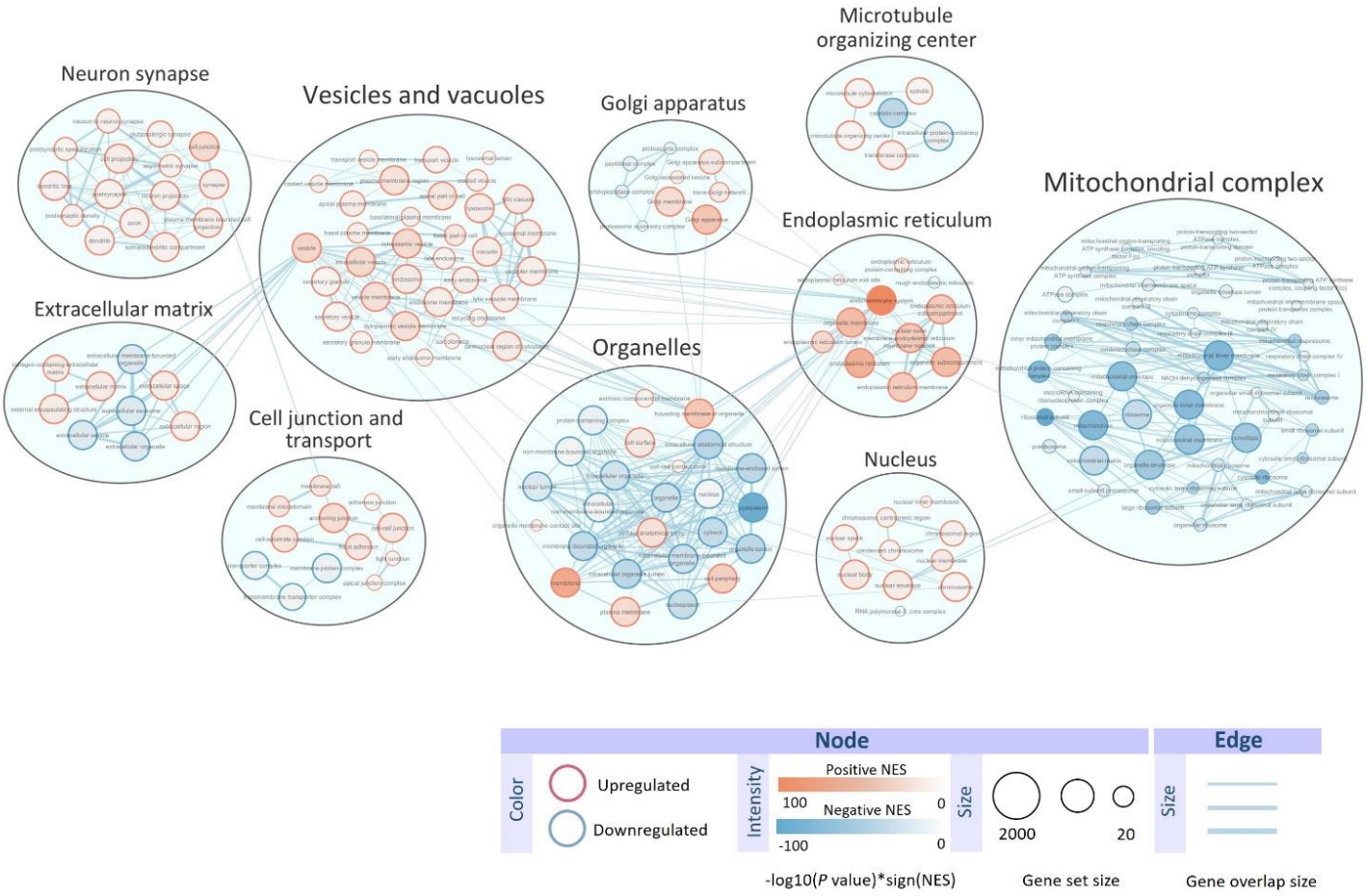

Figure 18. Transcriptomic enrichment map of cellular components affected in decidualized human endometrial stromal cells (d-hEnSCs) when progesterone receptor membrane component 2 (*PGRMC2*) was silenced. Transcriptomic enrichment map for gene ontology cellular components analyzed by g:Profiler in pairwise comparison of Sc d-hEnSCs and i*PGRMC2* d-hEnSCs. Node size represents the number of genes associated with a specific term. Node color represents the -$\log_{10}$(*P* value)*sign(NES). NES, Normalized Enrichment Score. Enrichment map was created with Cytoscape 3.10.2.

To validate the RNA-seq data and confirm the accuracy of our findings, we selected six DEGs for further analysis. These genes, including *HGF*, *COL1A1*, *TNC*, *CXCL14*, *DEPP1*, and *PGRMC2*, were chosen based on their potential relevance to female reproductive pathology and their association with key processes in decidualization and endometrial function, as previously mentioned.

After performing a RT-qPCR analysis on these six genes, our results confirmed significant downregulation of *PGRMC2* ($P < 0.0001$), *DEPP1* ($P < 0.05$), and *CXCL14* ($P < 0.05$), and significant upregulation of *TNC* ($P < 0.05$) in i*PGRMC2* d-hEnSCs compared with Sc d-hEnSCs (Figure 19A-D), consistent with the RNA-seq findings. However, no significant differences were detected in *COL1A1* and *HGF* mRNA expression (Figure 19E-F).

