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Identification of new regenerative therapies in reproductive medicine and their application as a future therapeutic approach for endometrial regeneration

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VNIVERSITAT DE VALÈNCIA

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Lucía de Miguel Gómez

Director:
Dr. Irene Cervelló Alcaraz

Valencia, September 2021

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Identification of new regenerative therapies in reproductive medicine and their application as a future therapeutic approach for endometrial regeneration

Author:
Lucía de Miguel Gómez

Bachelor's degree in Biotechnology
Master's degree in the Biotechnology of Assisted Human Reproduction

Supervised by:
Dr. Irene Cervelló Alcaraz (director)
Dr. José Bellver Pradas (tutor)

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An appendix about the SARS-CoV-2 potential impact over the endometrium is included.

El presente trabajo de tesis doctoral ha sido realizado principalmente en los laboratorios de la Fundación IVI, así como en las instalaciones de la Universitat de València (animalario de la Facultad de Medicina y sección de proteómica del Servicio Central de Soporte a la Investigación Experimental, SCSIE) y del Hospital Universitari i Politècnic La Fe. Gracias a la ayuda de un proyecto de investigación con la Universitat de València en la Facultad de Medicina, Departamento de Pediatría, Obstetricia y Ginecología que ha sido financiado por la ayuda PROMETEO de la Generalitat Valenciana para la realización de proyectos I+D para grupos de investigación (PROMETEO/2018/137), por la ayuda Acción Estratégica de Salud del Instituto de Salud Carlos III (CPI19/00149-PI17/01039) y por la ayuda FERRING COVID-19 INVESTIGATIONAL GRANT para investigación en medicina reproductiva y salud materna.

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La Dra. Irene Cervelló Alcaraz, Doctora en Biología, investigadora Miguel Servet del Instituto de Salud Carlos III, investigadora principal del área de Células Madre y Bioingeniería en Fundación IVI (FIVI) y coordinadora del laboratorio de FIVI

CERTIFICA:

Que el trabajo de investigación titulado: "**Identification of new regenerative therapies in reproductive medicine and their application as a future therapeutic approach for endometrial regeneration**", que incluye el apéndice sobre el impacto del SARS-CoV-2 en el endometrio, ha sido realizado íntegramente por Dña. Lucía de Miguel Gómez 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 29 de septiembre de 2021,

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ABBREVIATIONS

A	Activated
ABC	ATP-binding cassette
ADA2	Adenosine deaminase 2
ADMSC	Adipose-derived mesenchymal stem cell
ANG	Angiogenin
ANGPTL3	Angiopoietin-related protein 3
APOB100	Apolipoprotein B-100
AS	Asherman's Syndrome
ASC	Adult stem cell
ATPO	ATP synthase subunit O, mitochondrial
ATP1B3	Sodium/potassium-transporting ATPase subunit β-3
AUFI	Absolute uterine factor infertility
A2M	α-2-macroglobulin
BDNF	Brain-derived neurotrophic factor
BMDSC	Bone marrow-derived stem cell
BMMSC	Bone marrow-derived mesenchymal stem cell
BMP	Bone morphogenetic protein
bNGF	B-nerve growth factor
BP	Biological process
BrdU	5-bromo-2′-deoxyuridine
$CaCl_2$	Calcium chloride
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CCND1	Cyclin D1
CFU	Colony-forming cells/units
CKLF6	CKLF-like MARVEL transmembrane domain-containing protein 6
Cm	Centimeters
c-MYC	MYC proto-oncogene
CXCL8	C-X-C motif chemokine ligand 8/interleukin 8
C4B	Complement C4-B
DDA	Data-dependent analysis
DIA	Data-independent analysis
DNA	Deoxyribonucleic acid
E_2	Estradiol
EA	Endometrial atrophy
ECM	Extracellular matrix

EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
endoSSC	Endometrial stromal stem cell
endoEPC	Endometrial epithelial progenitor cell
EPC	Endothelial progenitor cell
FC	Fold change
FGA	Fibrinogen alpha-1 chain
FFPE	Formalin-fixed paraffin-embedded
FGF2	Fibroblast growth factor
FR	Fold regulation
FSH	Follicle-stimulating hormone
G	Relative centrifugal force
G-CSF	Granulocyte-colony stimulating factor
GF	Growth factor
GnRH	Gonadotropin-releasing hormone
GO	Gene ontology
GOLGA2	Golgin subfamily A member 2
hCG	Human chorionic gonadotropin
hESC	Human endometrial stromal cell
HGF	Hepatocyte growth factor
HOXA10	Homeobox protein Hox-A10
HIF1	Hypoxia-inducible factor 1
HPLC	High-performance liquid chromatography
HSC	Hematopoietic stem/progenitors cell
H2B-GPF	Histone 2B-green fluorescent protein
H&E	Hematoxylin and eosin
ICM	Inner cell mass
IFNγ	Interferon gamma
IGFBP4	Insulin-like growth factor-binding protein 4
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IL	Interleukin
IL1RA	Interleukin 1 receptor antagonist protein
IUA	Intrauterine adhesion
c-KIT	Tyrosine-protein kinase KIT
JAK-STAT	Janus kinase/signal transducer and activator of transcription
JUN	JUN proto-oncogene
KEGG	Kyoto encyclopedia of genes and genomes
KGF	Keratinocyte growth factor
KLF4	Kruppel-like factor 4

LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LGR5	Leucine-rich repeat-containing G-protein-coupled receptor 5
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharides
LRC	Label-retaining cell
MAPK	Mitogen-activated protein kinase
MCP1	Monocyte chemoattractant protein 1/C-C motif chemokine 2
MIF	Migration inhibitory factor
Min	Minutes
MIP1α	Macrophage inflammatory protein 1 α
MIP2	Macrophage inflammatory protein 2/C-X-C motif chemokine 2
Mm	Millimeters
MMP	Metalloproteinase
MMRN1	Multimerin 1
MSC	Mesenchymal stem cell
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
N	Population size
Na	Non-activated
NCAM1	Neural cell adhesion molecule 1
NDKB	Nucleoside diphosphate kinase B
NOD-SCID	Non-obese diabetic/severe combined immunodeficiency
NTPDase2	Ectonucleoside triphosphate diphosphohydrolase-2
OCT	Octamer-binding transcription factor
PDGF	Platelet-derived growth factor
PDGFRβ	Platelet-derived growth factor receptor β
PIGF	Placenta growth factor
PI3K-AKT	Phosphoinositide 3-kinase/protein kinase B
PKM	Pyruvate kinase
PPAR	Peroxisome proliferator-activated receptor
PPBP	Platelet basic protein
PPP	Platelet-poor plasma
PPP1CA	Serine/threonine-protein phosphatase PP1-α catalytic subunit
P_4	Progesterone
PRDX1	Peroxiredoxin 1
PRP	Platelet-rich plasma
PRPC	Platelet-rich plasma control
P85A	Phosphatidylinositol 3-kinase regulatory subunit α

qRT-PCR	Quantitative real-time polymerase chain reaction
Rap1	Ras-associated protein 1
RHOA	Transforming protein rhoA
RINI/RNH1	Ribonuclease inhibitor
RNA	Ribonucleic acid
SALL4	Sal-like protein 4
SCF	Stem cell factor
SC	Stem cell
SCN	Stem cell niche
SDF1/CXCL12	Stromal cell-derived factor 1/C-X-C motif chemokine 12
SERPINE1	Plasminogen activator inhibitor-1
SOX2	SRY-box transcription factor
SSEA1	Stage-specific embryonic antigen-1
STAT5A	Signal transducer and activator of transcription 5A
SUSD2	Sushi domain-containing-2
SWATH-MS	Sequential window acquisition of all theoretical mass spectrometry
TAC	Transit-amplifying cell
TE	Trophectoderm
TGFβ	Transforming growth factor beta
THY1	Thy-1 membrane glycoprotein/CD90 antigen
TSP1	Thrombospondin 1
TNFα	Tumor necrosis factor alpha
UBA3	NEDD8-activating enzyme E1 catalytic subunit
UCMSC	Umbilical cord mesenchymal stem cell
UCP	Umbilical cord plasma
VCAM1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
VSEL	Very small embryonic-like stem cell
vWF	Von Willebrand factor
Wnt	Wingless-type
YWHAG	14-3-3 protein gamma
2AAA	Pr65 subunit of protein phosphatase 2A
3D	Three-dimensional

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SPANISH SUMMARY

INTRODUCCIÓN

1. El sistema reproductor femenino

El útero, que constituye uno de los órganos internos principales del sistema reproductor femenino, consta de tres partes bien diferenciadas: fondo, cuerpo y cérvix. No obstante, la morfología uterina global varía según la especie, teniendo forma de pera en los humanos mientras que en los roedores es bicorne. En cualquier caso, el útero se compone de tres capas de tejido tisular: perimetrio (capa serosa y más externa), miometrio (capa muscular y más gruesa), y endometrio (capa mucosa y más interna, colindante con la cavidad intrauterina). Esta última capa, el endometrio, es la responsable directa de la implantación embrionaria. Para ello es necesario que presente una correcta morfología y grosor endometrial. Así, cualquier alteración que afecte a este tejido, dificultará o incluso llegará a impedir la correcta implantación del embrión y, por tanto, la consecución del embarazo, comprometiendo la fertilidad de la mujer.

Brevemente, el endometrio consta de cuatro tipos o componentes celulares (epitelio, estroma, red vascular y población de células inmunes residentes), que a su vez se organizan en una capa basal (adyacente al miometrio) y otra funcional (más superficial y en contacto con la cavidad intrauterina). La capa basal permanece relativamente constante a lo largo del ciclo menstrual mientras que la capa funcional se expande y vasculariza a lo largo de todo el ciclo, desprendiéndose durante la menstruación, en el caso de los humanos, o reabsorbiéndose, en el caso de los roedores y otras especies.

El ciclo hormonal que rige la función reproductiva de las mujeres se denomina ciclo menstrual (duración media de 28 días). Este ciclo comienza con la menstruación, iniciándose así la fase proliferativa, que coincide con la

maduración de los folículos o fase folicular del ciclo ovárico. Al final de la fase proliferativa, se produce la ovulación, tras la cual se inicia la fase secretora, que sucede paralelamente a la fase lútea ovárica. Este proceso cíclico está dirigido por los niveles de gonadotropinas (LH, del inglés *luteinizing hormone* y FSH, del inglés *follicle-stimulating hormone*) que a su vez regulan los niveles de estrógenos y progesterona, claves durante todo el ciclo. Durante la fase proliferativa predominan los estrógenos, de modo que el endometrio se engrosa. Cuando, tras la ovulación, se inicia la fase secretora, el tejido endometrial se vuelve secretor (producción de glicógeno y mucosidad) bajo el influjo de la progesterona secretada por el cuerpo lúteo, producto de la ovulación. Durante la fase secretora media, el tejido adquiere un fenotipo decidualizado (necesario para la implantación embrionaria) que, en caso de no producirse embarazo, se desprende al final de esta fase, como respuesta al descenso de estrógenos y progesterona, dando lugar a la menstruación e inicio de un nuevo ciclo.

En el caso de los roedores (el modelo animal en estudio a lo largo de esta tesis), el ciclo hormonal se denomina ciclo estral (duración media de entre 4 y 5 días). De la misma forma que en los humanos, el endometrio, bajo la influencia de las hormonas, sufre cambios morfológicos y funcionales a lo largo de toda su duración. Así pueden diferenciarse cuatro fases, que son proestro, estro, metaestro y diestro.

2. Células madre

Las células madre se describen como aquellas células indiferenciadas que tienen el potencial de perpetuarse a sí mismas durante un largo periodo de tiempo, mediante división celular. Este mecanismo de división puede ser simétrico, en caso de estar destinado a perpetuar la línea celular, o asimétrico, si el objetivo es diferenciarse, previa formación de las células de transición o progenitoras, a células maduras con funciones específicas.

En función de su potencial de diferenciación pueden clasificarse, de mayor a menor potencial, en: toti-, pluri-, multi-, oligo- o uni-potentes. Mientras que, en relación con su origen, puede hablarse de células madre primordiales, embrionarias, fetales, perinatales, adultas, pluripotentes inducidas y cancerosas.

2.1. Nichos de células madre adultas

Ha sido ampliamente descrito que las células madre adultas (CMAs) se organizan en micro-ambientes específicos, denominados nichos celulares, que, entre otras funciones, regulan los procesos de diferenciación y división de estas células. La presencia de estos nichos ha sido descrita en distintas localizaciones del organismo, como la médula ósea, el intestino o incluso el endometrio.

En el caso de la médula ósea, el nicho contiene CMAs tanto de linaje hematopoyético como mesenquimal, además de otro tipo de células maduras como osteoblastos o adipocitos. Cabría incidir aquí sobre la presencia de CMAs que expresan tanto el receptor VEGFR2 (del inglés *vascular endothelial growth factor receptor 2*) como el antígeno CD133, y que normalmente se denominan células madre $CD133^+$ derivadas de la médula ósea ($CD133^+$BMDSCs, del inglés *bone marrow-derived stem cells*). Estas células pueden ser liberadas al torrente sanguíneo mediante mecanismos de quimioatracción desencadenados por moléculas como SDF1 (del inglés, *stromal cell-derived factor 1*), o VEGF (del inglés *vascular endothelial growth factor*). Es por ello que han sido propuestas como opción terapéutica para distintas patologías, incluyendo desórdenes ginecológicos.

Dada la alta capacidad regenerativa del endometrio, la presencia de un nicho endógeno de CMAs siempre había sido propuesta. Pero no fue hasta inicios del siglo XXI cuando aparecieron los primeros trabajos que describían la presencia de una cierta población celular con capacidad clonogénica y alta capacidad proliferativa. A partir de aquí, se han identificado CMAs endometriales, tanto en

el endometrio humano como en el murino, con fenotipo estromal, epitelial y endotelial, así como la contribución de la médula ósea a este nicho endometrial, que constituiría una fuente exógena de CMAs.

La relevancia de esta población celular en el endometrio es tal, que un mal funcionamiento o regulación de la misma, ha sido relacionado con el desarrollo de ciertas patologías endometriales. Un crecimiento y patrón de división descontrolado de estas CMAs parece estar relacionado con la aparición de hiperplasia y carcinoma endometrial, endometriosis, o incluso adenomiosis. De la misma forma, una falta o descenso de su funcionalidad, propiciaría la aparición de la atrofia endometrial (AE) o el síndrome de Asherman (SA).

3. Patologías endometriales que afectan a la infertilidad y tratamientos disponibles

El endometrio puede ver comprometida su funcionalidad, y por lo tanto la fertilidad de la mujer, por distintas patologías como el síndrome de Mayer-Rokitansky, leiomiomas, adenomiosis, anomalías de los conductos de Müller, endometriosis, carcinoma, etc., si bien las dos de interés aquí son la AE y el SA.

La AE se caracteriza por un insuficiente grosor endometrial (normalmente menor a 7 mm) mientras que el SA es una enfermedad rara que presenta adhesiones intrauterinas, que aparecen cuando el tejido endometrial es reemplazado por tejido fibrótico. En cualquier caso, el tejido endometrial se encuentra degenerado, lo que dificulta, o incluso llega a impedir por completo, la implantación embrionaria y, consecuentemente, el embarazo.

A lo largo de los años, se han ido proponiendo distintas aproximaciones terapéuticas para ambas patologías. Se ha propuesto desde el uso de hormonas exógenas, hasta el uso de células madre, que parece haber resultado una de las opciones más efectivas, si bien su coste, complejidad y carácter invasivo para las pacientes, ha propiciado la búsqueda de otras opciones.

Una de estas opciones es el uso del plasma rico en plaquetas (PRP), un derivado sanguíneo de fácil obtención, pues solo requiere una extracción de sangre periférica. El PRP contiene una concentración de plaquetas superior a la fisiológica, obtenida mediante un proceso de centrifugación que enriquece esta fracción de plasma en factores de crecimiento y otras moléculas pro-regenerativas. Algunos de estos factores, contenidos en el interior de los gránulos α de las plaquetas, son PDGF (del inglés *platelet-derived growth factor*), EGF (del inglés *epidermal growth factor*) o HGF (del inglés *hepatocyte growth factor*).

HIPÓTESIS Y OBJETIVOS

La presente tesis doctoral gira en torno a la hipótesis de que ciertas moléculas con acción paracrina podrían ser usadas para tratar el endometrio patológico (principalmente de pacientes con AE o SA), y por ende causante de infertilidad. Siguiendo esta línea, el objetivo central fue el estudio de distintas aproximaciones terapéuticas para el tratamiento de este tipo de endometrio patológico.

Además, se establecieron objetivos concretos para cada uno de los cuatro manuscritos que componen esta tesis doctoral:

- Determinar los mecanismos genéticos y paracrinos detrás de la regeneración endometrial en pacientes con AE y SA tras la inyección autóloga de células madre derivadas de la médula ósea o $CD133^{+}$BMDSCs.
- Estudiar el efecto del plasma humano, bien derivado de sangre adulta o de cordón umbilical, en la regeneración endometrial mediante ensayos *in vitro* y un modelo murino de daño endometrial.
- Revisar y analizar los principales hitos en la identificación y caracterización de las CMAs endometriales (murinas y humanas).
- Recopilar y estudiar las diferentes opciones terapéuticas disponibles para aquellas pacientes con AE y SA, con énfasis en aquellos trabajos realizados en modelos animales de daño endometrial.

RESUMEN Y DISCUSIÓN DE LOS RESULTADOS

1. Manuscrito I: regeneración endometrial tras la inyección de células madre autólogas derivadas de la médula ósea

Hasta el momento, una de las opciones terapéuticas que ha resultado más prometedora para tratar pacientes con AE y SA es la inyección de células madre autólogas derivadas de la médula ósea, que parecen actuar de forma paracrina sobre el tejido dañado. Por ello, el primer objetivo de esta tesis fue evaluar como la inyección de $CD133^{+}$BMDSCs, que había resultado ser efectiva previamente tanto en un modelo humano como en uno animal, estaba modificando el endometrio a nivel molecular. Con el fin de intentar entender cuáles son los mecanismos paracrinos a través de los cuales llevan a cabo su acción terapéutica.

A través de las muestras procedentes del estudio humano (comparadas de forma pareada antes y tres meses después de la aplicación del tratamiento), este trabajo reveló que las células madre estaban induciendo la sobre-expresión de los genes *JUN*, *SERPINE1* y la interleuquina (IL) *IL4*, a la vez que reducían la expresión de la ciclina *CCND1* y interleuquina *CXCL8*. De hecho, este último gen, *CXCL8*, mostró la tasa de regulación (TR = -26.546) más acentuada tras la aplicación del tratamiento. Esta reducción de la expresión de *CXCL8*, involucrado en la activación de los neutrófilos, fue reforzada por la también expresión reducida de la proteína neutrófilo elastasa, secretada por los macrófagos y neutrófilos durante la inflamación. De modo que esta baja expresión de *CXCL8* y de la proteína neutrófilo elastasa sería indicativa del silenciamiento del ambiente inmunológico en el endometrio de las mujeres tratadas, promoviendo y facilitando así el desencadenamiento de eventos pro-regenerativos. Tales eventos estarían propiciados por la reducción de los niveles de *CCND1*, que mantendría las células en fase de síntesis de ADN y de preparación de la mitosis, y la expresión al alza de *JUN* (involucrado en la proliferación del epitelio), *SERPINE1* (involucrado en remodelación arterial, cicatrización de heridas cardíacas y

migración de queratinocitos), e *IL4* (correlacionada con una mayor proliferación, diferenciación y procesos anti-apoptóticos en diferentes tipos celulares). Asimismo, se comprobó cómo estos tres genes regulados al alza (*SERPINE1*, *JUN* e *IL4*) estaban involucrados en varias rutas de transducción de señal (rutas Wnt, MAPK, TNF, Wnt, HIPPO, TGFβ, JAK-STAT y PI3K-Akt), directa o indirectamente relacionadas con la regeneración tisular.

En las muestras procedentes del modelo animal, este trabajo evidenció, por un lado, que los resultados humanos eran compatibles con los del animal. Los genes *JUN* y *SERPINE1* también estaban sobre-expresados, mientras que *CCND1* había disminuido su expresión. Por otro lado, se identificó que la expresión de ciertas proteínas era más elevada en aquellos cuernos uterinos que habían recibido el tratamiento, comparados con los que no. Estas proteínas fueron IL18 y HGF, ambas descritas en procesos angiogénicos, y MCP1 (del inglés *monocyte chemoattractant protein 1*) y MIP2 (del inglés *macrophage inflammatory protein 2*), involucradas en la proliferación celular. Además, los niveles de HGF se han correlacionado directamente con la expresión de *SERPINE1* y *JUN*, lo que supondría la correlación entre la validación de los genes y la detección de las proteínas.

En resumen, estos resultados aportaron pruebas adicionales sobre la eficacia y el mecanismo de acción paracrino de las $CD133^{+}$BMDSCs sobre el endometrio. Es por ello que se decidió decidir seguir investigando con otros abordajes terapéuticos que involucrasen moléculas con acción paracrina, tal y como se describe en el manuscrito II.

2. Manuscrito II: plasma para la regeneración endometrial

Este segundo trabajo estudió el efecto del plasma procedente de cordón umbilical, así como el plasma rico en plaquetas (PRP) procedente de sangre periférica adulta en la regeneración de un endometrio dañado. Este artículo

evidenció como este derivado sanguíneo era capaz de promover procesos celulares involucrados en la regeneración tisular, como la migración y la proliferación de las células endometriales, así como eventos regenerativos en un modelo animal con daño endometrial inducido. Asimismo, se mostró como estos efectos fueron mayores en el caso de que el plasma proviniese de sangre de cordón umbilical en vez de sangre adulta.

Los ensayos *in vitro* revelaron que el plasma promovía tanto la proliferación como la migración de las células endometriales estromales. Las tasas de cambio (TC) en la ratio de proliferación demostraron como el efecto del tratamiento era más acentuado si provenía del plasma de cordón umbilical que del PRP adulto, tanto en células derivadas de una línea primaria (TC cordón = 1.797 vs. TC adulto = 1.296) como de una línea de células madre (TC cordón = 1.361 vs. TC adulto = 1.221). De la misma forma, en el plasma adulto, se corroboró como la fracción rica en plaquetas, era más efectiva que la fracción pobre (TC rico = 1.255 vs. TC pobre = 0.913). También se validó que el efecto de la activación de plasma (con respecto a la condición sin tratamiento aplicado) era estadísticamente significativo, mientras que la procedencia del PRP (mujer fértil o con AE/SA) no lo era. En los ensayos de migración celular, se obtuvieron resultados similares. El tratamiento con plasma de cordón umbilical, tanto en células primarias como madre, parecía estar induciendo una mayor ratio de cierre de la herida que la condición en la que se había aplicado PRP adulto, y por descontado, en la que no se había administrado ningún tratamiento.

En relación a estos dos procesos celulares, proliferación y migración, se ha descrito que, en el endometrio, pueden ser potenciados por factores de crecimiento como el PDGF o HGF, ambos presentes en el plasma. De hecho, el análisis ELISA multiplex de las muestras de plasma, reveló que estos dos factores presentaban una mayor concentración en el plasma de cordón umbilical con respecto al adulto, lo que reforzaría los resultados de los ensayos *in vitro*.

En cuanto al modelo animal, este experimento incluyó una primera prueba de concepto de sólo tres ratones en la que se demostró que el tratamiento con PRP aumentaba la expresión del marcador de proliferación Ki67 y del marcador de función endometrial HOXA10. Dados estos indicios de que se estaban produciendo eventos pro-regenerativos, se aumentó la población de ratones (n = 6 / grupo) y el número de grupos (4) del modelo, incluyendo plasma de cordón umbilical tanto activado como sin activar, además de la condición sin tratamiento y la de PRP adulto, como tratamientos en estudio.

El análisis proteómico de todos los cuernos uterinos reflejó una distribución diferencial de las proteínas en función del tratamiento aplicado y de los cuernos uterinos analizados (dañados o no dañados). Los cambios más significativos fueron encontrados en el grupo tratado con cordón umbilical activado.

Por un lado, la comparación de los cuernos uterinos dañados frente a los no dañados (controles) reveló la sobre-expresión de cuatro proteínas: P85A (del inglés *phosphatidylinositol 3-kinase regulatory subunit α*), 2AAA (del inglés *Pr65 subunit of protein phosphatase 2A*), RHOA (del inglés *transforming protein RhoA*) y STAT5A (del inglés *signal transducer and activator of transcription 5A*). Esta última, STAT5A, es un mediador de la respuesta celular al factor SCF (del inglés *stem cell factor*), entre otros factores de crecimiento. De hecho, los análisis ELISA multiplex realizados habían demostrado que este factor, SCF, estaba más concentrado en el plasma de cordón, tanto activado como no, que en el PRP adulto. Además, P85A y 2AAA participan en la ruta PI3K-Akt, implicada en la proliferación, diferenciación y migración celular. De hecho, la proteína PI3K, principal componente de esta ruta metabólica, puede ser activada por ciertos factores de crecimiento como EGF y PDGF, este último también más concentrado en el plasma de cordón umbilical. Por tanto, podría hipotetizarse que la sobre-expresión de estas cuatro proteínas podría ser debida a esta mayor concentración de ciertos factores de crecimiento en el plasma de cordón umbilical.

Por otra parte, la comparación de los cuernos uterinos dañados de los cuatro grupos de tratamiento, reveló también unos cambios más significativos en aquellos tratados con cordón umbilical. Entre las proteínas significativamente sobre-expresadas, cabe destacar tres. En primer lugar, UBA3 (una unidad catalizadora del enzima E1 activador de NEDD8), necesaria para la progresión del ciclo celular y descrita como esencial para la nedilación mediada por la proteína NEDD8, involucrada en la función endometrial humana normal (proliferación y decidualización). En segundo lugar, RINI (también denominado RNH1, del inglés *ribonuclease inhibitor*), un regulador de la neo-vascularización, sub-expresado en las glándulas endometriales en muestras de endometriosis en comparación con las de endometrios sanos. Estos procesos biológicos (proliferación, decidualización y vascularización endometrial) en los que están involucrados UBA3 y RINI también están influenciados por componentes plaquetarios específicos, como PDGF, HGF, VEGF y EGF. Curiosamente, RINI también se encontró sobre-expresada cuando se compararon los cuernos dañados tratados con plasma adulto frente a los controles de ese mismo grupo. Por último, cabe destacar la proteína THY1/CD90 (glicoproteína Thy1, también denominada antígeno CD90), que además de participar en las interacciones célula-célula o célula-ligando, es un conocido marcador de CMAs estromales del endometrio.

En base a estos resultados, este trabajo permite concluir que un endometrio dañado podría ser regenerado mediante la administración tanto de PRP procedente de sangre adulta como de plasma de cordón umbilical, siendo este último más eficaz. Además del aparente mayor potencial regenerativo del plasma de cordón, esta fuente de plasma presenta otras ventajas. Por un lado, la disponibilidad de las muestras, puesto que durante muchos años, el plasma se ha considerado un producto de desecho tras el procesamiento de la sangre de cordón umbilical para, por ejemplo, obtener progenitores hematopoyéticos. Asimismo, la obtención de plasma a través de biobancos permitiría garantizar, o

incluso estandarizar, una determinada concentración de plaquetas, ya que el principal inconveniente del PRP autólogo es la variabilidad entre pacientes.

3. Manuscrito III: células madre adultas en el endometrio

Sea cual sea la aproximación terapéutica de elección, se ha hipotetizado que la regeneración endometrial podría surgir de la estimulación del nicho de CMAs presente en la capa basal del endometrio. Por ello, el manuscrito III de esta tesis se centró en el estudio y recopilación de los trabajos, tanto en modelos murinos como humanos, relativos a esta población de CMAs. Para ello se llevó a cabo una revisión bibliográfica sobre las CMAs endometriales en ambos tipos de modelos.

Tanto en el endometrio humano como en el murino se han identificado CMAs de diferentes tipos: epiteliales, estromales y endoteliales. Asimismo, se ha descrito la presencia de una población exógena de CMAs derivadas de la médula ósea, que estaría contribuyendo al nicho endógeno endometrial. Sin embargo, no está del todo claro el mecanismo mediante el cual estas células exógenas migran hasta este tejido. Se ha hipotetizado tanto que lo hagan de manera habitual, en cada ciclo menstrual, como que lo hagan solo si hay un daño en el endometrio.

En el caso del endometrio murino, una de las técnicas más empleadas para identificar esta población celular está basada en el patrón de división lenta de las CMAs en comparación con aquellas diferenciadas, característica que, si bien no define su fenotipo, permite identificar CMAs indirectamente. Para ello se ha descrito tanto el uso de la bromodesoxiuridina, un análogo de la timidina, como el de la proteína fluorescente verde ligada a la histona 2 B.

Otra aproximación utilizada, tanto con células humanas como murinas, es la técnica de la *side population* (basado en el uso del colorante vital Hoescht 33342, que es más rápidamente expulsado por las CMAs, dada la mayor expresión de transportadores ABC en las mismas). También se han llevado a cabo ensayos funcionales basados en el potencial de diferenciación a múltiples linajes, la

capacidad de clonogenicidad (formación de colonias), y el potencial de auto-renovación característicos de las CMAs.

Por último, se han descrito marcadores específicos de CMAs más allá de los clásicos grupos de diferenciación (CD, del inglés *cluster of differentiation*), utilizados para diferenciar el fenotipo mesenquimal (CD44, CD90, CD105), endotelial (CD31, CD34) o hematopoyético (CD34, CD45), que permitirían aislar esta población celular mediante, por ejemplo, citometría de flujo. En ratones, se han utilizado marcadores como OCT4 (del inglés *octamer-binding transcription factor 4*), el proto-oncogén c-Kit o la proteína SALL4 (del inglés *sal-like protein 4*) para identificar las CMAs epiteliales. Mientras que en el endometrio humano, se han propuesto los marcadores CD146/PDGFRb (del inglés *platelet-derived growth factor receptor β*), SUSD2 (del inglés *sushi domain-containing-2*), LGR5 (del inglés *leucine-rich repeat-containing G-protein-coupled receptor 5*) y NTPDase2 (del inglés *ectonucleoside triphosphate diphosphohydrolase-2*) para aislar las CMAs endometriales estromales, y SSEA-1 (del inglés *stage-specific embryonic antigen-1*), N-cadherina y NTPDase2, para identificar las de fenotipo epitelial. Sin embargo, todavía no hay consenso acerca de qué marcadores son los más adecuados.

Todas estas aproximaciones de identificación y aislamiento han contribuido al uso de las CMAs como opción terapéutica para aquellas mujeres con AE o SA. Aun así, el uso de la terapia celular ha ido evolucionando hacia estrategias basadas en su acción paracrina y en otras técnicas emergentes, como aquellas derivadas de la bioingeniería, tal y como aborda el manuscrito IV.

4. Manuscrito IV: terapias disponibles para mujeres con atrofia endometrial y síndrome de Asherman

Finalmente, dada la mencionada falta de una terapia definitiva para las pacientes con AE y SA, este último manuscrito supuso la realización de una revisión bibliográfica actualizada sobre los enfoques terapéuticos disponibles para este

tipo de pacientes. Se revisaron tanto aquellas terapias que llevan incorporadas bastante tiempo en la rutina clínica como aquellas que, si bien parecen estar resultando bastante prometedoras, aún se encuentran en investigación.

Entre las terapias que vienen usándose desde hace décadas, y que suelen ser la primera línea de acción con estas pacientes, se encuentran el uso de hormonas exógenas (estrógenos, agonistas de la GnRH (del inglés *gonadotropin-releasing hormone*), hormona hCG (del inglés *human chorionic gonadotropin*)), tamoxifeno, o aspirina a bajas dosis para mujeres con AE, mientras que la adhesiolisis histeroscópica y también la aspirina, se han usado en aquellas con SA. Si bien, la falta de eficacia de estos tratamientos en todas las pacientes sumado a la escasez de ensayos clínicos aleatorios en humanos realizados con estas denominadas terapias ''clásicas'', ha incentivado la búsqueda de terapias alternativas.

Una de ellas es el tratamiento con células madre. Se han descrito terapias con CMAs derivadas de diversos tejidos como el amnios, el cordón umbilical, el tejido adiposo, la sangre menstrual o la médula ósea, siendo probablemente esta última la fuente de CMAs más estudiada. La acción terapéutica de estas células ha sido estudiada tanto en solitario como en combinación con factores pro-regenerativos como VEGF o SDF1. El empleo del factor G-CSF, que estimula la producción de células madre en la médula ósea, también ha sido estudiado.

Muchos de estos trabajos con CMAs han permitido esclarecer que estas células ejercen una acción paracrina en lugar de promover la regeneración del endometrio en base a su diferenciación y repoblación del tejido dañado. Por ello, algunos de los trabajos más recientes en el estudio de la regeneración endometrial han valorado el potencial terapéutico de derivados del secretoma de las CMAs, como son las vesículas extracelulares. Esta hipótesis de la acción paracrina de las células madre ha promovido otro tipo de terapias como son la aplicación dirigida de uno o varios factores de crecimiento o el uso del PRP.

Finalmente, el campo de la bioingeniería ha irrumpido en el manejo y estudio del endometrio patológico. En los últimos años se ha explorado el uso de biomoldes de colágeno o incluso derivados de matriz extracelular uterina descelularizada, tanto en solitario como funcionando de soporte y potenciador de la acción terapéutica de CMAs o factores de crecimiento. También se han empezado a usar hidrogeles, tanto como terapia única como en combinación con otras biomoléculas. Por último, y de forma más reciente, los últimos avances de la microfluídica y del cultivo tridimensional han demostrado su utilidad en la medicina reproductiva tanto como modelos de estudio *in vitro* como plataformas de cribado de fármacos. Esta transición del cultivo celular tradicional hacia uno tridimensional podría contribuir a la reducción del número de animales usados en experimentación, si bien aún queda camino por recorrer en este ámbito.

Paralelamente, esta revisión reveló una diversidad de métodos y enfoques en la inducción del daño endometrial (etanol, ácido tricloroacético, agujas, o electrocoagulación, entre otros métodos) y los parámetros seleccionados (resultados de fertilidad y marcadores de características morfológicas endometriales, funcionalidad y regeneración endometrial) para evaluar la eficacia de los tratamientos probados. Por ello, sigue resultando esencial establecer un modelo animal de daño endometrial lo más estandarizado posible.

En conclusión, los modelos animales e *in vitro* son esenciales como mecanismo de investigación previo a la traslación clínica, aunque los modelos actuales en la búsqueda del mejor tratamiento para las pacientes con AE y SA necesitan mejorar y estandarizarse. Teniendo en cuenta los últimos avances, parece que el campo de la bioingeniería está tomando fuerza, sola o en combinación con las aproximaciones terapéuticas ya existentes.

CONCLUSIONES

Tras discutir los cuatro manuscritos que componen la presente tesis doctoral, puede concluirse que:

I. Las células $CD133^{+}$BMDSCs promueven la regeneración del endometrio mediante la creación de un escenario inmunomodulador, probablemente dirigido por la baja expresión del gen *CXCL8*, que da paso a procesos pro-regenerativos, desencadenados por la regulación al alza de los genes *JUN*, *SERPINE1* e *IL4*, en el endometrio humano, y de las proteínas HGF, MCP1, MIP2 e IL18, en el tejido murino.

II. El PRP es capaz de promover procesos *in vitro* como la proliferación y la migración celular, tanto de células estromales endometriales primarias humanas como procedentes de líneas de células madre endometriales. Asimismo, este plasma promueve la proliferación celular, la expresión de marcadores de la función endometrial y otras proteínas implicadas en las rutas de señalización relacionadas con la regeneración tisular en un modelo de ratón con daño endometrial inducido. En ambos casos estos efectos pro-regenerativos son más acentuados si la fuente de plasma es la sangre del cordón umbilical en lugar de la sangre periférica adulta.

III. Es necesario seguir investigando en la caracterización y el aislamiento de las CMAs endometriales, ya que no hay consenso sobre cuáles son los mejores marcadores celulares específicos y, por lo tanto, normalmente se identifican de forma indirecta (demostración de las características inherentes a las células madre). Además, la contribución de la médula ósea al nicho endometrial y el papel específico del mismo en las patologías endometriales, como la AE y el SA, también precisan de más investigación.

IV. En los últimos años distintas estrategias terapéuticas novedosas como son aquellas derivadas del campo de la bioingeniería están comenzando a emerger con fuerza en el manejo del endometrio patológico, no sólo como

tratamientos por sí mismas sino también como potenciadores de las terapias ya existentes. Las nuevas plataformas *in vitro* (organoides, microfluídica) también están resultando prometedoras para estudiar en profundidad enfermedades endometriales como la AE y el SA.

APÉNDICE: EVALUACIÓN DEL IMPACTO POTENCIAL DEL VIRUS SARS-COV-2 EN EL ENDOMETRIO

Dada la incidencia de la pandemia global ocasionada por el virus SARS-CoV-2, causante de la COVID-19, esta tesis se complementó con el estudio de la posible afectación del virus sobre el endometrio humano. Para ello se analizaron biopsias endometriales procedentes de 15 mujeres en edad reproductiva (en distintas fases del ciclo menstrual) con COVID-19, en las que se había detectado el virus en muestras nasofaríngeas, mediante la reacción en cadena de la polimerasa (PCR), y que estaban ingresadas en el Hospital Universitari i Politècnic La Fe.

En todas las muestras se analizó tanto la presencia del material genético del virus, usando como diana la secuencia de la proteína de la nucleocápside viral, así como el receptor *ACE2* (del inglés *angiotensin-converting enzyme 2*), descrito como clave en el proceso de entrada del virus a las células humanas.

Los resultados de este trabajo demostraron que el virus no estaba presente en ninguna de las biopsias endometriales. Asimismo, el análisis del receptor *ACE2* evidenció la presencia del mismo en 10 de las 15 muestras estudiadas. Sin embargo, al comparar los niveles de expresión frente a los de un repositorio de muestras nasofaríngeas que habían demostrado estar infectadas con el virus, estos eran significativamente menores.

Por ello, aunque se trate de un estudio preliminar, estos resultados parecen estar indicando que el virus no penetra en el endometrio de pacientes infectadas, quizás, debido a la baja expresión del gen *ACE2* en el tejido endometrial.

I. INTRODUCTION

1. THE FEMALE REPRODUCTIVE SYSTEM

1.1. Anatomy of the female reproductive system

The female reproductive system is composed of external and internal reproductive organs. The external organs are the vulva, vagina, perineum, and urethra, while the internals ones include the ovaries, fallopian tubes, and uterus. This thesis dissertation focuses on the internal ones (Figure 1).

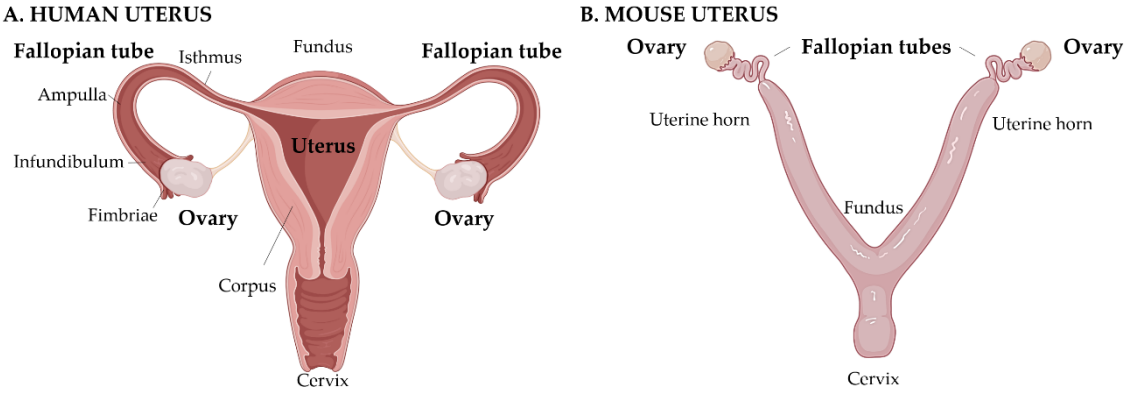


Figure 1. Internal organs of the female reproductive system. *The ovaries and the uterus, interconnected by the fallopian tubes or oviducts, are the two main internal female reproductive organs in humans (A) and mice (B) alike. Image created with www.BioRender.com.*

The ovaries, also known as the female gonads, host the oocytes and plus produce and secrete female sex hormones (mainly estrogen and progesterone, in addition to inhibin or relaxin, among others). The fallopian tubes (or oviducts) transport the ovulated oocyte(s) from the ovaries to the uterine cavity. During mating, sperm travel up to the ampulla, the middle section of the fallopian tubes, to fertilize the oocyte(s), and similarly the zygote would be transported to the uterine cavity. Lastly, the uterus accommodates the embryo and, ultimately, fetal development until birth (Graziottin and Gambini, 2015). The anatomy of the human internal female reproductive organs is illustrated in Figure 1A.

The human uterus, intrapelvically located between the bladder and the rectum, is a hollow, muscular, and inverted pear-shaped organ with thick walls. This organ usually measures 8 cm long, 5 cm wide, and 2.5 cm deep and consists of

three well-differentiated parts: the fundus, corpus, and cervix. The fundus is located above the fallopian tube entries and constitutes two-thirds of the whole uterus in conjunction with the corpus. The lower part, the cervix, communicates with the vagina and is separated from the corpus by a short isthmus (Ramirez-Gonzalez *et al.*, 2016).

Uterine anatomy differs slightly between humans and other mammalians. In particular, mice have a bicornuate uterus consisting of two lateral horns that join distally into a single body or corpus (Figure 1B), instead of a single triangular-shaped cavity like humans (Figure 1A; Rendi *et al.*, 2012). Nevertheless, in both species, the corpus uterine wall consists of three distinct layers (Figure 2): perimetrium, the external, serous layer; myometrium, the middle muscular and most extensive layer; and endometrium, the internal, mucous layer. 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.

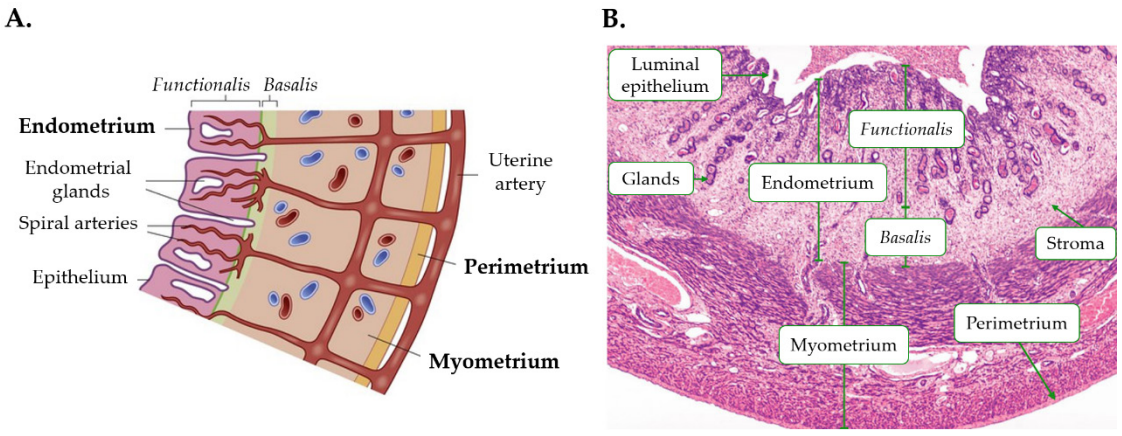


Figure 2. Uterine layers. *Diagram (A) and histological (hematoxylin and eosin staining) view (B) of the different compartments of the human uterine wall. The three uterine layers (myometrium, perimetrium, and endometrium) and the different compartments of the endometrium (stratum functionalis and basalis) are indicated. Images adapted with permission from www.basicmedicalkey.com.*

To fully comprehend infertility-causing endometrial pathologies such as endometrial atrophy and Asherman's Syndrome, it is essential to deepen understanding of the human endometrium. Furthermore, murine endometrium

characteristics also need to be addressed to better interpret results from murine models of uterine damage.

1.2. The human endometrium

The human endometrium is a dynamic, remodeling, and hormone-responsive tissue that, during a woman's reproductive life, goes through more than 400 cycles of growth, differentiation, shedding, and regeneration (Gargett *et al.*, 2009; Lessey, 2013).

1.2.1. The endometrium: cellular composition and organization

The endometrium is composed of epithelial, stromal, resident immune cells, and a vascular network. These four components are organized into two main compartments: *stratum basalis*, the basal layer adjacent to the myometrium, and *stratum functionalis*, the superficial layer adjacent to the endometrial cavity (Simón *et al.*, 2009; Figure 2). The *stratum basalis* remains relatively constant throughout the menstrual cycle, and contains adult stem cells (ASCs; further explained in Section 2.3), thought to be involved in the cyclic regeneration of the *functionalis* (Cervelló and Simón, 2009). On the other hand, the *stratum functionalis* thickens and becomes vascularized during the menstrual cycle, and is shed during menstruation if pregnancy does not occur (Cunningham *et al.*, 2015).

The endometrial epithelium consists of a monolayer of columnar polarized cells covering the uterine cavity and is organized in luminal and glandular components which undergo cyclical changes throughout the menstrual cycle (Simón *et al.*, 2009). The primary function of this arrangement is to regulate and allow embryo implantation. Meanwhile, the endometrial stroma mainly consists of fibroblast cells and extracellular matrix (ECM). Fibroblasts are implicated in remodeling of the ECM throughout the menstrual cycle, and play a pivotal role during decidualization (Simón *et al.*, 2009; Cunningham *et al.*, 2015).

The endometrial immune cell population, located in the stroma, is composed of natural killer cells (which predominate during the proliferative phase) macrophages, and T lymphocytes (which are involved in immunosuppression events and as such are increased after ovulation; Simón *et al.*, 2009).

Finally, the intrauterine vascular network initiates in the myometrium and leads to the endometrium, where vessels differentiate into basal spiral arteries that emerge and maintain the *basalis*, then ramify through the *stratum functionalis* (Figure 2A). This network and the regularly-occurring intrinsic angiogenesis (generation of new blood vessels from the existing ones) are vital throughout the whole menstrual cycle (Adair and Montani, 2010). Angiogenesis is essential for regenerating the vascular bed during menstruation, when the tissue grows and the arteries elongate during the proliferative phase and when the spiral arteries sprout in the secretory phase. Likewise, if fertilization occurs, angiogenesis takes place during embryo implantation and placentation (Cunningham *et al.*, 2015).

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 preceding pregnancy. The tissue requires a correct and functional morphology, with a minimum thickness of 6 mm, for the embryo to attach (Senturk and Erel, 2008; Galliano *et al.*, 2015). Anomalies regarding morphology and/or endometrial thickness can lead to endometrial disorders causing infertility (explained in depth in Section 3).

The human menstrual cycle, which lasts an average of 28 days, is regulated by the cyclic action of two main ovarian steroid hormones: estradiol (E_2) and progesterone (P_4; Baerwald *et al.*, 2012). Briefly, E_2 regulates the proliferation of the endometrial cells while P_4 counteracts this proliferative event by reducing the levels of estrogen receptors, inactivating the E_2, and interfering with the transcription of other estrogens. Accordingly, the menstrual cycle is divided into

proliferative and secretory phases, followed by menstruation itself, which coincide with the follicular and luteal phases of the ovarian cycle (Figure 3).

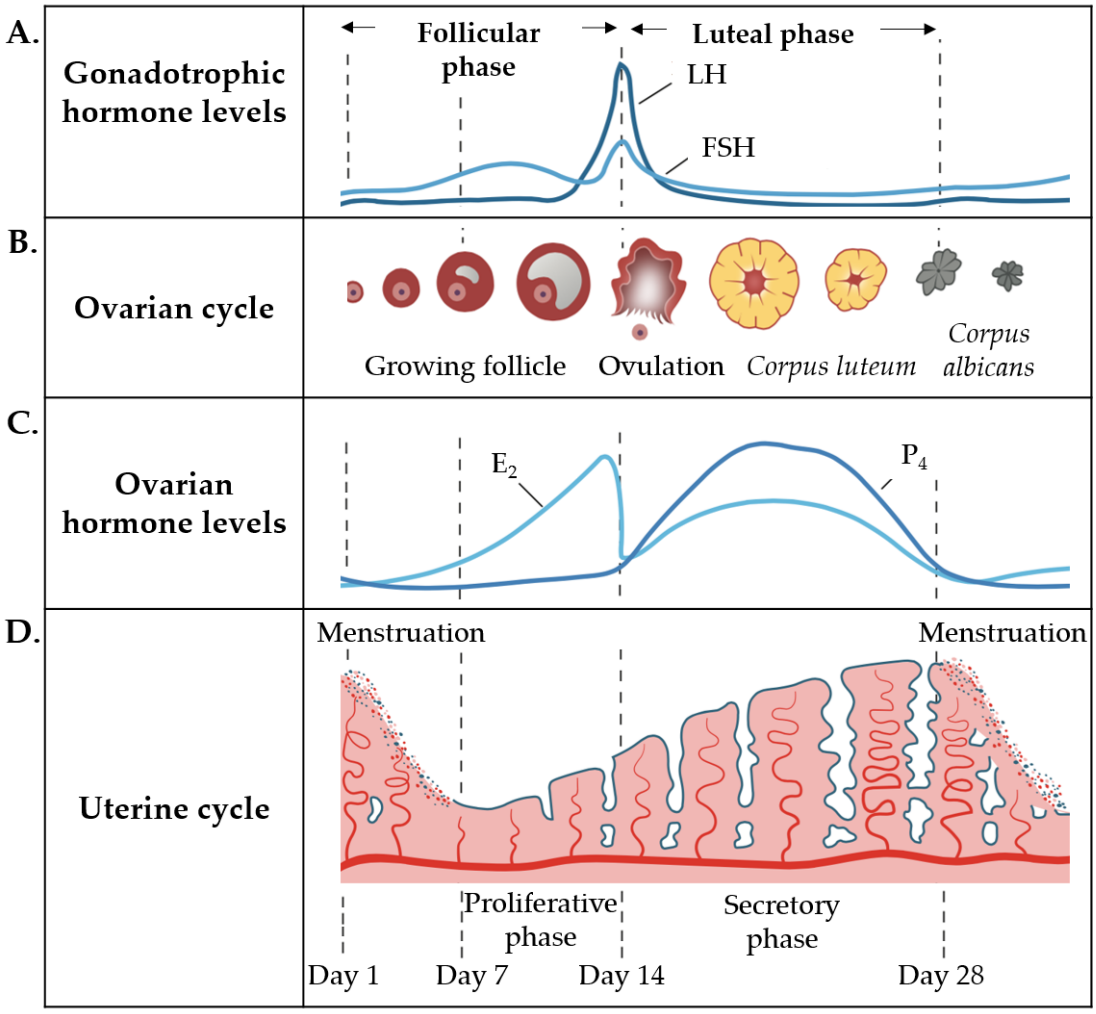


Figure 3. Dynamics of the menstrual cycle relating to hormone level, follicular development, and endometrial growth. *Schematic representation of the oscillations of (A) gonadotropic hormones (LH and FSH) and (C) ovarian hormones (E_2 and P_4) during the menstrual cycle. The (B) ovarian cycle (follicular and luteal phases) and the (D) endometrial phases (menstruation, proliferative, secretory phase, and menstruation) of the menstrual cycle are also shown. E_2: estradiol; FSH: follicle-stimulating hormone; LH: luteinizing hormone; P_4: progesterone. Image adapted with permission from www.viosfertility.com.*

During the follicular phase (or maturation of the ovarian follicles), the hypothalamus secretes gonadotropin-releasing hormone (GnRH). This hormone induces the secretion of the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), from the pituitary gland, which ultimately triggers follicle maturation and ovulation respectively (Figure 3A-B). The E_2 and P_4 produced in the ovaries as a result of these respective processes interact with

each other, affecting the ovarian cycle and endometrial growth, and reach minimal levels by menstruation (Figure 3C-D). Notably, the luteal phase is marked by a decrease in LH and FSH levels after ovulation (Figure 3A) and a progesterone-secreting *corpus luteum* (Figure 3B-C) responsible for preparing the endometrium for embryo implantation. Remarkably, the *corpus luteum* can produce P_4 until the fetus can adequate levels to sustain the pregnancy, or degenerate into a *corpus albican*s if pregnancy does not occur (Figure 3B; Hawkins and Matzuk, 2008; Lessey and Young, 2019).

In the absence of pregnancy, the P4 withdrawal signals the need to shed the decidualized uterine lining during menstruation and begin a new menstrual cycle. The first day of the menstruation is considered day one, and it generally lasts from four to six days (Figure 3D; Lessey and Young, 2019).

At the tissue level, the rise in E_2 with the wave of follicle development initiates the proliferative phase, which restores the epithelial surface of the endometrium (that is to say, the luminal epithelial cells develop microvilli) and the revascularization of the tissue after menstruation (Figure 4A-B). During the early proliferative phase, the endometrial thickness is usually less than 2 mm, and by day five of the menstrual cycle, the epithelial surface has usually been re-established (Hawkins and Matzuk, 2008). At this time, mitotic activity is noticeable in both the epithelium and stroma. The ASC population in the *stratum basalis* (see Section 2.3 for more details) presumably contributes to this tissue regeneration process. The glands during this early phase are narrow, straight, and tubular, lined with the *stratum basalis* (Figure 4B). During the late proliferative phase, the endometrium thickens due to glandular hyperplasia and increased stromal ECM. By the end of this phase, the glandular epithelial cells increase in size and become pseudostratified as ovulation approaches. In addition, gland density increases and they become more tortuous closer to the endometrium (Figure 4B; Lessey and Young, 2019).

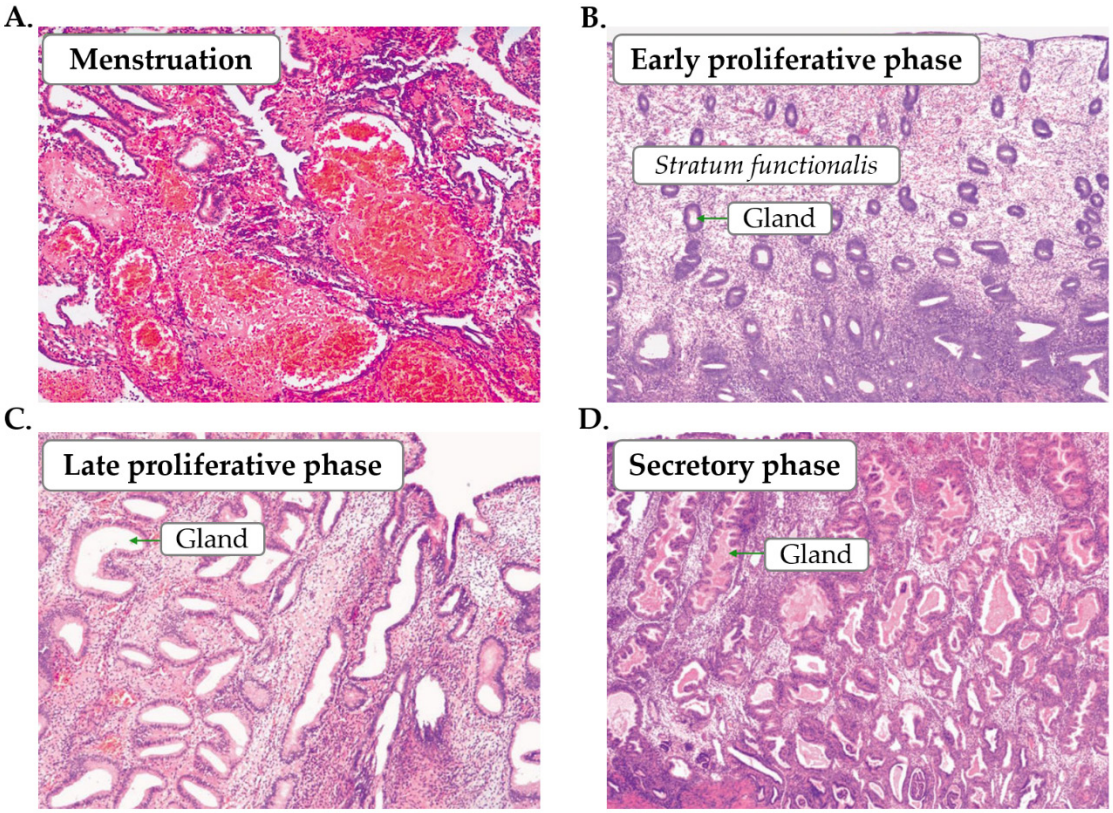


Figure 4. Photomicrographs of the endometrial tissue in menstrual the different cycle phases. *Hematoxylin and eosin staining demonstrates that after menstruation (A), the endometrium starts to grow (B), and epithelial glands increase in size along during the proliferative phase (C). Glands become secretory (D) at the end of the cycle. Image adapted with permission from www.basicmedicalkey.com.*

Ovulation marks the transition to the secretory phase. With increasing P_4 and decreasing E_2 during the early secretory phase, the secretion of glycogen and mucus is also increased. Mitotic activity of the endometrial cells only occurs in the first three days after ovulation, and then stromal edema contributes to thickening the endometrium. In the mid secretory phase, the spiral arteries become increasingly coiled and longer, contributing to decidualization (which in turn induces morphological change of the fibroblasts) and embryo receptivity during the window of implantation. The endometrial glands are tortuous in the mid and late secretory phases, and their secretory activity reaches a maximum around six days after ovulation (Figure 4D). In the absence of pregnancy, and the accompanying drop in E_2 and P_4, the arteries vasoconstrict, leading to involution of the endometrium during the late secretory phase. The cycle then repeats with

the beginning of another menstruation (Hawkins and Matzuk, 2008; Lessey and Young, 2019).

In this context, endometrial thickness is crucial. This parameter, usually measured by transvaginal sonography, is defined as the minimal distance between the echogenic interfaces at the junction of myometrium and endometrium and varies along the cycle (Galliano *et al.*, 2015). This distance usually ranges between 1-4 mm during menstruation, and reaches 8-12 mm by the end of the proliferative phase. In the late proliferative phase, the endometrium develops a trilaminar appearance (Figure 5A) with an outer surrounding echogenic *basalis* and hypoechoic and darker inner *functionalis* layer, separated by a thin echogenic median layer representing the lumen. This multilayered appearance usually disappears 48 hours after ovulation.

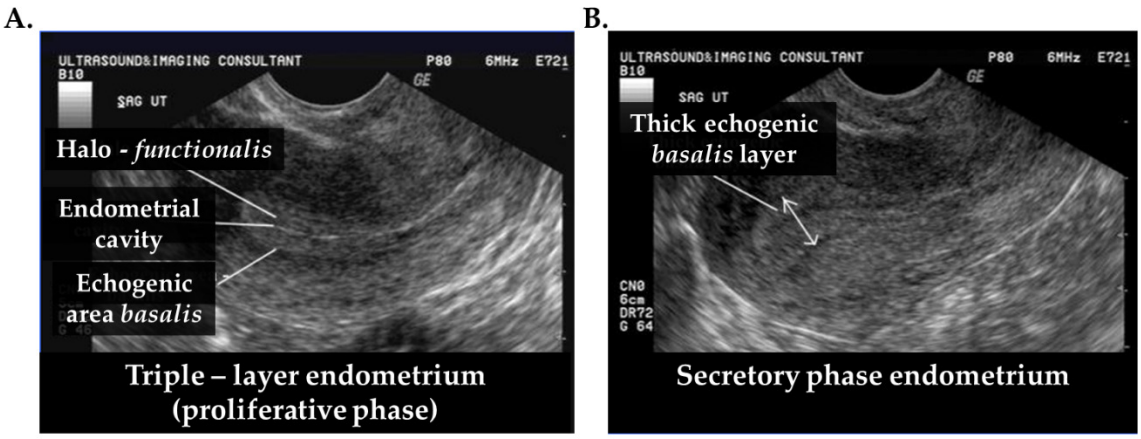


Figure 5. Ultrasound images of a normal human endometrium. *A. Trilaminar appearance of the endometrium during the proliferative phase with and outer surrounding echogenic basalis layer and hypoechoic and darker inner functional layer, separated by a thin echogenic median layer arising from the endometrial cavity. B. During the secretory phase the endometrium appears even thicker and more echogenic. Image adapted with permission from www.fetalultrasound.com.*

Finally, in the secretory phase, the endometrium becomes even thicker (up to 16 mm) and more echogenic (Figure 4B) due to stromal edema and endometrial glands distended with mucus and glycogen (Nalaboff *et al.*, 2001; Senturk and Erel, 2008). Several studies indicate that embryo implantation cannot be achieved

if the endometrial thickness is below a specific critical cut-off limit, generally 6 mm, however, there is controversy about the exact value (Senturk and Erel, 2008).

1.3. The mouse endometrium

Reproduction in the mature female mouse involves dependent hormonal and neural events (anterior pituitary, placental, and gonadal hormones), ensuring successful mating. This hormonal cycle (termed the estrous cycle) lasts from four to five days and occurs cyclically during the reproductive life span unless interrupted by pregnancy. In contrast to humans, the endometrial *stratum functionalis* is absorbed rather than shed at the end of the cycle (Bronson *et al.*, 1966; Rendi *et al.*, 2012).

1.3.1. Physiology, structure, and composition

The murine endometrium consists of an epithelial (luminal and glandular) and stromal compartment. The luminal epithelium is composed of simple columnar cells coating the inner part of the endometrium, while the glandular compartment extends into branched tubular glands surrounded by the endometrial stroma. The endometrial stroma consists of loosely arranged reticular connective tissue with many small polyhedral cells. Like humans, the murine endometrium is arranged in elevated transverse folds supplied by blood vessels and immune cells (Rendi *et al.*, 2012; Cousins *et al.*, 2021).

A fundamental difference with the human endometrium is that the mouse tissue requires a physical stimulus (an embryo) to undergo decidualization; it does not occur spontaneously (Cousins *et al.*, 2021). Nevertheless, both species share identical markers of endometrial receptivity such as homeobox protein Hox-A10 (HOXA10; a regulator of endometrial proliferation, differentiation, and decidualization), leukemia inhibitory factor (LIF; implicated in the implantation process), and integrin β3 (involved in cell adhesion; Chen *et al.*, 2016a).

1.3.2. Hormonal regulation: the estrous cycle

The mouse endometrium changes throughout the four stages of the estrous cycle (proestrus, estrus, metaestrus, and diestrus), even though the differences are not as apparent as in the human menstrual cycle (Figure 6). Similar to other rodents, the murine estrous cycle stage is usually evaluated by studying vaginal smears.

In proestrus (analogous to the human proliferative menstrual phase), the higher concentration of circulating E_2 (Figure 6A) induces leukocytes to infiltrate the endometrial stroma (Figure 6B), nucleated epithelial cells to proliferate and grow, and the uterus to become distended and hyperemic (Figure 6C). As occurs in humans, the endometrium becomes more vascular, and there is hypertrophy of the glands. These events lead to spikes in FSH and LH release that immediately decrease an E_2, trigger ovulation and the transition to estrus (Rendi *et al.*, 2012; Ajayi and Akhigbe, 2020).

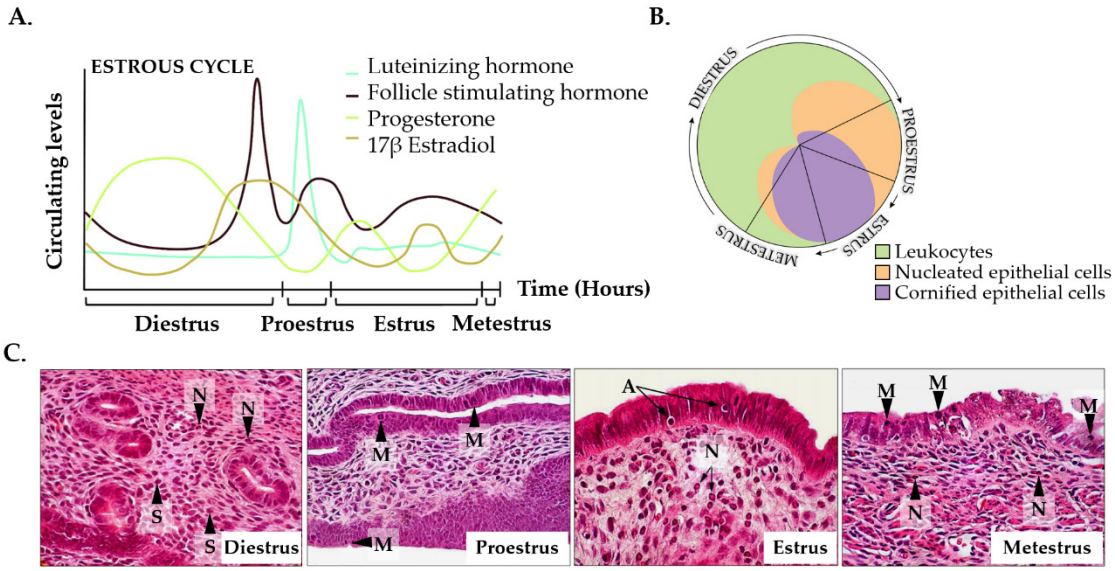


Figure 6. Estrous cycle in mice. *A. Graph showing the relative circulating levels of the four main hormones regulating the estrous cycle: luteinizing hormone, follicle-stimulating hormone, progesterone, and 17β estradiol. B. Diagram representing the different cell types and their relative proportion during the different estrous cycle stages. C. Histological images of the endometrial tissue in the different estrous cycle stages. A: apoptosis; M: mitotic activity; N: nucleated epithelial cells; P: epithelial cells; S: fibroblasts. Images adapted with permission (Hawkins and Matzuk, 2008; Wallace et al., 2018).*

During estrus, columnar epithelial cells and blood vessels reach their maximum development (Figure 6C). This phase is characterized by abundant cornified epithelial cells and scarcity of leukocytes (Figure 6B; Rendi *et al.*, 2012).

In metestrus (analogous to the human early secretory phase), levels of P_4 begin increasing and the uterine wall decreases in size and vascularity (Figure 6C), leukocytes are prominent in the stroma (Figure 6B), and epithelium continues vacuolar degeneration and replacement (Rendi *et al.*, 2012).

Finally, during diestrus (analogous to the human late secretory phase), while P_4 levels remain elevated, the uterine wall collapses, epithelial cells shrink, leukocyte infiltrates and regenerative changes are observed in the endometrium (Figure 6B-C; Wood *et al.*, 2007; Rendi *et al.*, 2012; Cousins *et al.*, 2021).

2. STEM CELLS

2.1. Stem cells: definition and classification

Stem cells (SCs) are defined as undifferentiated cells with the potential to perpetuate through self-renewal for a long period of time. They can divide into more SCs (by symmetric division) or, under certain physiologic or experimental conditions, give rise to specifically differentiated (mature) cells with particular functions (by asymmetric division or differentiation process; Alberts *et al.*, 2002).

Stem cells can be classified into five groups according to their degree of potency (Bindu *et al.*, 2011; Figure 7): totipotent SCs, which can differentiate into any tissue type (including extraembryonic tissues); pluripotent SCs, which have the ability to differentiate into cells from the three embryonic germ lineages (ectoderm, mesoderm, and endoderm) but not from the extraembryonic tissues (placenta, umbilical cord); multipotent SCs, which can only differentiate into cells from their specific embryonic germ lineage (e.g., hematopoietic SCs); oligopotent SCs, which can only differentiate into a few cell types from the specific embryonic

germ lineage they derive from (e.g., myeloid SCs); and, unipotent SCs, that can only differentiate into one cell type (e.g., epidermal SCs).

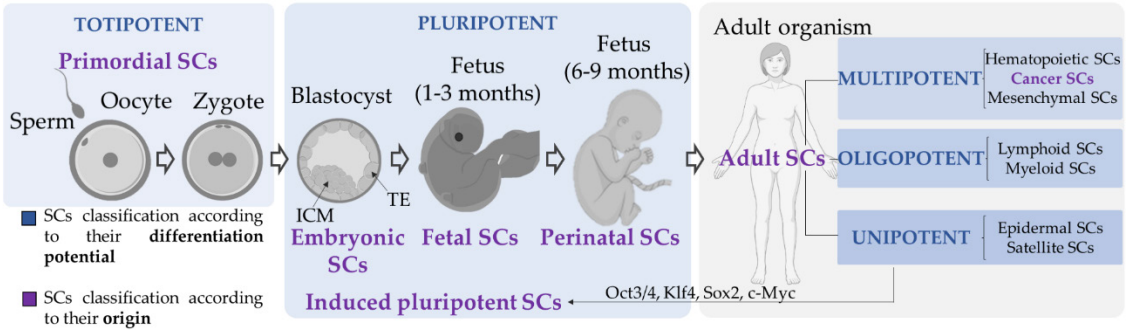


Figure 7. Schematic representation of the different types of human stem cells based on their differentiating potential and origin. *ICM: inner cell mass; KLF4: kruppel-like factor 4; c-MYC: MYC proto-oncogene; OCT3/4: octamer-binding transcription factor 3/4; SCs: stem cells; SOX2: SRY-Box transcription factor 2; TE: trophectoderm.*

According to their origin, SCs can be divided into the following seven groups (Ilic and Polak, 2011; Figure 7): primordial SCs, which differentiate into the gametes (spermatozoa or oocytes); embryonic SCs, pluripotent SCs derived from the blastocyst-stage embryo's inner cell mass (ICM) prior to implantation; fetal SCs, isolated from first-trimester fetuses; perinatal SCs, which can be obtained from the umbilical cord, placenta, or amniotic fluid; somatic or ASCs, which are multipotent SCs that have been identified in different organs and tissues (e.g., intestine or corneal epithelium) located in specific niches; induced pluripotent SCs, which are scientifically generated by modifying or reprogramming a differentiated cell to convert it back into a pluripotent cell with similar characteristics to the embryonic SCs; and cancer SCs, a small population of cells with stem-like properties, such as the capacity to differentiate into the diverse cell types that constitute the whole tumor (Ilic and Polak, 2011). More recently, another SC type named very small embryonic-like stem cells (VSELs), composed of early-development pluripotent stem cells persisting in adult tissues, has been included in this classification (Ratajczak *et al.*, 2017). These VSELs have been described in different adult tissues, including the reproductive ones (Virant-Klun *et al.*, 2019; Singh and Bhartiya, 2021).

2.2. Adult stem cells and their niches

Adult SCs have been widely described to be located in a particular protective and dynamic microenvironment, called niche (Scadden, 2006). These niches allow ASCs to maintain their SC characteristics and the specific tissue's renovation capacity. Schofield first postulated the concept of niches in 1978, with evidence that SCs from the bone marrow were located within a particular niche and if they left, they could become colony-forming unit cells but lose their immortality (Schofield, 1978; Figure 8).

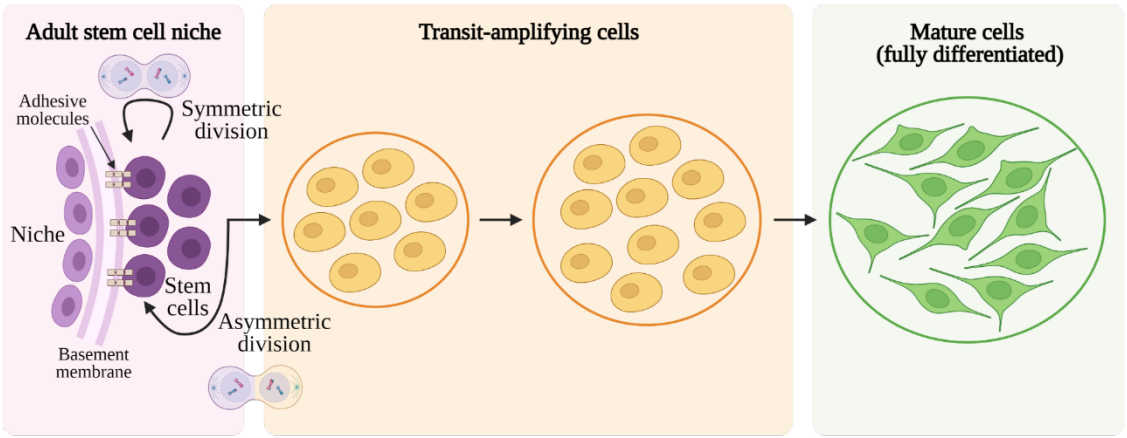


Figure 8. Adult stem cells and their niche. *Schematic representation of the structure and composition of an adult stem cell niche, including the basement membrane, stem cells, other non-stem cells composing the niche, and molecules (adhesive molecules, among others) contributing to the niche behavior. It is shown how the stem cells can undergo symmetric (to maintain the stem cell niche population) or asymmetric divisions (to produce transit-amplifying cells that will differentiate into mature cells in response to the tissue demands). Created with www.BioRender.com.*

In general, niche cells are embedded in an ECM that helps structure their microenvironment and has adhesive molecules that anchor the ASCs to the surrounding basement membrane (Figure 8). Other components such as soluble protein mediators of cellular response or metabolic products (both with paracrine activity) would also regulate the activity of the SCs. Finally, the interactions among all these components may also be critical for SCs' regulation (Spradling *et al.*, 2001).

The niche cells regulate the differentiation and division processes of the ASCs. By supporting SC activity (symmetric division) as well as tissue-specific needs for certain differentiated cells (asymmetric division), the niche remarkably maintains tissue homeostasis (Pardo-Saganta *et al.*, 2015; Chacón-Martínez *et al.*, 2018; Figure 8). Interestingly, ASCs in quiescent and active (dividing) states, can coexist in the same niche (Fuchs *et al.*, 2004). The niche orchestrates this regulation based on the generation of cell polarity and the orientation of the mitotic spindle (the microtubule-based bipolar structure that segregates the chromosomes during mitosis). Since the interactions between the SCs and the niche basement membrane seem to participate in polarizing the SC, if the signal produced by the polarization of the mitotic spindle emanates from the basement membrane, the SC will be asymmetrically divided, generating two different types of cells (stem and differentiated). However, if this signal originates from the sites of cell-cell contact, the SC will be symmetrically divided into two daughter SCs (Fuchs *et al.*, 2004; Pardo-Saganta *et al.*, 2015).

It is essential to point out that when the ASCs activate to produce differentiated (mature) cells, they first produce intermediate and undifferentiated cells meant to be progenitor or transit-amplifying cells (TACs; Figure 8). This type of cell, first described by Lajtha in 1979, is committed to producing the final mature cells after several rounds of division (Lajtha, 1979). Consequently, TACs are ephemeral cells and can rapidly amplify the population of differentiated cells produced after each SC division. These cell cycle dynamics (higher cell proliferation rates in TACs than in SCs) are among the main differences between SCs and TACs (Rangel-Huerta *et al.*, 2017).

Cell niches have been widely described in different human tissues such as the adult skeletal muscle (Collins *et al.*, 2005), epidermis (Tumbar *et al.*, 2004), small intestine (Barker *et al.*, 2007), brain (Temple, 2001), bone marrow (Schofield,

1978), and even the endometrium (Cervelló *et al.*, 2010; Gargett and Masuda, 2010; Figure 9).

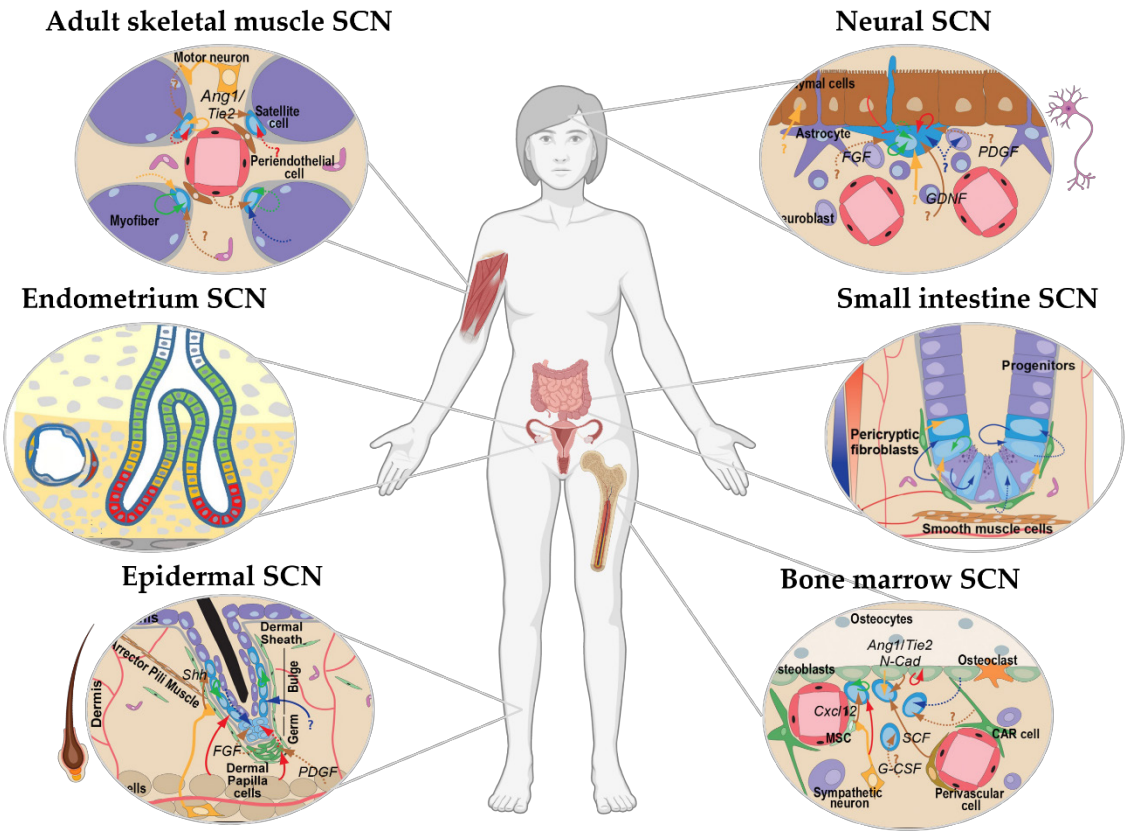


Figure 9. Stem cell niches in the woman's body. *Schematic representation of some of the ASCs niches identified in different woman's tissues. ANG: angiogenin; CAR: chimeric antigen receptor; CXCL12: C-X-C motif chemokine 12; FGF: fibroblast growth factor; G-CSF: granulocyte-colony stimulating factor; GDNF: glial cell line-derived neurotrophic factor; MSC: mesenchymal stem cell; N-Cad: N-cadherin; PDGF: platelet-derived growth factor; SCF: stem cell factor; SCN: stem cell niche. Created with www.BioRender.com; SCN images are included with permission (Rezza et al., 2014; Cousins et al., 2018).*

2.2.1. The bone marrow stem cell niche

The bone marrow stem cell niche is a heterogeneous microenvironment containing stem and progenitor cells of hematopoietic and non-hematopoietic (mesenchymal) lineages, in addition to mature cells such as osteoblasts, resident macrophages, or marrow adipocytes (Figure 10A; Reagan and Rosen, 2016). The hematopoietic stem/progenitor cells (HSCs), known for expressing the glycoprotein cluster of differentiation (CD) 34 (AbuSamra *et al.*, 2017), among other cell markers, produce most blood cells. Among the HSCs, there are

immature endothelial cells known as endothelial progenitor cells (EPCs) that can differentiate into mature endothelial cells, which in turn play a key role in neovascularization during wound healing (Borlongan *et al.*, 2011).

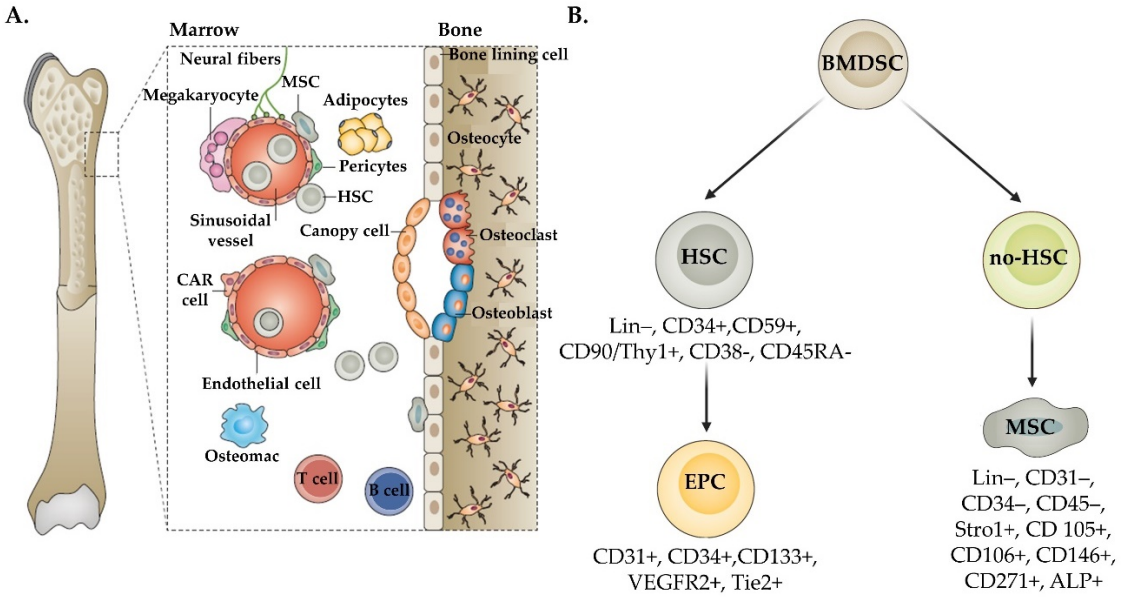


Figure 10. Human bone marrow stem cell niche. *A. Representation of the structure and the different types of cells (including stem and differentiated cells, both from either hematopoietic or mesenchymal lineages) composing the bone marrow stem cell niche. B. Schematic diagram showing the subpopulations of BMDSCs, including HSCs, MSCs, and EPCs. BMDSC: bone marrow-derived stem cell; CD: cluster of differentiation; EPC: endothelial progenitor cell; HSC: hematopoietic stem cell; MSC: mesenchymal stem cell; VEGFR2: vascular endothelial growth factor 2. Images are included with permission (Borlongan et al., 2011; Reagan and Rosen, 2016).*

Specific markers, such as vascular endothelial growth factor receptor 2 (VEGFR2) and CD133 (Peichev *et al.*, 2000), have been proposed to isolate EPCs, together with CD34 and the endothelial markers CD31 and Tie2 (Borlongan *et al.*, 2011; Reagan and Rosen, 2016; Figure 10B). These $CD133^+$ $VEGFR2^+$ EPCs, usually referred to as $CD133^+$ bone marrow-derived stem cells ($CD133^+$BMDSCs), can egress from the bone marrow and migrate to the bloodstream to incorporate into tissues, mainly in case of injury (Crosby *et al.*, 2000; Rafii and Lyden, 2003). The chemoattraction of these $CD133^+$BMDSCs to the bloodstream has been described to be led by several molecules such as stromal cell-derived factor 1 (SDF1), vascular endothelial growth factor (VEGF), or granulocyte-colony stimulating factor (G-CSF; Urbich *et al.*, 2003). Thus, $CD133^+$BMDSCs have been proposed as

a therapeutic approach for treating different pathologies such as myocardial infarctions (Mansour *et al.*, 2011), retinal pigment epithelium dysfunction (Harris *et al.*, 2009), and even gynaecologic disorders (Santamaría *et al.*, 2016; Herraiz *et al.*, 2018). It is worth mentioning here that the previously mentioned VSELs, which been detected in the human bone marrow, also express the SC marker CD133 (Ratajczak *et al.*, 2017). Thus, their potential involvement in the described $CD133^{+}$BMDSCs population should be further explored.

2.3. Adult stem cells in the human endometrium

The endometrium is a highly regenerative tissue (as previously described in Section 1.2) and as such, the presence of an ASC population has always been suspected. Although an early study by Prianishnikov proposed the existence of endometrial ASCs (Prianishnikov *et al.*, 1978), it was not until 2004 when this putative population was first evidenced. Two independent groups reported the presence of rare clonogenic epithelial and stromal cells with high proliferative potential (Chan *et al.*, 2004) and also proposed the role of BMDSCs in endometrial regeneration (Taylor, 2004).

Notably, the endometrial ASCs population has been described to be mainly located around the spiral arterioles of the endometrial *basalis* layer, which remains constant during menstruation (Murakami *et al.*, 2014; Cousins *et al.*, 2018).

Over the years, various SC markers have facilitated the isolation of different types of SCs (stromal, epithelial progenitors, and endothelial) in human endometrial tissue (Figure 11A). It might be mentioned here that in the last couple of years, the presence of VSELs in the murine endometrium has also been suggested, but there is still a need of research around this fact (Singh and Bhartiya, 2021).

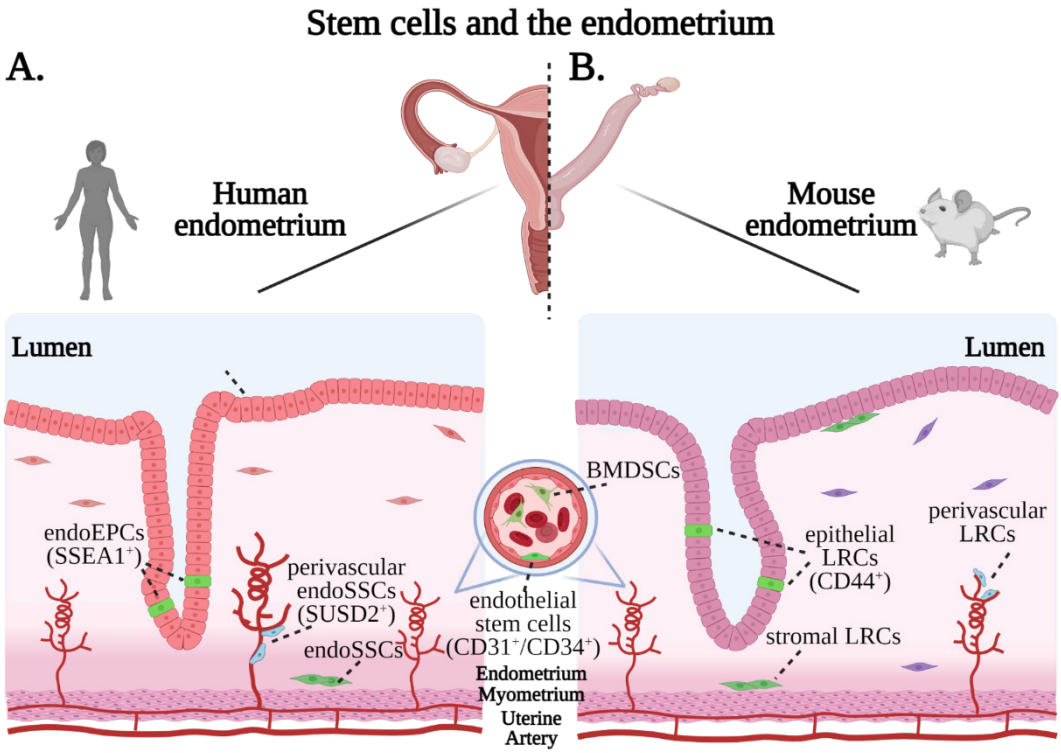


Figure 11. Stem cells and the endometrium. *Schematic representation of the different types of stem cells identified in the (A) human (namely, endoEPCs, perivascular endoSSCs, endoSSCs, endothelial stem cells, and BMDSCs) and (B) mouse (namely, epithelial LRCs, perivascular LRCs, stromal LRCs, endothelial stem cells, and BMDSCs) endometrium. The most described stem cell markers are indicated. BMDSCs: bone marrow-derived stem cells; CD: cluster of differentiation; endoEPCs: endometrial epithelial progenitor cells; endoSSCs: endometrial stromal stem cells; LRCs: label-retaining cells; SSEA1: stage-specific embryonic antigen-1; SUSD2: sushi domain-containing-2. Image adapted with permission from the graphical abstract of Manuscript III (de Miguel-Gómez et al., 2021) included in this thesis. Created with www.BioRender.com.*

The discovery of markers such as CD146 and the platelet-derived growth factor receptor β (PDGFRβ; Schwab and Gargett, 2007), sushi domain-containing-2 (SUSD2; Masuda *et al.*, 2012; Ulrich *et al.*, 2014), leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5; Sun *et al.*, 2009; Cervelló *et al.*, 2017), and ectonucleoside triphosphate diphosphohydrolase 2 (NTPDase2; Trapero *et al.*, 2019) have led to the identification of endometrial stromal cells in two different locations, perivascular spaces and in the *basalis* layer. In particular, NTPDase2, together with N-cadherin (Nguyen *et al.*, 2017) or stage-specific embryonic antigen-1 (SSEA1; Valentijn *et al.*, 2013) have become markers for endometrial epithelial progenitor cells. Whereas, CD31 and CD34 identify endothelial SCs in the side population of the endometrial fraction (Tsuji *et al.*, 2008; Masuda *et al.*,

2010). Nevertheless, it is important to note that despite all this evidence, there is still no consensus about specific and universal endometrial SC markers.

Adult SCs in the endometrium have been historically detected using functional assays based on cell cytometry and sorting. In humans, the side population approach, based on the SC's faster efflux of the vital dye Hoechst 33342, has been widely used to identify endometrial SCs (Kato *et al.*, 2007; Tsuji *et al.*, 2008; Cervelló *et al.*, 2010, 2011).

All the studies mentioned throughout this section have also used multilineage differentiation or clonogenicity assays to validate the stemness of these cells.

2.3.1. Role of endometrial adult stem cells in endometrial disorders

Endometrial ASCs have been described to participate in the development of endometrial disorders affecting fertility. Adult SCs seem to contribute to conditions characterized by an overgrowth of the endometrial tissue, such as endometrial hyperplasia, endometrial carcinoma (uncontrolled activity of ASCs which transforms them into cancer SCs; Hubbard *et al.*, 2009), endometriosis (ectopic location of endometrial tissue; Sasson and Taylor, 2008), or adenomyosis (myometrial location of endometrial tissue; García-Solares *et al.*, 2018). In contrast, the lack of functionality of these cells, or their niche, could also be responsible for disorders characterized by the absence of functional tissue, as occurs in endometrial atrophy or Asherman's Syndrome (Santamaría *et al.*, 2018).

2.4. Adult stem cells in murine models

Murine models have become fundamental and preliminary aids in describing the presence of ASCs in the mouse and human endometrium (Figure 11B). These findings were possible thanks to the use of label-retention methods using 5-bromo-2′-deoxyuridine (BrdU) or histone 2B-green fluorescent protein (H2B-GPF). Specifically, BrdU is an analog of thymidine that incorporates newly

synthesized deoxyribonucleic acid (DNA) during mitosis, and enabled the identification of stromal and epithelial label-retaining cells (LRCs; Chan and Gargett, 2006; Cervelló *et al.*, 2007; Chan *et al.*, 2012; Cao *et al.*, 2015). Studies with H2B-GFP also isolated both epithelial (Wang *et al.*, 2012; Patterson and Pru, 2013) and stromal LRCs (Kaitu'u-Lino *et al.*, 2012; Wang *et al.*, 2012) fractions of the postulated SC niche. In addition, these LRCs were also positive for different SC markers such as the proto-oncogene *c-KIT,* the octamer-binding transcription factor *OCT4* (Cervelló *et al.*, 2007) or *CD90/PDGFRb* (Cao *et al.*, 2015). The detection of *CD44*, an epithelial progenitor cell marker, has also allowed to identify murine endometrial SCs (Janzen *et al.*, 2013).

Apart from the essential role of endometrial ASCs in the regeneration of this tissue, it has been described that BMDSCs also contribute to the SC population located in the endometrial tissue (Figure 11B).

2.5. Contribution of the bone marrow to the endometrial stem cell population

The contribution of BMDSCs to the endometrial SC population and, consequently, to the endometrial regeneration, was first hypothesized in 2004 (Taylor, 2004). This group suggested that BMDSCs, which can differentiate into non-hematopoietic cells, may migrate towards the endometrium and, finally, differentiate into endometrial cells. However, the degree of contribution of these BMDSCs to the endometrium remains to be elucidated. For example, it is still unclear if these cells migrate towards the endometrium in each endometrial cycle, only after a bone marrow transplant or in specific injury situations.

Concerning this quandary, Ikoma and colleagues confirmed the implication of the BMDSCs in the endometrium of patients receiving a bone marrow transplant from a male donor (Ikoma *et al.*, 2009). This contrasts with results from Cervelló

et al. which suggested these cells were an exogenous source of SCs that could migrate towards the uterus in certain situations, but did not constitute the endogenous endometrial SC niche studied (Cervelló *et al.*, 2012).

Taken together, it could be postulated that the endometrial ASCs have two different origins (Figure 12): endogenous (constituted by the resident endometrial ASCs) and exogenous (formed by the BMDSCs that migrate and reach the endometrial tissue in response to certain stimuli).

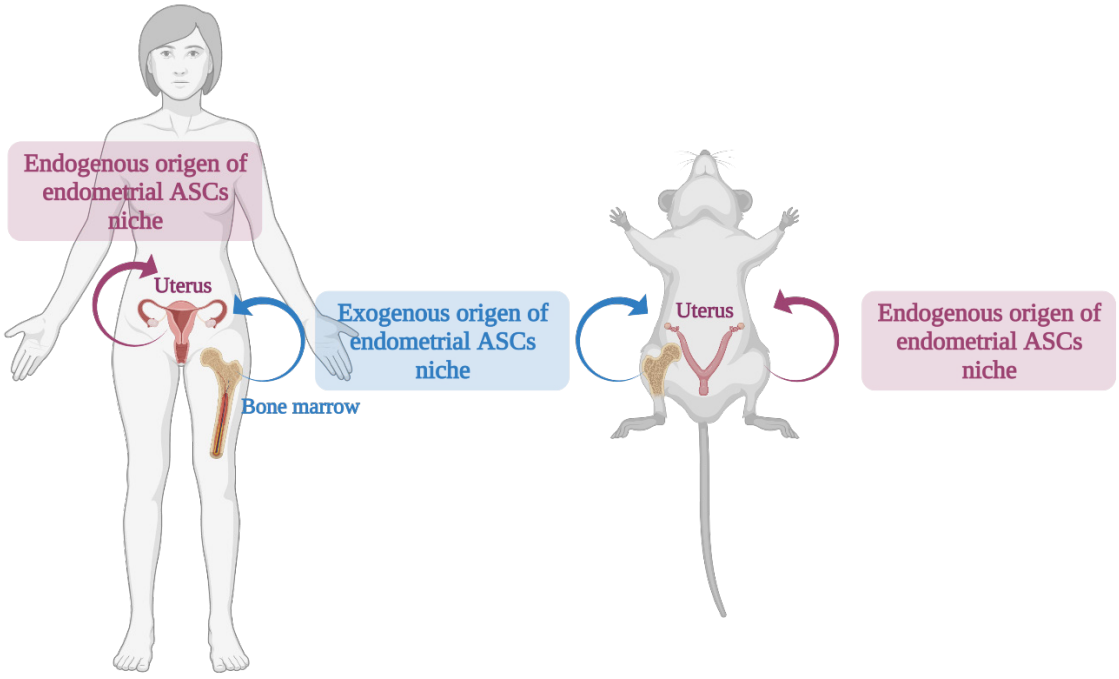


Figure 12. Proposed sources of ASCs to the endometrial stem cell niche: exogenous and endogenous. *In addition to the endogenous origin of endometrial ASCs (endometrium), the existence of an exogenous source of stem cells (bone marrow) has been postulated in both women and mice. In case of injury, the exogenous source would favor ASCs migration from the bone marrow towards the endometrium. ASCs: adult stem cells. Created with www.BioRender.com.*

According to this hypothesis, G-CSF (Xie *et al.*, 2017) and SDF1 (Ersoy *et al.*, 2017; Yi *et al.*, 2019), which participate in the chemoattraction of BMDSCs into the bloodstream, have been proposed for treating endometrial pathologies on the hypothesis that these factors would stimulate BMDSCs recruitment to the endometrium. To date, there are controversial results about the effectiveness of G-CSF (Barad *et al.*, 2014; Eftekhar *et al.*, 2014), and more studies are required to study the efficacy of SDF1.