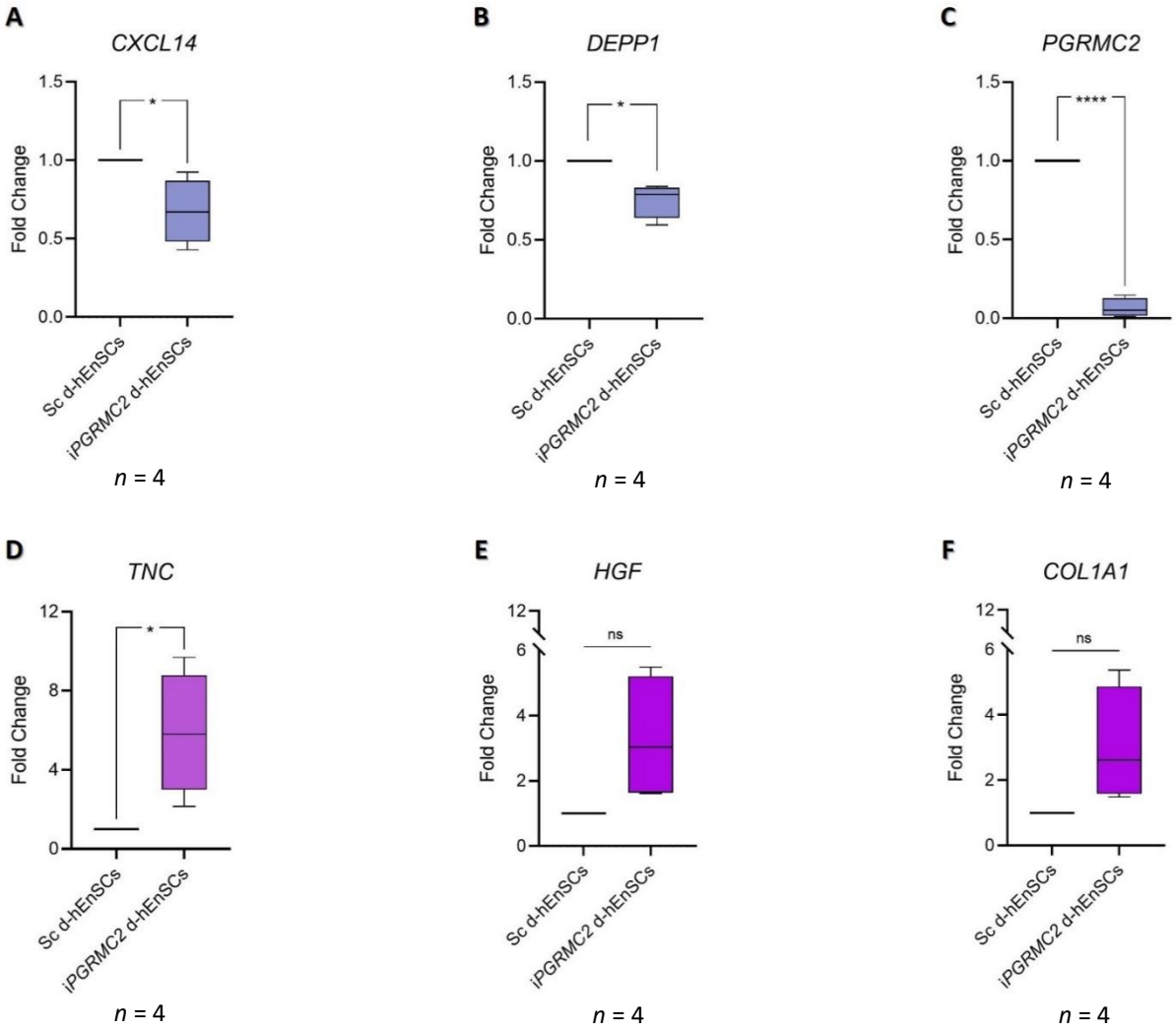

Figure 19. Validation of RNA-seq results by quantitative reverse transcription polymerase chain reaction (RT-qPCR). (A-F) Box and whiskers plots illustrate the fold change expression of C-X-C motif chemokine ligand 14 (*CXCL14*) (A), DEPP autophagy regulator 1 (*DEPP1*) (B), progesterone receptor membrane component 2 (*PGRMC2*) (C), tenascin C (*TNC*) (D), hepatocyte growth factor (*HGF*) (E), collagen type I alpha 1 chain (*COL1A1*) (F) mRNA between scramble control (Sc) and inhibited *PGRMC2* (i*PGRMC2*) decidual endometrial stromal cells. In the plots, central bars denote the median value, the box represents the 25th to 75th percentiles, and the whiskers mark the 5th and 95th percentiles. A total of $n = 4$ decidual human endometrial stromal cells (d-hEnSCs) were used; *$P < 0.05$; ****$P < 0.0001$; ns: not significant.

2.3.5 Proteomic effects of *PGRMC2* silencing during decidualization

As we observed significant differences in mRNA expression between the MS and LS phases of the menstrual cycle, but no changes at the protein level in the WB analysis, it became essential to explore the proteomic effects of *PGRMC2* silencing *in vitro*. Furthermore, the identification of DEGs in our transcriptomic analysis prompted us to explore whether these altered gene expressions were translated into functional protein alterations as well. To evaluate the changes in the global protein profile during the decidualization process in the absence of *PGRMC2*, we performed *PGRMC2* silencing in d-hEnSCs and compared i*PGRMC2* d-hEnSCs with negative control d-hEnSCs using SWATH-MS.

The effectiveness of the silencing assay by siRNAs was confirmed by WB, demonstrating approximately a 60% reduction in PGRMC2 protein abundance in hEnSCs transfected with *PGRMC2* siRNAs compared to negative control d-hEnSCs ($P < 0.001$; Figure 14B), validating that *PGRMC2* was effectively silenced at protein level as well.

The SWATH-MS analysis of negative control d-hEnSCs *versus* i*PGRMC2* d-hEnSCs ($n = 5$) identified 2070 different proteins, with a total of 28 DEPs when *PGRMC2* gene was silenced, with 4 being upregulated and 24 downregulated ($P < 0.05$) (Figure 20A & Supplementary Table S3). Functional enrichment analysis of the 28 DEPs revealed significant dysregulation in 40 biological processes in d-hEnSCs, primarily involving cellular respiration, cellular response, metabolism, ER protein localization, and ribonucleoside biosynthesis (Figure 20B & Supplementary Table S4). Additionally, 65 cellular components were identified, predominantly associated with organelle membranes and transport, vesicles, the extracellular matrix, ER, neurons and mitochondria components (Figure 21; Supplementary Table S4). In terms of molecular functions, the analysis indicated that molecule binding, transporter activity, and steroid biosynthesis were significantly altered in i*PGRMC2* d-hEnSCs (Supplementary Table S4).

A

	Analysis type	Total DEPs	Upregulated	Downregulated
i*PGRMC2* d-hEnSCs *vs* O d-hEnSCs	Proteomic (n = 5)	28	4	24