3. ENDOMETRIAL DISORDERS AFFECTING FERTILITY

The World Health Organization defines infertility as a disease of the reproductive system characterized by the inability to achieve a clinical pregnancy after one year (or longer) of regular unprotected sexual intercourse (Mascarenhas *et al.*, 2012). Various aspects can contribute to developing this disease, such as male factors, ovulation disorders, fallopian tube damage, and uterine pathologies. Here, we will introduce some of the uterine disorders causing infertility.

Absolute uterine factor infertility (AUFI) is defined by a problem entirely attributable to uterine absence or an abnormality leading to a total non-functional organ that prevents a successful pregnancy (Brännström, 2019). The principal leading cause is a hysterectomy during fertile age. The surgical removal of the uterus may be indicated for malignant uterine neoplasia, leiomyomas (uterine fibroids), or adenomyosis (Galliano *et al.*, 2015). On the other hand, congenital conditions, such as the Mayer-Rokitansky-Kuster-Hauser Syndrome, can lead to the complete absence of the organ (Bombard and Mousa, 2014). In the last few years, uterus transplantation, still in the early stages of clinical research, has emerged as a potential solution for AUFI patients (Brännström, 2019).

Other underlying causes can impair the possibility of pregnancy but do not necessarily imply AUFI. This category includes leiomyomas or adenomyosis that do not require hysterectomy, endometriosis, most of the Müllerian duct anomalies (errors in the Müllerian duct development during embryonic morphogenesis), and the majority of endometrial alterations (Galliano *et al.*, 2015).

3.1. Endometrial pathologies

Among pathologies directly affected by endometrial factors, there are disorders affecting the endometrial lining. These types of pathologies directly affect endometrial receptivity, negatively interfering with embryo implantation and, consequently, pregnancy (Galliano *et al.*, 2015). This affectation can be produced by lack of growth (such as endometrial atrophy or thin endometrium), or conversely, by overgrowth of the tissue (such as endometrial polyps (Lieng *et al.*, 2010), endometrial hyperplasia (Armstrong *et al.*, 2012), or endometrial carcinoma (Hubbard *et al.*, 2009)). Other disorders imply intrauterine adhesions, such as Asherman's Syndrome, which denotes the presence of fibrotic tissue.

For the purposes of this thesis dissertation, only endometrial atrophy and Asherman's Syndrome are studied in detail.

3.1.1. Endometrial atrophy

Endometrial atrophy (EA) is characterized by a diminished endometrial thickness (< 7 mm). This disorder is also referred to as thin or refractory endometrium and compromises endometrial receptivity (Figure 13A; Mahajan and Sharma, 2016). With an incidence of around 2.4% among patients undergoing an *in vitro* fertilization treatment (Kasius *et al.*, 2014) this rare pathology is most common in older women, reaching 25% in women beyond the age of forty (Sher *et al.*, 1991).

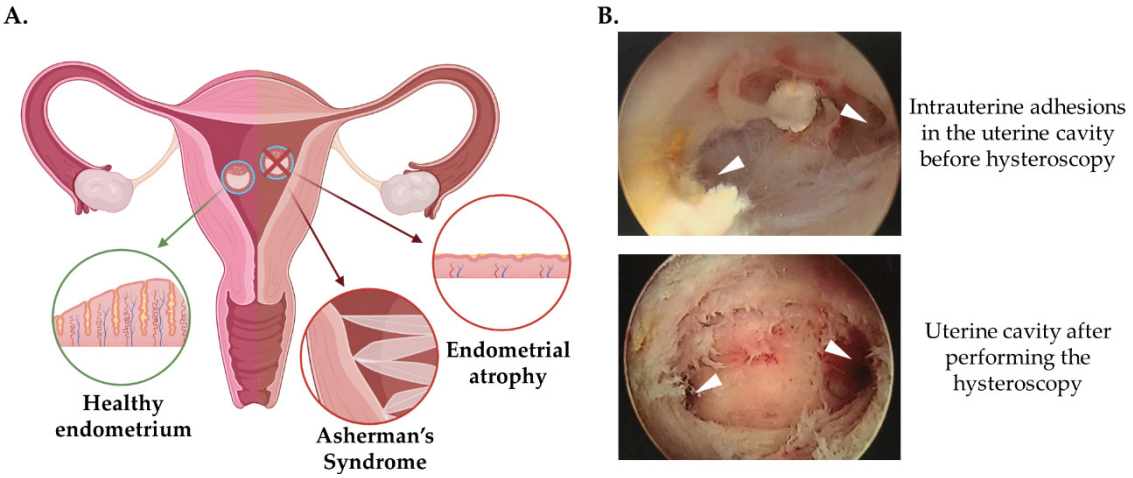


Figure 13. Endometrial atrophy and Asherman's Syndrome. *A. Diagram showing how a healthy endometrium allows the correct embryo implantation while a uterus affected by endometrial atrophy (insufficient endometrial thickness) or Asherman's Syndrome (presence of intrauterine adhesions) impedes this important event for a correct pregnancy achievement. B. Laparoscopic views of an Asherman's Syndrome patient's uterine cavity (fundus) taken during the hysteroscopy for removing intrauterine adhesions. Note that the fallopian tubes can be seen after the hysteroscopy (white arrows). Image A created with www.BioRender.com; images in B adapted with permission from www.drjennycook.com.*

The insufficient endometrial thickness in EA patients is usually accompanied by poor tissue vascularity, mainly of the glandular epithelium, and decreased VEGF expression (Miwa *et al.*, 2009). These abnormalities have been associated with iatrogenic causes such as repeated or vigorous curettages or myomectomies, or indiscriminate use of drugs such as clomiphene citrate (Mahajan and Sharma, 2016). Other proposed risk factors include acute or chronic infections, in which inflammation leads to the destruction of the *basalis* and the appearance of fibrotic tissue. Finally, EA origins may also be idiopathic, deriving from the intrinsic properties of the endometrium or the uterine architecture (Mahajan and Sharma, 2016).

The correct diagnosis of any pathology is essential for providing the best available therapeutic option. In this case, EA should be suspected in women with an insufficient endometrial thickness (usually evaluated by transvaginal sonography), implantation failure, and/or advanced age (Conforti *et al.*, 2013).

3.1.2. Asherman's Syndrome

Asherman's Syndrome (AS) is characterized by the presence of intrauterine adhesions (IUAs; Figure 13A-B). This rare disease occurs when the normal endometrium is replaced by fibrotic tissue inside the uterine cavity and/or endocervix (Yu *et al.*, 2008). The most common symptoms are menstrual abnormalities, pelvic pain, recurrent miscarriage, and abnormal placentation (Santamaría *et al.*, 2018). Asherman's Syndrome has also been postulated as a causal factor for a thin endometrium (Senturk and Erel, 2008).

There are different methods to classify AS patients, but the classification system described by the American Fertility Society in 1988 is probably the most commonly used. This system considers the extensiveness and morphology of the IUAs in addition to the menstrual pattern and as such classifies the AS patients into three groups (Panayotidis *et al.*, 2008): stage I or mild, with few adhesions involving less than one third of the uterine cavity accompanied by regular menses or occasional hypomenorrhea; stage II or moderate, with filmy and dense adhesions, in up to two-thirds of the cavity and hypomenorrhea; and finally stage III or severe, with dense adhesions involving more than two-thirds of the cavity in addition to amenorrhea.

Several risk factors contribute to the development of this pathology, usually related to iatrogenic trauma to the endometrium, such as postpartum curettages or hysteroscopic surgeries. Other causes can be a Müllerian duct malformation, uterine artery embolization, miscarriage, or, less frequently, genital infections, cesarean sections, or the insertion of an intrauterine device (Conforti *et al.*, 2013).

This syndrome should be suspected in those patients presenting hypomenorrhea, amenorrhea and/or infertility problems with a history of intrauterine surgery. For an accurate diagnosis, it is necessary to use imaging techniques of the uterine cavity. Nowadays, the best option is performing a hysteroscopy, which is usually

done on an outpatient basis. This technique uses a narrow telescope (called hysteroscope), introduced intravaginally, with a light and camera that provides a real-time view of the uterus. Other techniques, such as hysterosalpingography or sonohysterography, can also be used (Conforti *et al.*, 2013).

3.2. Murine models of endometrial damage

Murine models (mice and rats) used for translational research in reproduction present several advantages such as easy and low-cost maintenance, short gestation periods, and litter sizes of five to six in mice or up to nine in rats, which make them suitable for establishing models of gynaecological disorders (Andersen *et al.*, 2018). Over the years, different methods have been suggested for inducing endometrial injury imitating EA and AS alterations in these models (Figure 14).

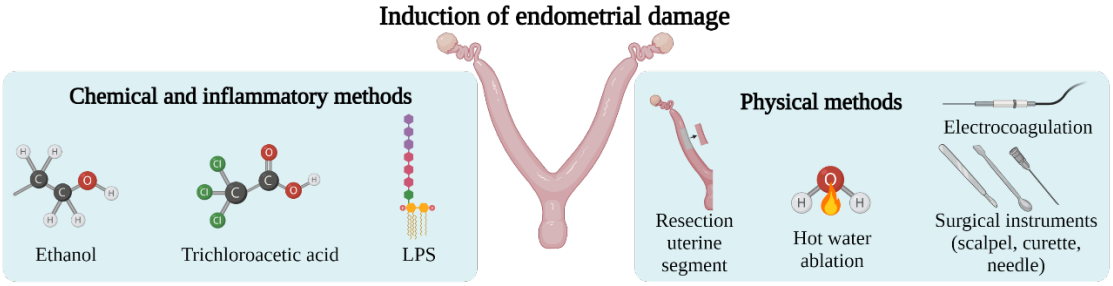


Figure 14. Methods to induce endometrial damage in murine models of EA/AS. *To simulate the human pathologies EA and AS, endometrial damage is induced in rodent models using chemical (infusion of ethanol, trichloroacetic acid, or LPS) and/or physical (resection of a uterine segment, hot water ablation, surgical instruments, electrocoagulation) methods. EA: endometrial atrophy; LPS: lipopolysaccharides; AS: Asherman's Syndrome. Created with www.BioRender.com.*

Proposed mechanical methods for inducing endometrial injury include the use of a curette (Zhang *et al.*, 2020), an electric scalpel (Zhao *et al.*, 2015), or a needle (Alawadhi *et al.*, 2014; Cervelló *et al.*, 2015). Although some studies have also reported the resection of a uterine segment to simulate endometrial damage (Ding *et al.*, 2014; Hellström *et al.*, 2016; Daryabari *et al.*, 2019), this approach is commonly used to reflect AUFI rather than EA or AS. In the last years, the use of

chemical methods, such as the infusion of ethanol (Jang *et al.*, 2017; Yi *et al.*, 2019) or even trichloroacetic acid (Kilic *et al.*, 2014), has become widely accepted over the physical procedures. Alternatively, injecting bacterial lipopolysaccharides (LPS) has also been proposed for reproducing inflammation-derived endometrial damage, however this approach has been used for studying the extent of damage in the endometrium rather than testing therapies (Wang *et al.*, 2017; Xiao *et al.*, 2017; Sun *et al.*, 2019). Overall, there is still a lack of standardization about which method is the most suitable.

4. TREATING ENDOMETRIAL ATROPHY AND ASHERMAN'S SYNDROME

4.1. Surgical management, hormonal, and non-hormonal treatments

Both EA and AS can be treated surgically, however, only AS patients commonly undergo surgery. The most frequently used technique is hysteroscopic adhesiolysis, which is usually accompanied by post-operative measures, such as the insertion of an intrauterine balloon stent (Lin *et al.*, 2013) or a Foley balloon (Amer *et al.*, 2005), due to the high recurrence rate of IUAs (Khan and Goldberg, 2018).

Therapeutic approaches using exogenous estrogens (Chen *et al.*, 2006; Coughlan *et al.*, 2014), GnRH agonists (Qublah *et al.*, 2008), human chorionic gonadotropin (hCG; Papanikolau *et al.* 2013; Davar *et al.*, 2016), or even tamoxifen (Reynolds *et al.*, 2010) have been suggested to increase endometrial thickness in EA patients. The use of exogenous estrogens prior to and after surgery may also be considered to prevent the formation of new IUAs (Tougerman *et al.*, 2001).

On the other hand, non-hormonal treatments are usually administered alongside hormone replacement therapy and sometimes in combination with estrogen

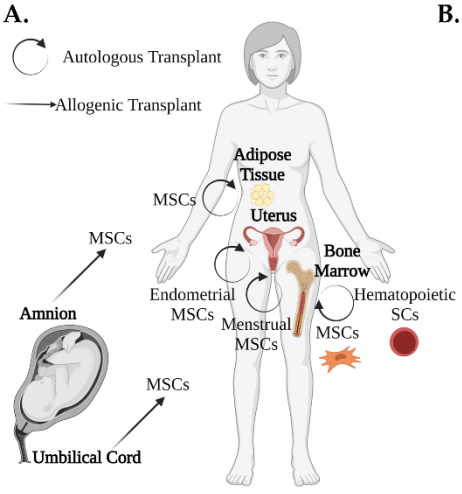
administration. Drugs like low-dose aspirin (Weckstein *et al.*, 1997; Urman *et al.*, 2000; Chen *et al.*, 2016b, 2017), vaginal sildenafil citrate (Sher and Fisch, 2000; Zinger *et al.*, 2006; Takasaki *et al.*, 2010), and pentoxifylline combined with vitamin E (Lédée-Bataille *et al.*, 2002; Letur-Konirsch and Delanian, 2003; Acharya *et al.*, 2009) have shown efficacy in EA/AS patients by improving endometrial vasculature and consequently the endometrial lining. However, there are also studies reporting no differences in endometrial thickness and/or reproductive outcomes after applying the before enlisted vasoactive substances (Check *et al.*, 1998; Hsieh *et al.*, 2000; Aleyasin *et al.*, 2009; Firouzabadi *et al.*, 2013).

The forementioned 'classical' therapeutic approaches remain ineffective for some AS or EA patients, which is why alternative techniques have become promising options for these patients.

4.2. Stem cell therapies

Several studies have reported using SCs obtained from various sources for treating either EA or AS in animal and human models (Figure 15).

A.



B.

Type of Stem Cells	Study Model	References
UCMSCs	Murine	Tang *et al.*, 2016; Zhang *et al.*, 2018a
UCMSCs + collagen scaffolds	Murine Human	Xu *et al.*, 2017; Xin *et al.*, 2019 Cao *et al.*, 2018
AmnioMSCs	Murine	Gan *et al.*, 2017; Li *et al.*, 2019; Bai *et al.*, 2020; Ouyang *et al.*, 2020
Adipose MSCs	Murine Human	Kilic *et al.*, 2014; Çil *et al.*, 2020; Yotsumoto *et al.*, 2020, Han *et al.*, 2020 Sudoma *et al.*, 2019; Lee *et al.*, 2020
Menstrual MSCs	Murine Human	Hu *et al.*, 2019; Zhang *et al.*, 2019; Chen *et al.*, 2020 Tan *et al.*, 2016; Zhang *et al.*, 2018b; Ma *et al.*, 2020
Endometrial MSCs	Human	Sapozhak *et al.*, 2020; Tersoglio *et al.*, 2020
BMDSCs: - whole fraction - MSCs - MSCs + scaffolds - Hematopoietic SCs - EPCs	 Murine Murine Murine Human Murine Human	 Alawhadi *et al.*, 2014 Zhao *et al.*, 2015; Gao *et al.*, 2018 Ding *et al.*, 2014; Yang *et al.*, 2017 Singh and Seth, 2014 Cervelló *et al.*, 2015 Santamaría *et al.*, 2016

Figure 15. Stem cell therapy in the management of EA/AS. *Schematic diagram (A) and summarizing table (B) of the main types of stem cells tested in EA and AS murine models and patients. AS: Asherman's Syndrome; BMDSCs: bone marrow-derived stem cells; EA: endometrial atrophy; EPCs: endothelial progenitor cells; MSCs: mesenchymal stem cells; SCs: stem cells; UCMSCs: umbilical cord-derived mesenchymal stem cells.*

4.2.1. Stem cells for treating endometrial atrophy and Asherman's Syndrome

Perinatal SCs for treating EA/AS can be collected from either the umbilical cord or the amnion. In AS murine models, treatment with mesenchymal stem cells (MSCs) derived from the umbilical cord (UCMSCs) increased glandular count, reduced endometrial fibrosis, and promoted cell proliferation (Tang *et al.*, 2016; Zhang *et al.*, 2018a). This cell therapy has also been used in conjunction with bioengineering (defined as the application of engineering and life science basis toward the development of biomaterials for restoring, maintaining, or improving tissue natural functions; Heineken & Skalak, 1991) techniques, which have been successfully implemented in AS murine models (Xu et al., 2017; Xin et al., 2019). Clinically, Cao and colleagues demonstrated how UCMSCs embedded within a collagen scaffold can be inserted after hysteroscopic adhesiolysis to increase endometrial thickness. This approach also improved the levels of healthy endometrium (via estrogen receptors and vimentin) and tissue regeneration markers (such as proliferation marker Ki67 and von Willebrand factor (vWF)) markers while reducing IUAs formation (Cao *et al.*, 2018). Similarly, MSCs derived from the amnion have also provided beneficial results in AS murine models. These SCs can repair endometrial injury by reducing fibrosis, improving endometrial morphology (Li *et al.*, 2019; Bai *et al.*, 2020; Ouyang *et al.*, 2020) and modulating immunological properties of this tissue (Gan *et al.*, 2017).

A second approach would be to use MSCs derived from adipose tissue (ADMSCs), which are relatively easy to access. Several studies have reported that ADMSCs restore endometrial morphology and reduce fibrosis (Kilic *et al.*, 2014), increase expression of pro-regenerative factors (such as VEGF and insulin-like growth factor 1 (IGF1); Çil *et al.*, 2020), and even improve embryo implantation (Yotsumoto *et al.*, 2020). Again, these SCs can benefit from the bioengineering. Adipose-derived MSCs loaded into an acellular human amniotic membrane improved angiogenesis in an AS rat model (Han *et al.*, 2020). Additionally, two

independent human studies have implemented this therapy, improving pregnancy rates in EA (Sudoma *et al.*, 2019) and AS patients (Lee *et al.*, 2020).

The uterus itself has also proved to be a source of therapeutic SCs. Groups have isolated MSCs from the menstrual blood and reported increased endometrial thickness not only in human studies (Tan *et al.*, 2016; Zhang *et al.*, 2018b; Ma *et al.*, 2020) but also in AS murine models (Hu *et al.*, 2019; Zhang *et al.*, 2019; Chen *et al.*, 2020). Alternatively, a few preliminary clinical studies have successfully demonstrated that endometrial biopsies can be used for isolating autologous ASCs (Sapozhak *et al.*, 2020; Tersoglio *et al.*, 2020).

Furthermore, in a recent study worth mentioning, urine cells were reprogrammed into induced pluripotent MSCs and combined with a hydrogel scaffold to regenerate the endometrium in a mouse model (Ji *et al.*, 2020).

Despite all the forementioned sources of SCs, the bone marrow is undoubtedly the most commonly used (or at least tested) source of SCs for treating EA and AS.

4.2.2. Bone marrow-derived stem cells for treating endometrial atrophy and Asherman's Syndrome

In 2014, Alawadhi and colleagues transplanted male mouse BMDSCs (whole fraction) in an AS murine model (Alawadhi *et al.*, 2014). Apart from the contribution of these cells to the endometrial SC niche (described in Section 2.5), this therapeutic approach improved the pregnancy outcomes of the group receiving BMDSCs. A year later, Zhao *et al.* also reported that mesenchymal BMDSCs improved endometrial thickness in a rat model with uterine damage via migration kinetics and immunomodulatory properties (Zhao *et al.*, 2015). In 2018, Gao and colleagues described the effectiveness of mesenchymal BMDSCs in an AS murine model through improved cell proliferation, increased expression of LIF (an endometrial receptivity marker), and reduced fibrosis in the damaged

endometrium (Gao *et al.*, 2018). Another study by Cervelló's group in 2015 showed similar results in murine models using human $CD133^+$BMDSCs (Cervelló *et al.*, 2015). The $CD133^+$BMDSCs were obtained from a pilot study of 16 EA and/or AS patients (Santamaría *et al.*, 2016), where cells were mobilized after G-CSF injection, collected through peripheral blood aphaeresis, and isolated based on $CD133^+$ expression and finally catheterized into the uterine spiral arterioles of each patient. The authors reported that these labeled SCs were present around endometrial blood vessels, inducing proliferation in surrounding cells and regulating the paracrine factors thrombospondin 1 (TSP1) and IGF1. The results suggest that autologous $CD133^+$BMDSCs provide an effective, although transient, endometrial repair (since the effect was maximized three months after injection and diminished after six months), allowing menstruation to resume, and improving endometrial morphology, thickness, neoangiogenesis, and pregnancy rates. Furthermore, Sing *et al.* reported that intrauterine infusion of autologous mononuclear $CD34^+$BMDSCs improves the endometrial thickness and menstrual pattern in six AS patients (Singh and Seth, 2014).

Bioengineering also exploits BMDSCs properties. An early study by Ding and colleagues reported that embedding BMDSCs in a collagen scaffold could increase the regenerative action of these SCs in an AS murine model (Ding *et al.*, 2014). More recently, another group ratified the synergy of a synthetic hydrogel over the activity of these types of SCs for treating uterine injury (Yang *et al.*, 2017).

Some of the studies presented above report that the engraftment of the administered SCs is relatively low (Cervelló *et al.*, 2015) and that these cells distribute around the blood vessels (Zhao *et al.*, 2015; Santamaría *et al.*, 2016), where the endometrial SC niche is supposedly located, rather than arriving at the injury area. Yang and colleagues describe how MSCs derived from the bone marrow secrete a variety of growth factors (GFs) involved in tissue regeneration processes, such as hepatocyte growth factor (HGF), IGF1, epidermal growth

factor (EGF), interleukins (ILs) 6 and 10, and tumor necrosis factor alpha (TNFα). These results, together with other studies evidencing the paracrine action of SCs in other medical specialties (Tögel *et al.*, 2007; Gneechi *et al.*, 2008; Schinköthe *et al.*, 2008), suggest that the injected BMDSCs act in a paracrine manner, and they secrete different molecules which seem to be the key players in the SC therapy action and/or stimulate the endogenous SC niche.

4.2.3. Paracrine action of the bone marrow-derived stem cells in tissue regeneration

Many studies advocate the importance of the paracrine action of the ASCs (including BMDSCs), postulating that the molecules synthesized and secreted by these cells may be essential. These biomolecules, usually referred to as the secretome, seem to be as important, if not more so, than differentiation and repopulation of the SCs in modulating the composition of the environment to evoke responses from resident cells, which ultimately repair tissue (Figure 16).

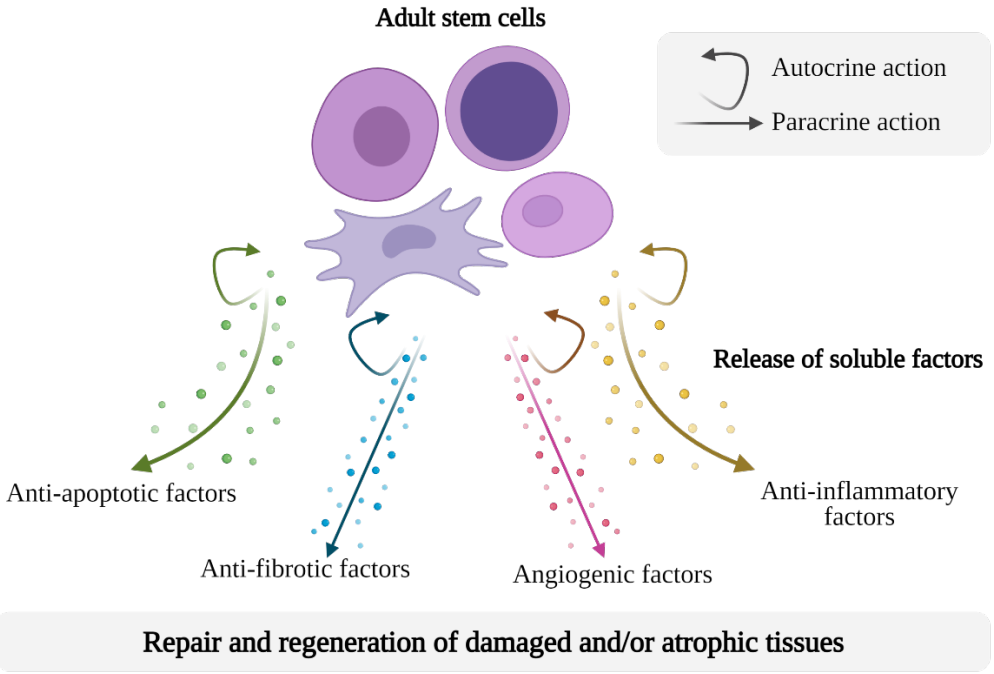


Figure 16. Schematic representation of the action of adult stem cells. *Somatic stem cells secrete a variety of soluble factors (in addition to extracellular vesicles, exosomes, and micro RNAs) that exert both autocrine (over themselves) and paracrine effects (over other cells, which are commonly damaged or degenerated and need the action of these secreted factors). Created with www.BioRender.com.*

Final effectors of the BMDSCs action include cytokines (such as IL1, IL6, and TNFα), GFs (namely, VEGF, brain-derived neurotrophic factor (BDNF), fibroblast growth factor (FGF2), and HGF), or ECM molecules (such as metalloproteinases (MMPs) and collagens). They are involved in angiogenesis (in particular VEGF, angiogenin, and MMP1/2), cell chemotaxis (mainly driven by chemokines), apoptosis, fibrosis, immunomodulation, and cell migration and proliferation (Gnecchi *et al.*, 2008, 2016; Andrzejewska *et al.*, 2019), which are important events for tissue regeneration (Krafts, 2010; Zhao *et al.*, 2016).

The paracrine action of the BMDSCs has been described in various organ processes, such as renal injury (Tsuji and Kitamura, 2015), brain damage (Rhee *et al.*, 2016), and cardiac repair (Khanabdali *et al.*, 2016). Notably, these paracrine effects have also been described in female reproductive organs and processes (apart from the endometrium). For example, BMDSCs have shown promising results in ovarian rejuvenation (Herraiz *et al.*, 2018), follicular restoration (Ghadami *et al.*, 2012), embryo culture (Kawamura *et al.*, 2012; Jasmin *et al.*, 2016), and treatment of chronic pelvic disease (Volkova *et al.*, 2017).

This paracrine premise suggests that applying only the biomolecules secreted by the SCs rather than the cells themselves could be enough to activate tissue regeneration. That being said, it may be possible to create a chemical *cocktail* without isolating and administrating SCs, which is usually very costly and invasive for the patients. Elucidating these factors has promising therapeutic potential for regenerative medicine.

Remarkably, non-invasive therapies have emerged as promising alternatives to SC therapy while pursuing these paracrine biomolecules. One example is platelet-rich plasma (PRP), a reservoir of growth and angiogenic factors (among other molecules) directly involved in tissue repair. Of note, some of the biomolecules that platelets encompass are also secreted by the BMDSCs.

4.3. Platelet-rich plasma

In recent years, PRP has emerged as a promising therapeutic option for EA and AS patients. Although the mechanism of action of PRP is not fully understood, its molecules are thought to promote tissue healing and regeneration by attracting undifferentiated cells in the injured area and triggering cell division, while suppressing pro-inflammatory cytokine release, limiting inflammation, and attracting macrophages (Lacci and Dardik, 2010; Jain and Gulati, 2016).

4.3.1. Platelets

Blood plasma serves as the liquid base for whole blood, due to it contains 91 - 92% of water and only 8 - 9% cells. The most abundant blood cells found in the plasma are platelets (also known as thrombocytes; Mathew *et al.*, 2018).

Platelets, which are small subcellular anuclear fragments circulating in the blood, usually range between 1.5×10^8/mL and 4.5×10^8/mL in humans. Since they have a small size and irregular discoid shape, the bloodstream continuously pushes the platelets against the blood vessels' edge, where they are optimally positioned to get caught in sites of endothelial injury. The platelet cytoplasm includes three main types of secretory granules (Boilard and Nigrovic, 2017): α-granules, which are the most abundant organelles, dense bodies (δ granules), and lysosomes.

4.3.2. Obtaining platelet-rich plasma

Perhaps the most significant advantage of PRP is how easily it can be obtained from the peripheral blood, requiring an ambulatory and minimally invasive procedure for blood collection. Even though PRP is usually autologously used, the plasma, considered the acellular fraction of the blood (Spanish National Biobank Network, 2012), should minimize immune problems when obtaining the PRP from a non-autologous (allogenic) human donor. However, some authors

suggest that the plasma must be centrifuged after activation to remove platelet debris and truly be considered an acellular product (Tan *et al.*, 2019).

After sample collection, an anticoagulant (e.g., acid citrate dextrose or sodium citrate) is immediately added to prevent blood coagulation and the premature activation of the platelets, a process that implies the break of its plasmatic membranes (Anitua *et al.*, 2012; Amable *et al.*, 2013).

The PRP fraction is then isolated from total blood using a centrifugation process which creates a gradient that separates the blood into three evident fractions, based on the diverse specific gravity of the blood components (Figure 17). From the bottom to the top, these three distinct layers are: red blood cells or erythrocytes, white blood cells or leukocytes (also called buffy layer, which can also contain platelets), and plasma. This plasma fraction further contains a platelet concentration gradient so that the lower fraction is usually referred to as the PRP and the upper part as the platelet-poor plasma (PPP; Sánchez *et al.*, 2019). As its name suggests, PRP is a plasma fraction with a supra-physiologic platelet concentration (around 200.000 platelets/µl).

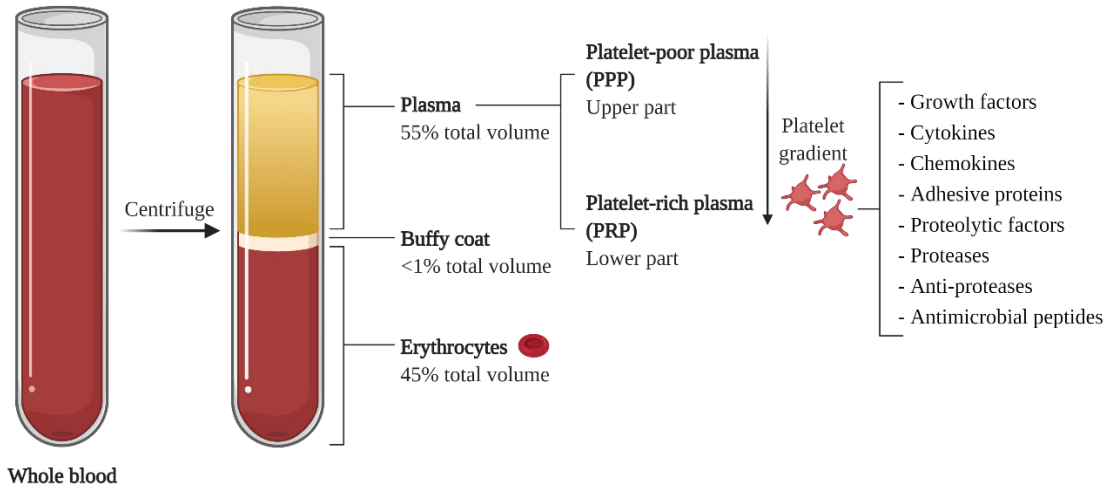


Figure 17. Platelet-rich plasma obtaining. *Schematic process of how the whole blood is separated into three different fractions (erythrocytes, buffy coat, and plasma), according to a density gradient, after centrifugation. Created with www.BioRender.com.*

The wealth of this product falls on the biologically active molecules located inside the platelets, which can be efficiently and locally released via degranulation of α-granules (a process described in detail in Section 4.3.3).

Given all the features which define PRP, it seems clear that this product is naturally heterogeneous (Qian *et al.*, 2017). The choice of the anticoagulant and platelet activator (see Section 4.3.3), the initial platelet concentration, and the centrifugation cycle's time and force can alter the final biological outcome of PRP treatment. Because PRP therapy has been widely used in different fields of medicine, a plethory of commercial PRP preparation kits for clinical applications has emerged. These kits allow standardization of the procedure, mainly in terms of final platelet concentration, and are closed systems, ensuring a sterile product.

4.3.3. Platelet-rich plasma activation and mechanism of action

Platelet-rich plasma is activated just before administration. The most commonly used PRP activators are thrombin or calcium chloride ($CaCl_2$; Cavallo *et al.*, 2016). However, other methods such as collagen (Cavallo *et al.*, 2016), freeze-thaw cycles (Sánchez *et al.*, 2019), and light exposure (Zhevago and Samoilova, 2006; Paterson *et al.*, 2016) have been described.

Activation releases the platelets α-granules in a process known as degranulation (Fréchette *et al.*, 2005). In turn, the α-granules then secrete different bioactive molecules by exocytosis, which will ultimately be implicated in the healing properties of the PRP. Remarkably, degranulation also induces the cleavage of fibrinogen, which then begins to form a platelet gel that confines the secretion of bioactive molecules at the site of injury.

Among all the components described inside the platelet α-granules, GFs are probably the most widely studied for their crucial role in tissue regeneration. The seven most relevant GFs and their general (Pavlovic *et al.*, 2016; Anitua *et al.*, 2018) and endometrial tissue-specific functions are described in Table I.

NAME	GENERAL FUNCTIONS	FUNCTION IN THE ENDOMETRIUM
Platelet-derived growth factor (PDGF)	- Collagen synthesis - Mitosis and chemotaxis of mesenchymal origin-cells - Macrophage activation - Promotion of angiogenesis and blood vessels repair	- Stimulation of proliferation and migration of endometrial stem and stromal cells (Matsumoto *et al.*, 2005; Gargett and Masuda, 2010) - Endometrial tissue contraction and remodeling support (Gargett and Masuda, 2010)
Transforming growth factor β (TGFβ)	- Collagen synthesis - Promotion of angiogenesis - Stimulation of immune cell chemotaxis - Enhancement of epithelial cells growth - Inhibition of osteoclast formation and bone resorption - Wound healing promotion	- Endometrial remodeling after menstruation: prevention of fibrosis and stimulation of collagen matrix contraction (Nasu *et al.*, 2005) - Involvement, via vasoconstriction, in the initiation of menstruation (Omwandho *et al.*, 2010) - Role in endometriosis, endometrial carcinoma, and hyperplasia (Nasu *et al.*, 2005; Omwandho *et al.*, 2010)
Epidermal growth factor (EGF)	- Mitosis, differentiation, and chemotaxis in epithelial and mesenchymal cells - Promotion of cytokine secretion	- Endometrial stem cell function and properties (promotion of CFU activity; Gargett and Masuda, 2010) - Mediation of decidualization (Chobotova *et al.*, 2005)
Vascular endothelial growth factor (VEGF)	- Promotion of angiogenesis and vessel permeability - Migration, chemotaxis, and proliferation of endothelial cells - Immune cell chemotaxis	- Endometrial angiogenesis: remodeling processes after partum and menstruation (Fan *et al.*, 2008) - Stimulation of endothelial cell migration, proliferation, assembly, and permeability (Okada *et al.*, 2014)
Insulin-like growth factor (IGF)	- Collagen synthesis - Migration, differentiation, and proliferation in different tissues - Wound healing promotion	- Placentation and embryo implantation (Forbes *et al.*, 2008) - Mediation of E_2 effects and mitosis of endometrial cells (Öner and Öner, 2007; Bruchim *et al.*, 2014)
Fibroblast growth factor (FGF2)	- Induction of proliferation, growth, and differentiation of mesenchymal cells, chondrocytes, and osteoblasts - Promotion of angiogenesis	- Endometrial stem cell function and properties (promotion of CFU activity; Gargett and Masuda, 2010) - Enhancement of endometrial receptivity (Paiva *et al.*, 2011)
Hepatocyte growth factor (HGF)	- Regulation of cell migration, growth, and morphogenesis - Antifibrotic effect - Wound healing regulation - Immune events modulation	- Enhancement of proliferation and migration of endometrial epithelial cells (Yoshida *et al.*, 2004) - Endometrial remodeling (Zhang, 2010)

Table I. Main growth factors present in platelet-rich plasma. *This table shows the principal biological functions of the mentioned growth factors and their reported functions in endometrial tissue. CFU: colony-forming cells/units; E_2: estradiol.*

In addition to these well-described factors, α-granules contain other factors such as bone morphogenetic proteins (BMPs) or keratinocyte growth factor (KGF), involved in tissue regeneration and modulation of inflammatory events (Anitua *et al.*, 2018). Other relevant components are cytokines (in particular IL6, TNFα, SDF1α, C-X-C motif chemokine ligand 8/interleukin 8 (CXCL8), or macrophage inflammatory protein 1 alpha (MIP1α)), which are mainly involved in the immune response but also play roles in angiogenesis and vascular modeling. Platelet α-granules also contain adhesive proteins (including vWF pro-peptide and laminins), involved in cell contact interactions, homeostasis, and ECM composition, in addition to clotting factors (such as factor V/Va and antithrombin III), involved in thrombin production and regulation. Furthermore, they contain fibrinolytic factors (such as α-2-macroglobulin (A2M) or plasminogen), related to plasmin production and vascular modeling, and proteases and anti-proteases (such as tissue inhibitor of MMPs 1–4) also related to vascular modeling in addition to angiogenesis, regulation of coagulation and cell behavior. These granules even contain antimicrobial peptides (Anitua *et al.*, 2010).

4.3.4. Use of platelet-rich plasma in gynaecology

Over the last two decades, PRP has been used in various medical specialties such as dental (Albanese *et al.*, 2013), plastic surgery (Cervelli *et al.*, 2009), orthopedics (Dragoo *et al.*, 2014), and dermatology (Leo *et al.*, 2015). However, PRP has only just recently emerged in gynaecology. New reports describe its beneficial effect in the ovary, for treating poor responders (Aflatoonian *et al.*, 2021) or polycystic ovarian syndrome patients (Anvari *et al.*, 2019), and the endometrium, for EA, AS, or even recovery after cesarean sections (Tehranian *et al.*, 2016).

Promising results of PRP treatment have been reported from *in vitro* experiments. Marini and colleagues pioneered the use of PRP in reproductive studies by culturing bovine endometrial cells with PRP supplementation, which increased

proliferation rates and the expression of genes related to a healthy endometrium (Marini *et al.*, 2016). In the same year, Anitua's group cultured human endometrial fibroblasts with PRP and reported increased cell proliferation and migration rates in addition to a higher expression of VEGF and procollagen type I, responsible for ECM remodeling (Anitua *et al.*, 2016). After applying PRP over human endometrial cells, proliferation and migration rates, in addition to the expression of MMPs and cytokines, were also evaluated by Aghajanova in 2018. This work ratified the positive effect of plasma activation and the higher regenerative potential of the PRP over the PPP fraction (Aghajanova *et al.*, 2018a).

Subsequently, studies using murine models of endometrial injury also supported the therapeutic benefits of PRP. For example, Jang's group reported a decreased fibrosis and increased expression of several markers of proliferation (Ki67), angiogenesis (*VEGF*), and normal endometrium function (cytokeratin, *HOXA10*) after the administration of autologous PRP (Jang *et al.*, 2017). Similarly, another group linked PRP administration with improved endometrial morphology, reduced fibrosis (lower expression of collagen type 1, TIMP metalloprotease inhibitor 1, and Tgfβ1), and better pregnancy rates (Kim *et al.*, 2020).

The enhanced action of PRP together with SCs has also been tested in rodent models. Platelet-rich plasma can increase the healing effect of menstrual blood-derived SCs (Zhang *et al.*, 2019) and BMDSCs (Zhou *et al.*, 2020) over endometrial morphology, expression of pro-regenerative factors, and fertility rates. Interestingly, the results from Zhang's group also elucidated that PRP promotes the SC paracrine action (as measured by the human *IGF1*, *SDF1*, and *TSP1* levels).

Finally, PRP therapy has been tested in EA and AS patients. Since the first report of five EA women successfully treated with PRP in 2015 (Chang *et al.*, 2015), a total of 12 different studies have described how the intrauterine administration of PRP can increase endometrial thickness, ultimately improving pregnancy rates. This compilation of studies includes randomized clinical trials (Eftekhar *et*

al., 2018; Nazari *et al.*, 2020; Javaheri *et al.*, 2020), cohort studies (Tandulwadkar *et al.*, 2017; Zadehmodarres *et al.*, 2017; Molina *et al.*, 2018; Chang *et al.*, 2019; Kim *et al.*, 2019; Frantz *et al.*, 2020), and case series (Farimani *et al.*, 2016; Colombo *et al.*, 2017; Aghajanova *et al.*, 2018a). Notably, Javaheri's work reported that PRP did not make any difference after treating AS (Javaheri *et al.*, 2020), suggesting that this treatment could benefit EA patients more than those with AS.

4.3.5. Platelet-rich plasma from other blood sources

The regenerative effects of PRP from peripheral adult blood could be enhanced if plasma derived from younger sources, such as the umbilical cord blood, as highlighted in different tissues (Orlando *et al.*, 2020). This new potential source of PRP is still widely unexplored but emerging as a promising therapeutic option.

Different *in vitro* studies have reflected how the most relevant biomolecules secreted by platelets (described in Section 4.3.3), such as platelet-derived growth factor (PDGF) or VEGF, are more concentrated in PRP from the umbilical cord than from adults (Parazzi *et al.*, 2010; Murphy *et al.*, 2012). On the other hand, the umbilical cord plasma contains lower concentrations of pro-inflammatory cytokines (Ehrhart *et al.*, 2018), which corroborates with its immune immaturity. This plasma source positively affects proliferation and migration rates of UCMSCs (Caseiro *et al.*, 2018), dental SCs (Lee *et al.*, 2011), or even fibroblasts (Hashemi et al., 2017). Furthermore, murine studies have reported the beneficial effect of cord plasma over hippocampal function (Castellano *et al.*, 2017) and aging (Bae *et al.*, 2019). Notably, several ongoing clinical trials are using this PRP for treating disorders such as retinitis pigmentosa (NCT04636853), neurotrophic keratopathy (NCT03084861), and frailty (NCT03229785). In gynaecology, Herraiz's group recently reported how this plasma source could effectively recover ovarian function in mice (Buigues *et al.*, 2021). Taken together, these studies substantiate the use of umbilical cord blood as a promising source of PRP.

II. HYPOTHESIS

HYPOTHESIS

The central hypothesis of this thesis dissertation was to demonstrate if specific biomolecules with paracrine effects could be used to treat infertility-causing endometrial pathologies, such as endometrial atrophy or Asherman's Syndrome.

More specifically, we hypothesized:

I. bone marrow-derived stem cells secrete specific paracrine molecules which could activate the endogenous endometrial adult stem cell niche to promote endometrial regeneration.

II. proteins and growth factors released from platelets isolated from human plasma (from various sources) can stimulate healing, growth, and regenerative processes in the pathologic endometrium.

Identifying these factors and elucidating their mechanisms of action, represents the first step in translating these promising therapies to widespread clinical use.

III. OBJECTIVES

MAIN OBJECTIVE

The main objective of the herein thesis dissertation was to explore alternative therapeutic approaches for treating the pathologic endometrium of women with endometrial atrophy and/or Asherman's Syndrome.

SPECIFIC OBJECTIVES

Each of the four manuscripts included in the herein Ph.D. dissertation had its own specific objective:

- **Manuscript I**: To determine the genetic and paracrine mechanisms behind endometrial regeneration in endometrial atrophy and/or Asherman's Syndrome patients after autologous $CD133^{+}$ bone marrow-derived stem cells transplantation.
- **Manuscript II**: To study the effect of human plasma, either from umbilical cord or adult peripheral blood, on the regeneration of endometrial damage using *in vitro* assays and a murine model of uterine damage.
- **Manuscript III**: To review and discuss the main milestones in the identification and characterization of endometrial adult stem cells in murine and human models.
- **Manuscript IV**: To compile and study the currently available therapeutic options for endometrial atrophy and/or Asherman's Syndrome patients, with a special focus on those testing different approaches in murine models of endometrial damage.

IV. METHODOLOGY: GUIDING LINE AMONG ARTICLES AND STUDY DESIGNS

1. RATIONALE AND COHERENCE AMONG THE MANUSCRIPTS

This section describes the coherence, unifying thread, and study designs of the four scientific articles (peer-reviewed) compiled in this Ph.D. dissertation. All four articles align with a well-defined and planned research work to study putative therapeutic solutions for two infertility-causing endometrial pathologies: EA and AS. In this line, the present Ph.D. dissertation mainly addresses the role of ASCs (as part of the endometrial SC niche and as a therapeutic approach) and PRP for treating these two endometrial disorders.

Manuscript I, entitled 'Stem cell paracrine actions in tissue regeneration and its potential therapeutic effect in human endometrium: a retrospective study,' focuses on determining gene and protein expression changes as well as paracrine mechanisms underlying endometrial regeneration in both EA/AS patients and an AS murine model after $CD133^{+}$BMDSCs injection. This study reaffirmed the significance of the paracrine mechanism of action of $CD133^{+}$BMDSCs: the molecules secreted by the SCs are the final effectors of the regenerative events rather than the cells themselves. Obtaining these results led us to consider whether other biological products containing similarly acting molecules could become alternatives to SC therapy. Despite its effectiveness, SC therapy is usually costly, an invasive methodology for the patients, and requires specific equipment. In this context, PRP is an easily-obtained blood derivative and seemed to be a promising alternative for EA/AS patients.

Manuscript II, entitled 'Comparison of different sources of platelet-rich plasma as a treatment option for infertility-causing endometrial pathologies,' aims to study the regenerative effect of human plasma (obtained from the umbilical cord and peripheral adult blood) on a damaged endometrium. This article demonstrates how adult PRP promotes different biological processes involved in

tissue regeneration (cell proliferation and migration) *in vitro*, and enhances endometrial regeneration in an AS murine model. Remarkably, these pro-regenerative effects of plasma were more apparent when the plasma was obtained from umbilical cord blood.

Manuscript III, entitled 'Stem cells and the endometrium: from the discovery of adult stem cells to pre-clinical models,' includes a literature review about the discovery of endometrial ASCs in mice and humans, describing the different ASC identification and isolation approaches and how findings have promoted the SC therapies for endometrial pathologies, in addition to the evolution towards paracrine-based strategies and other emerging techniques (including bioengineering applications).

Finally, **Manuscript IV**, entitled 'Strategies for managing Asherman´s Syndrome and endometrial atrophy: since the classical experimental models to the new bioengineering approaches' includes a bibliographic review about the current (including classical therapeutic management with estrogens or SCs) and emerging (for example, PRP, exosomes derived from SCs, bioengineering-based techniques, and endometrial organoids) therapeutic and experimental approaches for endometrial regeneration, with a special focus on EA/AS murine models to highlight the importance of the animal (preclinical) models preceding therapeutic implementation in humans.

2. STUDY DESIGNS OF THE EXPERIMENTAL MANUSCRIPTS

2.1. Manuscript I: Stem cell paracrine actions in tissue regeneration and potential therapeutic effect in human endometrium: a retrospective study

This article was based on the success of CD133⁺BMDSCs therapy in EA/AS patients (Santamaría *et al.*, 2016) and an AS murine model (Cervelló *et al.*, 2015). Using the uterine samples from these two previous studies (hence, the term retrospective in the title of the manuscript), a deeper analysis was designed to elucidate how the endometrial tissue responded to the SC treatment (Figure 18).

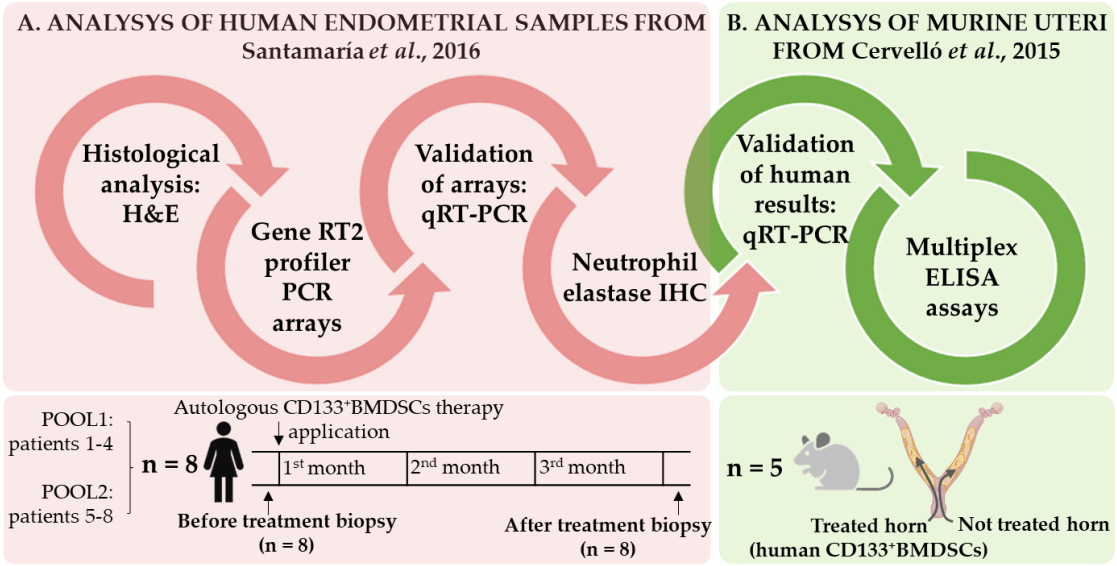


Figure 18. Experimental design of Manuscript I. *A. Experiments with the human endometrial samples from Santamaría et al., 2016. Starting from FFPE human endometrial tissue blocks, samples were stained with H&E to analyze the endometrial morphology. Then, RNA was extracted (n = 8 for both before and after treatment conditions; grouped in two pools of four samples each) to perform specific arrays containing gene targets related to tissue regeneration. Results were then validated by qRT-PCR. Finally, the detection of neutrophil elastase protein by IHC reaffirmed the down-regulation of CXCL8, detected in the arrays. B. Experiments using murine uterine samples from Cervelló et al., 2015. Starting from FFPE blocks, qRT-PCR was performed to validate the human arrays' results in the murine model. Subsequently, ELISA multiplex analyses were performed to evaluate the protein expression of specific regeneration-related cytokines, chemokines, and angiogenic factors in the murine endometrium. BMDSCs: bone marrow-derived stem cells; CD: cluster of differentiation; ELISA: enzyme-linked immunosorbent assay; FFPE:*

formalin-fixed paraffin-embedded; H&E: hematoxylin and eosin; IHC: immunohistochemistry; qRT-PCR: quantitative real-time polymerase chain reaction; SC: stem cells. Created with www.BioRender.com.

2.2. Manuscript II: Comparison of different sources of platelet-rich plasma as treatment option for infertility-causing endometrial pathologies

The figures below show a schematic representation of the samples collected for this study (Figure 19A) and the PRP obtaining process (Figure 19B).

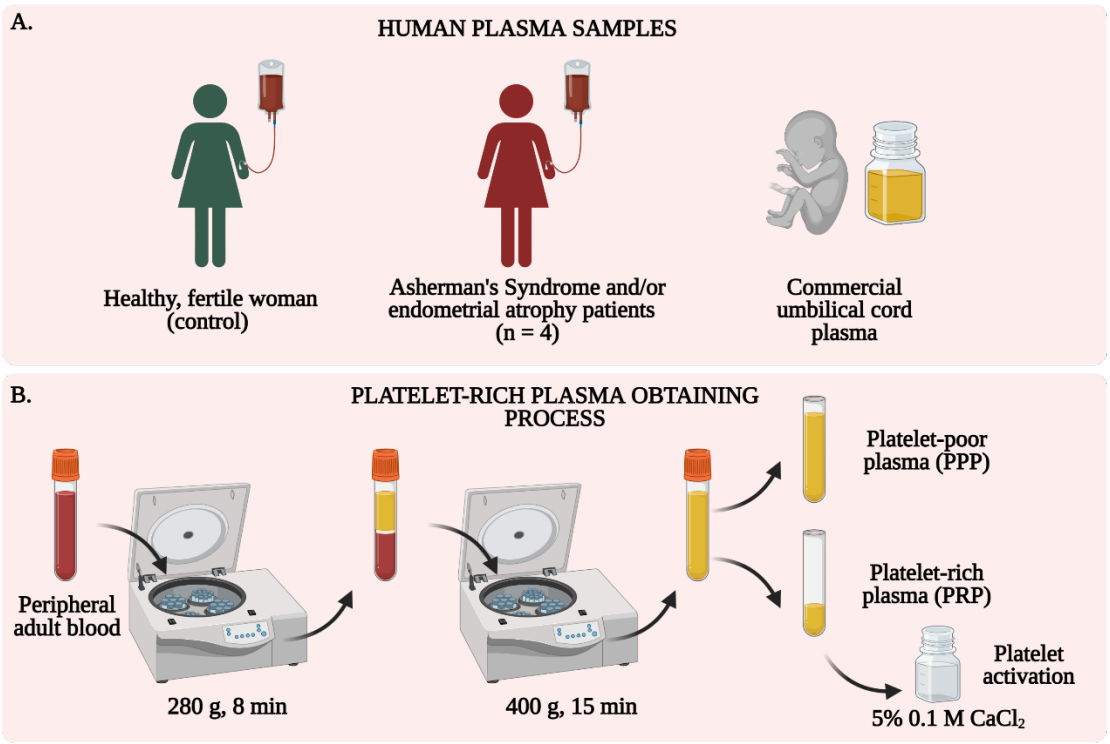


Figure 19. Experimental design of Manuscript II: sample collection and processing. *A. Compilation of the different human plasma samples collected for the study, either from adult blood (healthy, fertile woman and endometrial atrophy/Asherman's Syndrome patients) or the umbilical cord. B. Platelet-rich plasma obtaining process: two sequential centrifugations (280g/8min/room temperature followed by 400g/15min/room temperature) to obtain the poor (upper part) and the rich (lower part) platelet fractions. When required, plasma samples were activated using $CaCl_2$. $CaCl_2$: calcium chloride; g: relative centrifugal force; min: minutes. Created with www.BioRender.com.*

Protein composition of the plasma samples was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and multiplex enzyme-linked immunosorbent assays (ELISAs; Figure 20A), while *in vitro* models were used to assess the action of plasma over cell proliferation and

migration processes (Figure 20B), which are key events in tissue regeneration (Zhao *et al.*, 2016).

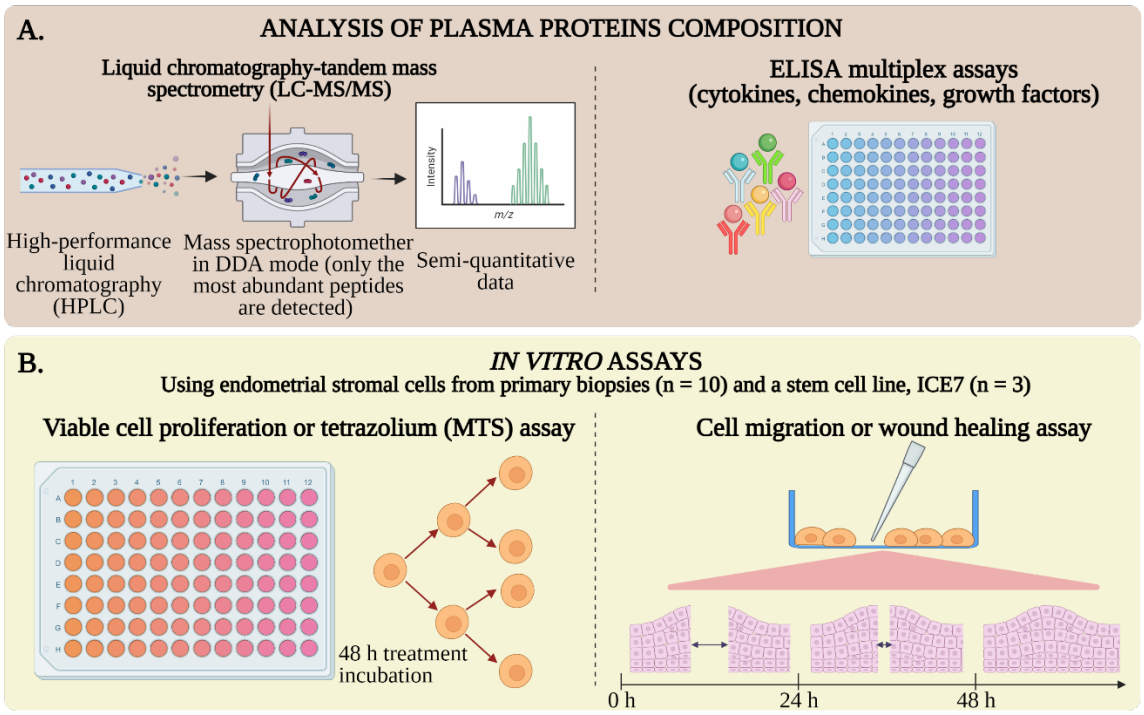


Figure 20. Experimental design of Manuscript II: plasma analysis and in vitro experiments. *A. Plasma protein content was analyzed using mass spectrometry (descriptive approach) and multiplex ELISA (quantitative approach). B. The regenerative action of plasma samples was evaluated using in vitro cell proliferation and migration assays. DDA: data-dependent acquisition. ELISA: enzyme-linked immunosorbent assay; MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt. Created with www.BioRender.com.*

Moreover, *in vivo* experiments were performed in an AS murine model (Figure 21).

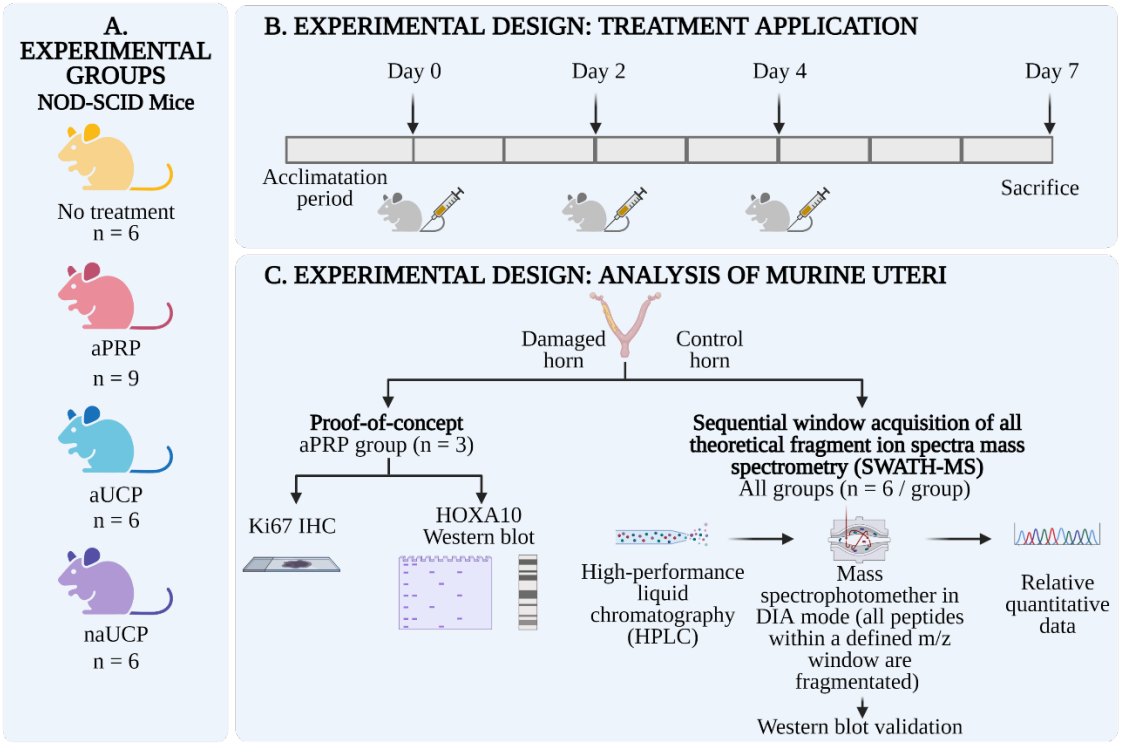


Figure 21. Experimental design of Manuscript II: AS murine model. *A. Experimental groups of NOD-SCID mice. B. Diagram of the treatment application protocol for all mice. Only left uterine horns were mechanically damaged (right horn remained as a control). C. Analysis of uterine horns: three mice from aPRP group were considered a proof-of-concept for evaluating the protein expression of Ki67 (IHC) and HOXA10 (western blot) while the rest of uterine horns (n = 6/group) were analyzed by mass spectrometry (proteomic results were subsequently validated by western blot). aPRP: activated platelet-rich plasma; aUCP: activated umbilical cord plasma; DIA: data-independent acquisition. naUCP: non-activated umbilical cord plasma; HOXA10: homeobox protein Hox-A10; IHC: immunohistochemistry; NOD-SCID: non-obese diabetic - severe combined immunodeficiency. Created with www.BioRender.com.*

2.3. Manuscript III: Stem cells and the endometrium: from the discovery of adult stem cells to pre-clinical models

The aim of this literature review was to summarize good-quality peer-reviewed studies regarding the characterization of the different types of endometrial SCs in the human and mouse endometrium. This article not only sheds light on the different approaches used to identify the forementioned cells in both models, but also describes how these findings promoted SC therapies for endometrial pathologies, evolving towards paracrine- and bioengineering-based approaches.

PubMed and Google Scholar databases were searched for articles, including both human and murine studies, published in English from 1978 to December 2020. The search included the following main keywords alone or in combination: 'adenomyosis', 'animal model', 'bone marrow', 'endometrial carcinoma', 'endometrial hyperplasia', 'endometriosis', 'endometrium', 'side population', 'label-retaining cells', 'stem cell markers', 'stem cell therapy', and 'stem cells'. Studies were selected according to their titles and abstracts. Articles were examined in detail, and a total of 74 full manuscripts were included in this review, including 41 articles addressing the identification of endometrial SCs in both murine models and humans, ten articles covering the role of SCs in endometrial pathologies, 15 articles about SC therapies for treating the endometrium, and eight articles discussing future perspectives and next steps. References from selected studies were used to identify and read additional literature.

2.4. Manuscript IV: Strategies for managing Asherman´s Syndrome and endometrial atrophy: since the classical experimental models to the new bioengineering approaches

This second bibliographic search summarizes the different available therapeutic options for treating EA and AS. The article first reviews the classical treatments for these two endometrial pathologies, then delves into the therapeutic use of SC therapies, growth factors, and other molecules, ending with an analysis of the latest therapeutic alternatives that have emerged (including PRP, tissue- and bio-engineering solutions, and organoids). Throughout the manuscript, the main focus lies on animal models of endometrial damage rather than human studies. The importance of establishing pre-clinical models for correctly translating these approaches into humans is pointed out throughout the text.

PubMed and Google Scholar databases were searched for articles published in English from 1997 to December 2020. The search included the following main keywords alone or in combination: 'animal model', 'Asherman's Syndrome', 'bioengineering', 'endometrial atrophy', 'endometrium', 'growth factors', 'hydrogel', 'microfluidics', 'murine model', 'organoids', 'platelet-rich plasma', 'scaffold', 'stem cells', and 'thin endometrium'. Studies were selected according to their titles and abstracts and a total of 88 full manuscripts were included in this review, including ten articles covering the classical management for EA and AS, 16 articles about SC therapies, 12 articles discussing the use of GFs and other molecules, 23 articles discussing the PRP approach, and 27 articles covering tissue- and bio- engineering approaches. Reference lists from selected articles and previous reviews in the field were used to identify additional literature.

V. STUDY RESULTS

1. MANUSCRIPT I

1.1. Citation

de Miguel-Gómez, Lucía*; Ferrero, Hortensia*; López-Martínez, Sara; Campo, Hannes; López-Pérez, Nuria; Faus, Amparo; Hervás, David; Santamaría, Xavier; Pellicer, Antonio; Cervelló, Irene. **Stem cell paracrine actions in tissue regeneration and potential therapeutic effect in human endometrium: a retrospective study.** BJOG: An international journal of obstetrics and gynaecology. 2020; 127(5):551-560. *Co-author-ship. Journal 2020 impact factor (IF): **6.531**; DOI: https://doi.org/10.1111/1471-0528.16088

A mini commentary by an external author accompanies this article in the journal: Taylor, Hugh S. **Therapeutic endometrial regeneration: clinical application of bone marrow-derived stem cells.** BJOG: An international journal of obstetrics and gynaecology. 2020; 127(5):561. DOI: https://doi.org/10.1111/1471-0528.16088

1.2. Summary of the results

The principal aim of this work was to study the molecular mechanisms of injected human $CD133^{+}$BMDSCs in endometrial repair. This study emerged from the previously reported paracrine action of these SCs (see section 4.2.3). This paracrine hypothesis arose from the following evidence reported in the animal model: (1) rather than arriving directly at the site of injury, the injected cells engrafted around the uterine blood vessels, where the endometrial SC niche location has been postulated; (2) there was minimal $CD133^{+}$BMDSCs engraftment (< 1%) in the endometrial tissue, which suggests that *in situ* SC replication and differentiation is not what triggers the regeneration process. Instead, these studies suggested the molecules these cells secrete and/or the endometrial factors they modulate may be stimulating the endogenous SC niche (Cervelló *et al.*, 2015).

Briefly, two different types of samples were analyzed, human (Santamaría *et al.*, 2016) and murine (Cervelló *et al.*, 2015), from two previous studies from the group. These studies were performed in parallel, with the human $CD133^{+}$BMDSCs used in the animal model originating from the patients enrolling in the pilot study.

1.2.1. Analysis of human endometrial samples

In the first part of the study (human model), all samples were analyzed in collaboration with an external pathologist (Figure 1 in Manuscript I). Staining with hematoxylin (which stains nuclei a blue-purple color) and eosin (which stains cytoplasm and ECM a pink color) revealed improved endometrial morphology three months after the SC therapy. The endometrial stroma appeared well-organized, with an enhancement in gland morphology, in the majority of the patients (patients 1, 3, 4, 5, and 8) suggesting that the endometrium might be returning to its functional form.

The results of the human gene arrays (including specific arrays for *EGF*/*PDGF* signaling pathway, human GFs, and human angiogenic GFs) disclosed the statistically differential expression of five genes (from a total of 252 genes) in both pools when comparing human samples before and after treatment. Concretely, JUN proto-oncogene (*JUN*; $P = 0.037$), plasminogen activator inhibitor-1 (*SERPINE1*; $P = 0.026$), and *IL4* ($P = 0.041$) were up-regulated after administrating the treatment, while cyclin D1 (*CCND1*; $P = 0.043$), and *CXCL8* ($P = 0.036$) were down-regulated. Notably, the up-regulated genes take part in seven signal transduction pathways: *JUN* is related to Wnt, MAPK, and TNF pathways; *SERPINE1* is linked to Wnt, HIPPO, and TGFβ pathways; and *IL4* is related to JAK-STAT, and PI3K-Akt pathways. See Figure 2A-B in Manuscript I for the detailed results.

A total of three of the five detected genes were validated by qRT-PCR in the human endometrial samples (to validate the array results), and in the murine uterine samples (to see if the regeneration process was comparable between models).

In the human samples, we validated the gene array results of the following three genes: *JUN* (fold regulation, FR = 1.429), *CXCL8* (FR = -26.546), and *CCND1* (FR = -1.434; Figure 2B in Manuscript I). Notably, *CXCL8*, involved in neutrophil activation, showed the most significant change in gene expression values (with the greatest absolute FR). To reinforce and test the effect of *CXCL8* down-regulation in the human endometrium, neutrophil elastase (a serine protease secreted by macrophages and neutrophils during inflammatory processes) expression was evaluated by immunohistochemistry. The results of this assay corroborated with the *CXCL8* down-regulation pattern after the SC treatment, showing a statistically significant decrease (P = 0.025) of neutrophil elastase expression after treatment in all human patients (n = 8; Figure 3 in Manuscript I).

Whereas in the murine samples, the following three genes were validated: *JUN* (FR = 1.215), *SERPINE1* (FR = 2.231), and *CCND1* (FR = -2.921). This final validation corroborated that the $CD133^{+}$BMDSCs triggered the same biological events as in the human endometrial tissue, by assessing the paracrine mechanism and permitting the extrapolation of the murine results to the human events (Figure S1 in Manuscript I).

1.2.2. Analysis of murine uterine samples

Lastly, two multiplex protein arrays were used to evaluate the expression of a total of 48 target proteins in both the treated (n = 5) and non-treated (n = 5) damaged murine horns. From all the proteins analyzed (n = 48), four of them showed a statistically significant higher expression in treated horns in comparison with the non-treated (control) ones: IL18 (P = 0.034), HGF (P = 0.024),

monocyte chemoattractant protein 1 (MCP1; otherwise known as C-C motif chemokine 2; P = 0.014), and macrophage inflammatory protein 2 (MIP2; otherwise known as C-X-C motif chemokine 2; P = 0.028). Other interesting proteins such as VEGFA, FGF2, β-cellulin, TNFα, and IL10 also tended towards a higher expression in the treated horn; however, the results were not statistically significant. See Figure 4 in Manuscript I for more detailed results.

The results of this study, together with the previous publications suggesting the paracrine action of the SCs (Cervelló *et al.*, 2015; Santamaría *et al.*, 2016), allows the postulation of the following hypothesis: "human CD133$^+$BMDSC activate several pro-regenerative factors through a paracrine mechanism to promote tissue regeneration, modifying endometrial behavior through an immunomodulatory milieu that precedes proliferation and angiogenic processes". According to this premise, the ASCs could be releasing specific paracrine factors that act on downstream target genes. The unique regulation pattern of these target genes, underlying the therapeutically potential of CD133$^+$BMDSCs, would describe an immunomodulatory scenario preceding a dynamic tissue regeneration. The observed decrease in the *CXCL8* gene and the human neutrophil elastase expression could probably be reflecting the shift in the inflammatory response immediately after treatment. Meanwhile, the up-regulation of *JUN*, *SERPINE1*, and *IL4*, could be related to the pro-regenerative events after the first phase of immunomodulation. The mouse model results supported this hypothesis with the detected up-regulation of key angiogenic and repair factors (IL18, HGF, MCP1, and MIP2).

In summary, SC therapy could create an immunomodulatory milieu (through the suppression of *CXCL8* and *CCND1* expression) which paves the way for pro-regenerative events (through the up-regulation of *JUN, SERPINE1,* and *IL4*) responsible for endometrial tissue remodeling.

2. MANUSCRIPT II

2.1. Citation

de Miguel-Gómez, Lucía; López-Martínez, Sara; Campo, Hannes; Francés-Herrero, Emilio; Faus, Amparo; Díaz, Ana; Pellicer, Antonio; Domínguez, Francisco; Cervelló, Irene. **Comparison of different sources of platelet-rich-plasma as treatment option for infertility-causing endometrial pathologies.** Fertility and sterility. 2021; 115(2):490-500. Journal 2020 IF: **7.329**; DOI: https://doi.org/10.1016/j.fertnstert.2020.07.053

A reflection by external authors accompanies this article in the journal: Subiran, Cristina; Kristensen, Stine Gry; Andersen, Claus Yding**. Umbilical cord blood-derived platelet-rich plasma: a clinically acceptable substitute for fetal bovine serum?** Fertility and sterility. 2021; 115(2):336-337. https://doi.org/10.1016/j.fertnstert.2020.10.027

2.2. Summary of the results

The main aim of this work was to study the potential effect of a blood derivate, PRP, on endometrial regeneration as a possible treatment for patients with EA/AS. With this purpose, the project was divided into the following three parts: PRP characterization, an *in vitro* study of the regenerative effect of PRP over human endometrial cells, and an *in vivo* study of the regenerative effect of PRP over the endometrium using an AS murine model.

Three main types of plasma samples were considered throughout the study: activated PRP from four EA/AS patients (aPRP1-4), activated commercial plasma from the umbilical cord (aUCP), and non-activated commercial plasma from the umbilical cord (naUCP). In addition, an extra control plasma fraction from a healthy, fertile woman, which was activated (aPRPC) or non-activated (naPRPC),

was used only to verify that EA/AS does not affect plasma from patients. Platelet-poor plasma fractions from the EA/AS patients' plasma were also considered at specific points of the study.

2.2.1. Analysis of platelet-rich plasma composition using proteomics and multiplex ELISAs

The LC-MS/MS proteomic results obtained from this study are uploaded in ZENODO, an open-access dataset repository (https://zenodo.org/record/5180821#.YRPpgYgzaUk).

The analysis of this data revealed the presence of 237 proteins in aPRP samples (n = 3, because the fourth aPRP sample was exclusively used in a proof-of-concept using the animal model; it is important to note that only proteins detected in at least two of the three aPRP samples were considered), 616 proteins in aUCP, and 331 in naUCP. Among all the proteins detected, TSP1, A2M, vWF, neural cell adhesion molecule 1 (NCAM1), or IGF2 were found in all plasma samples. Meanwhile, other relevant proteins such as CD109 antigen, angiopoietin-related protein 3 (ANGPTL3), and insulin-like growth factor-binding protein 4 (IGFBP4), were detected only in naUCP and multimerin 1 (MMRN1), pyruvate kinase (PKM), and peroxiredoxin 1 (PRDX1) were exclusively found in aUCP. Finally, adenosine deaminase 2 (ADA2), 14-3-3 protein gamma (YWHAG), and macrophage migration inhibitory factor (MIF) were only found in aPRP. See Figure 2A in Manuscript II for detailed results.

KEGG pathway analysis was performed for aUCP and aPRP samples (Figure 2B in Manuscript II). This analysis revealed that among others, the PI3K-Akt signaling pathway was shared by proteins detected in both samples, whereas the Hippo pathway was unique to the aUCP fraction, and MAPK and Rap1 pathways were exclusive to the aPRP fractions.

Despite not being included in Manuscript II, other results from this LC-MS/MS analysis are outlined here. Gene ontology (GO) analysis revealed that the most represented biological processes (BPs) were related to coagulation, complement activation, platelet aggregation, and cholesterol metabolic process in all samples, which are all intrinsic functions of the human blood. These BPs were the most represented due to proteins such as apolipoprotein B-100 (APOB100), complement C4-B (C4B), or fibrinogen α1 chain (FGA), which presented the highest Unused ProtScore values (protein confidence measurement for a detected protein, calculated from the peptide confidence for peptides from spectra that are not already wholly used by higher-scoring winning proteins) in all samples. Nevertheless, when searching for proteins involved in other functions, the attention was directed towards molecules involved in BPs related to tissue regeneration (Zhao *et al.*, 2016), such as wound healing or cell population proliferation in addition to proteins detected inside platelet α-granule lumens (Figure 22). As depicted in Figure 22, a higher number of proteins in UCP fractions (mainly aUCP) were involved in regeneration-related processes and had a higher significance (higher values of –Log10 *P* correspond with lower *P* values). Among these proteins, the previously mentioned TSP1, A2M, vWF, NCAM1, IGF2, CD109, ANGPTL3, IGFBP4, MMRN1, PKM, PRDX1, ADA2, YWHAG, and MIF were included.

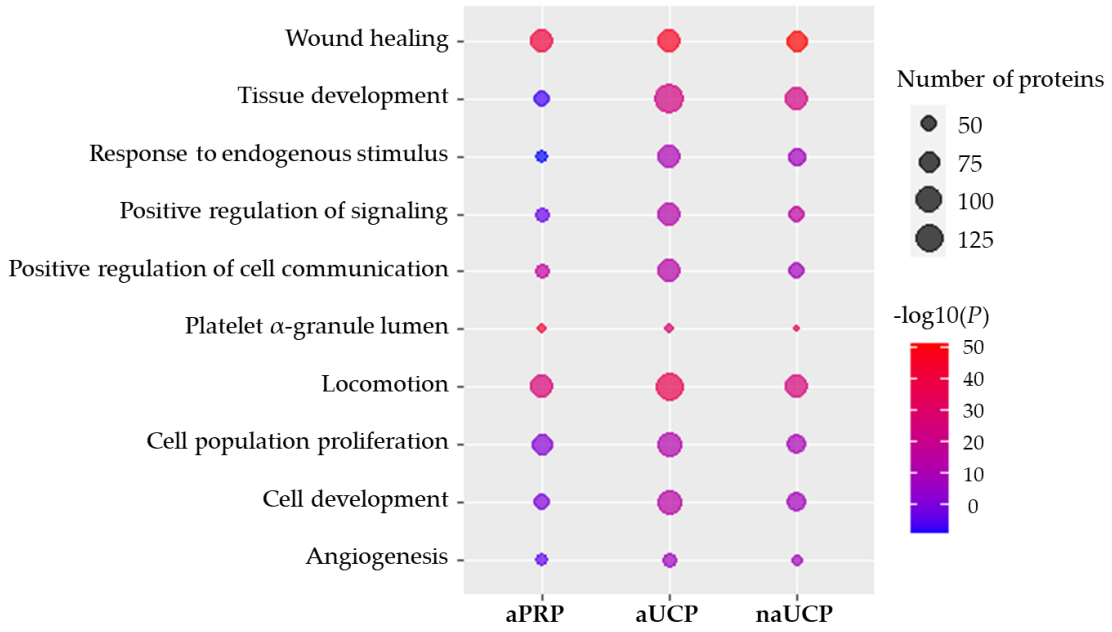


Figure 22. GO analysis of proteins detected in PRP, aUCP, and naUCP fractions by LC-MS/MS. *Bubble chart of the nine biological processes involved in tissue regeneration, according to Zhao et al., 2016, in addition to the cellular component term platelet α-granule lumen. Dot size is directly proportional to the number of proteins involved in each of the GO terms, while the color gradient correlates with the P-value for each GO term (values obtained after the functional enrichment performed with G-profiler software). aPRP: activated platelet-rich plasma; aUCP: activated umbilical cord plasma; LC-MS/MS: liquid chromatography-tandem mass spectrometry; naUCP: non-activated umbilical cord plasma; P: p-value. Graph created with RStudio environment.*

Notably, the LC-MS/MS results for the healthy, fertile patient plasma fractions (aPRPC and naPRPC) were not included in the manuscript. Figure 23 shows the descriptive composition (Figure 23A-B) and functional (Figure 23C-D) analyses comparing the aPRPC fraction to the EA/AS patients' plasma samples (aPRP). Remarkably, this analysis corroborated that plasma from patients with infertility-causing endometrial disorders was not distinguishable from plasma from a healthy, fertile woman. Comparisons were made by either considering all four samples individually (Figure 23A and C) or grouping the three patient samples (but only considering the proteins detected in at least two of the samples; Figure 23B and D). This analysis revealed that most of the proteins detected in the healthy, fertile woman's plasma overlapped with the ones detected in the EA/AS patients (Figure 23A-B), and unrelated proteins mostly included

immunoglobulin variable chains and isoforms from proteins already detected in the patients' plasma fractions. The KEGG functional analysis corroborated this apparent low relevance of the proteins exclusively detected in the aPRPC fractions. In addition, key metabolic pathways involved in pro-regenerative related-events were identified in all samples (Rap1 and PI3K-Akt signaling pathways) or at least in two of them (MAPK signaling pathway; Figure 23C-D).

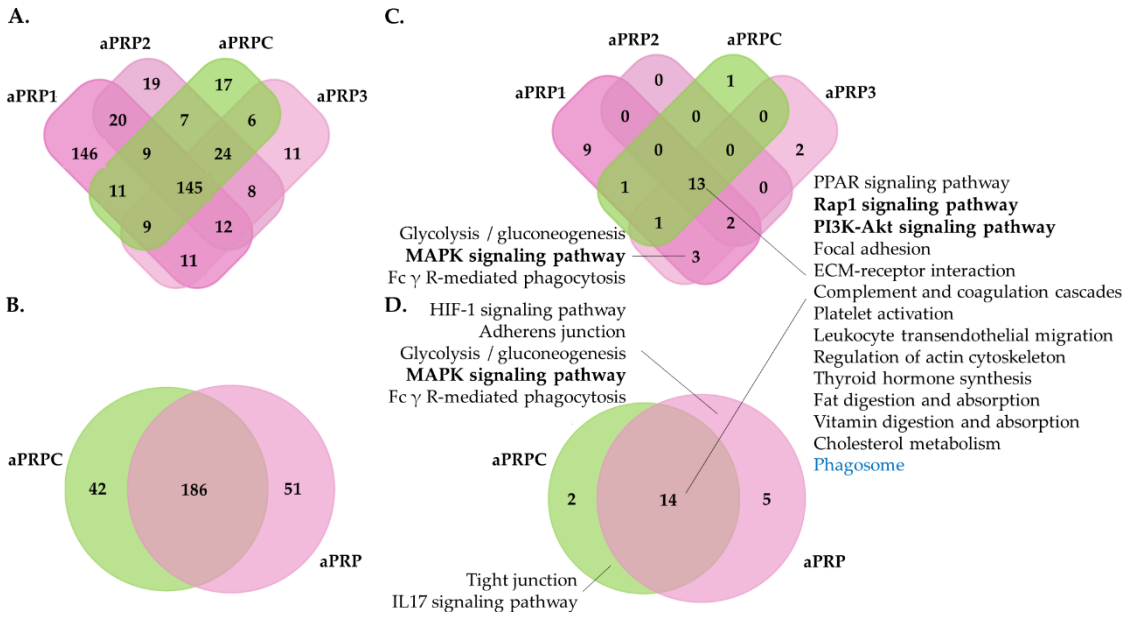


Figure 23. Composition and functional analysis of platelet-rich plasma samples based on the LC-MS/MS data. *A. Venn diagram showing the number of shared proteins between the aPRPC from a healthy fertile patient and the individual PRP fractions from three EA/AS patients. B. Venn diagram showing the number of shared proteins between the aPRPC from a healthy fertile patient and the aPRP fraction (including only proteins common to at least two of the three EA/AS patients). C. Venn diagram showing the shared KEGG pathways among the proteins detected in the aPRPC from a healthy fertile patient and the individual PRP fractions from EA/AS patients. D. Venn diagram showing the shared KEGG pathways among the proteins detected in the aPRPC and the aPRP fraction (common proteins in at least two of the three patients) from EA/AS patients. Note that the phagosome pathway marked in blue is attributable only to diagram D. Venn diagrams created with FunRich functional enrichment analysis tool. aPRP: activated platelet-rich plasma; aPRP: activated platelet-rich plasma control (healthy, fertile woman); ECM: extracellular matrix; HIF1: hypoxia-inducible factor 1; IL: interleukin; LC-MS/MS: liquid chromatography-tandem mass spectrometry; MAPK: mitogen-activated protein kinase; PI3K-Akt: phosphoinositide 3-kinase/protein kinase B; PPAR: peroxisome proliferator-activated receptor; Rap1: ras-associated protein 1.*

The analysis of GFs and cytokine concentrations in the plasma samples using multiplex ELISAs revealed a statistically higher concentration of certain GFs in

both aUCP and naUCP fractions than aPRP (Figure 2C and Supplemental Table I in Manuscript II). These GFs were HGF, PDGFBB, TGFβ, VEGFD, and stem cell factor (SCF, also known as Kit ligand). Notably, IL1 receptor antagonist protein (IL1RA) was more concentrated in the UCP fractions. Only IL15 presented the opposite trend, with a significantly higher concentration in aPRP than either UCP fractions. On the other hand, BDNF, β-nerve growth factor (bNGF), VEGFA, EGF, placental growth factor (PIGF), IL2, IL7, IL15, eotaxin, LIF, IL18, SDF1α, and MCP1 were detected in all samples, but differences were not statistically significant among fractions (see Manuscript II for the specific *P* values).

2.2.2. *In vitro* studies: evaluation of cell proliferation and migration

An *in vitro* study was used for evaluating cell proliferation and migration processes using primary (from endometrial biopsies collected from oocyte donors) and stem (ICE 7 line; Cervelló *et al.*, 2010) endometrial stromal cells. These approaches first required determining the optimal treatment concentration to add into the culture medium. With this aim, the ICE7 cell line was used to evaluate cell proliferation rates after applying different plasma concentrations, revealing the adequate and sufficient quantity of plasma to add was 1 % (Figure 3A in Manuscript II; please note that the X-axis should indicate % of treatment instead of $CaCl_2$).

First, the *in vitro* evaluation of the endometrial stromal cell proliferation rate revealed the significant positive effect of the plasma over this biological process, regardless of the source (plasma from the umbilical cord or EA/AS patients). However, this proliferation rate was even higher when the blood source was the umbilical cord instead of adult peripheral blood. The colorimetric assay, based on the reduction of the MTS tetrazolium salt by viable cells to generate a soluble colored formazan dye, revealed higher proliferation rates for the aUCP condition, in both the ICE7 cell line (n = 10; fold change, FC = 1.361) and hESCs (n = 3; FC =

1.797), rather than after treating with aPRP (FC = 1.221 and FC = 1.296 respectively). See Figure 3C-D in Manuscript II for more detailed results.

This approach was also used in validation steps prior to starting the experiments (see Figure 3B in the manuscript II). These initial validation probes were performed using the ICE7 cell line (n = 3). The preliminary studies corroborated the significantly increased effect ($P = 0.003$) of the aPRP (FC = 1.255) against the activated PPP fraction (FC = 0.913) over the proliferation rate. Furthermore, there was a noticeable effect of the platelet activation on cell proliferation: a significant change ($P = 0.017$) was observed when comparing aPRPC (FC = 1.202) with the No Treatment condition, while the difference was insignificant ($P > 0.050$) when comparing to the naPRPC fraction (FC = 1.135). Finally, cell proliferation results of aPRP1-3 from EA/AS patients (FC = 1.254) and aPRPC from a fertile, healthy patient (FC = 1.202) were comparable ($P > 0.050$).

Second, *in vitro* evaluation of cell migration was performed through a wound healing assay. The results obtained from this test appeared to corroborate the cell proliferation assay (Figure 3D in Manuscript II). There was a noticeably higher trend to cover the initial gap (in % of wound closure 24 hours after injury) when ICE7 cell line (n = 3) was treated with aPRP (42.196 %) or aUCP (49.400 %) in comparison with the No Treatment condition (38.770 %). This effect was reproducible after treating hESCs (n = 10), where aPRP (50.780 %) and aUCP (53.694 %) seemed to induce a higher wound closure rate than the No Treatment condition (42.522 %).

2.2.3. *In vivo* animal model of Asherman's Syndrome

The *in vivo* AS murine model first involved a preliminary group (n = 3) of mice treated with aPRP (using the fourth patient's sample which was not included in the *in vitro* analysis). Subsequently, in the main experiment, the uterine horns of mice from four different groups (n = 6 mice/group) corresponding to four distinct

conditions (No treatment, aPRP, naUCP, and aUCP) were analyzed by a large-scale quantitative proteomic technique: sequential window acquisition of all theoretical mass spectrometry (SWATH-MS).

The preliminary test showed that cell proliferation, measured by the immunodetection of the cell proliferation marker Ki67, was significantly increased (P = 0.011) in the damaged horns compared with the non-damaged (control) ones, suggesting active regenerative processes in the damaged area after aPRP administration. In addition, the expression of HOXA10, an important transcription factor for many genes involved in the endometrial function and development, showed a more intense signal (by immunohistochemistry) in damaged versus control horns of this proof-of-concept group. See Figure 4A in Manuscript II for these results.

In the main experiment, the SWATH-MS analysis of the damaged and non-damaged uterine horns (n = 48) from all groups (No treatment, aPRP, naUCP, aUCP) detected the expression of 2766 different proteins in the uterine tissues. This SWATH-MS raw data is uploaded in ZENODO, an open-access dataset repository (https://zenodo.org/record/5180621#.YRPpXogzaUk).

The comparison of the damaged versus control horns in the individual treatment groups (aPRP, naUCP, aUCP) revealed the following up-regulated proteins in the damaged horns of the aUCP group (Figure 4B in Manuscript II): phosphatidylinositol 3-kinase regulatory subunit α (P85A), Pr65 subunit of protein phosphatase 2A (2AAA), signal transducer and activator of transcription 5A (STAT5A) and, a small GTPase, the transforming protein RhoA (RHOA). All of which are known to be implicated in different events related to tissue regeneration, such as PI3K-Akt, TGFβ, or JAK-STAT signaling pathways. Notably, the up-regulated proteins in damaged horns from either aPRP or naUCP groups were unrelated to the mentioned pathways.

Comparing the damaged horns in each group against damaged ones in the other four groups showed how proteins group according to the treatment applied (Figure 4C in Manuscript II). Damaged uterine horns from the aUCP group showed up-regulation of NEDD8-activating enzyme E1 catalytic subunit (UBA3), Thy-1 membrane glycoprotein (THY1; also known as CD90 antigen), a ribonuclease inhibitor (RINI, also known as RNH1), and a mitochondrial membrane ATP synthase subunit O (ATPO). The last two proteins, together with serine/threonine-protein phosphatase PP1-alpha catalytic subunit (PPP1CA) and golgin subfamily A member 2 (GOLGA2), were up-regulated in the aPRP group. Sodium/potassium-transporting ATPase subunit beta-3 (ATP1B3) was up-regulated in naUCP group and a member of the chemokine-like factor superfamily, the CKLF-like MARVEL transmembrane domain-containing protein 6 (CKLF6), and the nucleoside diphosphate kinase B (NDKB), in the Not Treatment group.

Taken together, the results of this study suggest that adult PRP can promote endometrial regeneration and that these outcomes can improve if umbilical cord-derived plasma is used.

3. MANUSCRIPT III

3.1. Citation

de Miguel-Gómez, Lucía*; López-Martínez, Sara*; Francés-Herrero, Emilio*; Rodríguez-Eguren, Adolfo; Pellicer, Antonio; Cervelló, Irene. **Stem cells and the endometrium: from the discovery of adult stem cells to pre-clinical models**. Cells. 2021: 10(3): 595. *Co-author-ship. Journal 2020 IF: **6.600**; DOI: https://doi.org/10.3390/cells10030595

This work arose from an invitation for Dr. Irene Cervelló to collaborate in a **special issue** of the journal entitled '**Stem cell application in infertility**'.

3.2. Summary of the results

The main aim of this work was to review the different landmarks in the identification and isolation of ASCs in both murine and human endometrium (Figure 1 in Manuscript III). In addition, the article assesses the role of these cells in different endometrial pathologies and how SCs, not only from the endometrium but also from other sources, can have a therapeutic effect in regenerating the endometrial tissue, mainly in patients suffering from EA or AS, the two main pathologies focused on throughout this Ph.D. dissertation.

3.3. Endometrial stem cells and specific niches

Two main types of SCs have been identified in the human and murine endometrium: epithelial (usually referred to as epithelial progenitors in humans) and stromal (with both luminal and perivascular locations). In addition, the presence of endothelial SCs and the exogenous contribution of BMDSCs have been described.

As presented in Table I of Manuscript III, all reviewed procedures were summarized and classified based on endometrial SC identification and isolation techniques in both human and murine models.

In murine models, these cells are usually designated as LRCs. This singular description comes from their characteristic slow-cycling phenotype (compared to differentiated cells) that can be identified using either the BrdU DNA analog or the H2B-GFP labeling system. In both cases, the differentiated cells' faster proliferation and division cycles result in only the SCs retaining either BrdU or H2B-GFP after a given period of time. However, since longer retention of these compounds cannot exclusively be used to define stemness, and these label-retaining approaches strikingly cannot be performed in humans, other complementary methods, such as those described in the following paragraphs, need to be used.

The Hoescht 33342 side population approach is based on the abundant presence of verapamil-sensitive ATP-binding cassette (ABC) transporters in the SC membrane. Since the SCs can actively efflux the vital dye (Hoescht 33342) while differentiated cells retain it, these cell populations can be separated by flow cytometry.

Moreover, different functional assays can be used to evaluate characteristic features of the SCs, such as their multi-lineage differentiation potential (differentiation into adipocytes, osteoblasts, myocytes, and chondrocytes), the ability of single cells to survive, divide and form colonies (clonogenicity assay), and their indefinite self-renewal capability (long-term culture). Markedly, this last method has only been reported to isolate human ASCs, while the other two have also been described to identify murine endometrial ASCs.

Finally, specific SC markers beyond the classical CD used to differentiate the mesenchymal (CD44, CD90, CD105), endothelial (CD31, CD34), or hematopoietic

(CD34, CD45) phenotype have also been described. In mice, factors such as the binding transcription factor 4 (OCT-4), proto-oncogene c-Kit, or sal-like protein 4 (SALL4) have been used to identify epithelial LRCs. In the human endometrium, CD146/PDGFRb, SUSD2, LGR5, and NTPDase2 markers have been proposed for isolating endometrial stromal ASCs. While SSEA-1, N-cadherin, and NTPDase2, have been used to identify endometrial epithelial progenitor cells.

3.3.1. Stem cell therapy and the endometrium: the importance of basic research and pre-clinical models

The endometrial population of SCs, with a clear role in cyclic endometrial regeneration, has been described as key in developing different endometrial pathologies. This SCs' uncontrolled activity can lead to endometrial hyperplasia, contributing to endometrial carcinoma development if they shift to cancerous cells. Their potential involvement in endometriosis and adenomyosis pathogenesis, as contributors or initiators of ectopic endometrial tissue growth, has also been postulated.

3.3.2. Future perspectives and next steps

The article also incorporates the application of SC therapy for treating EA and AS in addition to how different types of cells (usually isolated using specific SC markers) have been used in animal models of endometrial injury. Finally, the manuscript includes some insights about emerging approaches that seem to improve or even replace the therapeutic action of SCs, mitigating some of their disadvantages, such as the risk of teratogenesis or low engraftment.

We gathered that despite the efforts and advances made by the scientific community to study the endometrial SC niche, there is still the need to find a universal and/or standardized methodology to correctly identify ASCs.