B

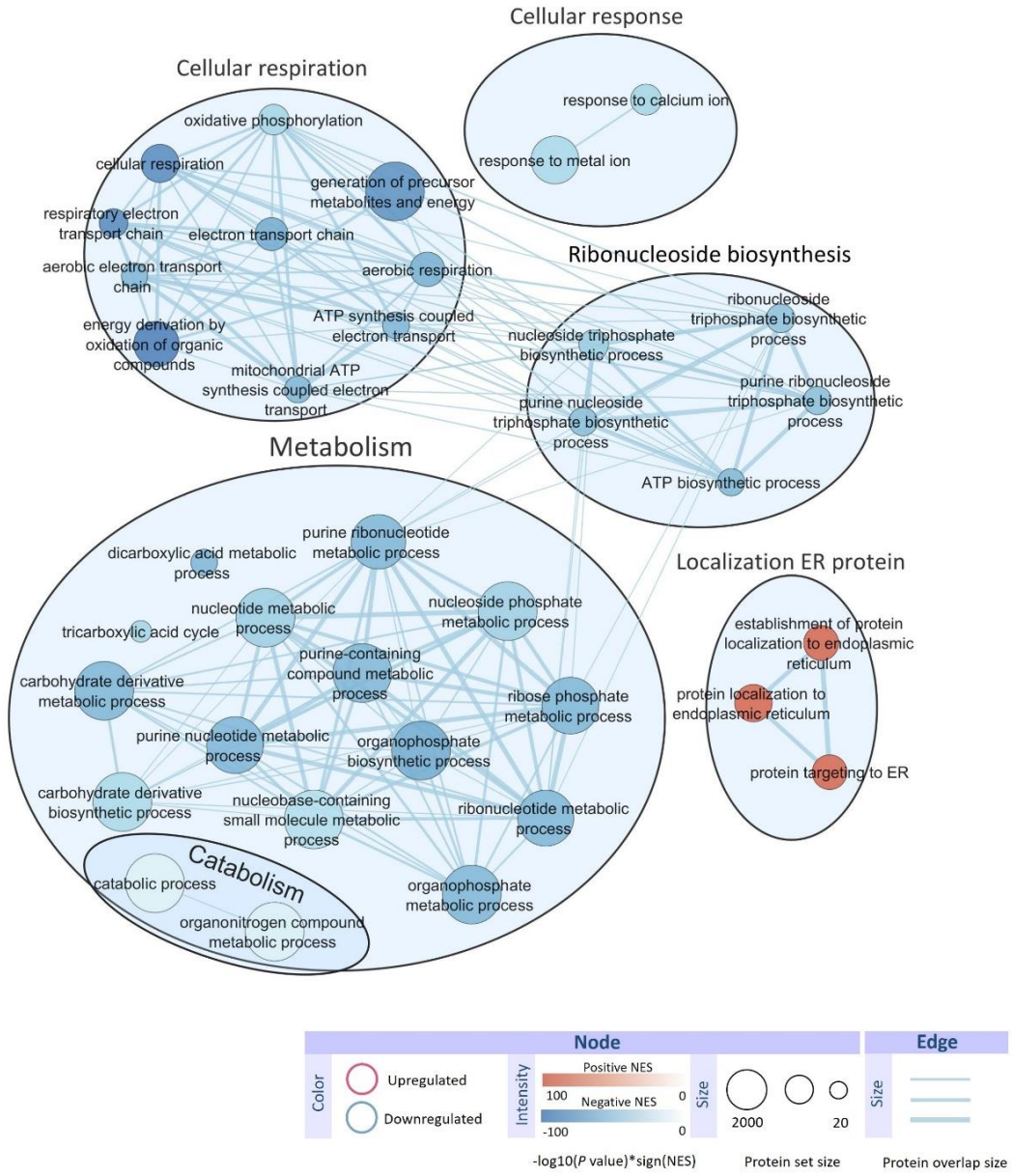

Figure 20. Proteomic analysis of decidualized human endometrial stromal cells (d-hEnSCs) when progesterone receptor membrane component 2 (*PGRMC2*) was silenced. (A) Differentially expressed proteins (DEPs) (total, upregulated and downregulated) in pairwise comparison of negative control d-hEnSCs (O d-hEnSCs) and d-hEnSCs with *PGRMC2* silenced (i*PGRMC2* d-hEnSCs) (n = 5). (B) Proteomic enrichment map for biological processes analyzed by g:Profiler (Benjamini-Hochberg FDR < 0.05) in pairwise comparison of O d-hEnSCs and i*PGRMC2* d-hEnSCs. Node size represents the number of proteins associated with a specific term. Node color represents the $-\log_{10}(P \text{ value})*\text{sign(NES)}$. ER, Endoplasmic Reticulum; NES, Normalized Enrichment Score. Enrichment map was created with Cytoscape 3.10.2.