4. MANUSCRIPT IV

4.1. Citation

de Miguel-Gómez, Lucía; Romeu, Mónica; Pellicer, Antonio; Cervelló, Irene. **Strategies for managing Asherman's Syndrome and endometrial atrophy: since the classical experimental models to the new bioengineering approaches**. Molecular reproduction and development. 2021; 88(8):527-543. Journal 2020 IF: **2.609**; DOI: https://doi.org/10.1002/mrd.23523

4.2. Summary of the results

This work emanated from the detected need for an updated compilation of all the EA/AS therapeutic approaches. Reviewing the literature, we found several published reviews, but all of them were focused on human studies. With our experience in murine models of endometrial damage, we thought it could be helpful to the scientific community if we provided a manuscript focusing on preclinical studies using animal models.

Hence, the objective of this work was to review all the therapeutic options (including approaches who are already being implemented in clinical practice as well as emerging ones) for EA/AS patients. Despite the review of some human studies, the main focus of this work is on the studies using animal models (mainly rodents) of endometrial damage. See Figure 1 in Manuscript IV for a global overview of the study.

4.2.1. Classical management of EA and AS

The classical or most widespread treatments for women with either EA (including exogenous estrogens, GnRH agonist, hCG, aspirin, or tamoxifen) or AS (including hysteroscopic adhesiolysis, and aspirin) are not always effective for all patients. In fact, some studies even report contradictory results, as is the

case of sildenafil citrate or the combination of pentoxifylline with vitamin E for both EA and AS patients.

4.2.2. Stem cell therapies, growth factors, and other molecules

In the last decade, SC therapy has probably been the approach that has been the most effective treatment for these patients. Different works report the beneficial effects of SCs derived from different sources such as the bone marrow (probably the most applied and studied), amnion, umbilical cord, adipose tissue, and menstrual blood over a damaged endometrium. While others report the synergic effect of the SCs when administered together with certain pro-regenerative factors such as SDF1 or VEGF. In addition, the use of the G-CSF, a chemoattractant of BMDSCs, has also been studied. Nevertheless, the majority of these works are in humans, and the results are sometimes contradictory.

As the study of SC therapy has progressed, the hypothesis of their paracrine action has strengthened (compared to previous dogma of their differentiation and repopulation being responsible for tissue regeneration). As such, there have been advances in the study of the secretome of these SCs, either as a whole or considering specific molecules, such as exosomes (a type of extracellular vesicles). This hypothesis also added value to older works exploring the pro-regenerative effect of different GFs (TGFβ, PDGF, or EGF) over the endometrium.

This SCs' paracrine effects hypothesis, together with the risks (such as teratogenesis, and ethical issues regarding use of specific SC sources) and disadvantages (such as the low retention of cells, and procedure invasiveness for patients) that this therapy entails, have probably favored the search for alternative therapeutic approaches.

4.2.3. Emerging therapeutic alternatives

In the last years, the use of PRP as a therapy has been gaining popularity. The GFs and other biomolecules located inside the α-granules of the platelets give wealth to this biological product, and have led to promising results.

More recently, therapeutic procedures based on tissue- and bio-engineering have arisen. For example, collagen scaffolds combined with different GFs (VEGF, FGF2) or even SCs, have been tested in animal models and some clinical trials including AS patients.

The use of scaffolds has also been explored using the decellularized uterine ECM as the prime material. Different works show the pro-regenerative effect of this material obtained from the decellularized uteri of different mammals (such as pig, sheep, rat, or humans). However, results are only currently available in *in vitro* systems or animal models.

Another bioengineering-derived approach is the use of hydrogels. Similar to 'solid' scaffolds, hydrogels have been derived from synthetic materials (such as hyaluronic acid) or decellularized ECM. In addition, these hydrogels have been studied in combination with other products, like estradiol, SDF1, or cells.

Finally, in the last couple of years, other technologies have surfaced, not directly as therapies, but as study models and screening systems for therapies or drugs for EA and AS patients. Examples of these systems are microfluidic technology (sometimes referred to as lab-on-a-chip) and the three-dimensional (3D) culture of organoids (sometimes defined as mini-organs).

In parallel, the review of all articles revealed a diversity of methods and approaches in the induction of endometrial damage (ethanol, trichloroacetic acid, needles, ablation with hot water, scalpels, electrocoagulation, resection of uterine segment) and the selected outcomes (fertility outcomes and markers of

morphological endometrial features, endometrial functionality and regeneration) to evaluate the efficacy of the tested treatments.

Overall, this study summarizes the different available therapeutic approaches for treating EA and AS and how different animal models have been used to test their effectiveness.

VI. DISCUSSION

This dissertation introduces novel approaches to managing two endometrial pathologies: EA, affecting 2.4% of women undergoing an *in vitro* fertilization treatment (data from 2014; Kasius *et al.*, 2014); and AS, affecting up to 19 % among women after miscarriage (data from 2014; Kasius *et al.*, 2014). It is important to point out that the incidences are highly variable depending on the studied subpopulation (Salazar *et al.*, 2017). Efforts to treat EA and AS patients typically aim to regenerate the endometrial tissue to restore its regular proliferation rate and thickness, ultimately enabling embryo implantation. In line with this goal, this dissertation work investigated the molecular changes induced in the endometrium by transplantation of BMDSCs (Manuscript I), and the use of an alternative plasma source —umbilical cord blood— for stimulating endometrial regeneration (Manuscript II). This original research is put into perspective by the overall changes in the field through a detailed literature review of endometrial ASCs (Manuscript III) and the available EA/AS therapeutic approaches (Manuscript IV). The literature reviews not only summarize existing knowledge in the field (consisting of papers, articles, books, and data), but also critically assess and analyze current dogmas and hypotheses.

1. ENDOMETRIAL RECOVERY AFTER BONE MARROW-DERIVED STEM CELLS TRANSPLANTATION

This study was based on the effectiveness of $CD133^{+}$BMDSCs therapy over an injured endometrium, either in women with EA or AS (Santamaría *et al.*, 2016) or in an AS murine model (Cervelló *et al.*, 2015). Both studies evidenced endometrial recovery: an increase in the endometrial thickness and fulfillment of healthy pregnancies in the human pilot study, while the murine model responded to therapy with stimulation of endometrial cell proliferation. Nevertheless, the

engraftment of the SCs around blood vessels rather than the site of injury, support the hypothesis that cells were exerting a reparative effect via different mechanisms (possibly paracrine) instead of differentiating themselves. At this point a new question arose: What exactly happens in the time frame between the SC administration and the noticeable tissue regeneration?

In the pursuit of answering this question in Manuscript I, we aimed to evaluate the endometrial tissue to elucidate how the transcriptome and/or protein profile changes in response to the action of the human $CD133^{+}$BMDSCs. Thus, we analyzed the resulting scenario after applying the treatment. We found that five human genes were differentially expressed after the treatment: *CXCL8* and *CCND1* were down-regulated, while *JUN*, *SERPINE1*, and *IL4* were up-regulated.

In consequence, we postulated some of the specific mechanisms that may be responsible for endometrial tissue repair in EA/AS patients after SC treatment. Fundamentally, we concluded that the injected SCs offered an immunomodulatory scenario after the therapy, which harbors the subsequent dynamic tissue regeneration.

Interestingly, *CXCL8*, the down-regulated gene with the highest change after treatment (qRT-PCR validation fold regulation value = -26.546), codifies an interleukin involved in neutrophil activation and T cell chemotactic activity (Akdis *et al.*, 2011), which prevents an effective immune response (Russo *et al.*, 2014) and presumably silences the immunologic milieu in treated women. The significant reduction in neutrophil elastase protein expression, a serine protease included in the extracellular traps that the neutrophils secrete (Papayannopoulos *et al.*, 2010), supported this statement. This expression trend of *CXCL8* also correlates with the down-regulation of the oncogenic cell-cycle regulator *CCND1* (Singh and Lokeshwar, 2009; Shao *et al.*, 2013), which is indicative of cells in S

(DNA synthesis) and G2-M (growth and preparation for mitosis and mitosis) phases rather than in the G1/G0 (growth/quiescent status) phase, consequently promoting a proper status tissue regeneration (Carlson *et al.*, 1999; Lehn *et al.*, 2010).

Regarding the up-regulated genes, *SERPINE1* has not only been described in arterial remodeling in cardiac wound healing (Creemers *et al.*, 2001) and keratinocyte migration during cutaneous injury repair (Providence *et al.*, 2008) but also in the endometrium, giving rise to vascular remodeling as well as morphological and functional changes in the stromal cells (Okada *et al.*, 2018) during decidualization (Lumbers *et al.*, 2015). Meanwhile, the increased expression of *IL4* has been widely correlated with higher proliferation, differentiation, and anti-apoptosis processes in several cell types (Akdis *et al.*, 2011; Turner *et al.*, 2014). Finally, the higher expression of *JUN* could be associated with the regeneration of the endometrial epithelium due to its role as an essential mediator of epithelial cell development and proliferation in the endometrium (Salmi *et al.*, 1998) and the skin (Szabowski *et al.*, 2000). Moreover, these three up-regulated genes have been identified in different signaling pathways involved in diverse pro-regenerative processes (cell cycle progression, angiogenesis, anti-apoptosis, cell differentiation, growth, proliferation, survival, cytokine production, and chemotaxis). Specifically, Wnt, MAPK, and TNF pathways correlated to *JUN*; Wnt, HIPPO, and TGFβ signaling pathways to *SERPINE1*; and JAK-STAT and PI3K-Akt to *IL4*.

In parallel, the analysis of murine uterine tissue identified four proteins with a significantly higher expression in treated horns than controls: IL18, MCP1, HGF, and MIP2. Of these, IL18 and MCP1 have been described in angiogenic processes (Lédée-bataille *et al.*, 2005; Deshmane *et al.*, 2009) resulting in tissue regeneration while HGF and MIP2 have been related to cell proliferation. In mice, HGF is implicated in endometrial remodeling during the estrous cycle and in epithelial

cell proliferation via paracrine mechanisms (Zhang, 2010; Li *et al.*, 2015). Remarkably, HGF levels have already been directly correlated with the expression of *SERPINE1* (Arteel, 2008) and *JUN* (Qin *et al.*, 2017) genes. Lastly, MIP2 has been described to enhance cell proliferation, mainly in hepatic tissue (Ren *et al.*, 2003).

Taken together, these results provided added evidence on the paracrine mechanism of action of the SCs, rather than their division and differentiation per se. We recommend that upcoming therapies for EA and AS be focused on less invasive treatments (compared to SC therapy) based on paracrine molecules (such as GFs, cytokines, chemokines, or anti-inflammatory factors) secreted by the SCs (Eleuteri and Fierabracci, 2019). Identifying the specific molecules most involved in endometrial regeneration could help develop a *cocktail* of selected factors that could mimic the regenerative effect of the SCs while reducing the risk of uncontrolled growth associated with the direct use of SCs. An example of this ambitious procedure can be found in the odontology field. Sakaguchi and colleagues reported the beneficial effect of a *cocktail* containing TGFβ1, IGF1, and VEGFA (Sakaguchi *et al.*, 2017) over periodontal tissue regeneration. However, this approach is immensely challenging due to the already forementioned complexity of the secretome's composition (Teixeira *et al.*, 2013) and the dependence on the SCs' culture conditions and the tissue from which they derive (Teixeira and Salgado, 2020). Alternative therapies based on this premise have emerged in recent years, with an excellent example being PRP. This biological product mitigates the previously mentioned disadvantages of both SCs and their secretome (or a specific *cocktail*), since PRP is an acellular, easy-obtaining blood derivative enriched in platelets (thus, its obtaining is immediate compared to the sometimes required weeks of SCs culture or time-consuming procedures for isolating specific SC populations before their transplantation), which are a natural source of biomolecules such as GFs and cytokines, also identified in the

SCs' secretome (Anitua *et al.*, 2018). Furthermore, PRP is ruled by a less strict regulation than SC therapy for its (almost) acellular and autologous origin and the minimal side effects reported (Albanese *et al.*, 2013).

In future experiments, the SCs' secretome could also be further analyzed to search for specific molecules or biological components responsible for triggering regeneration events.

2. PLASMA FOR REGENERATING THE ENDOMETRIUM

During the last decade, the gynaecological field has started to implement PRP in the treatment of different pathologies, including endometrial disorders such as EA (Chang *et al.*, 2019; Kim *et al.*, 2019) or AS (Zadehmodarres *et al.*, 2017). Given this trend and because of some controversial results in studies with AS patients (Javaheri *et al.*, 2020), we aimed to validate the hypothesis that plasma could be used to regenerate the endometrium (Manuscript II). What added values to our work was (1) the source of adult peripheral blood from EA/AS patients (we considered the use of autologous plasma), and (2) the use of umbilical cord plasma. In theory, the mechanism of action of PRP should not be affected by the endometrial pathologies, but we still wanted to confirm this and reproduce the most realistic scenario, as the treatment would be autologous in its clinical translation. Notably, both the composition analysis and the preliminary *in vitro* assays corroborated that there was no difference using plasma either from EA/AS patients or healthy, fertile donors.

We opted to include umbilical cord plasma as a positive control during the study design based on recent works suggesting the enhanced results of using a younger blood source (Castellano *et al.*, 2017; Bae *et al.*, 2019), and ultimately proposing a detrimental effect of aging in the plasma composition. On this basis, the umbilical

cord would be expected to be the plasma source with higher regenerative potential. In the end, something that was first intended to only serve as a positive internal control in our *in vitro* experiments ended up acquiring weight within the main project, mainly in the animal model, as later discussed.

The *in vitro* assays revealed that aPRP, and more intensively aUCP, promoted endometrial cell proliferation and migration, supporting previous studies in the field (Anitua *et al.*, 2016; Marini *et al.*, 2016; Aghajanova *et al.*, 2018b). These two BPs have been described to be enhanced in the endometrium by different GFs such as PDGF (Matsumoto *et al.*, 2005; Gargett and Masuda, 2010) and HGF (Yoshida *et al.*, 2004). In fact, after performing our plasma composition analysis, these two factors were significantly higher expressed in UCP, either activated or not, corroborating the *in vitro* results.

These *in vitro* results supported the establishment of an AS animal model for testing these treatments. This murine model first included a proof-of-concept of only three mice in which it was demonstrated that aPRP treatment increased the expression of Ki67 (a proliferation marker) and HOXA10 (a transcription factor involved in endometrial function regulation; Bagot *et al.*, 2001) proteins. These results were indicative of, at least partially, a regenerative effect of the aPRP. We subsequently increased the size of the group and included two more treatments: aUCP and naUCP.

The in-depth proteomic analysis of all uterine horns reflected a precise differential distribution of the proteins depending on the treatment applied and the uterine horns analyzed (damaged or undamaged/control). The discussion is focused on the up-regulated proteins in the aUCP group because this treatment showed the most relevant changes.

When comparing the damaged versus the undamaged uterine horns in the aUCP group four up-regulated proteins were highlighted: P85A, 2AAA, RHOA, and

STAT5A. The latter, STAT5A, is a mediator of cell response to SCF, among other GFs. In fact, SCF is a plasma component that we corroborated (by multiplex ELISAs) to be more concentrated in the UCP (either activated or not) than in adult plasma. Additionally, P85A and 2AAA, were revealed to participate in the PI3K-Akt pathway, involved in cell proliferation, differentiation, and migration. In parallel, the proteins shared between aUCP and aPRP (detected by LC-MS/MS) were detected in this route but downstream in the signaling cascade. Remarkably, the phosphatidylinositol 3-kinase (PI3K), the main component of this pathway, can be activated by certain GFs such as EGF and PDGF (Shi *et al.*, 2019). Thus, we could postulate that the UCP-induced up-regulation of P85A, 2AAA, RHOA, and STAT5A, could be led by the more concentrated GFs included in this type of plasma.

On the other hand, we evaluated the effects of the four treatments on the damaged uterine horns. Among the proteins significantly up-regulated in the aUCP group, there are three to be highlighted. First, UBA3, a catalytic subunit of the dimeric UBA3-NAE1 E1 enzyme, is required for cell cycle progression and described as essential for NEDD8-mediated neddylation needed for normal human endometrial function (proliferation and decidualization; Liao *et al.*, 2015). Second, RINI, a regulator of neovascularization, has also been described as down-regulated in endometrial glands in endometriotic versus healthy endometrium samples (Stephens *et al.*, 2010). The BPs covered by UBA3 and RINI are also influenced by specific plasma components, such as the forementioned PDGF, and HGF, which are involved in endometrial cell proliferation. VEGF (Fan *et al.*, 2008) and TGFβ1 (Lash *et al.*, 2012) are known to participate in endometrial angiogenesis, while EGF is involved in decidualization (Chobotova *et al.*, 2005). Interestingly, RINI was also up-regulated in damaged horns treated with aPRP, but only when compared to the control horns from the same group. Finally, THY1/CD90, which plays a role in cell-cell or cell-ligand interactions, is a well-

known endometrial stromal SC marker (Cervelló *et al.*, 2011). The increased expression of this last protein could indicate the stimulation of the endogenous endometrial ASCs niche by the aUCP, as will be further discussed in the text.

The results discussed herein indicate that out of the three types of plasma sources studied (aPRP, naUCP, and aUCP), aUCP seems to have the highest regenerative effect. These differences between treatments correlate with the differential concentration of biomolecules present in UCP compared to the other adult source.

Apart from the enhanced higher regenerative potential of UCP, this source of plasma has other appealing advantages. First of all, the availability of the samples: for many years, biobanks have considered plasma a waste product left over other therapeutic procedures, such as obtaining hematopoietic progenitors from the umbilical cord. Luckily, it has recently started to be considered as a potential source of PRP in different medical fields such as traumatology (Caiaffa *et al.*, 2021), dermatology (Gelmetti *et al.*, 2018), or ophthalmology (Giannaccare *et al.*, 2019). Most of the time, the injected platelets' concentration is unknown, relying on the patient's baseline levels, in contrast to the administration for stem cell therapy that allows the quantification of the injected cells despite the low engraftment rate. Since the main inconvenience in autologous PRP is this variability among patients, banking these samples could also guarantee, and/or standardize a particular concentration of platelets (Russell *et al.*, 2013).

Although using UCP will be considered an allogenic transplant or procedure, associated risks would be minimized by the acellular nature of plasma (even though it may contain traces of cellular contamination; Rieske *et al.*, 2020), and, the immune immaturity inherent to umbilical cord derivatives (Wang *et al.*, 2009). Our composition analysis corroborated with this immune immaturity, with anti-inflammatory molecules such as IL1RA being more concentrated in UCP samples

(compared to the adult ones), and pro-inflammatory cytokines (IL2, IL7, and IL15) being higher in adult plasma.

3. ADULT STEM CELLS IN THE ENDOMETRIUM

To discuss the potential role of the endometrial ASC niche in the pathogenesis of EA/AS or even as a therapeutic target for both $CD133^{+}$BMDSCs and PRP approaches, it is essential to understand this SC population.

Manuscript III starts by reviewing the different types of endometrial ASCs described in the literature and how they can be identified. After a careful revision of all the articles cited in the manuscript, it was clear that there is no unique or standardized procedure for identifying them. Even if different endometrial SC markers (including SSEA1, N-cadherin, CD146, PDGFRb, or SUSD2) have been proposed to find the endogenous source, the final confirmation of the ASC phenotype are the functional assays for stemness (namely, clonogenicity, survival, and maintenance of differentiation and proliferation potential in log-term culture, multi-lineage potential, and label-retention assays). Apart from these indirect identification procedures, we also corroborated the lack of standard nomenclature for the different types of SCs from various sources, including the endometrial ones that was pointed out by Gargett's group five years ago (Gargett *et al.*, 2016).

Another issue to consider in this work would be the contribution of the BMDSCs to the endometrial niche as an exogenous source. The HLA-type (Taylor, 2004) or Y chromosome (Ikoma *et al.*, 2009) has been used to search for and successfully locate BMDSCs in the endometrium after an HLA- or sex- mismatched transplantation, suggesting the contribution of this SC population to the endometrial cell niche. These early works were important for understanding the endometrial ASCs and implementing BMDSCs therapy in EA/AS patients.

Nevertheless, our group demonstrated years ago that BMDSCs transplant did not contribute to the human endometrial side population cells described as part of the endogenous niche (Cervelló *et al.*, 2012). In agreement, Du and colleagues (Du *et al.*, 2012) reported how BMDSCs only migrated to the uterus when normal function was disrupted. Subsequent, studies further corroborated this not only in the endometrium (Alawadhi *et al.*, 2014) but also in other tissues such as skin (Leitão *et al.*, 2019) and intraarticular tissue (Maerz *et al.*, 2017). Altogether, these studies reinforce why this niche could be the potential target for the treatments against EA/AS, but the role of the ASCs coming from bone marrow still needs clarification.

As reviewed in Section 2.3 (see Manuscript III), endometrial ASCs are potentially involved in certain disorders such as endometrial hyperplasia, endometrial carcinoma, endometriosis, and adenomyosis. It has also been postulated that this niche is altered in patients with EA/AS (Gargett *et al.*, 2012). Since most of the published studies focus on using ASCs (from different sources) as a therapeutic approach to restore the niche in these disorders, we established a separated section (number 3 in Manuscript III) and excluded EA and AS from Section 2.3 'Role of Stem Cells in Endometrial Pathologies' (Manuscript III). Based on our experience, we believe that the difficulty of collecting a proper endometrial biopsy in these patients is one of the reasons for this lack of knowledge of the actual basal status of the endometrial ASC niche (before applying any treatment).

The review article summarizes the implementation of SC therapy in EA/AS patients and the alternatives that have emerged in the last years. Overall, we concluded that there is still so much research to do on endometrial ASCs. The relevance of this research field and a complete understanding of this cell population could improve in the management of endometrial disorders, not only EA and AS, but also endometriosis, endometrial carcinoma, and adenomyosis.

4. AVAILABLE THERAPEUTIC OPTIONS FOR ENDOMETRIAL ATROPHY AND ASHERMAN'S SYNDROME

An effective and relatively non-invasive therapeutic approach is currently needed for EA/AS patients. In the two first manuscripts of the present Ph.D. thesis, we delved into two different alternatives: SC therapy (Manuscript I) and PRP (Manuscript II).

The inspiration for this review emanated from our internal bibliographic review when designing the PRP animal model (Manuscript II) and deciding the future steps of our research line. Throughout Manuscript IV, we reviewed the different therapeutic approaches currently tested in EA/AS patients, focusing on the animal models. We identified a lack of standardization in both the method to damage the endometrium and markers for endometrial repair.

Due to the lack of preclinical animal studies, we first briefly reviewed human studies regarding the oldest (classical) therapeutic approaches for EA/AS (including hysteroscopic adhesiolysis, exogenous hormones, tamoxifen, low-dose aspirin, sildenafil citrate, and pentoxifylline), which are still used as first-line treatments in some cases. Notably, results from these studies were inconsistent and sometimes contradictory. Strikingly, only 2/32 reviewed studies were defined as randomized clinical trials (Aleyasin *et al.*, 2009; Firouzabadi *et al.*, 2013), which are the gold standard of a treatment-based study design (Hariton and Locascio, 2018), the rest of the studies had low population sizes, or lacked of an adequate control group. Of course, this limitation goes hand in hand with the relative incidence of these pathologies compared with more commons ones such as endometriosis.

In the second block of the manuscript, we reviewed SC therapies. We were first surprised by the variety of SC sources, which made us question ourselves whether we would be able to extrapolate or normalize results after injecting SCs from different tissues. In fact, previous studies have reported differences in the proliferative capacity (Trivanović *et al.*, 2015) as well as in the proteome and transcriptome (Strioga *et al.*, 2012), depending on the source of the SCs (for example, adipose tissue, umbilical cord, bone marrow, or peripheral blood). Other issues we faced were the different methods for inducing endometrial damage (including using ethanol injection, mechanical damage, or hot water) and the plethora of assessments for endometrial regeneration. That being said, could a rat model damaged with hot water, treated with BMDSCs and evaluated for reproductive outcomes and new blood vessels (Gao *et al.*, 2018) be compared with a rat model injured with scalpel blades, treated with amniotic SCs, and evaluated for the expression of a compilation of GFs rather than its fertility and angiogenesis (Gan *et al.*, 2017)? There is no answer for this question; each research group must establish the best animal model possible and evaluate variables adequate and relevant for the expected outcome of the experiment. Despite the forementioned, SC therapy seems to be an effective treatment for endometrial damage, regardless of its source. The disadvantages (invasiveness and cost) come when translating this therapy to humans. As previously mentioned, the paracrine activity of the SCs, which helps mitigate these disadvantages, has motivated the exploration of SC-derived elements such as exosomes. However, this approach is still emerging, and we only found one study published last year (Zhao *et al.*, 2020).

The third and last block of the manuscript included reviewing studies regarding the most recent emergent therapies such as PRP, tissue- and bio-engineering based approaches. Studies with PRP presented the same problems as those discussed with the SCs. However, in this case, the heterogeneity lies in the

potential variability of the platelet concentration among individuals, the bioactive factors released, in addition to the diverse methods for isolating the PRP fractions. For example, Jang and colleagues reported a two-step centrifugation with the exclusion of the buffy coat (Jang *et al.*, 2017), while Kim's group used single centrifugation and included the buffy layer (Kim *et al.*, 2020). Furthermore, most published human studies refer to EA (with thin endometrium) rather than AS patients. Thus, the equipartition or comparison of the animal models with the current human studies cannot be totally established.

In addition, the use of collagen, decellularized scaffolds, and hydrogels are also emerging as vehicles and/or enhancers, of pre-existing techniques. Great examples are collagen scaffolds loaded with GFs (Sun *et al.*, 2011; Lin *et al.*, 2012) or SCs (Ding *et al.*, 2014; Song *et al.*, 2015) and hydrogels administered with estradiol (Yao *et al.*, 2020). The wealth of these applications relies on the properties of the ECM components which regulate cell behavior and BPs related to tissue regeneration (Theocharis *et al.*, 2016). Other emerging approaches also include microfluidic technology and the use of the organoids' 3D culture systems. The proper and extensive establishment of these methods would render the traditional two-dimensional culture systems obsolete and would better complement (or even reduce) the use of experimental animals.

In conclusion, animal and *in vitro* models are essential before clinical translation, even if the current models still need improvements and standardization. In the pursuit of the best treatment for EA/AS patients, further research is still needed. Given the latest advances, efforts should be put on the bioengineering-based approaches (alone or in combination with established classical approaches), either for testing treatments or a deeper understanding of the disorders.

In summary, all the results discussed herein provide a strong case that continuous research into improving therapeutic approaches for EA/AS patients

opens the door to new alternatives and study designs for incoming studies. The findings obtained in the present Ph.D. dissertation led us to the conclusions detailed in the next section.

VII. CONCLUSIONS

CONCLUSIONS

The following conclusions can be drawn from this Ph.D. thesis:

1. **Manuscript I**: $CD133^+$ bone marrow-derived stem cells promote endometrial healing by creating an immunomodulatory scenario in the human and the murine models, probably led by the down-regulation of the interleukin 8 gene. In consequence, pro-regenerative processes are triggered by the up-regulation of jun proto-oncogene, serpine 1, and interleukin 4 genes in the human endometrium, and hepatocyte growth factor, monocyte chemoattractant protein 1, macrophage inflammatory protein 2, and interleukin 18 proteins in the murine uterine tissue.

2. **Manuscript II**: Platelet-rich plasma (PRP) can promote *in vitro* proliferation and migration of human primary endometrial stromal cells and endometrial stromal stem cell lines. PRP also promotes *in vivo* cell proliferation, expression of markers of endometrial function, and other proteins involved in tissue regeneration-related biological pathways in a mouse model of endometrial damage. These pro-regenerative effects are enhanced when the plasma is obtained from human umbilical cord blood rather than human adult peripheral blood.

3. **Manuscript III**: Further research in the characterization and isolation of endometrial adult stem cells is needed, since there are no universal specific cell markers and they are usually identified with indirect methods (demonstrating characteristics of stemness). Moreover, the contribution of the bone marrow to the endometrial stem cell niche and the specific role of this niche in endometrial disorders such as endometrial atrophy and Asherman's Syndrome also requires clarification.

4. **Manuscript IV**: Emerging therapeutic approaches from the bioengineering field (including acellular scaffolds, hydrogels) are starting

to be implemented in endometrial management, not only as stand-alone treatments but also as enhancers of the pre-existing therapies, such as PRP, specific paracrine molecules, or stem cells. Novel *in* vitro platforms (namely organoids and microfluidics) are also promising for the deeper study of EA/AS pathogenesis.

5. Based on our experimental findings and our bibliographic studies, the future regenerative therapies for infertility-causing endometrial pathologies, like endometrial atrophy and Asherman's Syndrome, could be based on innovative procedures ranging from the classic paracrine effectors to the most innovative bioengineering tools.

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IX. ANNEXES

1. ANNEX I. MANUSCRIPT I

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Stem cell paracrine actions in tissue regeneration and potential therapeutic effect in human endometrium: a retrospective study

L de Miguel-Gómez,[a,*] H Ferrero,[a,*] S López-Martínez,[a] H Campo,[a] N López-Pérez,[a] A Faus,[a] D Hervás,[b] X Santamaría,[c,d] A Pellicer,[e,f] I Cervelló[a]

[a] Fundación Instituto Valenciano de Infertilidad (FIVI), Instituto de Investigación Sanitaria La Fe, Valencia, Spain [b] Data Science, Biostatistics and Bioinformatics, Instituto de Investigación Sanitaria La Fe, Valencia, Spain [c] Igenomix Academy, Valencia, Spain [d] IVIRMA, Barcelona, Barcelona, Spain [e] IVIRMA Valencia, Valencia, Spain [f] Reproductive Medicine Research Group, Instituto de Investigación Sanitaria La Fe, Valencia, Spain

Correspondence: I Cervelló, Fundación Instituto Valenciano de Infertilidad (FIVI), Instituto de Investigación Sanitaria La Fe, Avenida Fernando Abril Martorell, 106. Hospital La Fe, Torre A, Planta 1[a], Valencia 46026, Spain. Email: Irene.cervello@ivirma.com

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Objective Determining genetic and paracrine mechanisms behind endometrial regeneration in Asherman's syndrome and endometrial atrophy (AS/EA) patients after autologous CD133$^+$ bone marrow-derived stem cell (CD133$^+$BMDSC) transplantation.

Design Retrospective study using human endometrial biopsies and mouse models.

Setting Fundación-IVI, IIS-La Fe, Valencia, Spain.

Samples Endometrial biopsies collected before and after CD133$^+$BMDSC therapy, from eight women with AS/EA (NCT02144987) from the uterus of five mice with only left horns receiving CD133$^+$BMDSC therapy.

Methods In human samples, haematoxylin and eosin (H&E) staining, RNA arrays, PCR validation, and neutrophil elastase (NE) immunohistochemistry (IHQ). In mouse samples, PCR validation and protein immunoarrays.

Main outcome measures H&E microscopic evaluation, RNA expression levels, PCR, and growth/angiogenic factors quantification, NE IHQ signal.

Results Treatment improved endometrial morphology and thickness for all patients. In human samples, *Jun*, *Serpine1,* and *Il4* were up-regulated whereas *Ccnd1* and *Cxcl8* were down-regulated after treatment. The significant decrease of NE signal corroborated *Cxcl8* expression. Animal model analysis confirmed human results and revealed a higher expression of pro-angiogenic cytokines (IL18, HGF, MCP-1, MIP2) in treated uterine horns.

Conclusions CD133$^+$BMDSC seems to activate several factors through a paracrine mechanism to help tissue regeneration, modifying endometrial behaviour through an immunomodulatory milieu that precedes proliferation and angiogenic processes. Insight into these processes could bring us one step closer to a non-invasive treatment for AS/EA patients.

Keywords Asherman's syndrome, bone marrow-derived stem cells, endometrial atrophy, endometrial regeneration, paracrine mechanisms.

Tweetable abstract CD133$^+$BMDSC therapy regenerates endometrium, modifying the immunological milieu that precedes proliferation and angiogenesis.

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Introduction

Stem cell therapy is a widely used technique in regenerative medicine that has provided promising results. Therapies using autologous stem cells can successfully treat diseases such as limb ischaemia[1] and multiple myeloma.[2] While adult/somatic stem cells are present in many tissues,[3] adult bone marrow is a well-known reservoir of mesenchymal stem cells and endothelial progenitor cells (EPCs).[4,5] CD133 is a surface antigen that defines a broad population of adult/somatic stem cells, including EPCs.[6] The regenerative properties of CD133$^+$ haematopoietic bone marrow-derived stem cells (CD133$^+$BMDSCs) have been

*These authors contributed equally to this work.

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demonstrated in many fields, in particular in ischaemic heart conditions.[7]

Recent evidence supports that paracrine actions provoked by these cells play an essential role in mediating regeneration, releasing biologically active factors.[8] Baraniak and McDevitt described this concept as follows: 'a recent paradigm shift has emerged suggesting that beneficial effects of stem cells may not be restricted to cell restoration alone, but also due to their transient paracrine actions'.[9]

Of all endometrial pathologies, Asherman's syndrome (AS) and endometrial atrophy (EA) are some of the most relevant for assisted reproduction. AS is characterised by intrauterine adhesions caused by curettage or uterine traumas, leading to a lack of a functional endometrium.[10] EA is caused by poor endometrial growth resulting from several risk factors (lack of estrogens, surgical interventions or idiopathic causes). Women with AS/EA have the same final effect: lack of a functional endometrium with insufficient endometrial growth, resulting in a higher risk of impaired implantation, early miscarriage, and diminished pregnancy rate.[11] Though different treatments have been tried (exogenous estrogen, low-dose aspirin, vaginal sildenafil citrate),[12] only stem cell therapy has demonstrated effectivity.[13–18] Moreover, BMDSC and their paracrine effects have shown promising results in ovarian rejuvenation,[19] follicular restoration,[20] embryo culture,[21,22] and chronic pelvic disease treatment.[23]

In this context, our group has recently completed an innovative study showing the effects of $CD133^{+}$BMDSC therapy in human[18] and murine[15] AS/EA models. The low frequency of stem cell engraftment in our animal model appeared insufficient to explain the significant endometrial regeneration described. This observation supports the above premise that the final effectors of the regenerative process are soluble factors released by the transplanted $CD133^{+}$BMDSCs.[24]

This report represents a continuation of these previous studies,[15,18] further investigating different factors and mechanisms induced by $CD133^{+}$BMDSCs that probably assist in endometrial recovery. The identification of these transient effects could be valuable to learn the specific patterns of endometrial regeneration and possibly create non-invasive therapies for AS/EA.

Material and methods

Study participants, experimental design, and histological analysis

Eight patients from our previous pilot study (ClinicalTrials.gov NCT02144987)[18] were selected for this project; note that these patients were recruited for the Santamaría *et al.* study and the current work is a retrospective study analysing endometrial biopsies from the pilot study.[18] A detailed description of these patients is given in Table S1. Each patient operated as her own control, as we collected endometrial biopsies before and after the injection of $CD133^{+}$BMDSCs.

Samples used for this study were human endometrial formalin-fixed and paraffin-embedded (FFPE) biopsies obtained before and 3 months after autologous $CD133^{+}$BMDSC injection. All biopsies were taken during the proliferative phase under hormonal replacement therapy cycles (estradiol alone before progesterone). The experimental design is detailed in Figure 1.

Haematoxylin and eosin (H&E) staining using standard protocols was performed: morphological, microanatomical, and histological analyses of individual samples were carried out and compared individually in both groups (Figure 1).

RNA isolation and reverse transcription

Human endometrial tissues were cut into 5-microm sections per block and condition (before/after treatment). Samples were randomly joined into two pools: patients #1–4 (pool 1) and patients #5–8 (pool 2). These pools were treated for RNA isolation according to the RNeasy FFPE Handbook protocol (Qiagen, Hilden, Germany).

For reverse transcription, the First-Strand cDNA Synthesis protocol from FFPE samples (Qiagen) was used.

Molecular analysis and gene expression arrays

Before performing the arrays, cDNA was evaluated with the housekeeping gene *Gapdh* (Qiagen) by quantitative real-time PCR (qRT-PCR). Three qRT-PCRs with RT2 Profiler PCR Arrays format C (Qiagen) were then carried out before and after treatment (pools 1 and 2): PAHS-040ZC—Human EGF/PDGF Signalling Pathway, PAHS-041ZC—Human Growth Factors, and PAHS-072ZC—Human Angiogenic Growth Factors. These three arrays were selected based on previous results which suggested proregenerative and proangiogenic effects as a result of the stem cell therapy.[15,18]

Bioinformatics data analysis

Analysis of the qRT-PCR data was performed following the approach of Yuan *et al.*[25] A *t*-test was then calculated for each gene comparing ΔCT values between both groups. CT values of statistically significant genes were represented in a heatmap with rows and columns ordered using hierarchical clustering.

After analysing gene expression arrays, the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway database was used with manual annotation of the genes.[26]

Human gene array validation

To verify the results, the expression of *Jun* (jun proto-oncogene, c-Jun), *Ccnd1* (cyclin D1) and *Cxcl8* (C-X-C

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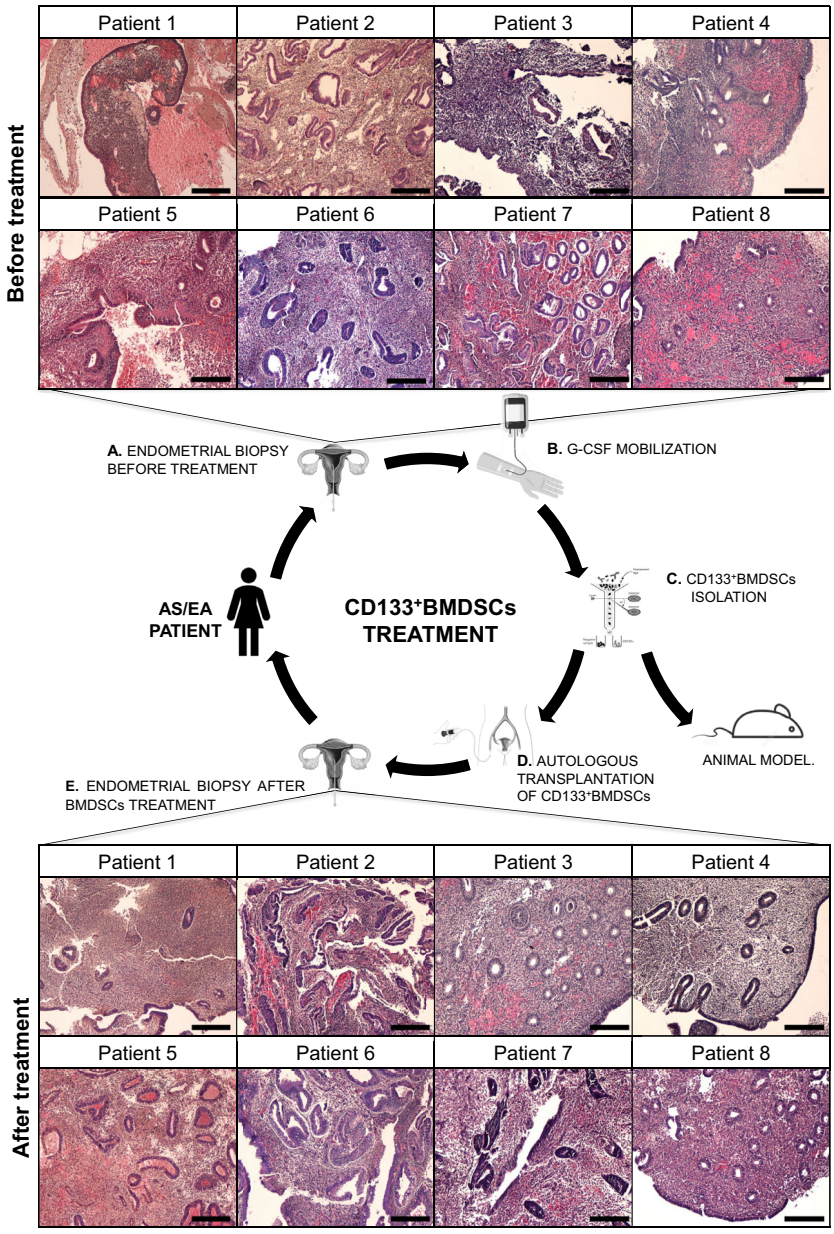

Figure 1. Study design. (A) Before any treatment, an endometrial biopsy was obtained from women with Asherman's syndrome (AS) and/or endometrial atrophy (EA). Histology and microanatomy were analysed by haematoxylin and eosin (H&E) staining (pictures showed in the upper panel, 10×; scale bar 0.2 μm). (B,C) After that, human CD133+ bone marrow-derived stem cells (CD133+BMDSCs) were mobilised (by G-CSF, granulocyte colony-stimulating factor) and isolated from these patients by flow cytometry. (D) Isolated cells were autotransplanted in the same women and (E) another biopsy was obtained 3 months after the intervention (lower panel showing H&E staining after treatment, 10×; scale bar 0.2 μm). In parallel, these CD133+BMDSC were also used for an animal model represented in the right side of the diagram (Cervelló *et al.*, 2015).[15]

motif chemokine ligand 8) was analysed. qRT-PCR was performed using specific primers (Thermo Fisher Scientific, Waltham, MA, USA) (Table S2). For this validation, we used samples from all patients in pool 1 and pool 2 (detailed in *RNA isolation and reverse transcription* section) before and after treatment. Relative gene expression levels were determined by the ΔΔCt and normalised to *Gapdh*. Qiagen Data Analysis Software (https://www.qiagen.com/shop/genes-and-pathways/data-analysis-center-overview-page/) was used to calculate fold regulation (FR).

Neutrophil elastase protein expression

Immunohistochemistry for neutrophil elastase (NE) in the human samples was performed. Deparaffinised tissue

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sections were incubated with monoclonal mouse anti-human NE (1:100; M0752 Dako, Agilent, Carpinteria, CA, USA); human tonsil was used as positive control. Following this, the Envision HRP system was used (K4065, Dako).

Randomly chosen areas at X20 magnification of NE stained slides were evaluated by three blinded observers. An average of 2400 cells was counted, by IMAGE-PRO PLUS Software v6.3 (MediaCybernetics, Rockville, MD, USA), to analyse stained cells in before/after treatment samples. Total NE expression was presented as the mean percentage of positive signals versus total cells with their corresponding standard deviation (SD).

Murine models with Asherman's syndrome and human CD133$^+$BMDSC transplantation: validation of human results and protein immunoarrays

Uteri ($n = 5$) from previously published work were used; female nonobese diabetic mice, NOD-SCID (strain code 394; NOD.CB17-Prkdcscid/NcrCrl; Charles River Laboratories, Saint Germain Nuelles, France) were used.[15] Here, both horns were mechanically damaged and the left horns were treated by intrauterine injection with human CD133$^+$BMDSC, with the damaged right horns maintained as controls (Figure 4A).

First, the differentially expressed human genes *Jun*, *Serpine1* (PAI-1, plasminogen activator inhibitor-1), and *Ccnd1* were validated in the mouse model. *Il4* and *Cxcl8* were not tested as the former cannot be detected in NOD-SCID mice due to its dynamic activity in allograft rejection via T cells[27] and the latter is not expressed in mice.[28] RNA extraction and qRT-PCR were performed as detailed before; specific primers are given in Table S2. Secondly, cytokine profile and growth and angiogenesis factors in the uterine tissue were measured. After deparaffination and rehydration, total protein extraction was performed using Qproteome FFPE Tissue Kit (Qiagen) in all samples from both horns of the five mice. Similar to the human model, two multiplex immunoarrays were done to investigate molecules involved in regeneration and angiogenic processes: Mouse Cytokine & Chemokine 26-plex ProcartaPlex Panel (Thermo Fisher Scientific) and MILLIPLEX MAP Mouse Angiogenesis/Growth Factor Magnetic Bead Panel (Merck, Darmstadt, Germany). Quantification was carried out using a Luminex MagPix system and Luminex XPONENT Software (Austin, TX, USA).

Statistical data analysis

Statistical analysis was performed using GraphPad PRISM 7.04 software (La Jolla, CA, USA). Data are presented as mean ± SD. A paired sample *t*-test was used to analyse NE signals in before/after treatment samples and in the immunoarray data. A *P*-value <0.05 was considered significant.

Results

Endometrial reconstruction after cell therapy with CD133$^+$BMDSC

H&E staining of the human endometrium before treatment showed stromal compaction and a non-functional secretory glandular morphology in most of the samples (#1, #3, #4, #5, #8) (Figure 1, upper panel). In contrast, 3 months after treatment with CD133$^+$BMDSCs, the endometrium displayed clear stromal organization and the morphology of the glands varied from inactive to secretory (Figure 1, lower panel). In all the cases, the histological pattern of the endometrial samples after treatment was improved from a functional point of view, with exceptional results in patients #1–4, #7, and #8.

Endometrial thickness in all patients was between 3 and 5 mm before treatment; after treatment this widened to between 5 to 12 mm. More details can be found in Table S1.

Gene expression arrays in samples before and after treatment

A total of 252 genes were analysed from the three different gene arrays used. Note that we are only taking those genes into account that have highly restricted significant expression patterns in both pools. Therefore, only six genes had a significantly different expression in the treatment group: *Jun* ($P = 0.037$), *Araf* ($P = 0.049$), and *Ccnd1* ($P = 0.043$) from the Human EGF/PDGF Signalling Pathway array; *Il4* ($P = 0.041$) from the Human Growth Factors array; and *Cxcl8* ($P = 0.036$) and *Serpine1* ($P = 0.026$) from the Human Angiogenic Growth Factors array (Figure 2A). We discarded *Araf* because it was up-regulated in pool 1 but down-regulated in pool 2. The other five genes were up- or down-regulated in both pools. *Jun*, an oncogene, *Serpine1*, an inhibitor of fibrinolysis, and *Il4*, involved in immune response, were down-regulated but became up-regulated after treatment. *Ccnd1*, a regulator of CDk4 kinase, and *Cxcl8*, a potent mediator of the inflammatory response, were down-regulated after treatment. As seen in Figure 2B, *Cxcl8* was the gene with the highest FR between conditions.

Selection and validation of reference genes

Validation of the three genes selected from the gene array results corroborated that in human samples *Jun* was up-regulated after the treatment (FR = 1.429), whereas *Ccnd1* and *Cxcl8* were down-regulated (FR = −1.434 and −26.546, respectively) (Figure 2B).