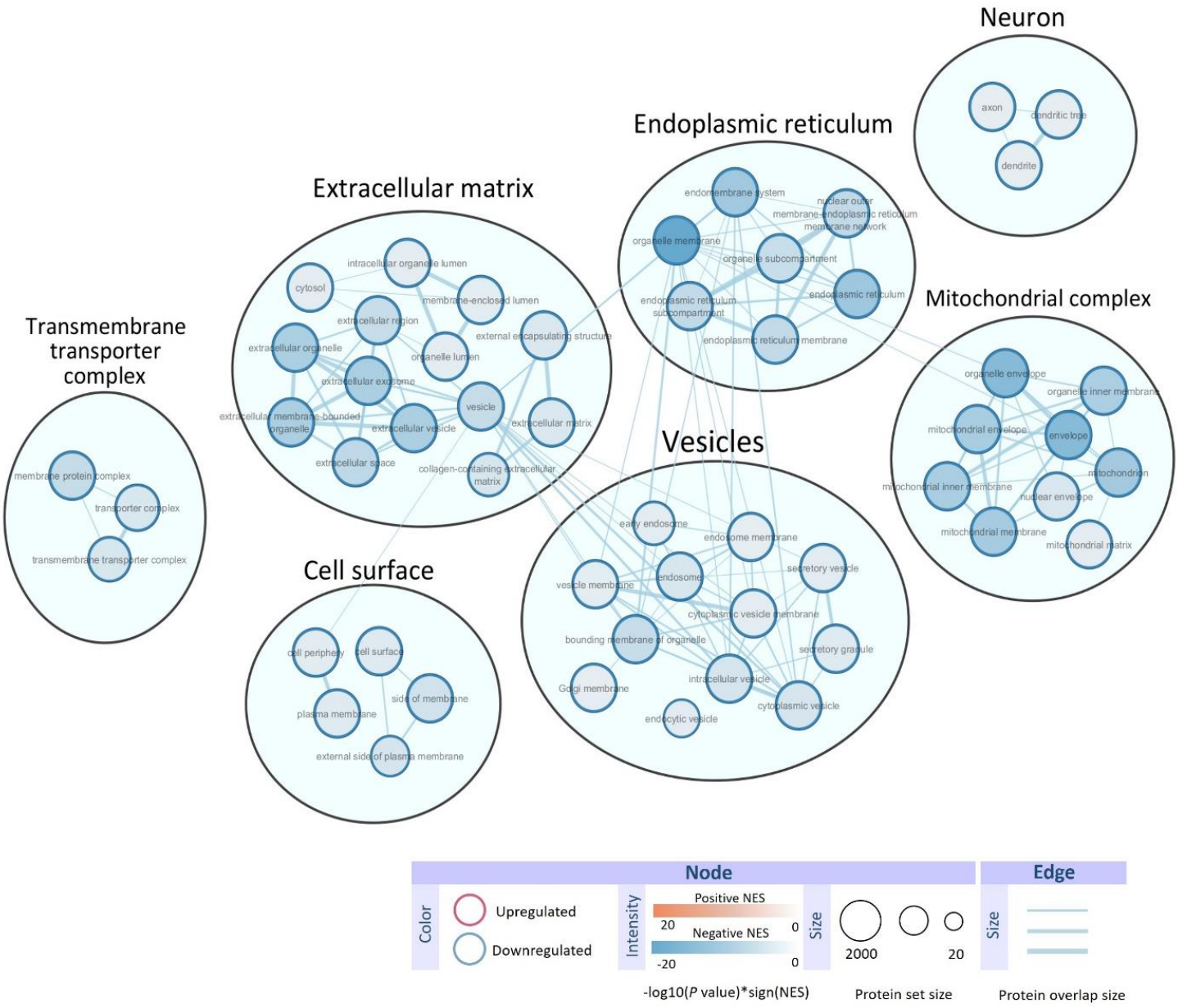

Figure 21. Proteomic enrichment map of cellular components affected in decidualized human endometrial stromal cells (d-hEnSCs) when progesterone receptor membrane component 2 (*PGRMC2*) was silenced. Proteomic enrichment map for cellular components analyzed by g:Profiler (Benjamini-Hochberg FDR < 0.05) in pairwise comparison of O d-hEnSCs and i*PGRMC2* d-hEnSCs. Node size represents the number of proteins associated with a specific term. Node color represents the $-\log_{10}$(*P* value)*sign(NES). NES, Normalized Enrichment Score. Enrichment map was created with Cytoscape 3.10.2.

2.3.6 *PGRMC2* silencing effects during trophoblast invasion

Due to the outcomes derived from the transcriptomic and proteomic analyses in d-hEnSCs when *PGRMC2* was silenced, which highlighted alterations in cellular pathways associated

with cell migration, cell adhesion, and extracellular matrix remodelling, we conducted further experiments to explore the potential impact of these changes on trophoblast invasion. To elucidate the role of PGRMC2 in this process, an *in vitro* invasion model was carried out wherein control, Sc and i*PGRMC2* d-hEnSCs were co-cultured with JEG-3 trophoblast spheroids. This model allowed us to directly assess the effects of *PGRMC2* silencing on trophoblast behavior and its invasive capacity into the hEnSCs.

The trophoblast spheroids invaded the hEnSCs monolayer at different expansion rates between control, Sc, and i*PGRMC2* d-hEnSCs over 24 and 48 hours of co-culture. Optical and fluorescence measurements revealed a significantly decrease in trophoblast spheroids expansion in i*PGRMC2* d-hEnSCs compared to control ($P < 0.001$) and Sc ($P < 0.01$) d-hEnSCs (Figure 22D). In fact, a similar area of the labelled spheroids in i*PGRMC2* d-hEnSCs between 24h and 48h can be appreciated (Figure 22C).

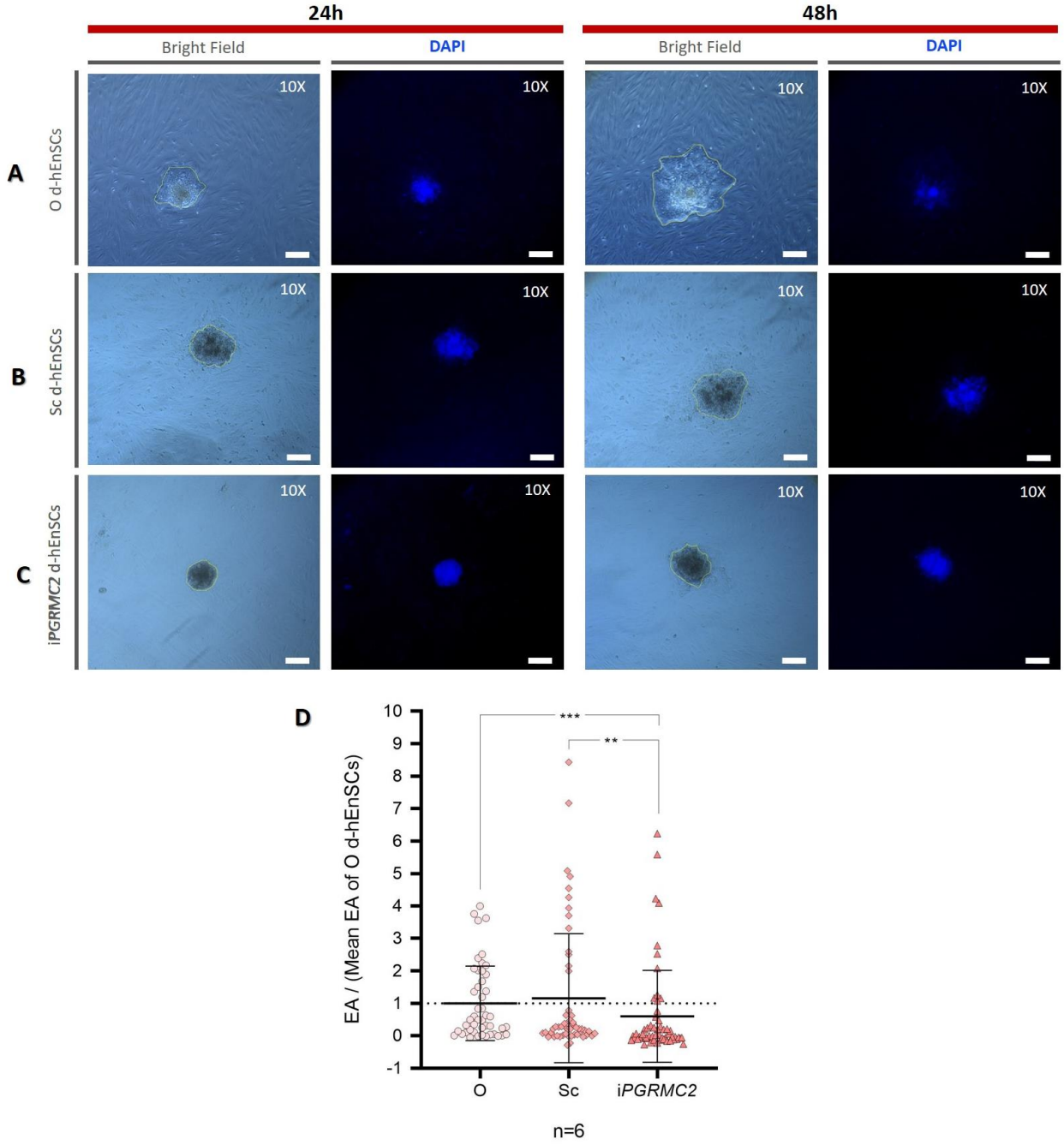

Figure 22. Differences in the trophoblast outgrowth area at 24 and 48h of co-culture when endometrial stromal progesterone receptor membrane component 2 (*PGRMC2*) was silenced. Optical and fluorescence photographs show JEG-3 trophoblastic spheroids labelled in blue invading the stromal monolayer at different expansion area (EA) rates between decidual negative control (O d-hEnSCs; A), decidual scramble control (Sc d-hEnSCs; B) and decidual *PGRMC2*-silenced stromal cells (i*PGRMC2* d-hEnSCs; C). Photographs were acquired at 24 and 48h of co-culture and at ×10 magnification. Scale bars: 100 µm. (D) Quantification of expanded area (EA) differences between negative control (O; *n* = 47 spheroids), scramble control (Sc; *n* = 53 spheroids) and inhibited *PGRMC2* (i*PGRMC2*; *n* = 56 spheroids) decidual endometrial stromal cells. A total of *n* = 6 decidual human endometrial stromal cells (d-hEnSCs) were used. Data are represented as normalized EA ± SD; ***P* < 0.01; ****P* < 0.001.