Gene expression pattern analysis

The KEGG pathway database was used to characterise the differentially up-regulated gene functions.[26] Here, it became

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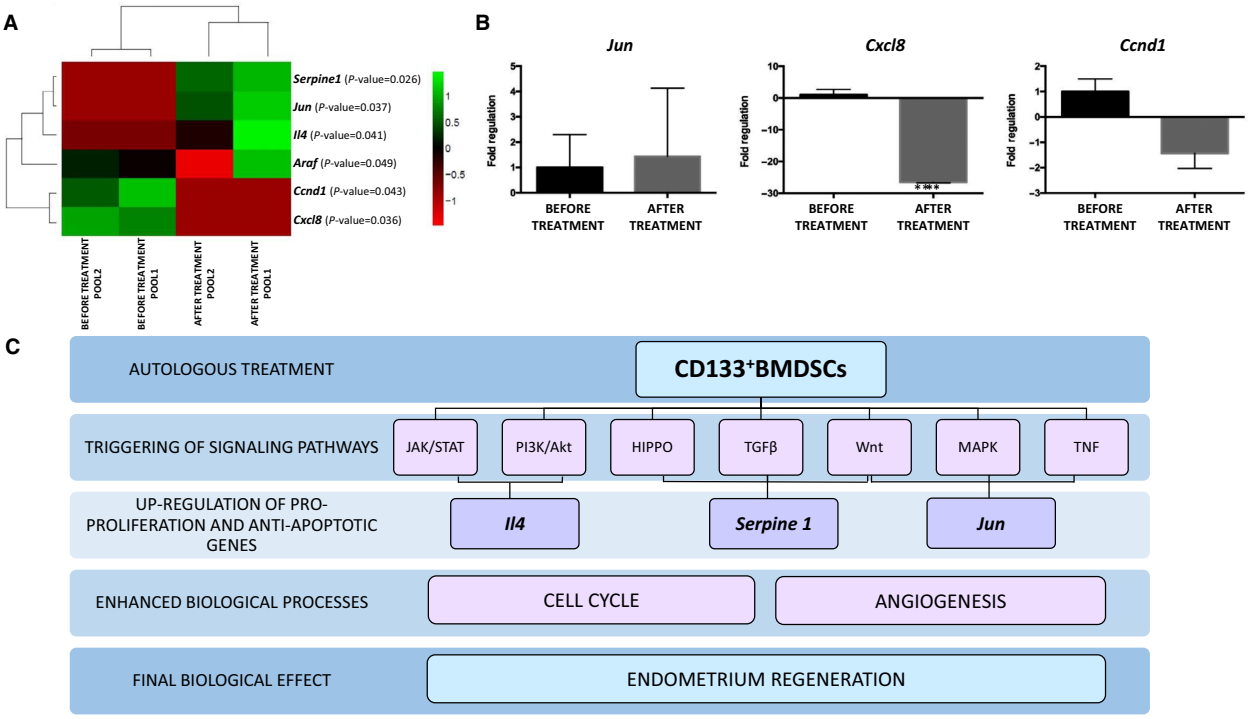

Figure 2. Comparison of endometrial gene expression profile before/after CD133+BMDSCs therapy in patients with AS and EA. (A) Heat map showing genes with significant different expression before/after treatment conditions. *Serpine1*, *Jun*, and *Il4* proved to be up-regulated after the treatment. Conversely, *Ccnd1* and *Cxcl8* were down-regulated after the treatment. Fold regulation value is shown with a typical colour gradation, in green for up-regulation and in red for down-regulation situations, as shown on the right side of the figure. (B) qRT-PCR array data validation of selected genes (*Jun, Ccnd1,* and *Cxcl8*) was performed in samples before/after treatment by qRT-PCR (to note that we have analysed two pools per each condition; $n = 4$ patients per pool). Gene expression is represented as fold regulation; **fold regulation < -2. (C) Schematic overview of up-regulated genes, metabolic pathways in which they are involved, and biological processes they trigger.

apparent that *Jun*, *Serpine*, and *Il4* could fundamentally influence cell cycle progression and angiogenesis, playing roles in anti-apoptosis, cell differentiation, proliferation and survival, cytokine production, cellular growth, and chemotaxis. Seven signal transduction pathways in which these genes take part were identified: Wnt, MAPK and TNF pathways correlated to *Jun*; Wnt, HIPPO and TGFβ pathways correlated to *Serpine1;* and JAK-STAT and PI3K-AKT pathways correlated to *Il4* (Figure 2C).

Neutrophil elastase protein expression before and after treatment

To demonstrate the effect of *Cxcl8* down-regulation, a neutrophil chemoattractant, NE immunohistochemistry was performed. After counting all positive signals, we detected a statistically significant decrease after treatment in all patients ($P = 0.025$) (Figure 3).

Validation of human gene results and protein expression in murine models

It was confirmed in murine samples that *Jun* and *Serpine1* genes were up-regulated after treatment (FR = 1.215 and 2.231, respectively), whereas *Ccnd1* was down-regulated (FR = −2.921) (Figure S1).

Multiplex immunoarrays of the uterine horns were performed (injected–treated and non-injected–not treated with human CD133+BMDSC) (Figure 4A). The expression of all proteins in the treated ($n = 5$) and not treated ($n = 5$) horns can be found in Table S3. From the 48 target proteins analysed, four showed a statistically significantly higher expression in treated horns: IL18, HGF (hepatocyte growth factor), MCP-1 (C-C motif chemokine 2), and MIP2 (C-X-C motif chemokine 2) (Figure 4B,C). Other interesting proteins such as VEGFA (vascular endothelial growth factor A), FGF2 (fibroblast growth factor 2), betacellulin, tumour necrosis factor (TNF)α and IL10 also showed a tendency towards a higher expression in the treated horn without this being significant (Table S3).

Discussion

Main findings

Although this study is based on findings and samples previously obtained by our group,[15,18] all the results discussed

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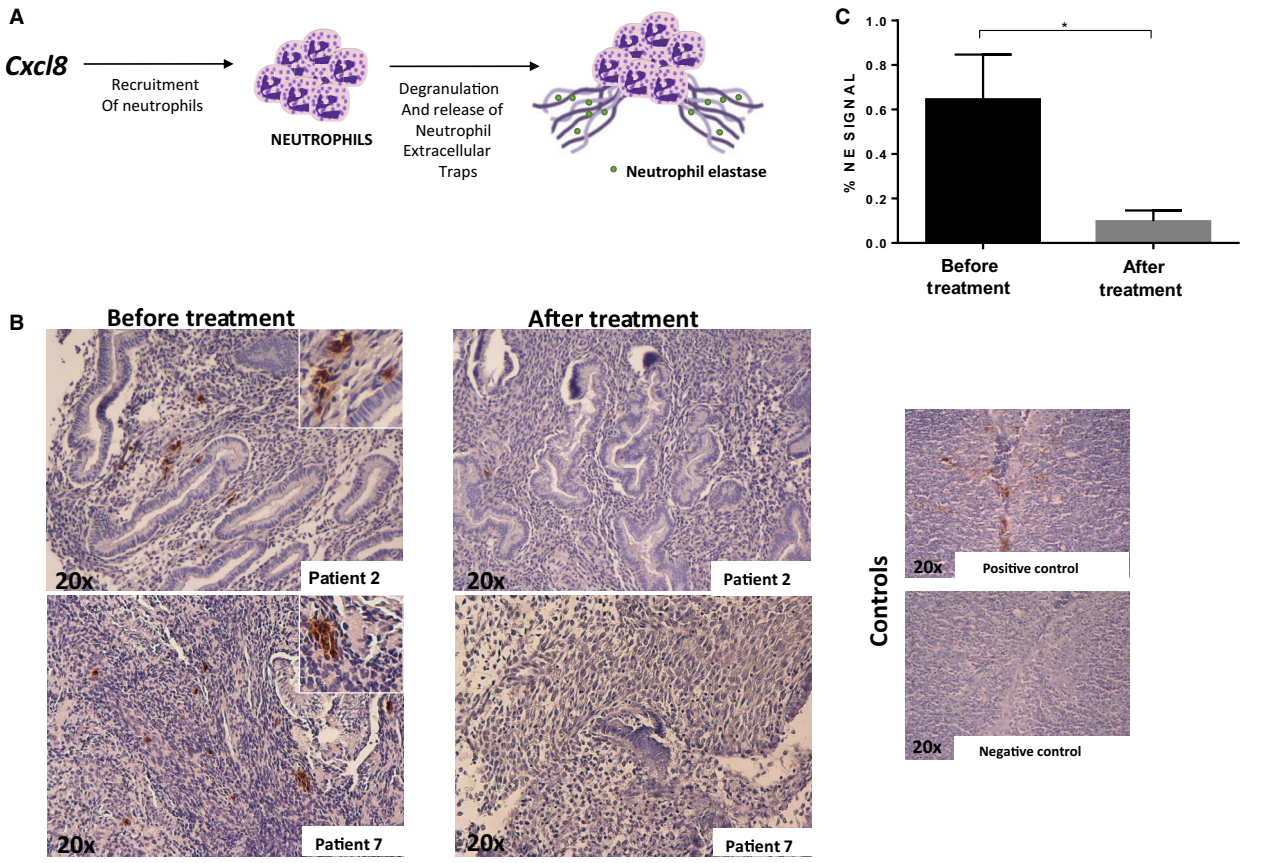

Figure 3. Neutrophil elastase endometrial protein expression in patients with AS and EA before/after cell therapy with autologous CD133^{+}BMDSCs. (A) Schematic overview of the relation established among *Cxcl8* gene, neutrophils recruitment and neutrophil elastase (NE) expression at protein level. (B) Immunohistochemistry against NE of two representative histological samples (at 20× magnification) before/after treatment. Positive (human tonsil) and negative (absence of primary antibody) controls were used for NE immunohistochemistry. (C) A graphic showing the statistically significant difference in NE signal is showed; *Paired samples *t*-test indicated significant differences <0.05 (*P*-value = 0.025).

here are completely new, reinforcing interesting and new concepts in the regenerative medicine field mainly in endometrial regeneration after CD133^{+}BMDSC therapy.

We elucidate for the first time some of the specific mechanisms responsible for endometrial tissue repair in AS/EA patients after specific autologous stem cell treatment. The identification of five differentially expressed genes (*Jun*, *Serpine1*, *Il4*, *Ccnd1*, *Cxcl8*) related to the therapeutically potential of CD133^{+}BMDSC describes an immunomodulatory scenario and a subsequent dynamic regeneration. We also observed a decrease in human NE expression influencing probably the inflammatory responses after treatment. After validating a number of these genes in both women and mice, mouse horns were shown to overexpress crucial angiogenic and reparative factors (IL18, HGF, MCP-1, MIP2) after stem cell administration, reinforcing its regenerative potential.

AS/EA are pathological conditions strongly related to subfertility and recurrent implantation failure.[12,29] The implication of BMDSC in endometrial tissue recovery has been widely documented in mouse models,[17,30] macaques,[31] and humans;[32–34] however, the specific events by which this grafting may improve the restoration still remain unknown. Current research efforts include elucidating the systems implicated in tissue regeneration driven by BMDSCs.[8,9,35] Our aim is to decipher the stem cell mechanisms and paracrine signals implicated in the recovery and regeneration of pathological endometrium after BMDSC treatment in humans and mice.

The silencing of the immunological milieu in treated women could be led mainly by the noteworthy down-regulation of *Cxcl8* gene, described as a cytokine involved in neutrophil activation and T cell chemotactic activity[36] avoiding the production of an effective immune response.[37] We corroborated this by the arrest of neutrophils due to the significant reduction of NE expression.[38] Interestingly, some studies correlated the decrease of *Ccnd1* gene expression, an oncogenic cell-cycle regulator which varies with

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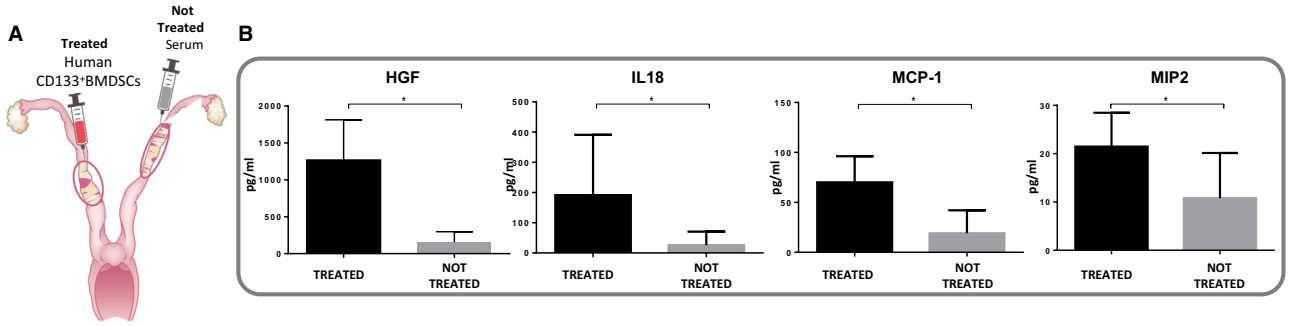

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Accession number	Molecules	Name	Protein concentration			*P*-value	Molecular function	Reference
			Detection limit (pg/ml)	Treated	Not treated			
Q08048	HGF	Hepatocyte growth factor	137.174	1260.960±545.593	164.756±129.916	0.024	Chemoattractant activity. growth factor activity	Zhang. 2010;[58] Li *et al*. 2015.[59]
P70380	IL18	Interleukin-18	38.599	278.078±214.744	25.830±44.739	0.034	Cytokine activity	Ledee-Bataille *et al.* 2005.[57]
P10148	MCP1	C-C motif chemokine 2	8.862	70.018±26.231	19.007±23.027	0.014	Cytokine activity. chemokine activity	Low *et al*. 2001;[64] Deshman *et al*. 2009.[63]
P10889	MIP2	C-X-C motif chemokine 2	0.586	21.467±6.988	10.782±9.348	0.028	Chemokine activity	Ren *et al*. 2003.[66]

Figure 4. Comparison of protein expression profile in treated and not treated uterine horns (with human CD133+BMDSCs) in a mouse model with damaged uterus. (A) Diagram summarising the methodology used in our animal model, where left horn was damaged and intrauterine injection performed with BMDSCs (named as treated), and right horn only with the damage (not treated). (B) Proteins showing a statistically significant difference in tissue expression when treated and not treated uterine horns were compared: IL18, HGF, MCP-1 and MIP2; *paired samples *t*-test indicated significant differences (*P*-value < 0.05). **paired samples *t*-test indicated significant differences (*P*-value < 0.01). To note: the difference in expression of all proteins in treated horns showed to be at least twice as much as in the not treated horns. (C) Table with the main characteristics of IL18, HGF, MCP-1, and MIP2.

the phase of the cell cycle in normal cells,[39] with the down-regulation of *Cxcl8*.[39–41] A decline of *Ccnd1* indicates that cells are in S (synthesis) and G2-M (growth and mitosis) phases, promoting a proper status for proliferation and functional endometrial recovery.[39,42,43]

This hypothesis is supported further by the up-regulation of the *Serpine1* gene.[44] *Serpine1*, described to be mainly produced by the endothelium,[45] is implicated in arterial remodelling in cardiac wound healing[46] and is required for keratinocyte migration during cutaneous injury repair.[47] In the human endometrium, the increased expression of *Serpine1* was described throughout decidualisation,[48] giving rise to vascular remodelling, and morphological and functional changes in the stromal cells.[49] In our context it is likely that *Serpine1* may influence differentiation and neovascularization during the stromal department regeneration. The increase of *Il4* expression has been correlated with higher proliferation, differentiation, and anti-apoptosis actions in several cell types including cancer cells;[36,50] probably inferred here in the treated endometrium by a cascade of regenerative events.

Beyond the events described above, affecting endothelial and stromal compartments of human endometrium, these effects were accompanied by the epithelial endometrial differentiation, presumably guided by *Jun*.[51–54] The moderate up-regulation of this gene in treated patients was associated with the regeneration of the epithelial endometrial compartment due to its role as an important mediator of epithelial cell development and proliferation. The central role of *Jun* in proliferation and differentiation of primary human keratinocytes was shown by the formation of an aberrant epithelium in the murine epidermis when *c-Jun* is not expressed.[55] Moreover, Salmi *et al.* described how *Jun* expression appeared to be associated with the proliferation of endometrial epithelial cells but remained relatively unchanged in the stromal compartment in human endometrium.[56]

To support our study, we attempted to identify the repertoire of secreted factors in the animal model.[15] Several detected human genes were also validated in mouse uterine tissue, increasing the possibility that the events observed in the animal model could also be taking place in humans. From all the selected factors analysed (Table S3), IL18, HGF, MCP-1 and MIP2 showed a higher expression pattern on treated horns when compared with controls.

IL18, commonly described as a pro-inflammatory cytokine, can also operate as an angiogenic factor,[57] suggesting its role promoting neovascularization after tissue injury. Furthermore, HGF is implicated not only in endometrial remodelling during the estrous cycle but also in cell proliferation via auto/paracrine mechanisms in mouse endometrium, mainly in epithelial cells.[58,59] In addition, HGF has been postulated to regulate its own activation by up-

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regulation of the protein product from *Serpine1* gene.[60,61] It has also been described to be up-regulated when *Jun* is overexpressed.[62] Additionally, MCP-1 has been widely described in tissue repair, remodelling, and angiogenic processes.[63,64] Butler *et al.*[65] described MCP-1 and HGF (and VEGFA) as angiocrine factors, defined as factors from vascular endothelial cells that have a paracrine action. In addition, MIP-2 has been described to enhance cell proliferation, mainly in hepatic tissue.[66] Interestingly, the pro-angiogenic properties shared by these four factors are correlated with the neovascularisation and regenerative evidence we found in the human model.

Strengths and limitations

This study provides detailed information to explain complex mechanisms at gene and protein levels that are related to human endometrial regeneration after stem cell therapy. This novel molecular approach elucidates $CD133^{+}$BMDSC therapy effectiveness. Findings can be generalised because the selection process is well-designed, and samples are representative of the study population; moreover, the mouse model confirmed the results obtained in human patients. However, we know that although AS and EA patients share the same endometrial complication, these disorders have different aetiologies. As each patient acted as her own control, we did not include an untreated control group. Nevertheless, in future studies, molecules identified in the murine model should be tested in human endometrial tissue (probably by *in vitro* studies with primary cultures of human endometrial cells) to assess our preliminary results as a non-invasive therapy in AS/EA patients. Several factors identified in the murine model were related to the inflammatory response but as we are working with NOD-SCID mice, we did not focus our discussion on that.

Interpretation

With the elucidation of certain transient paracrine actions, it is possible that these factors could be used in the future to enhance the therapeutic efficacy of stem cell approaches. Thus, this study clarifies the mechanisms of the effectiveness of stem cell therapy. In general, the mechanisms sustained by the transplanted stem cells were quite similar in human and murine models: the finding of a more immunotolerant environment, favouring regenerative events, and the respective proliferation of the endothelial, stromal and epithelial compartments guided by very different and specific patterns. These are accompanied by the global neovascularization process carried out by the well-named angiocrine factors.

Conclusion

In conclusion, successful human endometrial regeneration after autologous $CD133^{+}$BMDSC therapy seems to depend on the ability of the immune system to become tolerant and receptive as well as on the capability of resident cells to promote tissue regeneration and neo-vascularisation, all via paracrine actions. The next steps would be the validation of these factors as true effectors in both mouse and human AS/EA models and investigating whether pregnancy and delivery rates could be improved.

Disclosure of interests

LdM-G, HF, SL-M, NL-P, HC, DH, and AF report no conflicts of interest. XS, AP, and IC have a patent to declare: STEM CELL THERAPY ON ENDOMETRIAL PATHOLOGIES (Application number: 62013121). Completed disclosure of interest forms are available to view online as supporting information.

Contribution to authorship

LDm-G and HF: experimental studies and procedures, manuscript drafting, analysis. SL-M and NL-P: experimental studies. HC: analysis, manuscript drafting, and critical discussion. AF: experimental studies and procedures. DH: bioinformatics procedures and analysis, manuscript drafting. XS: study design. AP: study design and critical discussion. IC: experimental studies and procedures, study design, analysis, manuscript drafting, and critical discussion.

Details of ethics approval

The samples used in this study came from two studies: Cervelló *et al.* (2015; Ethics Committee A1329228834285, University of Valencia)[15] and Santamaría *et al.* (2016; ClinicalTrials.gov NCT02144987).[18]

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. PCR array data validation of selected genes in murine uterine tissue. *Jun, Ccnd1* and *Serpine1* were validated (as performed in human samples) in treated and not treated uterine horns by qRT-PCR (to note we have analysed two pools per each condition, n= 4 patients per pool).

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Table S1. Study participants.

Table S2. Specific primers used for the validation of *Jun*, *Ccnd1*, *Cxcl8*, *Serpine1* and *Gapdh*.

Table S3. Multiplex immunoarray data from animal model. ■

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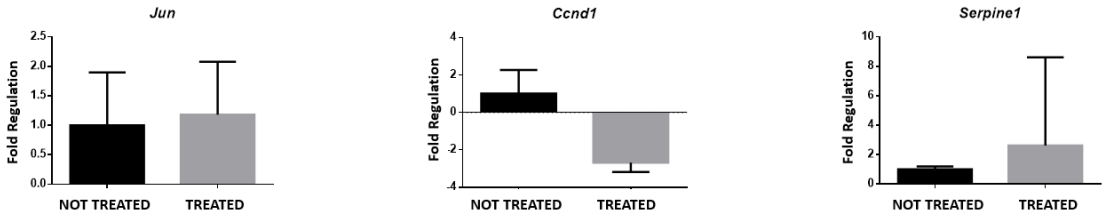
SUPPLEMENTAL MATERIAL OF MANUSCRIPT I

Supplemental Figure 1



Supplemental Figure 1. PCR array data validation of selected genes in murine uterine tissue. *Jun, Ccnd1 and Serpine1 were validated (as performed in human samples) in treated and not treated uterine horns by qRT-PCR (to note we have analyzed two pools per each condition, n= 4 patients per pool). qRT-PCR: quantitative real-time polymerase chain reaction.*

Supplemental Table I

PATIENT	AGE	PATHOLOGY	MAXIMUM BEFORE TREATMENT ENDOMETRIAL THICKNESS (MM)	MAXIMUM AFTER TREATMENT ENDOMETRIAL THICKNESS (MM)
1	42	EA with intramural myoma	5	6.8
2	34	EA + AS tage II with myoma and 80% fibrotic adhesions	3.5	5
3	35	EA	4.3	5.7
4	43	EA + AS Stage 2	3	8
5	45	EA+ AS Stage 1	5	5
6	35	EA+ AS Stage 2	3.5	7.1
7	40	AS Stage 3	4.7	12
8	38	EA	4	7

Supplemental Table I. Study participants. *Clinical characteristics of the 8 selected patients with Asherman's Syndrome (AS) and endometrial atrophy (EA). The Asherman's Syndrome Classification by 'The American Fertility Society classification of intrauterine adhesions, 1988'.*

Supplemental Table II

GENE	PRIMERS' SEQUENCES
Hu/Ms JUN Fw	CAA CAT GCT CAG GGA ACA
Hu/Ms JUN Rv	TGC GTT AGC ATG AGT TGG
Hu/Ms CCND1 Fw	CAA GCT CAA GTG GAA CCT
Hu/Ms CCND1 Rv	ACT TCA CAT CTG TGG CA
Hu CXCL8 Fw	CTG TGT GAA GGT GCA GTT
Hu CXCL8 Rv	TCC ACT CTC AAT CAC TCT CA
Ms SERPINE 1 Fw	GAA GTG GAA AGA GCC AGA TT
Ms SERPINE 1 Rv	CCA TCA GAC TTG TGG AAG AG
Hu/Ms GAPDH Fw	TCA AGA AGG TGG TGA AGC AGG
Hu/Ms GAPDH Rv	ACC AGG AAA TGA GCT TGA CAA A

Supplemental Table 2. Specific primers used for the validation of JUN, CCND1, CXCL8, CCND1 and GAPDH. *GAPDH was used as housekeeping gene. JUN, CCND1 and GAPDH primers were common for both species, human (Hu) and mouse (Ms).*

Supplemental Table III

TARGET	DETECTION LIMIT (pg/ml)	TREATED HORN MEAN±SD (pg/ml)	NOT TREATED HORN MEAN±SD (pg/ml)	*P*-value
Amphiregulin	6.859	UDL	UDL	-
Angiopoietin-2	27.435	UDL	UDL	-
Betacellulin	6.859	61.787±107.018	45.239±59.159	0.215
EGF	27.435	21.502±8.038	26.002±8.866	0.059
Endoglin	27.435	ND	ND	-
Endothelin	1.372	ND	ND	-
Eotaxin	0.757	UDL	UDL	-
FGF-2	68.587	6074.119±6422.586	8656.657±4380.677	0.350
Follistatin	137.174	ND	ND	-
GM-CSF	2.759	1.961±3.396	UDL	-
Gro-alpha/KC	1.294	3.108±3.939	1.517±1.314	0.142
HGF	**137.174**	**1260.960±545.593**	**164.756±129.916**	**0.024***
IFN-Gamma	1.123	1.204±0.822	1.825±0.163	0.122
IL-1	13.717	19.547±33.857	UDL	-
IL-10ǂ	1.953	13.868±14.447	7.706±11.312	0.306
IL-12p70ǂ	1.721	8.829±8.792	2.281±3.650	0.174
IL-13	2.515	UDL	UDL	-
IL-17A	1.221	UDL	UDL	-
IL-18	**38.599**	**278.078±214.744**	**25.830±44.739**	**0.034***
IL-1β	1.196	3.046±4.421	0.212±0.212	0.133
IL-2	1.465	2.095±3.629	UDL	-
IL-22ǂ	10.156	10.758±18.634	UDL	-
IL-23ǂ	7.52	34.079±41.639	9.246±8.007	0.199
IL-27ǂ	1.538	5.788±3.855	3.879±3.901	0.336
IL-4	1.172	UDL	UDL	-
IL-5	2.148	4.368±6.887	UDL	-
IL-6	5.64	5.719±9.906	UDL	-
IL-9	18.115	UDL	UDL	-
IP-10	0.488	UDL	0.755±1.308	-
Leptinǂ	13.717	54.219±50.938	25.016±5.898	0.344
MCP-1	**8.862**	**70.018±26.231**	**19.007±23.027**	**0.014****
MCP-3	0.22	0.262±0.299	0.240±0.416	0.461
MIP-1	6.859	UDL	UDL	-
MIP-1α	0.513	UDL	UDL	-
MIP-1β	0.952	12.367±15.056	5.725±8.688	0.294
MIP-2	**0.586**	**21.467±6.988**	**10.782±9.348**	**0.028***
PLGF-2	2.743	UDL	UDL	-
Prolactinǂ	68.587	34.978±60.584	UDL	-
RANTES	2.197	UDL	UDL	-
s-ALK1	13.717	341.588±291.877	231.011±368.948	0.158
sCD31/PECAM	13.717	ND	ND	-
SDF-1	137.174	1834.075±1091.400	2060.224±488.059	0.481
sFasLǂ	68.587	70.663±122.391	UDL	-
TNF-α	2.71	6.623±7.400	8.365±14.489	0.354
VEGFAǂ	5.487	110.476±191.350	UDL	-
VEGFC	27.435	ND	ND	-
VEGFD	68.587	UDL	UDL	-

Supplemental Table III. Multiplex immunoarrays data from the animal model. *All targets analyzed are shown, including detection limit, mean concentration expressed in pg/ml (with SD) in treated and not treated uterine horns, and P-value. Targets in bold are those showing statistically significant differences; *paired samples t-test indicated significant differences (P < 0.050); **paired samples t-test indicated significant differences (P < 0.010). [1]Targets showing an upper trend without being significant. ND: not detected. SD: standard deviation; UDL: under detection limit.*

2. ANNEX II. MANUSCRIPT II

Check for updates

Comparison of different sources of platelet-rich plasma as treatment option for infertility-causing endometrial pathologies

Lucía de Miguel–Gómez, M.Sc.,[a,b] Sara López-Martínez, M.Sc.,[a] Hannes Campo, Ph.D.,[a] Emilio Francés-Herrero, B.Sc.,[a,b] Amparo Faus, B.Sc.,[a] Ana Díaz, Ph.D.,[b] Antonio Pellicer, M.D., Ph.D.,[c] Francisco Domínguez, Ph.D.,[a] and Irene Cervelló, Ph.D.[a]

[a] Fundación Instituto Valenciano de Infertilidad, Instituto de Investigación Sanitaria La Fe; [b] University of Valencia, Valencia, Spain; and [c] IVIRMA, Rome, Italy

Objective: To study the effect of human plasma from different sources, namely, umbilical cord blood and adult blood platelet-rich plasma (PRP), on the regeneration of endometrial damage.
Design: Composition analysis, in vitro approaches, and a preclinical murine model using plasma to promote endometrial regeneration.
Setting: Hospital and university laboratories.
Patient(s)/Animal(s): Adult plasma from four Asherman syndrome/endometrial atrophy patients and one fertile woman, commercial umbilical cord plasma, and uterine-damaged NOD/SCID mice model were used.
Intervention(s): Endometrial stromal cells from primary culture and an endometrial stem cell line were cultured in vitro, and uterine-damaged NOD/SCID mice were treated with plasma samples from several origins.
Main Outcome Measure(s): To investigate the possible beneficial effects of PRP from Asherman syndrome/endometrial atrophy patients. To test if plasma from human umbilical cord blood had a stronger effect than adult PRP in endometrial regeneration. To demonstrate if PRP from Asherman syndrome/endometrial atrophy patients was as effective as PRP from a healthy woman and could therefore be used for autologous treatment.
Result(s): All plasma samples contained molecules with a high potential for regeneration (stem cell factor, platelet-derived growth factor BB, thrombospondin-1, von Willebrand factor). Furthermore, the highest increase in in vitro proliferation and migration rate was found when endometrial stromal cells were treated with umbilical cord plasma; adult PRP also revealed a significant increment. In the mouse model, a higher expression of Ki67 and Hoxa10 in the endometrium was detected after applying adult PRP, and the proteomic analysis revealed a specific protein expression profile depending on the treatment. The damaged uterine tissue showed more proregenerative markers after applying umbilical cord plasma (Stat5a, Uba3, Thy1) compared with the other treatments (nonactivated umbilical cord plasma, activated adult PRP, and no treatment).
Conclusion(s): Human PRP possesses regeneration properties usable for endometrial pathologies. Besides that, these regenerative effects seem to be more apparent when the source of obtaining is umbilical cord blood. (Fertil Steril® 2021;115:490-500. ©2020 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Asherman syndrome, endometrial atrophy, platelet-rich plasma, umbilical cord blood, regenerative medicine

Discuss: You can discuss this article with its authors and other readers at https://www.fertstertdialog.com/posts/30507

The endometrium is an extremely regenerative and complex tissue lining the uterus. It is responsible for embryo implantation and the success of the future pregnancy during the female reproductive life (1, 2). However, some disorders affecting this tissue can complicate the implantation process (3–5). In this context, Asherman syndrome (AS) and endometrial atrophy (EA) are relevant endometrial pathologies causing these types of fertility problems. AS is

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Reprint requests: Irene Cervelló, Ph.D., Fundación Instituto Valenciano de Infertilidad (FIVI), Instituto de Investigación Sanitaria La Fe, Avenida Fernando Abril Martorell, 106. Hospital La Fe, Torre A, Planta 1[a], Valencia 46026, Spain (E-mail: irene.cervello@ivirma.com).

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defined by the presence of intrauterine adhesions, which contribute to a partial or complete absence of functional endometrium (6), while EA is characterized by insufficient endometrial growth (7). Women suffering from AS or EA have a lower probability of a successful pregnancy owing to impaired implantation and early abortion (8). However, until now, there has not been a completely effective and reliable therapy. Different treatments have been proposed (7), but only stem cell therapy has shown any effectiveness (9–14). Nevertheless, this kind of treatment implies invasive and expensive procedures. For this reason, the medical and scientific community is still looking for alternate and cost-effective therapies. In recent years, platelet-rich plasma (PRP) has been proposed as a promising alternative. PRP can easily be isolated and applied noninvasively, and moreover, this can be done autologously.

Over the past two decades, PRP has been used in different medical fields ranging from dermatology to dental surgery (15–17). PRP is a plasma fraction with a platelet concentration above the normal average (~200,000/μL) (18). It is known that platelet α-granules deliver, after activation, cytokines, chemokines, and different growth factors, such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), transforming growth factor beta (TGFβ), or stromal cell–derived factor 1 alpha (SDF1α). Therefore, PRP is enriched in these factors (18, 19), which play a crucial role in the recruitment of different cell types that proliferate and migrate toward the injured site, promoting tissue regeneration and angiogenesis (18, 20).

More recently, PRP has started to be used in reproductive medicine. Different groups have shown positive results with in vitro experiments using human endometrial cells (21–23). Furthermore, rodent models have shown promising results, describing how PRP administration in damaged uterine horns enhances cell proliferation, reduces fibrosis, and even increases implantation sites (24, 25). Some "proof-of-concept" studies in humans have been carried out, instilling autologous PRP in patients with a thin endometrium (26–28). However, there is still a need for a profound understanding of its mechanism of action.

Going one step forward, PRP can be obtained not only from adult peripheral blood, but also from umbilical cord blood (UCB). It has been generally considered that the younger the source, the higher its regenerative potential is. It is thought to be linked to the higher concentration of growth and proangiogenic factors found compared with plasma from an older source (29). This has been illustrated functionally with the use of in vitro (30, 31) and in vivo (32, 33) assays. There are also several ongoing clinical trials using umbilical cord PRP (34–36).

In the present pilot study, we developed novel in vitro and in vivo models to investigate the possible beneficial effects of PRP from AS/EA patients. Using both approaches, we aimed to test if plasma from human UCB had a stronger effect than adult PRP in promoting proregenerative events in endometrial afflictions. We also aimed to test if PRP from AS/EA patients was as effective as PRP from a healthy woman and could therefore be used for autologous treatment.

MATERIALS AND METHODS

Study Design

An overview of our study is detailed in Figure 1. After extracting peripheral blood (PB) from patients with endometrial pathologies (n = 4) and a control (fertile female; n = 1), PRP was prepared (Fig. 1A). Commercial umbilical cord plasma (Stemcell Technologies; catalog no. 70020.2, lot no. 1706230142) was also prepared (Fig. 1B). The product information specified a white donor who tested negative for human immunodeficiency viruses 1 and 2 and hepatitis B and C viruses. The product was obtained with the use of consent forms and protocols approved by either the U.S. Food and Drug Administration or an institutional review board. Parts of these samples were activated with the use of 0.1 mol/L calcium chloride ($CaCl_2$) (Sigma Aldrich; catalog no. 21115), for inducing platelet degranulation and growth factors and other signaling molecules secretion. Then, samples were used for 3 different experiments (Fig. 1B). First, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and multiplex protein immunoassays were used for analyzing the specific plasma composition. Second, an in vitro approach for measuring cell proliferation and migration was developed to demonstrate the plasma effect over key cellular processes involved in tissue regeneration. Finally, an AS murine model was used, in which the uterine horns were analyzed by Sequential Window Acquisition of All Theoretical-Mass Spectra (SWATH-MS) to evaluate if plasma was able to recover damaged uterine horns.

Human Plasma Samples and PRP Preparation

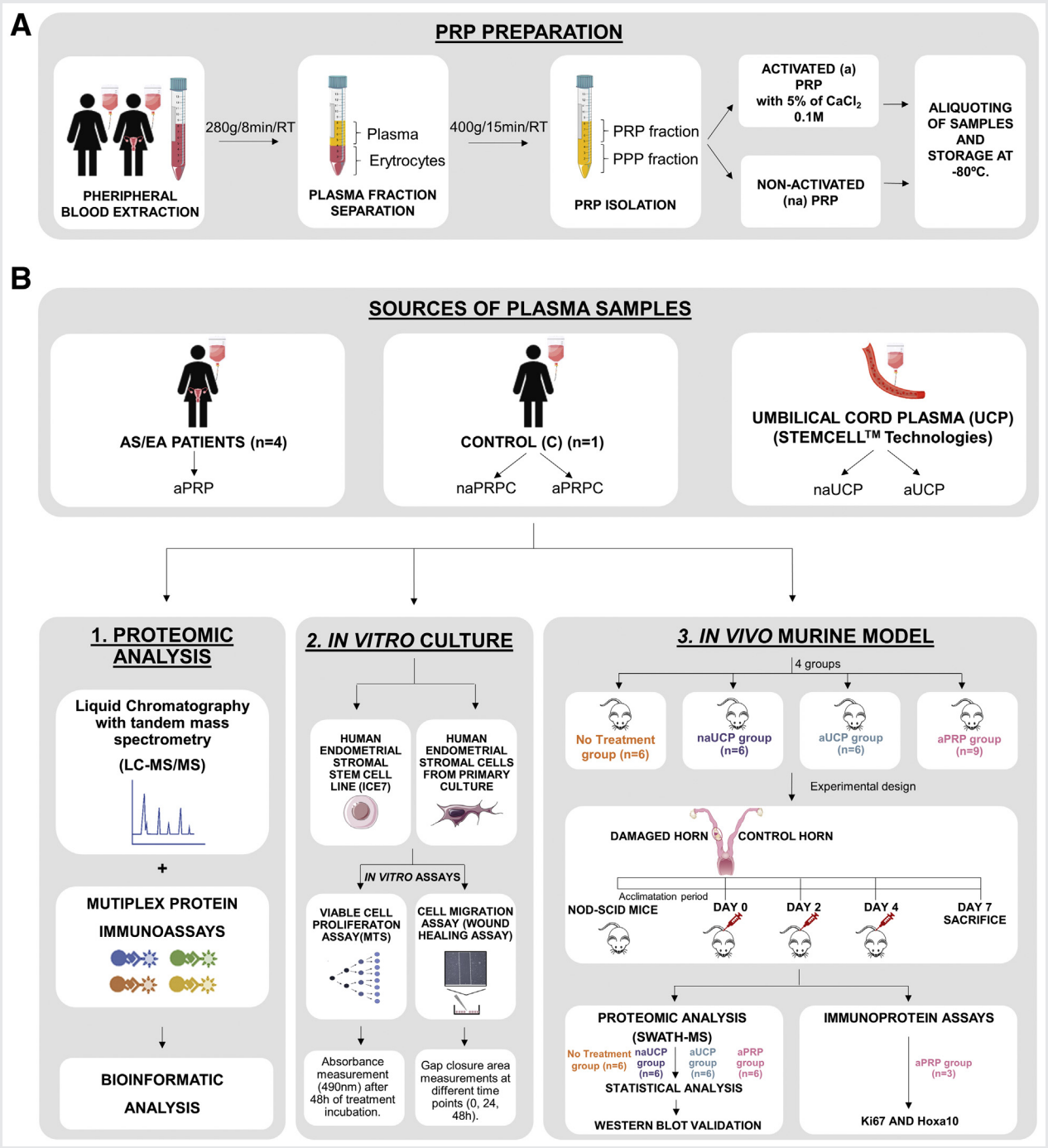
PB samples were collected from four different AS/EA patients (diagnosed by hysteroscopy in proliferative phase) and from a female control with proven fertility; all of them were from 34 to 49 years old and provided informed consent. The study was approved by the Clinical Ethics Committee of IVI Valencia (1707-FIVI-003-IC).

To isolate PRP, all PB samples were sequentially centrifuged (280*g* for 8 min at room temperature followed by 400*g* for 15 min at room temperature) and the PRP (upper part) and platelet-poor plasma (PPP; lower part) fractions were collected (Fig. 1A). Then, all patient samples (aPRP), part of the PRP control (aPRPC), and part of the umbilical cord plasma (aUCP) were activated with the use of 5% 0.1 mol/L $CaCl_2$.

Adult PRP and UCP Analysis by Proteomic Analysis

LC-MS/MS technique was performed as described elsewhere (37). Briefly, all samples were loaded into a one-dimensional sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) gel and LC-MS/MS analysis was performed, eluted peptides were analyzed in a nanoelectrospray-ionization quadrupole time-of-flight

FIGURE 1



Study design. (**A**) Platelet-rich plasma (PRP) preparation procedure from peripheral blood. Once isolated, it was activated (if necessary) with the use of 5% 0.1 mol/L $CaCl_2$, aliquoted, and stored at −80°C. (**B**) Overview of the different assays and analyses performed with the use of PRP from three different sources: commercial plasma from umbilical cord blood, four different Asherman syndrome (AS)/endometrial atrophy (EA) patients, and a control (healthy) patient. Briefly, all plasmas were analyzed by means of liquid chromatography–tandem mass spectrometry (LC-MS/MS) and multiplex protein assays and used for two different in vitro assays (MTS and wound healing) with the use of two different human endometrial stromal cell types (from a stem cell line and from primary culture) and for an animal model (mice uterine horns were analyzed by means of Sequential Window Acquisition of All Theoretical-Mass Spectra [SWATH-MS] proteomic procedure and several protein assays).

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(nanoESI QqTOF) mass spectrometer, and all the fragments were combined in a single search with the use of ProteinPilot v.5.0 and the Swissprot database.

Two immunoassays, covering 46 different analytes (Supplemental Table 1, available online at www.fertstert.org), were performed in all samples: Cytokine/Chemokine/Growth Factor 45-Plex Human ProcartaPlex Panel 1 and TGF-B1 Human ProcartaPlex Simplex Kit (Thermo Fisher Scientific; catalog nos. EPX450-12171-901 and EPX01A-10249-901). Quantification was carried out with the use of a Luminex MagPix system and Luminex xPonent software.

In Vitro Evaluation of Cell Proliferation and Migration Assays

Both assays were performed with two different cell types: a human stromal stem cell line, ICE7 (n = 3), obtained by means of the Hoescht methodology described previously (1); and primary human endometrial stromal cells (hESCs) obtained from endometrial biopsies from healthy oocyte donors (n = 10) (38). In both assays, three conditions (No Treatment, aPRP, and aUCP) were studied. Furthermore, in the cell proliferation assay using ICE7 cells, we performed some preliminary tests: three more conditions were included (aPRPC, nonactivated [na] PRPC, and aPPP) to determine the optimal concentration (1%, 5%, 10%, or 20%) of treatment in the culture medium.

For viable cell proliferation assay, cells were seeded in a 96-well plate and incubated overnight with Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Thereafter, CellTiter 96 AQueous One Solution Reagent (Promega; catalog no. G3580), containing a tetrazolium compound (MTS), was added and absorbance (490 nm) measured to evaluate viable cell proliferation.

For the wound healing assay, cells were seeded in 24-well plates with DMEM supplemented with 10% FBS and grown to confluence. The monolayer was then scratched with the use of a pipette tip. After washing the wells with phosphate-buffered saline solution for removing detached cells, corresponding treatments were added and cells were photographed 0, 24, and 48 hours after scratching and the wound area quantified with the use of ImageJ software.

In Vivo Experiments: Proteomic Profiles and Endometrial Regeneration in an AS Animal Model

In vivo procedures were performed in female 8-week-old NOD-SCID mice (Charles River Laboratories), according to guidelines of the Ethics Committee for Animal Welfare (A1483088947170) of the University of Valencia. An adaptation from our previous protocol (12) was followed: Damage was induced inside the lumen of left uterine horns (damaged) with the use of a needle; right horns were left undamaged (control). Mice were distributed in four groups and all of them underwent the same protocol: After three successive injections (days 0, 2, and 4) in the tail vein of 100 μL plasma, they were killed on day 7 (Fig. 1B). The four groups were: No Treatment (n = 6; milliQ H_2O), naUCP (n = 6), aUCP (n = 6), and aPRP (n = 9). Mice from the aPRP group were treated with aPRP from four different patients (three aPRP samples were injected in two animals each for SWATH-MS and the fourth aPRP sample in the remaining animals). Endometrial tissue from both horns of six mice per group (n = 48) was analyzed by means of SWATH-MS. We used the remaining mice (n = 3) from the aPRP group for a preliminary test before proceeding with the SWATH-MS analysis. Moreover, endometrial tissues were tested for Ki-67 (a cell proliferation marker) and Hoxa10 (a transcription factor directly related to endometrium function and development) signals, to demonstrate the proregenerative events.

Ki67 signal (1:100; Abcam; catalog no. ab833) was measured with the use of immunohistochemistry and Hoxa10 with the use of Western blot (WB; 1:100; Santa Cruz; catalog no. sc-281428). Image ProPlus (Media Cybernetics) and ImageJ software, respectively, were used for quantification.

Relative Quantification of Murine Protein Endometrial Tissues by SWATH

SWATH-MS procedure was carried out as described elsewhere (39). Briefly, all samples were pooled to build the spectral library from a one-dimensional SDS-PAGE gel, and resulting peptides were analyzed in a nanoESI qQTOF mass spectrometer. Then, triple TOF was operated in SWATH mode for individual samples. Data were analyzed with the use of Peak View 2.1 and Marker View. Protein areas were normalized by the total sum of the areas of all the quantified proteins. After statistical analysis, results were validated by means of WB.

Functional In Silico Analysis

For proteomic data analysis, Gene Ontology (GO) (40) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (41) databases were considered through g:Profiler tool set (42), Genemania (43) and, KEGG mapper–Search pathway web tools.

Statistical Analysis

Data were analyzed with the use of GraphPad Prism 7.04 software and presented as mean ± SD or median with interquartile range when appropriate. An analysis of variance was used to analyze cell proliferation and wound closure rates and growth factor/cytokine/chemokine concentrations. A Mann-Whitney test was used to analyze Ki67 and Hoxa10 signals. $P<.05$ was considered to be statistically significant.

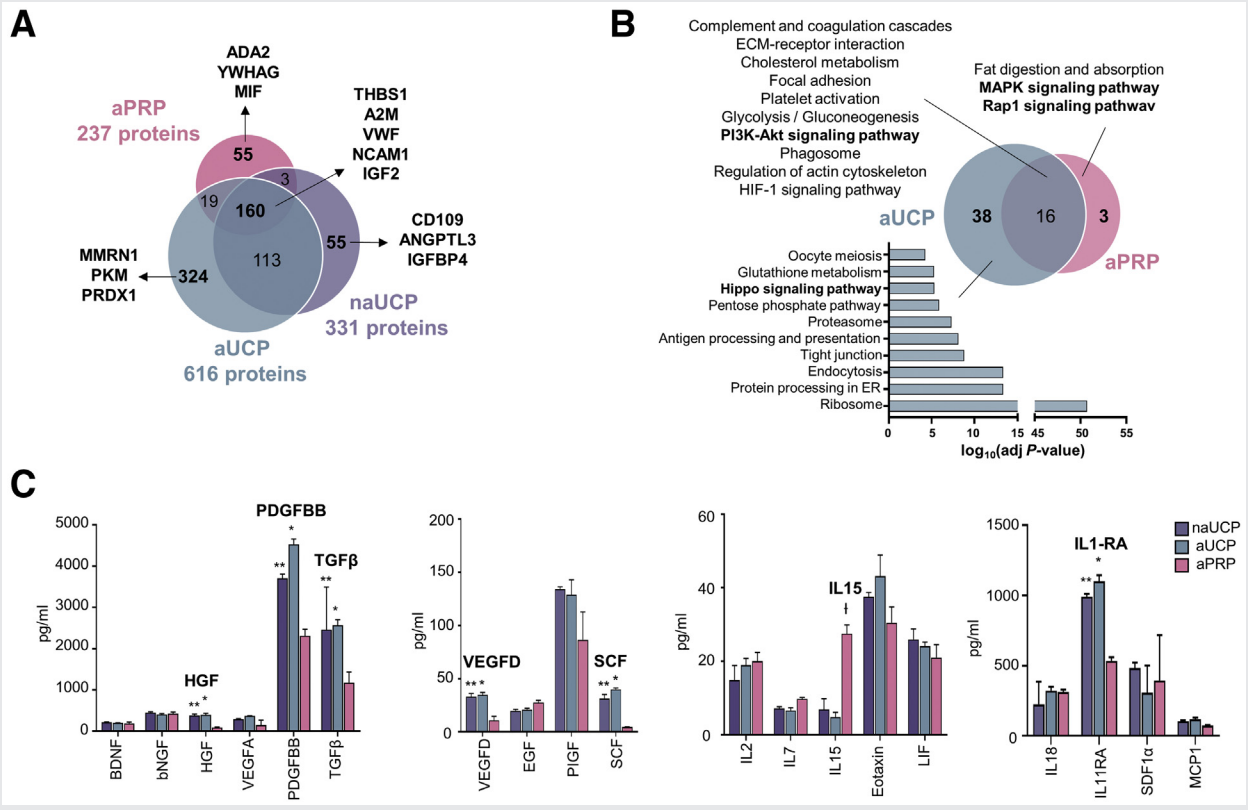
SWATH-MS data were analyzed by means of multinomial regression with Elastic Net penalization (Elastic Net penalty value, $\alpha = 0.5$) (44).