VII. DISCUSSION

VII. DISCUSSION

This thesis delves into the role of PGRMC2 in the human endometrium during the menstrual cycle and *in vitro* decidualization. In the uterus, both classical and non-classical PGRs exhibit fluctuating expression levels throughout the menstrual cycle regulating crucial events for endometrial receptivity and pregnancy establishment (Medina-Laver *et al.*, 2021). Despite classical PGRs contributing to the majority of female reproductive processes (Luetjens *et al.*, 2006; Kowalik *et al.*, 2013a), it has been observed that cells lacking classical PGRs still respond to P4 (Frye and Vongher, 1999).

PGRMC2, a non-classical PGR, has been implicated in the inhibition of antral follicle development (Griffin *et al.*, 2014; Will *et al.*, 2017) and participates in meiotic spindle assembly (Jühlen *et al.*, 2016). Reduced PGRMC2 expression has been noted in women with poor ovarian reserve, endometrial hyperplasia, and endometriosis (Skiadas et al., 2012; Bunch et al., 2013; Vázquez-Martínez et al., 2020) whereas PGRMC2 absence altered the expression of vascular endothelial growth factor A (VEGF-A), Kit ligand, and AMH (Peluso *et al.*, 2018). However, the temporal expression patterns and specific roles of PGRMC2 remain elusive in the human endometrium, and particularly, during decidualization.

To fill this knowledge gap, we first examined PGRMC2 mRNA and protein expression throughout the menstrual cycle in healthy human endometrial tissue.

We observed that endometrial epithelial glands showed the strongest expression of PGRMC2 across the menstrual cycle, with a marked decrease during the LS phase, aligning with previous findings in macaques and patients with endometriosis (Keator *et al.*, 2012; Bunch *et al.*, 2013). Additionally, we noted oscillating *PGRMC2* gene expression in the hEnSCs throughout the menstrual cycle, with a decrease in the LS phase compared to the MS phase, suggesting a potential function of PGRMC2 during embryo implantation. In contrast to *PGRMC1* (Garrido-Gómez *et al.*, 2014; Salsano *et al.*, 2017), *PGRMC2* mRNA expression was significantly higher in the endometrium during the MS phase with respect to the LS phase, corroborating that *PGRMC1* mirror the expression patterns of classical *PGRs* (Wang et al.,

1998). The complementary expression profiles of endometrial PGRMC1/2 and classical PGRs during the WOI suggests that PGRMC2 may play a pivotal role in embryo implantation, as was previously proposed in macaques (Keator et al., 2012).

The significant discrepancy observed between the gene expression levels of *PGRMC2* and its protein abundance is noteworthy. This suggest that *PGRMC2* may be acting at the genomic level similarly to *PGRMC1*, influencing gene expression and cellular processes without necessarily correlating with significant changes in protein abundance detectable by WB. This regulatory mechanism could involve PGRMC2 modulating the expression of target genes through transcriptional regulation, rather than simply increasing its own protein levels. The lack of detectable differences in protein levels via WB analysis might be attributed to the dynamic nature of protein interactions and functions within the endometrial tissue. While WB provides a quantitative assessment of total protein levels, it may not capture transient fluctuations or localized expressions that could occur at the cellular level. In contrast, immunohistochemistry staining allows for the visualization of PGRMC2 within the context of tissue architecture, revealing changes in intensity that may reflect its functional activity in different phases of the menstrual cycle. This complexity in protein regulation may also explain the fewer DEPs identified in comparison to DEGs observed. Thus, while PGRMC2 may not exhibit significant variations in overall protein abundance, its influence on gene expression and activity could still be prominent, as evidenced by the immunohistochemistry results. Therefore, the protein-level results obtained through WB for PGRMC2 should be interpreted with caution, as they may not fully represent the complex regulatory roles that PGRMC2 plays at both genomic and functional levels within the endometrium.

The comparison of *PGRMC2* with other progesterone receptors, both classical and non-classical, highlights the uniqueness of its regulation. In the traditional paradigm, classical PGRs drive decidualization, however, our findings evidence additional contributions of non-classical PGRs. Previous studies have shown that PGRMC1 expression changes in the receptive endometrium with respect to the non-receptive endometrium (Garrido-Gómez *et al.*, 2014), PGRMC1 overexpression during the secretory phases inhibited decidualization (Salsano *et al.*, 2017), PGRMC1 inhibition in d-hEnSCs disrupts the expression of numerous decidualization-related genes (Salsano *et al.*, 2021) and PGRMC1 also plays a crucial role in regulating

decidualization and endometrial receptivity through its regulation of COX2 (Tsuru *et al.*, 2023), a key enzyme for prostaglandin production, particularly PGE2. The increase in PGE2 is essential for promoting decidualization, a process vital for embryo implantation and pregnancy maintenance. In view of these findings, we hypothesized that PGRMC2 might similarly contribute to endometrial decidualization through similar molecular mechanisms. In our *in vitro* model of human endometrial decidualization, PGRMC2 expression was enhanced in decidualized cells suggesting PGRMC2 deficiency during human decidualization or embryo attachment may lead to reproductive failure, as documented in studies involving mice (Mccallum *et al.*, 2016; Clark *et al.*, 2017) and zebrafish (Wu *et al.*, 2019a).

Our transcriptomic and proteomic findings supported this hypothesis, revealing that targeted PGRMC2 knockdown substantially altered the expression patterns in d-hEnSCs.

Decidualization is a dynamic process characterized by physiological changes that prepare the endometrium to receive an embryo. Among these changes is endometrial angiogenesis, the P4-mediated vascular maturation that occurs during the secretory phase of the menstrual cycle. The d-hEnSCs support the remodeling and development of the endometrial vasculature, which not only plays a critical role in embryonic growth and survival but also has critical roles in homeostasis, immune defense, oxygen transport, nutrition, excretion, and fluid balance (Okada *et al.*, 2018). Notably, our transcriptomic analysis revealed that *PGRMC2* knockdown in the d-hEnSCs significantly dysregulated genes previously linked to decidualization and angiogenesis, such as *ADAMTS-5* (Zhu *et al.*, 2007), *S100A10* (Bissonnette *et al.*, 2016), *LEPR* (Kitawaki *et al.*, 2000) and *ITGB8* (Kumar *et al.*, 2017).