RESULTS

Comprehensive Proteomic Evaluation of Plasma from Different Sources

The LC-MS/MS proteomic analysis showed the presence of 616 proteins in aUCP, 331 in naUCP, and 237 in aPRP. Only proteins detected in at least two of the aPRP samples were considered (Fig. 2A). The functional in silico analysis revealed no relevant differences among PRP samples from patients and control (data not included), revealing that plasma from AS/EA

FIGURE 2



Composition analysis of plasmas. (**A**) Venn diagram showing the number of common proteins between the activated and nonactivated fractions in umbilical cord plasma (UCP) and the proteins present in all activated platelet-rich plasma (aPRP) samples. (**B**) Venn diagram showing the shared KEGG pathways among the proteins detected by liquid chromatography–tandem mass spectrometry in the aUCP and aPRP. (**C**) Multiplex protein assays results for cytokines, chemokines, and growth factors are also shown for the different samples analyzed. Data are presented as mean ± SD. *$P<.05$, aUCP vs. aPRP; **$P<.05$, naUCP vs. aPRP; †$P<.05$, aPRP vs. a/naUCP.

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patients is not different from a healthy woman and could be used as autologous treatment.

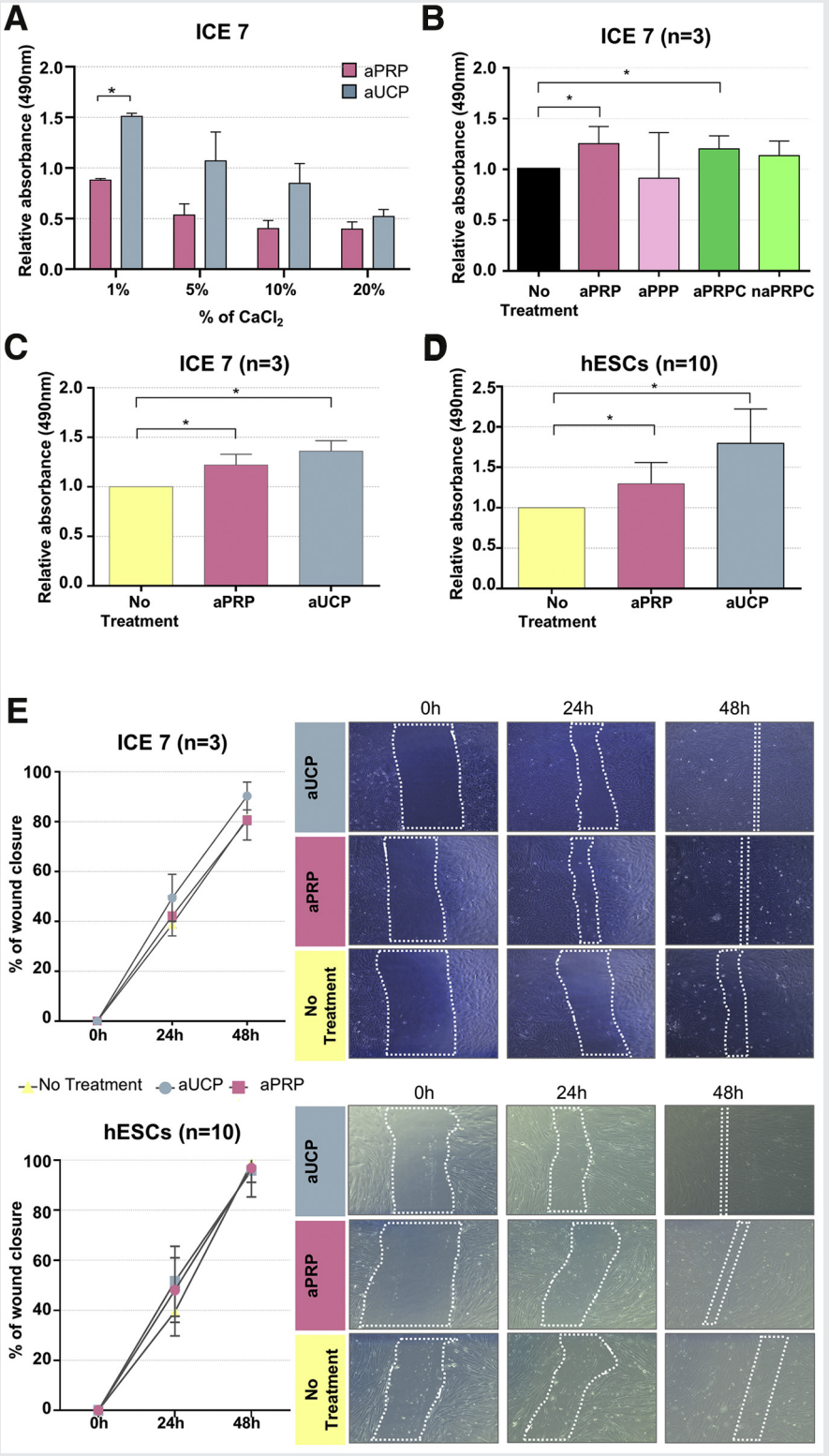
According to the objective of this study, we were interested in proteins related to tissue regeneration (45, 46), specifically those localized inside platelet α-granules. Among all the proteins detected, thrombospondin-1, α-2-macroglobulin, von Willebrand factor, neural cell adhesion molecule 1, or insulin-like growth factor II was found in all plasma samples. Meanwhile, other interesting proteins, such as CD109 antigen, insulin-like growth factor–binding protein 4, and angiopoietin-related protein 3 were detected only in naUCP; multimerin-1, pyruvate kinase, and peroxiredoxin-1 were found only in aUCP, and adenosine deaminase 2, 14-3-3 protein gamma, and macrophage migration inhibitory factor were only found in aPRP (Fig. 2A). Owing to the big difference between aUCP and naUCP composition, we continued our analysis with only the activated fraction.

KEGG pathway analysis was performed only for aUCP and aPRP samples. The analysis revealed that among others, PI3K-Akt signaling pathway was shared by proteins detected in both samples. Hippo pathway appeared only in the aUCP fraction, and MAPK and Rap1pathways appeared only in the aPRP fractions (Fig. 2B).

Growth Factors, Interleukins, and Chemokines in Plasma Samples

The results of the arrays revealed a statistically higher concentration of several growth factors in both aUCP and naUCP fractions when compared with aPRP. These growth factors were TGFβ (respectively, $P=.0048$, $P=.0024$), kit ligand or stem cell factor ($P=.0001$, $P<.0001$), platelet-derived growth factor BB ($P=.0008$, $P=.0024$), HGF ($P=.0005$, $P=.0003$), and VEGFD ($P=.0011$, $P=.0006$; Fig. 2C). From all the cytokines and chemokines studied, we detected interleukin (IL) 2, IL7, IL15, IL18, eotaxin, leukemia inhibitory factor, monocyte chemoattractant protein 1, and SDF1α in all samples. However, only IL1-RA ($P=.0060$, $P=.0010$) and IL15 ($P=.0026$) presented statistically differential results; of note, IL15 was the only molecule with a significantly higher concentration in aPRP (Fig. 2C). A complete list of the analyzed molecules and the values obtained can be found in Supplemental Table 1 (available online at www.fertstert.org).

FIGURE 3



In vitro assays: cell proliferation and migration assays. (**A**) Assessment of optimal concentration to supplement culture media. aPRP = activated platelet-rich plasma; aUCP = activated umbilical cord plasma. (**B**) Proof-of-concept MTS (proliferation assay) results in ICE7 stem cell line: comparison of cell proliferation between aPRP and activated platelet-poor plasma (aPPP) fractions and activated and nonactivated PRP control (a/naPRPC) samples. MTS results using (**C**) human endometrial stromal stem cells (ICE7; n = 3) and (**D**) human endometrial stromal cells (hESCs; n = 10). (**E**) Wound healing (cell migration) assays results using ICE7 stem cell line (n = 3) and hESCs (n = 10). Data are presented as mean ± SD. *$P<.05$.

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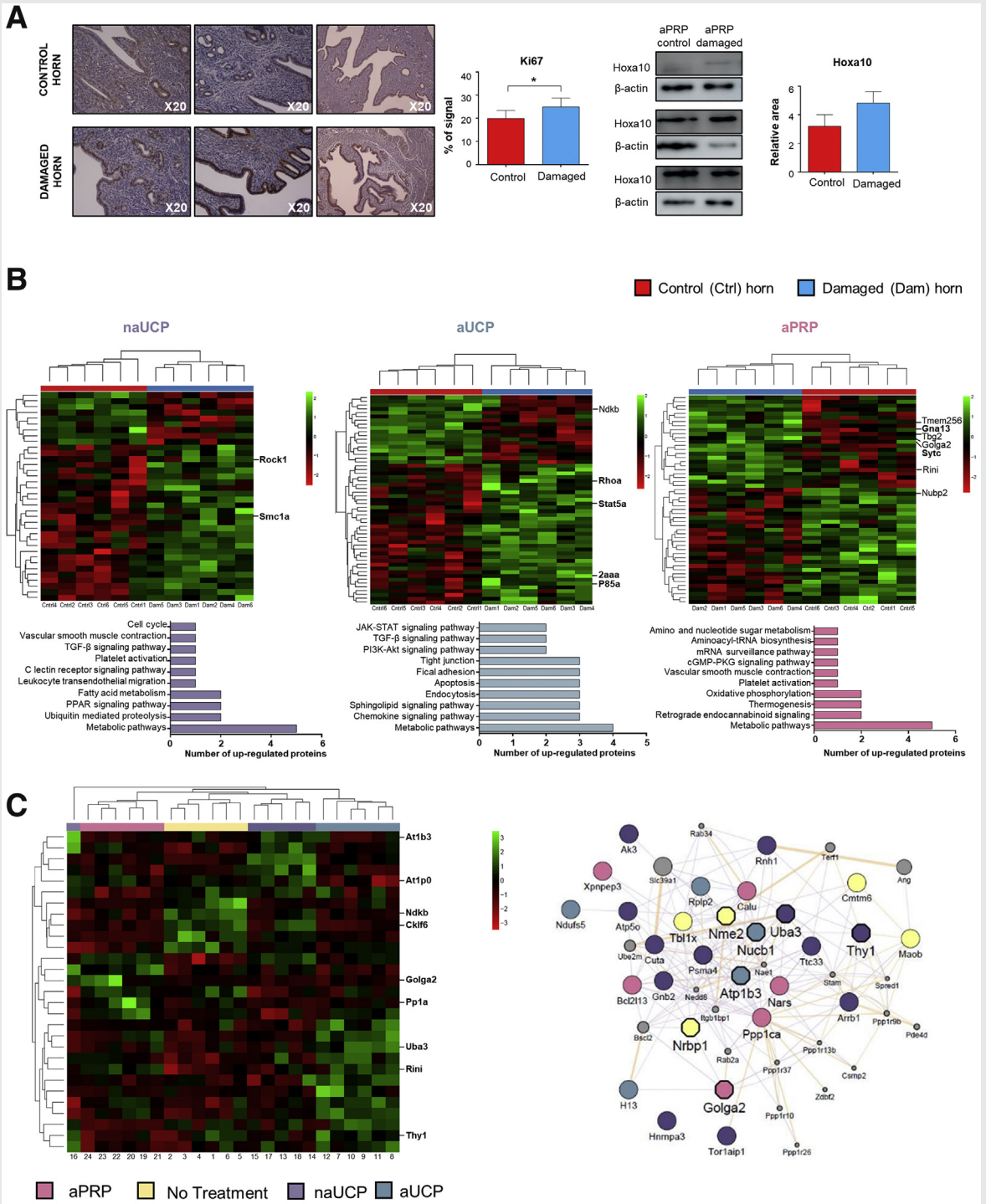
FIGURE 4



In vivo assays: Asherman syndrome/endometrial atrophy murine model. (**A**) Proof of concept: After activated platelet-rich plasma (aPRP) treatment, Ki67 immunostaining was performed in uterine tissue sections (×20 magnification) and Hoxa10 Western blot performed from protein extracts of uterine horns. Data are presented as median with interquartile range. (**B**) Heatmaps of Sequential Window Acquisition of All Theoretical-Mass Spectra (SWATH-MS) data, showing protein presence between damaged and undamaged horns. Up-regulated proteins related to the KEGG pathways in the graphs below are highlighted in bold. The other proteins were also detected in the comparison among the four types of damaged horns or validated by Western blot. (**C**) Left: Heatmap of SWATH-MS results after Elastic Net penalization comparing damaged horns from the four groups. Proteins related to proregenerative events are highlighted in bold. Right: network (GeneMania) showing the interactions among the differentially expressed proteins in the different groups. *$P<.05$.

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Increased In Vitro Cell Proliferation and Migration After Treating Cells with Plasma

To measure viable cell proliferation rates, we first performed MTS assays. The preliminary tests using ICE7 cells revealed that the highest rate was found in the presence of 1% plasma in the culture medium ($P=.0015$; Fig. 3A) and reaffirmed the higher potential of aPRP ($P=.0034$) to stimulate cell proliferation compared with aPPP (Fig. 3B). These initial tests also reinforced the positive effect of the activation process, when comparing aPRPC ($P=.0171$) and naPRPC with the No Treatment condition (Fig. 3B). As seen in Figure 3B, aPRPC (fold change [FC] = 1.20) seems to have an effect similar to aPRP (FC = 1.26), confirming that plasma from AS/EA patients is not affected.

Second, in the main proliferation assay using ICE7, proliferation rates from aPRP ($P=.0380$) and aUCP ($P=.0042$) conditions were significantly higher compared with the No Treatment condition. Furthermore, this increment was higher when applying aUCP (FC = 1.36) instead of aPRP (FC = 1.22; Fig. 3C). These results were reproducible when using primary stromal cells (hESCs). Cell proliferation was significantly incremented with the addition of aPRP ($P=.0394$) and aUCP ($P<.0001$) to the culture medium. In this case, aPRP proliferation rate increased by a 1.30-fold change and aUCP by 1.80-fold change (Fig. 3D).

Regarding the wound healing assay, results appeared to generally corroborate these results, despite being not statistically significant. There was a noticeably higher trend to cover the initial gap (% of gap closure 24 hours after the wound) when cells were treated with aPRP (42.20%) or aUCP (49.40%) compared with No Treatment (38.77%), with the use of ICE7 cells (Fig. 3E, upper part). This effect was also reproducible with hESCs: aPRP (50.78%) and aUCP (53.69%) seemed to induce a higher wound closure rate than No Treatment (42.52%; Fig. 3E, lower part).

In Vivo Increased Expression of Cell Proliferation and Endometrial Markers After Applying aPRP

A preliminary test performed in the animal model revealed that aPRP was likely promoting tissue regeneration. The Ki67 immunoassay, which evaluates the cell proliferation rate, showed an increased significant signal ($P=.0106$) in the damaged horns compared with the control horns in the aPRP group (Fig. 4A, left). Hoxa10, an important transcription factor for many genes involved in endometrial function and development, as measured by means of WB, also showed a more intense signal in damaged versus control horns (Fig. 4A, right), without being statistically significant.

Relative Protein Quantification in Murine Uterine Horns Revealed aUCP as the Criterion-Standard Treatment

The SWATH-MS analysis of the right and left uterine horns (n = 48) from all mice groups (No Treatment, aPRP, naUCP, and aUCP) revealed the presence of 2,766 different tissue proteins. We performed a statistical analysis using two different approaches: First, we compared the damaged versus control horns in aPRP, naUCP, and aUCP groups separately, and second, we compared the damaged horns in each group versus the damaged ones in the other groups. A detailed list of the differentially expressed proteins after performing both approaches is presented in Supplemental Table 2 (available online at www.fertstert.org).

When we globally analyzed the abundance of proteins in both horns, the damaged and control group profiles were clearly distinguishable (Fig. 4B). Interestingly, we found that the up-regulated proteins in the damaged horns of the aUCP group were implicated in different events related to tissue regeneration, such as PI3K-Akt, TGFβ, or JAK-STAT signaling pathways. These proteins were P85α (a subunit of phosphatidylinositol 3-kinase), 2aaa (Pr65 subunit of protein phosphatase 2A), Stat5a (a signal transducer and activator of transcription), and Rhoa (a small guanosine triphosphatase). The up-regulated proteins in the damaged horns of the other groups, aPRP and aUCP, were not involved in these signaling pathways.

When we analyzed only the damaged horns, proteins seemed to group depending on the treatment applied (Fig. 4C). When analyzing these proteins in depth, damaged horns in the aUCP group showed an up-regulation of Uba3 (NEDD8-activating enzyme E1 catalytic subunit), Thy1 (CD90 antigen), Rnh1 (ribonuclease inhibitor), and Atpo (mitochondrial membrane ATP synthase). These last 2 proteins, together with Ppp1ca (protein phosphatase) and Golga2 (Golgin subfamily A member 2), were up-regulated in the aPRP group. Atp1b3 (a subunit of a sodium/potassium-transporting ATPase) was up-regulated in the naUCP group, and Cklf6 (member of the chemokine-like factor superfamily) and Ndkb (nucleoside diphosphate kinase B) were up-regulated in the No Treatment group.

WB analysis of several of these differentially expressed proteins, in both approaches, corroborated the regulation pattern of these proteins (Supplemental Fig. 1, available online at www.fertstert.org). The selected proteins were Cklg6, Golga2, Uba3, Atp1b3, the cytosolic Fe-S cluster assembly factor Nubp2, tubulin γ2, and the transmembrane protein 256.

DISCUSSION

In this study, we confirmed the regenerative potential of plasma depending on its specific composition or source. This was done via assessing endometrial cell proliferation and migration in in vitro assays and tissue regeneration in an AS murine model. We also demonstrated via proteomic analysis the superior potential of UCB over adult blood as a source of regenerative plasma.

The main goal in patients affected with AS or EA is to regenerate the endometrial tissue, restoring its normal proliferation rate and thickness. Thus, it is necessary to trigger the complex regeneration process, which includes distinct events such as angiogenesis, cell recruitment, matrix deposition, and inflammation (47). We postulate that the presence and concentrations of several growth factors, chemokines, and cytokines change with the age of the plasma and that PRP could play a role in these kinds of processes. We demonstrated

that the origin (adult blood or UCB) affected the concentration of some of these molecules. Among these, a potent mitogen (HGF) and other growth factors involved in cell chemotaxis and proliferative activities (PDGFBB, SCF), events involving angiogenesis (TGFβ, VEGFD), and the inflammatory response (IL1-RA) had a statistically significant higher concentration in UCP (in both aUCP and naUCP groups) (29, 30), providing a higher regenerative ability, whereas IL2, IL7, and IL15 were more concentrated in adult PRP. The increased concentration of these proinflammatory cytokines could be explained by the immune immaturity of the UCB (29, 48), a characteristic that, added to the fact that plasma is an acellular blood fraction, minimizes any kind of immunologic issue, that is, a rejection reaction when using UCB (49). These results complement previous studies where a detrimental effect of aging in plasma was shown (32, 50). Castellano et al. demonstrated that UCP could regenerate hippocampal function in aged mice (32) better than adult plasma via specific key factors. These proteins were also detected only (or with a higher score) in the UCP samples, corroborating the results of that study. The in silico functional analysis of these plasma proteins showed different signaling pathways involved in cell proliferation, differentiation, and migration, such as the PI3K-Akt pathway, that could be triggering the regeneration processes. The identification of these pathways also correlates with our in vitro results, where an increase in cell proliferation and migration rates was seen when adding PRP to the medium, with UCP outperforming the other groups.

Some studies already demonstrated the beneficial effect of adult PRP over animal models with induced uterine damage (24, 25), but none included the younger UCP as done in this study. In our AS murine model, deep analysis of the protein patterns of the uterine tissue established that aUCP produced major changes in the protein abundance tissue pattern. The functional analysis revealed that the upregulated proteins in the aUCP group were mainly involved in the regulation of angiogenesis and mitotic cell cycle: Uba3, described as essential for NEDD8-mediated neddylation, which is required for normal human endometrial function (51); Thy1/CD90, a well known endometrial stromal cell marker (2); and Rini, a regulator of neovascularization that has also been described as down-regulated in endometrial glands in endometriotic versus healthy endometrium samples (52). Furthermore, when comparing the damaged versus the control uterine horns in the aUCP group, upregulated proteins, such as Stat5a (a mediator of cell response to SCF and other growth factors), were also involved in PI3K-Akt and JAK-STAT pathways, as were the plasma proteins, but in further steps of the cascades.

Considering that this study pioneers the use of UCP in the endometrial field, even in preclinical murine models, we are aware of several limitations. Because plasma samples were not very easy to obtain, our study size was small. Despite the wound healing assay being the most widely used, several restrictions, such as injury, stress, and nonproliferation processes, affecting cell migration have been described after performing the scratch. Other options related to this issue should be further explored, either with longer exposure times or with other types of migration assays.

CONCLUSION

Given the specific mechanisms described above, it becomes apparent that PRP is a promising regenerative enhancer, especially when using UCB. Activated UCP was revealed as the best treatment in three different scenarios: analysis of plasma composition (higher concentrations of key molecules involved in proregenerative processes), in vitro evaluation of cell proliferation, and migration and uterine tissue analysis in an AS murine model. Nonetheless, despite the fact that autologous PRP has already started to be used in human patients suffering from AS/EA (27, 28, 53–55), specific clinical studies are needed to reinforce the higher effectiveness of UCP versus adult PRP.

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Comparación de plasma rico en plaquetas de diferentes orígenes como opción de tratamiento para patologías endometriales causantes de infertilidad.

Objetivo: Estudiar el efecto del plasma humano de diferentes orígenes, sangre de cordón umbilical y plasma rico en plaquetas (PRP) de sangre adulta, sobre la regeneración del daño endometrial.

Diseño: Análisis de composición, ensayos *in vitro* y modelo murino preclínico que utiliza los diversos plasmas para promover la regeneración endometrial.

Entorno: Laboratorios hospitalarios y universitarios.

Paciente (s) / Animal (es): Se utilizó plasma adulto de cuatro pacientes con síndrome de Asherman/atrofia endometrial y uno de una mujer fértil, plasma comercial de cordón umbilical y el modelo de ratones NOD/SCID con daño uterino.

Intervención (es): Se cultivaron *in vitro* células del estroma endometrial procedentes de cultivo primario y la línea celular de células madre endometriales, además se trataron ratones NOD/SCID con daño uterino con muestras de plasma con diferentes orígenes.

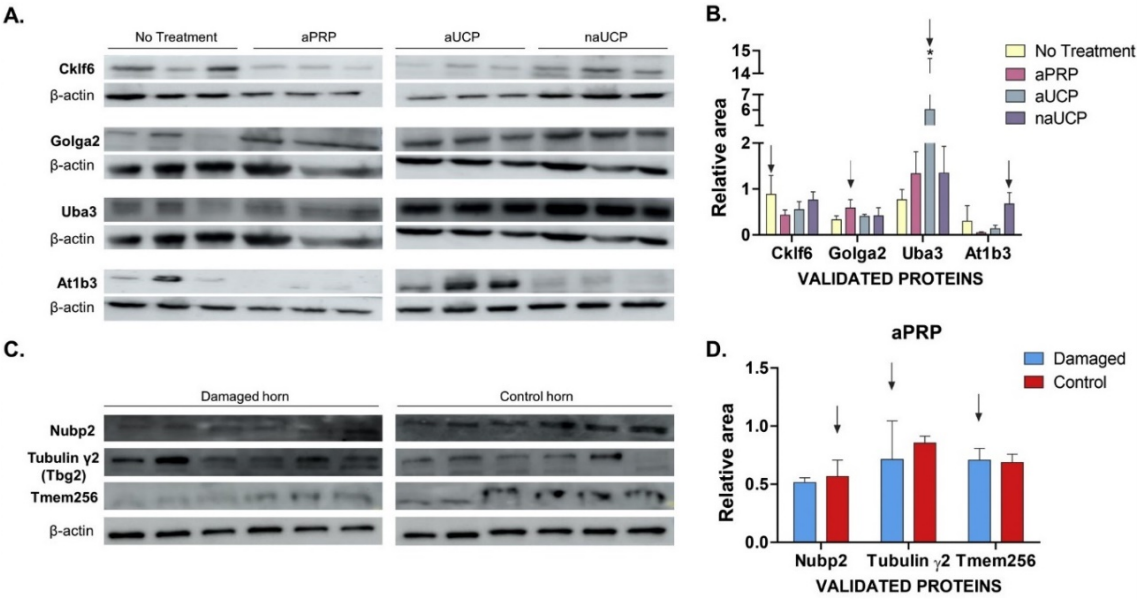
Principales medidas de resultado: Investigar los posibles efectos beneficiosos del PRP en pacientes con síndrome de Asherman/atrofia endometrial. Probar si el plasma de la sangre del cordón umbilical humano tiene un efecto más fuerte que el PRP adulto en la regeneración endometrial. Demostrar si el PRP de pacientes con síndrome de Asherman/atrofia endometrial fue tan eficaz como el PRP de una mujer sana y, por tanto, podría utilizarse como tratamiento autólogo.

Resultado (s): Todas las muestras de plasma contenían moléculas con un alto potencial de regeneración (factor de células madre, factor de crecimiento derivado de plaquetas BB, trombospondina-1, factor de von Willebrand). Además, el mayor aumento en la tasa de proliferación y migración en los cultivos *in vitro* se encontró cuando las células del estroma endometrial se trataron con plasma de cordón umbilical; el PRP adulto también reveló un incremento significativo. En el modelo de ratón, se detectó una mayor expresión en el endometrio dañado de Ki67 y Hoxa10 después de aplicar PRP adulto, y el análisis proteómico reveló un perfil de expresión de proteínas específico según el tratamiento. El tejido uterino dañado expresó más marcadores pro-regenerativos después de aplicar plasma de cordón umbilical (Stat5a, Uba3, Thy1) en comparación con los otros tratamientos (plasma de cordón umbilical no activado, PRP adulto activado y ningún tratamiento).

Conclusión (es): El PRP humano posee propiedades de regeneración aplicables a patologías endometriales. Además de eso, estos efectos regenerativos parecen ser más evidentes cuando el origen o fuente de obtención es la sangre del cordón umbilical.

SUPPLEMENTAL MATERIAL OF MANUSCRIPT II

Supplemental Figure 1



Supplemental Figure 1. Validation of Elastic Net regularization Sequential Window Acquisition of All Theoretical-Mass Spectra (SWATH-MS) results with the use of Western blot. *A. Western blot bands for one validated protein among the up-regulated ones in each of the groups (comparison between damaged horns from the four groups): Cklf6 (No Treatment), Golga2 (activated platelet-rich plasma; aPRP), Uba3 (activated umbilical cord plasma; aUCP), and At1b3 (activated umbilical cord plasma; naUCP). B. Graph showing the relative area measurements (ImageJ software). C. Western blot bands for three validated proteins (one down-regulated and two up-regulated when comparing damaged vs. control horns) in the aPRP group (comparison between both horns from naUCP, aUCP, and aPRP groups): Nubp2 (down), tubulin γ2 (up), and Tmem256 (up). D. Graph showing the relative area measurements (ImageJ software). Data are presented as mean ± SD. ∗P < 0.050. Arrows indicate the group (B) or horn (D) in which the protein expression value was higher.*

Supplemental Table I

TARGET PROTEINS	COMPLETE NAME	aUCP	naUCP	aPRP
GM CSF	Granulocyte-macrophage colony-stimulating factor	UDL	UDL	UDL
IFN γ	Interferon gamma	UDL	UDL	UDL
IL1 β	Interleukin-1 beta	2.631	3.252	6.489
IL 12 p70	Interleukin-12, p70	UDL	UDL	UDL
IL13	Interleukin-13	17.650	UDL	UDL
IL18	Interleukin-18	225.043	322.307	312.571
IL2	Interleukin-2	14.932	18.994	20.059
IL4	Interleukin-4	UDL	UDL	UDL
IL5	Interleukin-5	18.883	6.296	UDL
IL6	Interleukin-6	UDL	UDL	UDL

TNF α	Tumor necrosis factor alpha	UDL	UDL	UDL
IL10	Interleukin-10	UDL	UDL	UDL
IL17A	Interleukin-17A	17.552	8.072	UDL
IL21	Interleukin-21	UDL	UDL	UDL
IL22	Interleukin-22	UDL	UDL	UDL
IL23	Interleukin-23	UDL	UDL	UDL
IL27	Interleukin-7	UDL	UDL	UDL
IL9	Interleukin-9	UDL	UDL	UDL
IFN α	Interferon alpha	UDL	UDL	UDL
IL1 α	Interleukin-1 alpha	UDL	UDL	UDL
IL1RA	Interleukin-1 receptor type 1	991.067	1101.370	534.291
IL15	Interleukin-15	6.942	4.863	27.540
IL31	Interleukin-31	UDL	UDL	UDL
IL7	Interleukin-7	7.189	6.694	9.830
TNF beta	Lymphotoxin-alpha	NA	NA	NA
eotaxin	Eotaxin	37.535	43.134	33.230
gro alpha/KC	Growth-regulated alpha protein	UDL	UDL	UDL
IL8	Interleukin-8	UDL	UDL	UDL
IP10	C-X-C motif chemokine 10	UDL	50.824	60.033
MCP1	C-C motif chemokine 2	104.826	118.202	74.710
MIP1 α	C-C motif chemokine 3	UDL	UDL	UDL
MIP1 β	C-C motif chemokine 4	UDL	UDL	UDL
RANTES	Regulated on activation, normal T cell expressed and secreted, C-C motif chemokine 5	NA	NA	NA
SDF1 α	Stromal cell-derived factor 1	483.637	308.482	588.130
BDNF	Brain-derived neurotrophic factor	217.209	205.151	425.956
b NGF	Beta-nerve growth factor	311.380	308.400	756.450
EGF	Epidermal growth factor	19.760	20.947	63.870
FGF2	Fibroblast growth factor 2	9.274	UDL	UDL
HGF	Hepatocyte growth factor receptor	382.793	398.939	87.560
LIF	Leukemia inhibitory factor	25.951	24.160	25.860
PDGF BB	Platelet-derived growth factor subunit B	3702.540	4523.905	2312.272
PIGF	Phosphatidylinositol-glycan biosynthesis class F protein	134.133	128.939	160.930
SCF	Kit ligand	31.250	40.129	6.840
VEGF A	Vascular endothelial growth factor A	291.082	371.897	192.140
VEGF D	Vascular endothelial growth factor D	33.225	34.913	16.150
TGF β	Transforming growth factor beta	2449.059	441.121	1340.786

Supplemental Table I. Multiplex protein assays results of the analyzed plasmas. *Protein targets included in the multiplex assays and the concentration values detected in analyzed samples. Blue wells show those factors that were statistically significant among the umbilical cord samples and the adult.*

Supplemental Table II

Proteins	Coefficient naUCP	Coefficient aUCP	Coefficient aPRP	Coefficient No Treatment
Q9DB20|ATPO	4.16E-07	-4.92E-07	3.63E-08	4.00E-08
Q9D6K7|TTC33	2.80E-05	2.47E-04	-3.22E-04	4.77E-05
Q9QXE7|TBL1X	1.99E-06	4.85E-07	-4.56E-06	2.09E-06
P01831|THY1	2.29E-07	6.66E-07	-1.14E-06	2.41E-07
Q9D8V0|HM13	7.59E-06	-1.64E-05	-7.84E-05	8.72E-05
Q9CZ69|CKLF6	4.47E-06	-8.90E-06	-8.31E-06	1.27E-05

Q9CQ89|CUTA	8.49E-07	-2.31E-06	2.47E-06	-1.01E-06
O35887|CALU	9.52E-06	-7.37E-07	1.58E-05	-2.46E-05
Q9WTP7|KAD3	7.63E-07	1.75E-06	-1.98E-06	-5.32E-07
P97370|AT1B3	4.01E-07	-1.35E-07	-2.25E-07	-4.09E-08
Q99LY9|NDUS5	1.60E-05	-4.40E-06	-9.91E-06	-1.67E-06
P99027|RLA2	1.85E-05	-2.88E-06	-6.77E-06	-8.83E-06
Q02819|NUCB1	1.44E-05	-3.23E-06	-1.47E-06	-9.66E-06
Q8BP47|SYNC	-1.78E-05	-2.65E-06	1.04E-05	1.01E-05
Q8BW75|AOFB	-6.82E-06	-2.75E-06	-8.29E-06	1.79E-05
Q01768|NDKB	-3.34E-07	-6.70E-07	-1.06E-06	2.06E-06
Q99J45|NRBP	-5.63E-07	8.71E-08	-1.39E-06	1.87E-06
Q91VI7|RINI	-4.35E-07	5.94E-07	7.91E-07	-9.49E-07
P59017|B2L13	-2.35E-05	4.43E-06	2.14E-05	-2.41E-06
Q8BWG8|ARRB1	-1.09E-05	1.78E-05	3.59E-06	-1.05E-05
P62137|PP1A	-1.84E-06	-1.06E-06	3.32E-06	-4.15E-07
Q921M4|GOGA2	-5.60E-06	-7.07E-06	1.83E-05	-5.59E-06
Q8BG05|ROA3	-1.32E-07	3.03E-07	-1.31E-08	-1.57E-07
P62880|GBB2	-1.06E-06	1.66E-06	-1.89E-07	-4.13E-07
Q9R1P0|PSA4	-1.11E-06	4.24E-06	-1.91E-06	-1.23E-06
Q8C878|UBA3	-1.98E-06	4.23E-06	-7.11E-07	-1.54E-06
Q8C1A5|THOP1	-2.56E-06	5.58E-06	-9.06E-08	-2.94E-06
Q921T2|TOIP1	-2.87E-07	1.46E-05	-9.26E-07	-1.34E-05
B7ZMP1|XPP3	-4.09E-05	9.74E-05	-4.01E-05	-1.65E-05

Supplemental Table II-A. SWATH-MS statistics results. *Enumeration, including the ElasticNet coefficient, of the statistically differentiated proteins when performing the intergroup comparison among all damaged horns. In blue, coefficients above one, which indicate up regulation of the protein.*

Non activated UCP		**Activated UCP**		**Activated PRP**	
Proteins	**Coefficient**	**Proteins**	**Coefficient**	**Proteins**	**Coefficient**
Q9JII5|DAZP1	8.68E-01	**Q8BG05|ROA3**	1.19E+00	**A2ASQ1|AGRIN**	5.22E-01
P35282|RAB21	8.51E-01	**Q76MZ3|2AAA**	6.66E-01	**Q921M4|GOGA2**	1.68E-01
P14824|ANXA6	8.38E-01	**Q9CZD3|GARS**	4.28E-01	**Q9D0R2|SYTC**	1.61E-01
Q9D892|ITPA	5.41E-01	**Q8K1M6|DNM1L**	4.08E-01	**Q8K4L3|SVIL**	1.30E-01
Q99LY9|NDUS5	4.83E-01	**P50544|ACADV**	3.79E-01	**Q8VCK3|TBG2**	9.76E-02
Q61074|PPM1G	4.71E-01	**P56399|UBP5**	3.76E-01	**P27601|GNA13**	8.94E-02
Q8C460|ERI3	4.64E-01	**Q8VCW8|ACSF2**	3.31E-01	**P97470|PP4C**	8.84E-02
Q8C7R4|UBA6	2.61E-01	**P68368|TBA4A**	3.16E-01	**Q99KJ8|DCTN2**	8.68E-02
P19973|LSP1	2.29E-01	**Q91WQ3|SYYC**	2.25E-01	**Q80ZJ1|RAP2A**	8.27E-02
Q9D6Y7|MSRA	1.80E-01	**P08775|RPB1**	1.75E-01	**P47934|CACP**	7.14E-02
P36916|GNL1	1.73E-01	**Q9CWZ3|RBM8A**	1.70E-01	**Q91VW3|SH3L3**	6.79E-02
P32020|NLTP	1.63E-01	**Q9ER88|RT29**	1.59E-01	**Q8VE47|UBA5**	6.71E-02
P61089|UBE2N	1.48E-01	**Q9D880|TIM50**	1.59E-01	**Q9JLI6|SCLY**	6.31E-02

Q9CQM5|TXD17	1.42E-01	**O08734|BAK**	1.57E-01	**Q8VCF0|MAVS**	5.61E-02
Q3TLP5|ECHD2	1.18E-01	**P53996|CNBP**	1.52E-01	**Q9CQ91|NDUA3**	5.27E-02
Q9D6F9|TBB4A	1.05E-01	**P26450|P85A**	1.36E-01	**Q91VI7|RINI**	4.65E-02
Q80YR5|SAFB2	7.21E-02	**P10107|ANXA1**	1.20E-01	**Q8BWY3|ERF1**	3.71E-02
P97390|VPS45	5.94E-02	**Q9D379|HYEP**	9.30E-02	**Q78XR0|TPC6A**	3.30E-02
Q9JKY0|CNOT9	4.76E-02	**Q3U2P1|SC24A**	6.88E-02	**Q9JK38|GNA1**	3.22E-02
Q9QYY8|SPAST	4.16E-02	**P21958|TAP1**	4.85E-02	**Q9JMA1|UBP14**	2.76E-02
Q6P8J2|SAT2	3.07E-02	**Q71FD7|FBLI1**	4.83E-02	**Q5F285|TM256**	1.01E-02
Q9CQR6|PPP6	2.72E-02	**Q9CR86|CHSP1**	4.64E-02	**Q99JT9|MTND**	7.72E-03
Q8VCN9|TBCC	2.24E-02	**Q922L6|NELFD**	4.35E-02	**O35683|NDUA1**	9.12E-04
Q9CU62|SMC1A	1.78E-02	**Q8BI72|CARF**	4.32E-02	**P28076|PSB9**	-1.50E-03
Q99PU5|ACBG1	1.42E-02	**Q8K2B3|SDHA**	3.78E-02	**P14131|RS16**	-1.93E-03
P70335|ROCK1	1.08E-02	**P63242|IF5A1**	3.77E-02	**Q60766|IRGM1**	-2.34E-03
Q80TL7|MON2	4.50E-03	**P36552|HEM6**	3.19E-02	**Q7TNP2|2AAB**	-3.58E-03
Q922P9|GLYR1	-1.61E-02	**P42230|STA5A**	2.71E-02	**Q9CRC8|LRC40**	-4.00E-03
O55028|BCKD	-1.98E-02	**Q8BWG8|ARRB1**	2.58E-02	**Q80Y17|L2GL1**	-1.23E-02
Q6P2B1|TNPO3	-3.61E-02	**P14824|ANXA6**	2.53E-02	**Q9DBG1|CP27A**	-1.60E-02
Q6NV83|SR140	-4.85E-02	**Q8K3C3|LZIC**	2.14E-02	**Q8JZM8|MUC4**	-2.04E-02
P49615|CDK5	-5.63E-02	**Q62523|ZYX**	1.94E-02	**Q9D0R8|LSM12**	-2.39E-02
Q9ET01|PYGL	-2.20E-01	**Q9QUI0|RHOA**	1.37E-02	**Q811D0|DLG1**	-3.94E-02
Q9R0N0|GALK1	-3.73E-01	**P47968|RPIA**	1.34E-02	**P09470|ACE**	-7.97E-02
Q8CHP8|PGP	-9.68E-01	**Q07113|MPRI**	1.14E-02	**Q8C7K6|PCYXL**	-8.07E-02
Q9ESZ8|GTF2I	-1.85E+00	**Q9Z2Q6|SEPT5**	9.45E-03	**P61211|ARL1**	-8.15E-02
		Q05186|RCN1	6.31E-03	**Q99LG2|TNPO2**	-1.09E-01
		P31786|ACBP	-1.61E-03	**A2ABV5|MED14**	-1.16E-01
		Q61789|LAMA3	-1.58E-02	**Q8R5F8|ES8L1**	-1.19E-01
		P60843|IF4A1	-1.92E-02	**Q62470|ITA3**	-1.36E-01
		P99026|PSB4	-2.81E-02	**Q924K8|MTA3**	-1.39E-01
		Q9D8X2|CC124	-4.62E-02	**Q8BMG7|RBGPR**	-1.54E-01
		Q06185|ATP5I	-5.77E-02	**Q9D8V0|HM13**	-1.59E-01
		Q9D7B7|GPX8	-6.39E-02	**Q9R062|GLYG**	-1.75E-01
		Q3UZ39|LRRF1	-7.65E-02	**O35972|RM23**	-1.82E-01
		Q921Y0|MOB1A	-1.19E-01	**P53810|PIPNA**	-1.92E-01
		Q9WV98|TIM9	-2.46E-01	**Q2EMV9|PAR14**	-2.77E-01
		Q9R0P5|DEST	-3.09E-01	**Q9R061|NUBP2**	-2.98E-01
		P62309|RUXG	-3.96E-01	**Q9CQL5|RM18**	-2.98E-01
		Q8R016|BLMH	-4.26E-01	**Q91V09|WDR13**	-3.41E-01
		Q60692|PSB6	-4.46E-01	**P84104|SRSF3**	-3.65E-01
		P56391|CX6B1	-4.63E-01	**Q61510|TRI25**	-4.75E-01
		Q01768|NDKB	-4.80E-01		
		P63168|DYL1	-5.89E-01		
		P48758|CBR1	-7.08E-01		

Supplemental Table II-B. SWATH-MS statistics results. *Enumeration, including the ElasticNet coefficient, of the statistically differentiated proteins when performing the intragroup comparison in the naUCP, aUCP and aPRP groups. In blue, coefficients above 1, which indicate up regulation of the protein.*

3. ANNEX III. MANUSCRIPT III

cells

MDPI

Review

Stem Cells and the Endometrium: From the Discovery of Adult Stem Cells to Pre-Clinical Models

Lucía de Miguel-Gómez [1,2,†], Sara López-Martínez [1,†], Emilio Francés-Herrero [1,2,†], Adolfo Rodríguez-Eguren [1], Antonio Pellicer [2,3] and Irene Cervelló [1,*]

[1] IVI Foundation, Health Research Institute La Fe, 46026 Valencia, Spain; lucia.demiguel@ivirma.com (L.d.M.-G.); sara.lopezma@ivirma.com (S.L.-M.); emilio.frances@ivirma.com (E.F.-H.); adolfo.rodriguez@ivirma.com (A.R.-E.)
[2] Department of Pediatrics, Obstetrics, and Gynaecology, School of Medicine, University of Valencia, 46010 Valencia, Spain; antonio.pellicer@uv.es
[3] IVIRMA Rome Parioli, 00197 Rome, Italy
* Correspondence: irene.cervello@ivirma.com; Tel.: +34-963-903-305
† These authors contributed equally to this work.

Abstract: Adult stem cells (ASCs) were long suspected to exist in the endometrium. Indeed, several types of endometrial ASCs were identified in rodents and humans through diverse isolation and characterization techniques. Putative stromal and epithelial stem cell niches were identified in murine models using label-retention techniques. In humans, functional methods (clonogenicity, long-term culture, and multi-lineage differentiation assays) and stem cell markers (CD146, SUSD2/W5C5, LGR5, NTPDase2, SSEA-1, or N-cadherin) facilitated the identification of three main types of endogenous endometrial ASCs: stromal, epithelial progenitor, and endothelial stem cells. Further, exogenous populations of stem cells derived from bone marrow may act as key effectors of the endometrial ASC niche. These findings are promoting the development of stem cell therapies for endometrial pathologies, with an evolution towards paracrine approaches. At the same time, promising therapeutic alternatives based on bioengineering have been proposed.

Keywords: endometrium; niche; stem cells; animal models; regeneration

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1. Introduction

Stem cells are undifferentiated cells capable of simultaneously self-renewing and differentiating into multiple tissue-specific cell types under appropriate stimuli [1,2]. Traditionally, stem cells can be classified according to their location and differentiation potency. The most potent stem cells are zygotes, classified as totipotent stem cells, which have the ability to generate a whole embryo and extra-embryonic structures. Then, pluripotent stem cells such as embryonic stem cells give rise to the three primary germ layers, namely endoderm, ectoderm, and mesoderm. Induced pluripotent stem cells, generated by the reprogramming of somatic cells, are included in this group. Unipotent stem cells have the narrowest differentiation capability and divide themselves repeatedly into a single cell type. Finally, multipotent cells give rise to specific lineages [3]. Adult stem cells (ASCs), also referred to as somatic stem cells, are a genre of multipotent stem cells located in specific differentiated organs and can differentiate into a limited type of mature cell to maintain tissue homeostasis [2]. The necessary conditions are provided by the specific anatomical location surrounding the ASCs. This microenvironment, called the stem cell niche, gives rise to autocrine, paracrine, and systemic signals that enable stem cell maintenance and differentiation into specific cell types that participate in tissue repair or regeneration [4,5]. Most of the described ASCs reside in the bone marrow, but they are also detected in several organs and play a crucial role in tissue homeostasis, renewal, and repair [6]. Consequently, this transdifferentiation capacity enables research into therapeutic approaches in tissues such as the blood [7], intestine [8], skin [9], muscle [10], brain [11], and endometrium [12].

The endometrium is the innermost lining of the uterus and its main function is preparing for implantation and attracting the blastocyst towards the uterus. The human endometrium is divided into two different layers with different properties. First, the functionalis, which is the upper layer, is formed by luminal epithelium and subjacent stroma as well as microvasculature. Second, the basalis is constituted by glands and stroma that are preserved throughout the female's life [13]. During menstruation, the functionalis layer is removed from the body through the menstrual blood, while the basalis remains as an endometrial supply for regeneration of a new functional layer in the next cycle. The human endometrium has some features including menstruation that make it physiologically unique from the murine models. The human menstrual cycle consists of three stages: growth, differentiation, and shedding, which occur around 400 times until menopause [14]. In the mouse, the estrous cycle is divided into four stages (proestrus, estrus, metestrus, and diestrus) occurring every 4 to 5 days, but does not involve menstruation and regeneration of the functionalis layer [15]. Mice undergo up to 80 estrous cycles and can give rise to 8-10 litters during their reproductive life, so active repair and regeneration mechanisms in the endometrial mucosa are critically important [16]. This pronounced remodeling ability and the later proliferative changes seen in adult mammals have driven the hypothesis that there is a niche of ASCs in the endometrium that is activated in every cycle [17,18]. Accordingly, alterations in this endogenous niche could be responsible for endometrial pathology, causing fertility problems [19]. Endometrial pathologies such as Asherman syndrome (AS), caused by the presence of intrauterine adhesions [20], or endometrial atrophy (EA), characterized by an atrophic and usually thin endometrium [21], could originate from insufficient production of endogenous cells and/or a non-functional ASC niche. Other gynecological pathologies such as endometriosis could also be caused by variations in endogenous endometrial ASC activity [19].

Even though endometrial ASCs have been described in different mammals, such as cows [22], pigs [23], sheep [24], horses [25], and non-human primates [26], in this review we focus on the discovery of stem cells in mice and humans, pointing out how these ASCs were identified and describing the different techniques employed (Figure 1). In addition, we highlight the value of pre-clinical models of stem cell therapy to treat AS and EA.