Further, we found that downregulated genes were associated with aerobic respiration, mitochondrial energy production and protein synthesis (particularly ribosome function, translation processes, and protein localization in the ER). Aligned with this, repressed proteins were linked also to mitochondrial complex, cellular respiration, biosynthesis, and metabolic responses. Mitochondrial defects are known to cause pathophysiological disorders, including infertility and reproductive pathologies (Gabriel *et al.*, 2012; Benkhalifa *et al.*, 2014). Furthermore, shorter mitochondria have recently been associated with decreased reliance on oxidative phosphorylation and lower ATP concentrations in endometrial decidual stromal cells

(Ryu *et al.*, 2020). Decidual cells are influenced by the number of mitochondria they contain and adapt to oxidative stress by expressing various intra- and extracellular free radical scavengers and by disabling stress-dependent proapoptotic signalling pathways (Gellersen *et al.*, 2007; Ryu *et al.*, 2020).

In line with this, our results show also that *PGRMC2* knockdown significantly altered the pathways of proteins involved in oxidative processes, including MIRO1 (Tseng *et al.*, 2021), QCR9 or UQCR10 (Geldon *et al.*, 2021), SDHB (Goncalves *et al.*, 2021), CMC1 or SLC25A12 (Falk *et al.*, 2014) and DLST or ODO2 (Ohta and Ohsawa, 2006), manifesting that there is no compensatory mechanism in gene expression for the reduced protein expression in the mitochondrial complex. Interestingly, an upregulation of genes related to the ER complex were shown, besides others of the endomembrane system as the Golgi apparatus, the plasma membrane and cell junctions, potentially impairing protein synthesis. On the other hand, genes related to homeostasis, such as *ABCA1* (Yu and Tang, 2022) and *CCR1* (Jones *et al.*, 2005), were also significantly affected by *PGRMC2* knockdown. This suggests that beyond its role in mitochondrial function and oxidative processes, PGRMC2 might also play a key role in maintaining cellular structure and homeostasis necessary for endometrial stromal cell function, potentially affecting their adaptability and response to environmental and hormonal signals.

The proteins encoded by the upregulated genes are mainly involved in biological processes related to morphogenesis, angiogenesis, cell migration and cell adhesion, as well as cellular processes such as ER function, vesicle and vacuoles, cell junction and transport and organelles. Proteomically, an overexpression of ER-located proteins was detected in d-hEnSCs after *PGRMC2* silencing.

Taken together, the differential gene expression in the d-hEnSCs organelles and membranous systems as well as in the transporting complex corroborate the role of PGRMC2 in the extensive secretory remodelling occurring during decidualization (Anelli *et al.*, 2022). Our observations of PGRMC2 localization in membranes and organelles confirm suggested associations for PGRMC1/2 (Albrecht *et al.*, 2012; Bunch *et al.*, 2013; Griffin *et al.*, 2014; Peluso *et al.*, 2014; Jühlen *et al.*, 2016).

Inhibition of *PGRMC1*, which has complementary expression to PGRMC2, upregulated genes related to ATP generation and transport, protein and lipid biosynthesis, post-transcriptional processing, vesicle trafficking, and oxidative stress alleviation (Salsano *et al.*, 2020, 2021), indicating both non-classical receptors are involved in the extensive cytoplasmic remodelling during decidualization. Additionally, the role of PGRMC2 in lipid metabolism and biosynthesis has potential implications for endometrial receptivity. Lipids and lipid-derived molecules are known to be crucial in cell signalling and membrane dynamics, which are vital for the structural and functional changes during decidualization. PGRMC2's involvement in these pathways suggests that it might help regulate the lipid environment of the endometrium, thereby influencing cellular communication and the implantation process.

In addition, silencing of *PGRMC2* in d-hEnSCs showed DEGs and DEPs associated with neuronal synapses. The downregulation of neuronal synapse-related genes in proteomic and, consequently, its upregulation in transcriptomic as a compensatory mechanism may indicate that similar mechanisms of cell communication and signal transduction, as seen in neurons, are involved in the cellular processes of the endometrium where said pathways are affected by *PGRMC2* silencing.

This unexpected finding suggests a potential role for neuronal signalling pathways in the regulation of endometrial function. Neuronal synapse-related genes are known to play crucial roles in cell-to-cell communication, signal transduction, and plasticity in neurons (Südhof, 2021). These genes may perform similar functions in endometrial stromal cells, facilitating communication and coordination among cells, which is essential for processes such as decidualization and tissue remodelling. The presence of these genes suggests that endometrial stromal cells may have a degree of plasticity and adaptability, allowing them to respond dynamically to hormonal and environmental cues (Luddi *et al.*, 2019; Li *et al.*, 2024). Other studies have also observed a relationship between genes involved in neuronal synapses and uterine infertility (Carbajo-García *et al.*, 2022).

Inhibiting classical *PGRs* during decidualization upregulates genes related to proliferation, angiogenesis, apoptosis, cell migration, adhesion, cytoskeletal organization, cytokine and chemokine secretion (Kaya *et al.*, 2015; Mazur *et al.*, 2015). While some of these

decidualization processes (such as proliferation and apoptosis) are regulated exclusively by classical PGRs, angiogenesis, cell migration, and adhesion appear to be regulated by both non-classical (PGRMC2) and classical PGRs. This finding is further supported by the Ki-67 analysis, which confirmed that the transfection process did not affect cell proliferation, ensuring that the observed changes in the transcriptomic profiles are specifically due to *PGRMC2* silencing rather than non-specific effects on cell viability or proliferation.

PGRMC2 also seems to have key ER-related functions, given its expression in the endomembrane system and protection against ER stress. Based on observations from the knockout of other transcription factors regulating decidualization and required for ER and Golgi apparatus remodelling (Zhao *et al.*, 2020; Anelli *et al.*, 2022; Pittari *et al.*, 2022), *PGRMC2* silencing appears to impede protein biosynthesis and cellular energy production. *PGRMC2* silencing enhanced ER stress, extracellular matrix-receptor interactions, and focal adhesion signalling pathways through post-transcriptional regulation in either the ER or Golgi apparatus (Zhao *et al.*, 2020; Pittari *et al.*, 2022). Further analysis of the DEGs and DEPs implicated several key signalling pathways, including the PI3K/AKT pathway, known for its role in cell proliferation, survival, and metabolism. PGRMC2's involvement in these pathways suggests it fine-tunes cellular responses necessary for successful decidualization and implantation. Additionally, PGRMC2 appears to influence the expression of various cytokines and growth factors, which are crucial for creating a receptive endometrial environment and facilitating embryo-maternal communication.

On the other hand, due to the altered expression of embryo invasion biomarkers [i.e., *S100A10* (Bissonnette *et al.*, 2016), *SERPINE1* (Gurung *et al.*, 2021), *CXCL12* (Laird *et al.*, 2011), *CXCL14* (Haibin *et al.*, 2009; Leach *et al.*, 2012; Kawamura *et al.*, 2021) and *SPP1* (Kramer *et al.*, 2021)] upon *PGRMC2* silencing in d-hEnSCs, we performed a functional assay with trophoblast spheroids to corroborate the implication of endometrial PGRMC2 in the post-implantation stages. Successful trophoblast invasion, essential for pregnancy initiation and maintenance, depends on optimal endometrial receptivity (Singh *et al.*, 2011). Invasion involves morphological, biochemical, and vascular changes in the endometrium, alongside adequate decidualization of hEnSCs. Our transcriptomic findings showed *PGRMC2* silencing

upregulates genes, but not proteins, related to angiogenesis, cell migration and adhesion which are essential for trophoblastic invasion (Muter *et al.*, 2023).

Thus, we hypothesize the RNA overexpression may be a compensatory behaviour to the insufficient protein biosynthesis. Indeed, PGRMC2 altered the expression of migration genes [i.e., *S100A10* and *CCR1* (Bissonnette *et al.*, 2016; Li *et al.*, 2020; Sang *et al.*, 2022)]. A similar response was previously reported in SKOV-3 ovarian cancer cells with limited migration (Albrecht *et al.*, 2012). Our *in vitro* outgrowth model supports our transcriptomic findings, suggesting that PGRMC2 regulates hEnSCs cell migration, but not proliferation, during embryo invasion into the d-hEnSCs. Considering the elevated ER stress in d-hEnSCs associated with early pregnancy loss (Liu *et al.*, 2011; Gao *et al.*, 2012; Soczewski *et al.*, 2020), this mechanism of action may explain why the lack of PGRMC2 is associated with post-implantation failure (Mccallum *et al.*, 2016; Clark *et al.*, 2017).

In addition, the increase of PRL in i*PGRMC2* d-hEnSCs could be also a compensatory mechanism. For instance, PRL has been shown to interact with various cytokines and growth factors, which are crucial for the regulation of decidualization and trophoblastic invasion (Dos Santos *et al.*, 2012; Santos *et al.*, 2021). This suggests that the absence of PGRMC2 could disrupt these interactions and PRL may not effectively promote trophoblastic invasion or decidualization.

Finally, the differential overexpression of non-classical *versus* classical PGRs in various menstrual phases and in hEnSCs highlights the prominent role of non-classical PGRs in reproductive functions. Moreover, the complementary expression patterns of PGRMC1 and PGRMC2 in both proliferative and secretory phases, as well as in non-decidualized and decidualized cells, suggests a parallel role in embryo implantation, and potentially, in maintaining pregnancy.

Given the limited sample sizes in both transcriptomic and proteomic analyses, as well as in the outgrowth model and Ki-67 analysis, our conclusions are preliminary and should be interpreted cautiously. Transcriptomic measures mRNA levels, which do not always correlate with protein expression levels due to post-transcriptional modifications and regulatory mechanisms. Proteomics has its own limitations, particularly in detecting low-abundance

proteins. This limitation could be responsible for the relatively low number of DEPs identified in our study, as the sensitivity of current proteomic techniques may not be sufficient to capture proteins with low expression levels. Additionally, the complexity of protein post-translational modifications and the dynamic nature of the proteome can further complicate the interpretation of proteomic data. Notwithstanding the limitations associated with the low number of DEPs, the consistency of these findings with the transcriptomic results supports the robustness of our data, indicating that the observed changes are biologically meaningful and relevant. Overall, while both methods offer valuable insights, their inherent limitations highlight the need for complementary approaches and advanced techniques to achieve a more comprehensive understanding of the underlying biological processes.

Additionally, the use of a primary endometrial stromal population differentiated *in vitro* does not fully represent the heterogeneity of the *in vivo* endometrium, nor does it account for the paracrine communications between endometrial cell types. Moreover, the protein-level results obtained through WB for PGRMC2 should be interpreted cautiously, as they may not fully reflect the complex regulatory roles that PGRMC2 plays at functional levels within the endometrium. Furthermore, our *in vitro* study has only analyzed PGRMC2 in the hEnSCs, and its role in the epithelial compartment remains to be elucidated.

Despite these limitations, the results obtained have significant implications for the field of assisted reproduction. Identifying PGRMC2 as a key regulator in decidualization and embryo invasion opens new avenues for developing targeted therapies that could improve fertility treatment outcomes, identifying new biomarkers in endometrial physiology, and personalizing treatments. Furthermore, PGRMC2 might serve as a potential therapeutic target for conditions associated with impaired decidualization and embryo invasion, such as RIF and endometriosis. By modulating PGRMC2 activity or uncovering the triggers behind its lack of expression, it might be possible to enhance endometrial receptivity and improve pregnancy rates in patients undergoing assisted reproductive technologies.

Future research should focus on expanding the sample size to validate these findings and further explore the interactions between PGRMC2 and other endometrial microenvironment components. Additionally, *in vivo* studies evaluating the impact of PGRMC2 modulation in

animal models could provide valuable insights into its role in implantation and pregnancy maintenance. Specifically, investigating the mechanisms by which PGRMC2 regulates the trophoblast-endometrium interaction and employing advanced techniques like single-cell RNA sequencing could delineate the cell-specific roles of PGRMC2 in the endometrium. Additionally, exploring the epigenetic regulation of PGRMC2 expression might uncover new layers of complexity in its function and regulation.

This study presents the first *in vivo* characterization of PGRMC2 expression throughout the human menstrual cycle and in *in vitro* decidualization of hEnSCs. We provide a comparative analysis between the classical and non-classical PGRs systems, along with a novel transcriptomic and proteomic approach to assess the functional implications of targeted *PGRMC2* knockdown in hEnSCs. Nevertheless, additional studies are needed to validate PGRMC2's mechanism of action and its intertwining relationship(s) with other non-classical and classical PGRs during decidualization, to fully understand the role of these proteins in female reproduction.

VIII. CONCLUSIONS

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The conclusions drawn from this PhD thesis are:

1. *PGRMC2* gene expression fluctuates in the human endometrium throughout the menstrual cycle, significantly peaking during the mid-secretory phase compared to the late secretory phase.

2. No changes in PGRMC2 protein abundance were detected along the human menstrual cycle, despite significant mRNA changes. In contrast, immunohistochemistry analysis revealed variations in PGRMC2 intensity, particularly in glandular and stromal cells.

3. After *in vitro* decidualization, PGRMC2 gene expression and protein abundance significantly increased in human endometrial stromal cells, principally in cell membranes and organelles.

4. The expression of non-classical *PGRs* was significantly higher than the classical *PGRs* along the menstrual cycle and in both non-decidualized and decidualized human endometrial stromal cells. While *PGRMC1* and classical *PGRs* gene expression significantly decreased during the secretory phases, *PGRMC2* is the most expressed receptor in the late secretory phase.

5. The *PGRMC2* silencing in decidualized human endometrial stromal cells resulted in significant changes at transcriptomic and proteomic levels. These changes affected a range of biological processes, such as angiogenesis, cell migration, cell adhesion, and cellular response; as well as cellular components like the ER, vesicles, the extracellular matrix, organelles and plasma membranes, and mitochondria components.

6. *PGRMC2* silencing impairs the ability of decidualized human endometrial stromal cells to support trophoblast invasion, underscoring the importance of PGRMC2 in facilitating stromal cells-trophoblast interactions.

IX. REFERENCES

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X. ANNEX

X. ANNEX

1. SUPPLEMENTARY MATERIAL

Supplementary Table S1. Differentially expressed genes for i*PGRMC2* d-hEnSCs vs. Sc d-hEnSCs comparison.

Supplementary Table S2. Gene ontology term enrichment for differentially expressed genes in d-hEnSCs after *PGRMC2* silencing.

Supplementary Table S3. Differentially expressed proteins for negative control d-hEnSCs vs. i*PGRMC2* d-hEnSCs comparison.

Supplementary Table S4. Protein ontology term enrichment for differentially expressed proteins in d-hEnSCs after *PGRMC2* silencing.

Supplementary material is available at the following link:

"Medina Laver, Yassmin (2024), "Characterization and function of progesterone receptor membrane component 2 (PGRMC2) in the human endometrium - Annex 1.", Mendeley Data, V1, doi: 10.17632/5s4bs476zz.1".

All raw sequencing data are available through NCBI's Gene Expression Omnibus (GEO) under GEO Series accession number GSE251843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi? acc=GSE251843). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository under accession number PXD048494.

2. SCIENTIFIC PRODUCTION

2.1 SCIENTIFIC ARTICLES DIRECTLY RELATED WITH THE PRESENT PHD THESIS

Medina-Laver Y, González-Martín R, de Castro P, Díaz-Hernandez I, Alamá P, Quiñonero A, Palomar A, Domínguez F. Deciphering the role of PGRMC2 in the human endometrium during the menstrual cycle and *in vitro* decidualization using an *in vitro* approach. Human Reproduction 2024;**39**(5):1042–1056. https://doi.org/10.1093/humrep/deae044

Medina-Laver Y, Rodríguez-Varela C, Salsano S, Labarta E, Domínguez F. What do we know about classical and non- classical progesterone receptors in the human female reproductive tract? A review. *International Journal of Molecular Sciences* 2021;**22**:11278. https://doi.org/10.3390/ijms222011278

2.2 WORKS SUBMITTED TO INTERNATIONAL CONFERENCES DIRECTLY RELATED WITH THE PRESENT PHD THESIS

Medina-Laver Y, de Castro P, Palomar A, González-Martín R, Quiñonero A, Domínguez F. "Unraveling PGRMC2 functions in decidualized human endometrial stromal cells by RNA sequencing." Oral presentation. SRI 70th Annual Scientific Meeting. Brisbane, Australia. March 21st-25th, 2023.

Medina-Laver Y, Palomar A, de Castro P, González-Martín R, Quiñonero A, Domínguez F. "Deciphering the role of PGRMC2 in decidualization and trophoblast invasion using primary *in vitro* models". Oral presentation. SRI 69th Annual Scientific Meeting. Denver, US. March 15th-19th, 2022.

Medina-Laver Y, Diaz-Hernandez I, Alama P, Gonzalez-Martin R, Palomar A, Quiñonero A, Dominguez F. "Characterization of the non-classic progesterone receptor membrane component 2 (PGRMC2) during the human menstrual cycle and *in vitro* decidualization". Oral presentation. SRI 68th Annual Scientific Meeting. Boston, US. July 6th-9th, 2021.

2.3 AWARDS RELATED WITH THE PRESENT PHD THESIS

SRI President's Presenter Award (2022) to **Medina-Laver Y**. for "Deciphering the role of PGRMC2 in decidualization and trophoblast invasion using primary *in vitro* models". Oral presentation.

SRI President's Presenter Award (2021) to **Medina-Laver Y**. for "Characterization of the non- classic progesterone receptor membrane component 2 (PGRMC2) during the human menstrual cycle and *in vitro* decidualization". Oral presentation.

2.4 SCIENTIFIC ARTICLES NOT RELATED WITH THE PRESENT PHD THESIS

Oh Y, Quiroz E, Wang T, **Medina-Laver Y**, Redecke SM, Dominguez F, Lydon JP, DeMayo FJ, Wu SP. The NR2F2-HAND2 signalling axis regulates progesterone actions in the uterus at early pregnancy. *Front Endocrinol* 2023;**14**:1229033. doi: 10.3389/fendo.2023.1229033.

Palomar A, Quiñonero A, **Medina-Laver Y**, Gonzalez-Martin R, Pérez-Debén S, Alama P, Domínguez F. Antioxidant supplementation alleviates mercury-induced cytotoxicity and restores the implantation-related functions of primary human endometrial cells. *Int J Mol Sci*. 2023;**24**(10):8799. doi: 10.3390/ijms24108799.

González-Martín R, Palomar A, **Medina-Laver Y**, Quiñonero A, Domínguez F. Endometrial cells acutely exposed to phthalates *in vitro* do not phenocopy endometriosis. *Int J Mol Sci*. 2022;**23**(19):11041. doi: 10.3390/ijms231911041.

Palomar A, González-Martín R, Pérez-Debén S, **Medina-Laver Y**, Quiñonero A, Domínguez F. Mercury impairs human primary endometrial stromal cell function. *Biol Reprod*. 2022;**106**(5):1022-1032. doi: 10.1093/biolre/ioac016.

2.5 WORKS SUBMITTED TO INTERNATIONAL CONFERENCES NOT RELATED WITH THE PRESENT PHD THESIS

Palomar A, **Medina-Laver Y**, Quiñonero A, González-Martín R, Domínguez F. “Antioxidant treatment revert mercury-induced damage in an *in vitro* human primary stromal cells (hEnSC) invasion model”. Poster presentation. SRI 70th Annual Scientific Meeting. Brisbane, Australia. March 21st-25th, 2023.

Palomar A, Quiñonero A, González-Martín R, **Medina-Laver Y**, Domínguez F. Assessment of decidualization and oxidative stress in primary human endometrial stromal cells (hEnSC) after mercury-induced damage. Poster presentation. SRI 70th Annual Scientific Meeting. Brisbane, Australia. March 21st-25th, 2023.

Palomar A, Quiñonero A, **Medina-Laver Y**, González-Martín R, Salsano S, Domínguez F. "Entosis act through the Rho-ROCK signalling pathway during human embryo implantation”. Oral presentation. SRI 69th Annual Scientific Meeting. Denver, US. March 15th-19th, 2022.

Palomar A, González-Martín R, Quiñonero A, **Medina-Laver Y**, Pérez-Debén S, Domínguez F. “Antioxidant treatment ameliorates mercury-induced damage on viability and function of human endometrial epithelial cells”. Poster presentation. SRI 69th Annual Scientific Meeting. Denver, US. March 15th-19th, 2022.