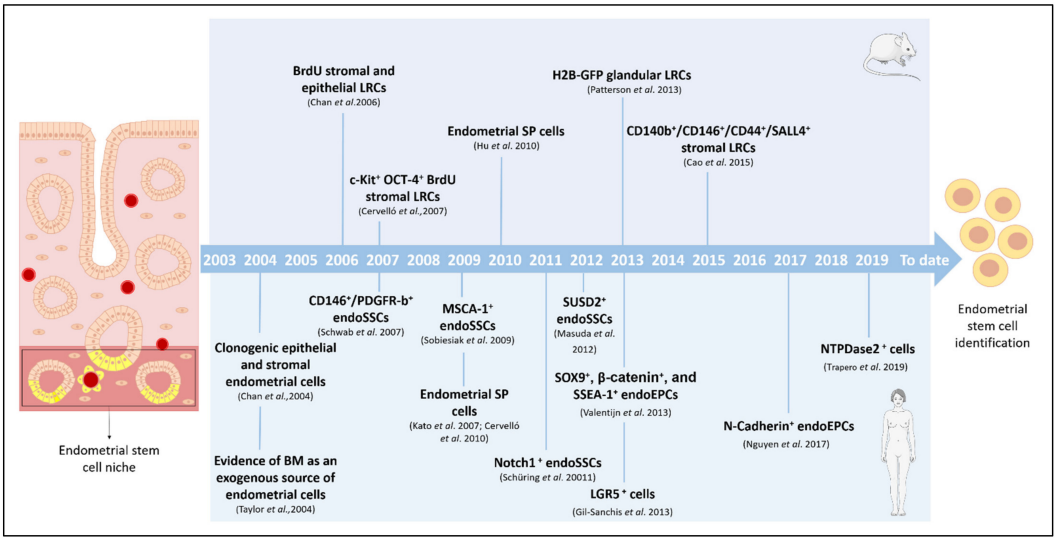

Figure 1. Milestones in the identification, isolation, and characterization of endometrial stem cells. Timeline of the principal findings concerning endometrial stem cells and specific isolation techniques in murine and human models. BM: bone marrow; BrdU: bromodeoxyuridine; CD: cluster of differentiation; endoSSCs: endometrial stromal stem cells; endoEPCs: endometrial epithelial progenitor cells; GFP: green fluorescent protein; H2B: histone 2B; LRCs: label-retaining cells; LGR: leucine-rich repeat containing G-protein-coupled receptor; MenSCs: menstrual blood stem cells; NTPDase2: ectonucleoside triphosphate diphosphohydrolase-2; SSEA-1: stage-specific embryonic antigen-1; SP: side population; SUSD2: sushi domain-containing-2.

2. Endometrial Stem Cells and Specific Niches

In 1978, Schofield proposed the concept of a stem cell niche for the first time, referring to an anatomically defined compartment in which stem cells reside and are able to renew and/or remain undifferentiated [27]. We know now how the niche is specialized and the dynamic microenvironment in which stem cells interact with differentiated cells, secreted factors, and/or components of the extracellular matrix (ECM) [28]. These stimuli determine the behavior (rate and pattern of division) of the stem cells in each specific tissue [29]. Prianishnikov was the first to propose the existence of ASCs in the endometrium [30] as an immature hormone-independent population, residing in the deepest basalis layer with the capability of differentiating towards hormone-responsible endometrial cells. The constant maintenance of this layer along the menstrual (or estrous) cycle makes it a reasonable candidate to be a reservoir of ASCs, even if some stem cells are also found in the functionalis [31]. In 1991, Padykula claimed that the population of stem-like cells migrates and produces different progenitor cells that differentiate specifically into vascular, epithelial, or stromal compartments, thanks to their specific niches [32]. Over the years, these endogenous endometrial ASCs have been proposed not only to be responsible for the cyclic endometrial growth and, consequently, for certain gynecological disorders, but also as useful in therapeutic approaches. According to the origin of the stem cells, endometrial stem cell niches include epithelial, stromal, and endothelial cells, and are likely to contribute to endometrial regeneration [33].

2.1. Identifying Endometrial Stem Cells in Murine Models

Our current knowledge about ASCs in the human endometrium would not be possible without reproductive biology studies in animal models, mainly murine ones. Given the absence of specific mouse endometrial stem cell markers, label-retention assays provided critical tools to identify, characterize, and localize these cell populations in vivo (Table 1).

Table 1. Endometrial stem cell identification and isolation techniques in human and murine models. All procedures reviewed in the text are summarized and classified.

Identification/Isolation Method		Main Characteristic	Application in Murine Endometrial ASCs	Application in Human Endometrial ASCs	References
Label-retention methods	BrdU	DNA analog Pulse-chase assays	YES	NO	[34–41]
	H2B-GFP	Transgenic system Allows detection of viable cells	YES	NO	[42,43]
Side population		ABC-transporter-positive cell identification	YES	YES	[44–51]
Stem cell marker identification	Flow cytometry	Allows identification and isolation; Compatible with multiple-marker profiles	YES	YES	[41,44,45,47–49,51–67]
	MACS	Magnetic labeling-based cell isolation method; Preferable for single marker procedures	NO	YES	[49,53,55,57,61,63,64]
Clonogenicity assays		Evaluates the ability of a single cell to produce a colony	YES	YES	[41,44,45,47–49,51–53,56,57,61,62,64,68–75]
Long-term culture		Stem cells exhibit long-term proliferative potential	NO	YES	[45,53,62,75]
Multi-lineage differentiation		MSCs can differentiate into adipocytes, osteoblasts, myocytes, and chondrocytes in vivo and in vitro	YES	YES	[44–46,52–58,62,65,67,74]

ASCs: adult stem cells; BrdU: bromodeoxyuridine label-retaining cells; H2B-GFP: histone 2B-green fluorescent protein; MACS: magnetic-activated cell sorting; MSCs: mesenchymal stem cells.

2.1.1. Label-Retention Methods in Murine Models

Bromodeoxyuridine Label-Retaining Cells

Label-retention assays are widely used to identify slow-cycling cells in multiple tissues. The concept derives from the quiescent or slow-cycling phenotype shared by most adult or somatic stem cells to preserve their proliferative potential and reduce errors during DNA duplication. The assay consists of the delivery of a pulse of a DNA analog, such as bromodeoxyuridine (BrdU), followed by a chase period in which the analog is absent. This method is not applicable for humans, but is very useful in animal models. When mice are injected with BrdU, all proliferating cells are marked, but only the quiescent ones maintain BrdU during the chase (or a period of time), and are identified as label-retaining cells (LRCs) [76]. This technique identified LRCs in the mouse endometrium [34–40] to unveil the biology of this tissue and pathologies causing infertility problems.

(1) Stromal BrdU-LRCs

Between 2006 and 2007, two independent studies described the presence of LRCs in the mouse endometrium for the first time [34,35]. After 12 weeks of BrdU injection, Chan et al. identified a small population of stromal LRCs (6%) at the endometrial–myometrial junction, beneath the luminal epithelium, or in a perivascular location near $CD31^+$ cells. These LRCs did not express stem cell antigen 1 (SCA-I) or the cluster of differentiation (CD) 45 [77], indicating a non-hematopoietic origin and demonstrating that they were not infiltrating leukocytes. Nevertheless, some LRCs expressed alpha-smooth muscle actin (α-SMA) and estrogen receptor alpha (ER-α) (16%), suggesting they were perivascular cells and responsive to hormonal stimulation.

Moreover, after 8-10 weeks of BrdU injection, Cervelló et al. identified a stromal LRC population expressing the stem cell factor receptor c-Kit and the pluripotent stem cell marker octamer-binding transcription factor 4 (OCT-4), also known as POU5FI. Expression of c-Kit and OCT-4 was restricted to cells located in the lower region of the endometrial stroma, representing 0.32% and 0.19% of the LRCs, respectively [35]. A recent study described stromal LRCs expressing PDGFR-b, CD146, CD44, CD90, and sal-like protein (SALL4) after 6 weeks of BrdU injection. Furthermore, the stromal LRCs persisted during pregnancy and proliferated after delivery, returning to their quiescent status after postpartum repair [36].

(2) Epithelial BrdU-LRCs

In contrast to stromal LRCs, epithelial LRCs have a short persistence in the endometrium of postnatal and prepubertal mice. After 3-4 weeks of BrdU injection, the presence of epithelial LRCs is residual due to greater proliferation of epithelial cells with the initiation of the estrous cycle [34,35]. Chan et al. reported that the epithelial LRCs were mainly located in the luminal epithelium, with cells rarely observed in the glandular epithelium. These cells did not express the leukocyte marker CD45, hematopoietic stem cell antigen SCA-I, or ER-α. This contrasts with the proliferative capacity of epithelial LRCs in response to estrogen, suggesting the existence of indirect stimuli by neighboring ER-α^+ cells [37].

Histone 2B-Green Fluorescent Protein-Label-Retaining Cells

Notably, label-retention assays do not define stemness. Consequently, conclusions can only be drawn about patterns of cell division and tissue regeneration. Therefore, evaluating the functional properties of the identified LRCs is essential.

Recently, the transgenic histone 2B-green fluorescent protein (H2B-GFP) system has allowed us to overcome the limitations of the BrdU label-retention system, since it allows us to isolate viable LRCs. In the H2B-GFP system, cell labeling is done through antibiotic-induced expression of GFP-labeled histones. Two independent studies identified stromal LRCs after using the H2B-GFP system for 3–8 weeks during embryonic development, early postnatal stages, and adulthood [42,43]. Wang et al. reported the presence of epithelial LRCs in the endocervical transition zone and the distal oviduct after 9–13 weeks when the

expression of H2B-GFP was induced during embryonic development and until 21 days post-natal [42]. Patterson et al. identified glandular LRCs after 8 months in mice labeled at the peripubertal stage that persisted through various pregnancies. However, this effect was not observed when adult mice were labeled, suggesting that some glandular development is completed the in peripubertal stages [43].

Several studies based on LRC assays showed a higher rate of renewal of the luminal epithelium compared to the glandular (basalis) and stromal fractions of the murine endometrium [38,39,43]. This higher turnover during development facilitates cell labeling and subsequent dilution. These findings are consistent with the idea that the glandular epithelium or a population of stromal stem cells replenishes the luminal epithelium. This added to the identification of stromal LRCs close to the luminal epithelium [34] and led to the proposed existence of a "mesenchymal-to-epithelial transition" phenomenon [39,43]. Likewise, the perivascular distribution of other stromal populations of LRCs [76] is reminiscent of perivascularly located mesenchymal stem cells (MSCs) in humans (see Section 2.2.1), and these LRCs could be involved in regeneration of the vascular stroma [40].

2.1.2. Other Approaches to Identify Murine Endometrial Stem Cells

Side Population Cells

ASCs can be identified by differential staining based on the efflux of fluorescent vital dyes, such as Hoechst 33342, thanks to the presence of special ATP-binding cassette (ABC) transporters. Dual-wavelength flow cytometric analysis of these cells reveals a "side population" (SP) [78]. Unlike the findings in humans [44–50], no SP cells have been identified in the normal cycling endometrium of mice. However, Hu et al. reported the existence of a SP in the postpartum endometrial stroma [51]. This population did not express endothelial, hematopoietic, or mesenchymal stem cell markers, but some cells were positive for SCA-1, c-Kit, and ER-α. Furthermore, based on ER-α expression, these authors suggested the possibility that the SP could differentiate in vitro into tissue-specific endometrial cells under the effect of estrogens.

Progenitor Cell Markers in the Mouse Endometrium

Several types of stem cells, such as mesenchymal, hematopoietic, and cancer cells, express the transmembrane protein CD44 [68]. In 2013, Janzen et al. reported that $CD44^+$ epithelial cells compose an epithelial progenitor population [52]. In addition to being ER-α and progesterone receptor negative and surviving hormonal deprivation, these cells generate more gland-like structures than do $CD44^-$ cells in immunosuppressed mice. In a more recent study, Daene et al. used mice containing a GFP reporter under the control of the telomerase reverse transcriptase promoter (mTert-GFP) to identify potential stem cells in the endometrium [41]. They detected mTert-GFP^+ populations from epithelial, hematopoietic, and endothelial lineages. Interestingly, mTert-GFP expression did not predict a label-retaining or proliferative phenotype, indicating that these cells are distinct from the previously described slow-cycling cells.

2.2. Identifying Endometrial Stem Cells in Humans

In humans, the presence of stem cells in the endometrium was first evidenced in 2004 by two different research groups, both reporting the existence of clonogenic endometrial cells (epithelial and stromal fractions) with high proliferative potential [69] and the specific role of bone marrow-derived stem cells (BMDSCs) in the regeneration of this tissue [70]. As is the case with murine models, due to the lack of well-described specific markers, the first approaches to isolate and identify human endometrial stem cells focused on verification of the universal definition of stemness: self-renewal and differentiation. Differentiation capability is usually demonstrated by multilineage differentiation (usually adipogenic, chondrogenic, and osteogenic lineages) and in vivo tissue reconstruction assays [33]. The gold-standard techniques to demonstrate self-renewal are the demonstration of clonogenicity and long-term culturing capabilities. Other common approaches include searching

for cell-surface markers expressed in other known stem cells, such as immunoglobulins or clusters of differentiation. These approaches use cell sorting techniques to select the cell subpopulation of interest. The most common cell sorting techniques are fluorescence- and magnetic-activated cell sorting, referred to as FACS and MACS respectively [71]. The previously described SP method is also commonly used. These techniques (see Table 1) have been implemented in the search for different types of human endometrial stem cells: stromal, epithelial progenitor, and endothelial stem cells. These cells are detected not only in situ, in the endometrium, but also in the menstrual blood. The bone marrow has been revealed as an exogenous source of stem cells contributing to the endogenous endometrial stem cell niche. Thus, when studying this type of stem cell in depth, reference is usually made to two different sources of endometrial stem cells: endogenous and exogenous.

2.2.1. Endogenous Endometrial Stem Cells

In general, three different types of endogenous ASCs (stromal, epithelial progenitor, and endothelial cells) are described in the human endometrium. Any of these endogenous ASC types in the human endometrium could be the cause of endometrial dysfunction.

Endometrial Stromal Stem Cells

Like almost all human tissues, the human endometrium contains a small resident population of stromal stem cells (endoSSCs). Typical markers of the mesenchymal phenotype are CD44, CD73, CD90, CD105, and CD106. The absence of other markers such as CD34 or CD45 is also indicative of this type of stem cell [72]. In the endometrium, endoSSCs are located adjacent to the endothelial cells in the microvessels within both the basalis and functionalis layers, suggesting they are also discarded through menstruation [53,54,73]. Several candidates for endoSSCs have been studied in the human endometrium, including the CD146/platelet-derived growth factor receptor beta (PDGFR-b) marker [54], sushi domain-containing-2 (SUSD2) marker [55], SP method [31,44,45], leucine-rich repeat containing G-protein-coupled receptor 5 (LGR5) marker [79], and ectonucleoside triphosphate diphosphohydrolase-2 (NTPDase2) marker [80].

The endoSSCs were first identified as expressing CD146 and PDGFR-b, conventional markers of pericytes [54]. $CD146^+$ $PDGFR\text{-}b^+$ cells express typical markers of MSCs (i.e., CD73, CD90, CD105, and STRO-1) [81] and lack fibroblastic, hematopoietic, and other well-described mesenchymal cell markers, indicating they are perivascular endoSSCs [31,56,82]. To identify a single marker for endoSSCs, human endometrial stromal cell fractions were screened by flow cytometry. SUSD2, also referred to as W5C5, permitted a good isolation of endoSSCs by magnetic bead sorting [53,57]. However, co-expression of SUSD2 with CD146/$PDGFR\text{-}b^-$ was not consistent [83]. On the other hand, a recent article showed that even $CD146^+$ pericytes isolated by FACS present a mesenchymal phenotype and limited potential to regenerate the endometrium [58].

Human endometrial SP cells obtained by the SP technique are hormone-independent with an intermediate telomerase activity and MSC phenotype, allowing for the neoformation of human endometrium in vivo [44,45,48,59]. Recently, our group showed that human endometrial SP cell lines could enhance endometrial reconstitution of $SUSD2^+/ICAM^+$ cells in vivo. We hypothesized that the stromal fraction, which participates in the heterogeneous endometrial SP cell fraction, was acting as the stem cell niche [59].

The universal stem cell marker LGR5 was also detected in the perivascular regions of the functionalis layer, suggesting $LGR5^+$ cells are a putative stem cell population [60]. However, the weakness of the in vivo endometrial reconstitution together with CD163 expression and a possible hematopoietic origin suggested that these cells had a more macrophage-like phenotype [79]. Alternatively, NTPDase2 has been detected in perivascular $SUSD2^+$ cells but not in the rest of the stromal fraction, and thus has been proposed as a marker for endoSSCs, located in the endometrial basal layer [80].

Other relevant candidates for endoSSC markers are mesenchymal stem cell antigen-1 (MSCA-1), also known as tissue non-specific alkaline phosphatase or TNAP [61], and

Notch1 [62]. However, their utility is limited as they are not suitable cell markers for endoSSC isolation [84].

Finally, precursors of human decidual stromal cells from decidua-endometrial biopsies, obtained from first-trimester pregnancies, exhibit features compatible with perivascular endoSSCs (also expressing CD146 and PDGFR-b) in addition to the ability to decidualize in vitro, suggesting they are decidual MSCs involved in the mechanisms of maternal–fetal immune tolerance [74].

Endometrial Epithelial Progenitor Cells

Endometrial epithelial progenitor cells (endoEPCs) are a subpopulation of cells located at the base of the glands in the basalis layer [63,64,83]. Although some studies have proposed that endoEPCs could derive from endometrial stromal cells [39,85], epithelial stem cells have been identified in the human endometrium. In addition, endoEPCs have been obtained from epithelial SP fractions, presenting the epithelial cell marker CD9 and producing gland-like structures in vivo [44,45].

Stage-specific embryonic antigen-1 (SSEA-1) can also identify the human glandular epithelial cells of the basalis, distinguishing them from those in the functionalis. SSEA-1^+ endoEPCs are quiescent, with long telomeres, and can produce endometrial gland-like spheroids in three-dimensional (3D) in vitro culture [63]. The atrophic menopausal endometrium is also SSEA-1^+ and regenerates itself to produce a functional layer under estrogen supply, suggesting that endoEPCs last after menopause [55,63,64]. The genes SOX9 and beta-catenin [63] have also been studied as putative cell markers of endoEPCs together with SSEA-1, although they usually require co-detection with other specific markers, and additional studies are needed.

Recently, N-cadherin, a protein previously related to the stem cell niche in other organs [86–89], has been found in quiescent cells from the deep basalis [63,64]. N-cadherin$^+$ epithelial cells are more clonogenic than are N-cadherin$^-$/cytokeratin$^+$ epithelium and can be differentiated to gland-like organoids. However, N-cadherin did not immunolocalize with SSEA-1 [64]. Nguyen et al. suggested a differentiation pyramid hypothesis initialized by the higher abundance of immature N-cadherin$^+$/SSEA-1^- endoEPCs at the base of the glands, adjacent to the myometrium. As these primitive N-cadherin$^+$/SSEA-1^- cells move through the basalis, they gradually lose N-cadherin expression and differentiate into transit N-cadherin$^-$/SSEA-1^+ cells, which completely differentiate when they arrive at the functionalis [64]. However, more functional studies are required to clarify this hypothesis.

Finally, the presence of NTPDase2 in surrounding basal glands, but not in functional glands, could be linked to the putative action of NTPDase2 in the preservation of endoEPCs [80].

Endothelial Stem Cells

Endothelial stem cells have also been postulated to be part of the endometrial stem cell niche. These cells are CD31$^+$/CD34$^+$ (classical endothelial markers) and have been detected among the SP endometrial cells (isolated using the ATP-binding cassette subfamily G member 2 or ABCG2 marker) located not only in the basalis but also in the vascular endothelium [47]. This heterogeneous endometrial subpopulation of SP cells also has a higher expression of endothelial markers than do non-SP cells [48].

2.2.2. Exogenous Endometrial Stem Cells

The bone marrow, which contains a heterologous cell population including progenitors, hematopoietic, and non-hematopoietic stem cells [90], is proposed as an external source of ASCs, contributing to the endometrial stem cell niche.

Contribution of Bone Marrow to the Endometrial Stem Cell Niche

BMDSCs are MSCs that serve as an exogenous source of stem cells. BMDSCs are found in several tissues and have the capability to repopulate non-hematopoietic tissues.

Some authors postulate that these BMDSCs could shift towards the endometrial stem cell niche and differentiate into tissue-specific stem cells [70]. In both human and mouse models, BMDSCs are able to differentiate into non-hematopoietic endometrial cells such as epithelial, stromal, and endothelial cells [65,66,70]. Cervelló et al. confirmed BMDSCs migrate towards the endometrium and contribute to the cell composition of the stroma and the epithelial compartment in patients receiving bone marrow transplants, although bone marrow is proposed to be a limited exogenous source rather than a cyclic source of BMDSCs [50]. The contribution of this exogenous source of stem cells to the endothelial cells after angiogenic pro-regenerative events was also reported [91].

Additionally, there is no agreement on established markers and their characterization due to the complexity of the endometrium and the high likelihood of coexistence of several niches in other tissues [92]. In general, MSCs express varied cell surface markers such as CD29, CD44, CD73, CD90, and/or CD105 and do not express CD14, CD34, CD45, and/or HLA-DR, nor is the expression of these epitopes clearly observed during transdifferentiation processes in other tissues [93]. Clonogenic endometrial cells from humans show similar properties in vitro to those of mesenchymal stem cells [44], thus making it questionable whether these identifying properties are valid or not [83].

Consequently, the contribution of this source of stem cells in the endometrium is still under debate: It is unsettled whether they have a role in the remodeling process or if they just show a uterine phenotype after migrating towards the endometrium [83]. Nevertheless, the exogenous source offers great potential for treatment of infertility caused by endometrial alterations.

2.3. Role of Stem Cells in Endometrial Pathologies

Stem cells can participate in the development of infertility-causing diseases directly related to the uterus and/or endometrium, such as endometrial hyperplasia, endometrial carcinoma, endometriosis, adenomyosis, and leiomyomas.

Endometrial hyperplasia occurs when the endometrial lining is too thick, and it is a risk factor for endometrial carcinoma, characterized by endometrial epithelial neoplasia [75]. In this type of carcinoma, endogenous ASCs are thought to transform into cancer stem cells (CSCs) through a series of genetic alterations and/or epigenetic mutations, which could be responsible for tumor formation [75]. Additionally, a SP subpopulation was isolated from endometrial carcinoma samples [94], while CD44, CD55, and CD133 were used to isolate these CSCs [95].

The role of stem cells has also been suggested in the pathogenesis, though still unknown, of endometriosis [96], implying the presence or development of endometrial tissue in ectopic locations, and adenomyosis [97], characterized by the presence, focalized or diffused, of ectopic endometrial tissue in the myometrium. In endometriosis, a multifactorial disease, three main hypotheses concerning ASCs have been postulated [31]. The first, and most accepted one, is Sampson's theory of retrograde menstruation. This hypothesis implies that putative menstrual blood stem cells arriving at ectopic locations are the origin of the endometriotic lesions [96]. The second one is that endometriosis in prepubertal girls is explained by endometrial ASCs from neonatal uterine bleeding caused by maternal hormone withdrawal right after delivery [98]. The third and last theory is that the relationship between endometrial stem cells and progesterone resistance of the endometrial stromal cells impairs their ability to decidualize [99]. Whatever the mechanism by which the endometrial stem cells arrive at and generate endometriotic lesions, these stem cells can express different stem cell markers such as SEEA-1 and N-cadherin [31].

Migration of menstrual blood stem cells from retrograde menstruation into the peritoneal cavity could also generate focal adenomyosis [97]. Further, endometrial ASCs in the basalis layer of patients with adenomyosis present more pseudopods and thus may more easily migrate towards the endometrium, generating adenomyotic lesions [100].

3. Stem Cell Therapy and the Endometrium: The Importance of Basic Research and Pre-Clinical Models

The knowledge acquired during the search for an endogenous endometrial stem cell population has contributed to stem cell therapy, widely used in other medical fields, for endometrial pathologies. Stem cell therapy is based on the capacity of the stem cells to arrive at the damaged site, self-renew, and differentiate into target tissue cells to empower the repopulation and regeneration of, in this case, the endometrium. However, with the growing implementation of stem cell therapies and low engraftment of the cells observed in some studies [101,102], other mechanisms of action have been proposed.

3.1. Paracrine Action of Stem Cells: Main Findings in the Endometrium

Currently, the paracrine hypothesis is probably the most documented and accepted one. Following this premise, stem cells secrete different biomolecules such as growth, angiogenic, or immunosuppressive factors, as well as chemokines and exosomes, which contribute to regeneration of the injured tissue [103]. In line with these secreted immunosuppressive factors, the stem cells are also thought to have an immunomodulatory potential to manage the inflammation status of the injured site and prepare the tissue for the incoming regenerative events [104]. Different studies in animal models corroborate this paracrine hypothesis by elucidating low stem cell engraftment when applied to a damaged endometrium [105]. In addition, this therapy has similar effectiveness whether it is administered through the tail vein or directly into the uterine horns [105,106]. These findings support the hypothesis that the regenerative potential of stem cells might not be due to proliferation of the administered stem cells themselves but to the paracrine factors they secrete or stimulate in the receptor organism. Interestingly, injected stem cells engraft into the spiral arterioles of the endometrium where the stem cell niche is thought to be located [107]. This finding reinforces the existence of this stem cell niche and the paracrine effect that the endogenous cells exert to attract the therapy (stem cells in this case) to directly act over the niche and promote tissue regeneration.

The immunomodulatory action of these stem cells in the target tissue was also elucidated in the endometrial milieu. Downregulation of some genes such as chemokine *CXCL8* is hypothesized to reduce the inflammation status and induce the expression of other factors such as *SERPINE1* or proto-oncogene *c-JUN*, preparing the tissue for the incoming regeneration processes [107].

3.2. Stem Cell Therapy for Treating Endometrial Pathologies

To treat AS and EA, different sources of stem cells have been used. The most explored sources for endometrial regeneration are the umbilical cord [108,109], amniotic membrane [110,111], bone marrow and adipose tissue [112], in addition to menstrual blood [113], or even autologous endometrial biopsies [114,115]. These last four types of stem cells usually have an autologous origin. Thus, complications such as graft-versus-host disease, associated with allogenic stem cell transplant, do not apply; however, they can involve other disadvantages such as an insufficient number of cells [116]. This could explain why most published works in the field, most of them in the form of pilot studies or case reports, directly try the therapy in humans [117]. However, using pre-clinical models, usually rodents (mice and rats), mimicking either the adhesions and fibrotic tissue characteristic of AS patients or the thin and/or atrophic endometrium of patients with EA, is important before translating medical approaches to women [118], which is always the final goal. The importance of these animal models resides in their capacity to predict the outcome of future clinical trials.

Pre-Clinical Models of Endometrial Injury

Bone marrow is an excellent source for obtaining stem cells to treat endometrial pathologies. The contribution of these stem cells to the endometrial stem cell niche has probably encouraged their use. As mentioned before, the bone marrow contains a heterolo-

gous cell population, [90], thus, it is important to distinguish which subpopulation of stem cells is administered into murine models of AS or injured endometrium. Some studies use the non-hematopoietic or stromal BMDSCs that are positive for CD29, CD44, CD73, and/or CD90, but negative for CD45 or CD34 [106,119–121]. Other research groups inject hematopoietic BMDSCs cells using different markers such as CD133 antigen [105]. Furthermore, other studies report the use of the whole bone marrow stem cell fractions [122,123].

Studies using other stem cell sources [108–115] to treat animal models of endometrial injury (umbilical cord, adipose tissue, amniotic membrane, menstrual blood, and endometrial biopsies) mainly explored the mesenchymal stem cell type using markers such as CD29, CD44, CD73, CD90, and CD105.

4. Future Perspectives and Next Steps

There is still a lack of knowledge in the basic biology of the endometrial stem cell niche and more efforts should be directed to find specific stem cell markers. Understanding the human endometrial stem cell niche is important not only to moving forward in the gynecological field, which is the main objective, but also to potentially progressing other medical areas. The potential of endometrial stem cells, either obtained from the menstrual blood or the endometrial tissue, has been described in other medical fields. Indeed, studies propose the use of endometrial stem cells for treating Parkinson's disease in primates [124] and a mouse model of encephalomyelitis [125].

However, currently, the trend is in refining stem cell therapy to treat endometrial pathologies in combination with other approaches to enhance its pro-regenerative action. In some studies, chemokines or growth factors are applied in combination with MSCs [67,123]. Other works make use of a very promising biological product in the regenerative medicine field, platelet-rich plasma [126]. Bioengineering approaches such as hydrogels [121] or collagen scaffolds [127] obtained from the ECM have also been explored. The ECM contains a variety of growth factors and other molecules involved in regeneration processes [128]. Finally, in line with the paracrine action hypothesis, derivatives from the stem cells themselves, such as the stem cell secretome [129] or exosomes [130], alone or in combination with other approaches, have also been revealed as promising for restoring the endometrial tissue in murine models of endometrial injury. In parallel, there are also research projects focused on optimizing the isolation of stem cells derived from menstrual blood [131,132], which has emerged as a promising source of stem cells.

5. Conclusions

Stem cells are key to the normal functionality of the endometrium. Therefore, complete understanding of their activity is essential in management of endometrial pathologies, not only as of the cause of these disorders, but also as the solution (either as the therapy or the target of other approaches).

As reported here, the presence of endogenous stem cells in the endometrium has been widely described and accepted. However, there is still a lack of standardization in detection and isolation methods for this specific population of ASCs. Future studies will likely find specific stem cell markers or validate those that have already been proposed.

Stem cell identification methods commonly used in animal models, such as the BrdU-based assay, have the major disadvantage of not allowing functional assays due to the required fixation procedure. These assays also differ from the methods used historically in the pursuit of endometrial stem cells in humans. Thus, further studies focused on developing methods for identification of endometrial stem cells in animal models would help to equate and compare findings with the human models and consequently to facilitate clinical translation of incoming therapies. These therapies for endometrial management have evolved along the years from classic stem cell therapy to emerging approaches based on the paracrine activity of the stem cells and bioengineering.

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4. ANNEX IV. MANUSCRIPT IV

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REVIEW ARTICLE

Strategies for managing Asherman's syndrome and endometrial atrophy: Since the classical experimental models to the new bioengineering approach

Lucía de Miguel-Gómez[1,2] | Mónica Romeu[3,4] | Antonio Pellicer[2,5] | Irene Cervelló[1]

[1]Fundación Instituto Valenciano de Infertilidad (FIVI), La Fe Health Research Institute, Valencia, Spain

[2]University of Valencia, Valencia, Spain

[3]Reproductive Medicine Research Group, La Fe Health Research Institute, La Fe University Hospital, Valencia, Spain

[4]Women's Health Area, Human Reproduction Unit, La Fe University Hospital, Valencia, Spain

[5]IVIRMA Roma, Rome, Italy

Correspondence
Lucía de Miguel Gómez, Department of Pediatrics, Obstetrics and Gynaecology, Faculty of Medicine and Dentistry, Avenida Blasco Ibañez, 15, Valencia 46010, Spain.
Email: lucia.miguel@uv.es

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ABSTRACT

Endometrial function is essential for embryo implantation and pregnancy, but managing endometrial thickness that is too thin to support pregnancy or an endometrium of compromised functionality due to intrauterine adhesions is an ongoing challenge in reproductive medicine. Here, we review current and emerging therapeutic and experimental options for endometrial regeneration with a focus on animal models used to study solutions for Asherman's syndrome and endometrial atrophy, which both involve a damaged endometrium. A review of existing literature was performed that confirmed the lack of consensus on endometrial therapeutic options, though promising new alternatives have emerged in recent years (platelet-rich plasma, exosomes derived from stem cells, bioengineering-based techniques, endometrial organoids, among others). In the future, basic research using established experimental models of endometrial pathologies (combined with new high-tech solutions) and human clinical trials with large population sizes are needed to evaluate these emerging and new endometrial therapies.

KEYWORDS
bioengineering, endometrial regeneration, platelet-rich plasma, stem cells

1 | INTRODUCTION

The uterus, ovaries, and fallopian tubes compose the internal female reproductive tract. The human uterus is a hollow and inverted pear-shaped organ, while in species like rodents, ruminants, and pigs, two lateral uterine horns join distally into a single body or corpus (Rendi et al., 2012). Regardless of shape, each uterus has differentiated layers: the perimetrium (the serous and most external layer), myometrium (the thickest and muscular layer, located in the middle), and endometrium (the mucous and most internal layer) (Simón et al., 2009).

The endometrium supports embryo implantation and fetal nutrition in pregnancy and is highly dynamic and regenerative, undergoing more than 400 cyclic changes through proliferation, differentiation, and shedding (if there is no embryo) each menstrual cycle for the duration of the reproductive years of a woman (Gargett et al., 2012). In humans, in each menstrual cycle, endometrial thickness progressively increases and decreases in response to estrogen and progesterone produced by the ovaries. Estrogen causes the endometrium to grow and thicken to prepare the uterus for pregnancy during the proliferative phase and after ovulation in the middle of the cycle, progesterone increases to prepare the tissue for embryo implantation in the secretory phase. In the absence of a fertilized egg, both hormones decrease and menstruation occurs. Once the endometrial lining has completely shed, a new menstrual cycle begins (Cunningham et al., 2015). This cyclic regeneration is postulated to

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be driven by the somatic stem cell population located in the niche of the basal layer of the human endometrium (Gargett et al., 2010; Cervelló et al., 2013). In rodents (mice and rats), the main animal model used in reproductive research, the reproductive cycle is called the estrous cycle and contains four phases (proestrus, estrus, metaestrus, and diestrus), occurring every 4 to 5 days. Unlikely humans and other primates, rodents do not menstruate (Goldman et al., 2007).

Endometrial anomalies impact fertility and can reduce the chance of pregnancy. Absolute uterine factor infertility results from the absence of a uterus or a nonfunctional uterus (Brännström et al., 2015) while less severe conditions, such as leiomyomas, adenomyosis, Müllerian duct anomalies, and endometrial alterations, impair reproductive outcomes but do not necessarily imply absolute infertility. Among the pathologies directly related to endometrial factor, some affect the endometrial lining, such as endometrial atrophy (EA), and others involve intrauterine adhesions (IUAs) or scar tissue formation, which in the most severe cases can completely obliterate the uterine cavity leading to Asherman's syndrome (AS) (Galliano et al., 2015). Women with EA have a thin endometrium, usually defined by an endometrial thickness, at the time of hCG administration (in an in vitro fertilization procedure), measured by ultrasonography of less than 6–8 mm. At the same time, women with AS syndrome have a severe degree of IUAs, accompanied by menstrual disturbances, infertility, recurrent pregnancy loss, and/or placental abnormalities (Conforti et al., 2013). In any case, this thin and/or fibrotic endometrium may impair implantation and lead to early pregnancy loss or diminish the probability of pregnancy (Mahajan & Sharma, 2016; Senturk & Erel, 2008). Therefore, regenerating the endometrial tissue in EA and AS patients, either to restore the endometrial integrity from fibrotic lesions or to thicken it, is a therapeutic option to allow for embryo implantation.

As detailed above, animal models have been developed to elucidate possible solutions and treatments for endometrial alterations. However, effective and standardized options are lacking. Stem cells, platelet-rich plasma, and bioengineering-derived methodologies may be useful in place of traditional surgical treatment methods and hormonal treatments, but further studies are needed to bring these techniques into clinical practice (García-Velasco et al., 2016). Different models are used to test and study mechanisms of action of these treatments, as well as to understand the pathogenesis of the endometrial variations before clinical translation to humans (Andersen et al., 2018), but a well-established uterine-damaged animal model is needed to test treatment options for endometrial regeneration. Mechanical damage using a needle (Alawadhi et al., 2014; Cervelló et al., 2015), a curette (Huberlant et al., 2014; Feng et al., 2020), or an electric scalpel (Xu et al., 2018) are proposed as ways to model uterine injury. Another option is to damage the endometrium by chemical methods, such as ethanol (Jang et al., 2017), which has gained wide acceptance over the years (Sun et al., 2019).

In this review, we summarize classical and emerging advances in experimental models, mainly rodents (mice and rats) of endometrial regeneration. The therapeutic alternatives for treating AS and EA, based on animal research, are shown in Table 1. These studies are grouped into two areas: stem cell therapies, growth factors, and other molecules, and emerging therapeutic alternatives (including platelet-rich plasma, tissue engineering, bioengineering solutions, and organoids). Different variables related to either endometrial regeneration evaluation or fertility restoration verification are also listed (Table 1). Among the methods discussed, bioengineering-derived techniques are the most promising in the management of an injured endometrium, as in AS and EA. To translate these techniques to clinical use, a well-established model of endometrial injury is essential. All the studies cited along with this study support the effectiveness of the different treatments to regenerate the endometrium in animal models (mainly rodents) of uterine damage. However, not all of them induce uterine damage using the same protocol.

Thus, this study is focused on the importance of animal models before translating novel therapies to human patients.

2 | MATERIAL AND METHODS

The PubMed database and Google Scholar were searched to identify studies published through December 2020 assessing therapeutic options for endometrial pathologies. We used the following search terms: animal model, Asherman's syndrome, bioengineering, endometrial atrophy, endometrium, growth factors, hydrogel, microfluidics, murine model, organoids, platelet-rich plasma, scaffold, stem cells, and thin endometrium. Additional studies were found in the bibliographies of selected works. Only original articles in English were included. Studies from bovine, murine, ovine, and porcine models were reviewed as well as some human studies.

3 | RESULTS

3.1 | Classical management of AS and EA

These classical techniques have been mainly described in humans. Hysteroscopic adhesiolysis is the most common treatment for human AS (Khan & Goldberg, 2017; Roge et al., 1997). However, surgery is not always effective, and often (20% to 62.5%) the IUAs reappear (Hanstede et al., 2015). Thus, postoperative measures are frequently needed, such as the insertion of an intrauterine device, a Foley balloon, or hyaluronic acid treatment (Amer et al., 2005; Lin et al., 2013; March, 2011). But still, a systematic review from 2017 concluded that there is no clear evidence on the safety and effectiveness of anti-adhesion treatment after hysteroscopy for improving the reproductive outcomes rates or for decreasing reappearance of IUAs (Bosteels et al., 2017).

Regarding EA patients, and also in those with AS, other less invasive therapeutic approaches have been tested, including the use of exogenous estrogens (Coughlan et al., 2014; Cheng et al., 2006; Shen et al., 2013; Tourgeman et al., 2001), a gonadotropin-releasing hormone agonist (Qublah et al., 2008), human chorionic gonadotropin (Davar et al., 2016; Papanikolaou et al., 2013), or tamoxifen (Reynolds et al., 2010).

TABLE 1 Therapeutic alternatives for treating endometrial pathologies: animal model approaches.

					Endometrial regeneration					
Therapeutic approach		**Study**	**Animal model**	**Endometrial damage method**	**Endometrial thickness**	**Fibrotic area**	**Endometrial glands**	**Blood vessels**	**Markers of endometrial functionality and regeneration**	**Fertility outcomes**
Stem cell therapies, growth factors, and other molecules	Bone marrow–derived stem cells	Zhao et al., 2015	Rat	95% ethanol injection	↑	NR	↑	↑	↑ bFGF, IL6, VIM, CK, ITGβ3, LIF ↓ TNF-α, IL1β	NR
	Bone marrow–derived stem cells	Gao et al., 2018	Rat	Endometrial ablation with 85°C hot water	NR	↓	↑	↑	↑ CK, LIF	↑
	$CD133^+$ bone marrow–derived stem cells	Cervelló et al., 2015	Mouse	Mechanical damage (24G-needle)	NR	↓	NR	NR	↑ Ki67, TSP1 ↓ IGF1	NR
	Umbilical cord-derived mesenchymal stem cells	Tang et al., 2016	Rat	Mechanical damage (curette)	NR	↓	↑	NR	↑ Ki67, VEGF, VIM, CK ↓COL1A, TGFβ1, FGF2, CTGF	NR
	Umbilical cord-derived mesenchymal stem cells	Zhang et al., 2018	Rat	95% ethanol injection	↑	↓	↑	↑	↑ Ki67, VIM, CK, MMP9, VEGFA, CD31 ↓ αSMA, TGFβ1, TNF-α. IL2, IFNγ	NR
	Ammniotic mesenchymal stem cells	Gan et al., 2017	Rat	Mechanical damage (surgical scalpel blades)	↑	↓	↑	NR	↑ bFGF, IL6, VEGF, CK ↓ TNF-α, IL1β, TGFβ1, PDGFBB, TIMP, COL1A1	NR
	Amniotic mesenchymal stem cells	Ouyang et al., 2020	Rat	Mechanical damage (curette)	↑	↓	↑	↑	↑ bFGF, VEGF, IGF1, WNT5a, SNAI2 ↓ TGFβ1, TIMP1, COL1A1, PDGF-C, TSP1, CTGF	↑

(Continues)

TABLE 1 (Continued)

Therapeutic approach		Study	Animal model	Endometrial damage method	Endometrial regeneration					
					Endometrial thickness	Fibrotic area	Endometrial glands	Blood vessels	Markers of endometrial functionality and regeneration	Fertility outcomes
	Adipose mesenchymal stem cells + estradiol	Kilic et al., 2014	Rat	Trichloroacetic acid injection	↑	↓	NR	↑	↑ PCNA, Ki67, VEGF	NR
	Menstrual mesenchymal stem cells	Hu et al., 2019	Mouse	Mechanical damage (4G-needle)	↑	NR	NR	NR	↑ VEGF, VIM, CK	↑
	Stromal cell-derived factor 1 + bone marrow–derived stem cells	Yi et al., 2019	Mouse	95% ethanol injection	↑	↓	↑	NR	↑ Ki67, CD31, LIF, IL6, ITGβ3, MMP2, MMP9	NR
	Stromal cell-derived factor 1	Ersoy et al., 2017	Mouse	Mechanical damage (needle)	NR	↓	NR	NR	NR	↑
Emerging therapeutic alternatives: platelet-rich plasma	Platelet-rich plasma from adult blood	Jang et al., 2017	Rat	95% ethanol injection	↑	↓	↑	NR	↑ CK, HOXA10, VEGF, Ki67, cKIT,	NR
	Platelet-rich plasma from adult blood	Kim et al., 2020	Mouse	Mechanical damage	NR	↓	↑	NR	↓ TGFβ1, TIMP1, COL1A1	↑
	Platelet-rich plasma from adult and umbilical cord blood	De Miguel-Gómez et al., 2021	Mouse	Mechanical damage (24G-needle)	NR	NR	NR	NR	↑ Ki67, HOXA10, UBA3, THY1, STAT5a	NR
Emerging therapeutic alternatives: tissue engineering solutions and bioengineering	Collagen scaffolds + basic fibroblast growth factor	Sun et al., 2011	Rat	Resection of uterine segment	↑	↓	↑	↑	↑ αSMA, vWF, Ki67	↑
	Collagen scaffolds + vascular endothelial growth	Lin et al., 2012	Rat	Resection of uterine segment	↑	↓	↑	↑	↑ αSMA, vWF	↑
	Collagen scaffolds + bone marrow–derived stem cells	Ding et al., 2014	Rat	Resection of uterine segment	↑	↓	↑	↑	↑ αSMA, vWF, bFGF, IGF1, TGFβ1, VEGF	↑
	Collagen scaffolds + embryo derived stem cells	Song et al., 2015	Rat	Resection of uterine segment	↑	↓	↑	↑	NR	↑
	Decellularized scaffolds from rat uterus	Miyazaki & Maruyama, 2014	Rat	Resection of uterine segment	↑	↓	↑	↑	↑ VIM, CK, αSMA. PR	↑

TABLE 1 (Continued)

Therapeutic approach	Study	Animal model	Endometrial damage method	Endometrial regeneration					
				Endometrial thickness	Fibrotic area	Endometrial glands	Blood vessels	Markers of endometrial functionality and regeneration	Fertility outcomes
Decellularized scaffolds from rat uterus	Hellström et al., 2016	Rat	Resection of uterine segment	NR	NR	NR	↑	↑ αSMA, eCAD, vWF, HOXA11, BCL2	NR
Decellularized scaffolds from ovine uterus	Daryabari et al., 2019	Rat	Resection of uterine segment	↑	NR	NR	NR	↑ αSMA, CD31, Ki67	NR
Aloe-poloxamer hydrogel + estradiol + decellularized rat uterus derived nanoparticles	Yao et al., 2020	Rat	Mechanical damage (surgical scalpel blades)	↑	↓	↑	NR	↑ Ki67, ERβ ↓ TGFβ1, TNF-α	NR
Hydrogels Heparin-poloxamer hydrogel + estradiol	Zhang et al., 2020	Rat	Mechanical damage (curette)	↑	↓	↑	NR	↑ bFGF, PCNA, BCL2, KISS1 ↓ CASP3, BAX	↑
Hydrogel + Stromal cell-derived factor 1	Wenbo et al., 2020	Rat	Mechanical damage (curette)	↑	↓	↑	NR	↑ TGFβ1, VEGF, CD31, CK	↑
Hydrogel + bone marrow–derived stem cells	Yang et al., 2017	Rat	Mechanical damage (scraping spoon)	↑	↓	↑	↑	↑ CK, vWF ↓ IL1β	NR
Hydrogel + endometrial stromal cells	Kim, Park et al., 2019	Rat	Mechanical damage	↑	↓	↑	NR	↑ Ki67, CD44, PECAM, IGF1, VEGF, LIF	↑
Hyaluronic acid hydrogel + stem cell secretome	Liu et al., 2019	Rat	Electrocoagulation	↑	NR	↑	↑	NR	↑

Note: Studies are grouped into two areas: stem cell therapies, growth factors, and other molecules, and, emerging therapeutic alternatives (including platelet-rich plasma and tissue engineering solutions and bioengineering). Sub-approaches are included. Different variables related to either endometrial regeneration evaluation or fertility restoration verification are also listed. BAX: apoptosis regulator BAX; BCL2: apoptosis regulator Bcl-2; eCAD: e-cadherin; CASP3: caspase 3; CD: cluster of differentiation; CK: cytokeratin; COL1A1: collagen alpha-1(I) chain; CTGF: connective tissue growth factor; ECM: extracellular matrix; EGF: epidermal growth factor; ER: estrogen receptor; bFGF: basic fibroblast growth factor; G-CSF: granulocyte colony-stimulating factor; HGF: hepatocyte growth factor; HOXA10: homeobox A10; IFNγ: interferon gamma; IGF: insulin-like growth factor; IL: interleukin; KISS1: metastasis-suppressor KiSS-1; MMP: matrix metallipeptidase; NR: not reported; ITGβ3: integrin beta 3; LIF: leukemia inhibitory factor; PCNA: proliferating cell nuclear antigen; PDGF: platelet-derived growth factor; PECAM: Platelet endothelial cell adhesion molecule; PR: progesterone receptor; αSMA: alpha smooth muscle actin; SNAI2: zinc finger protein SNAI2; STAT5a: signal transducer and activator of transcription 5 A; TGFβ: transforming growth factor beta; THY1: Thy-1 membrane glycoprotein; TIMP: metalloproteinase inhibitor 1; TSP1: Thrombospondin-1; TNF-α: tumor necrosis factor alpha; UBA3: NEDD8-activating enzyme E1 catalytic subunit; VEGF: vascular endothelial growth factor; VIM: vimentin; vWF: von Willebrand factor; WNT5a: protein Wnt-5a. ↑: increased; ↓: diminished.

Other classical approaches include the use of vasoactive substances to increase endometrial blood flood in AS and EA patients (Miwa et al., 2009; Ng et al., 2007). Some studies report higher implantation and clinical pregnancy rates after administration of low-dose aspirin in patients with EA (Urman et al., 2000; Weckstein et al., 1997) while other investigations reported any improvement (Check et al., 1998; Hsieh et al., 2000). In AS, an improvement in endometrial thickness has been reported after aspirin administration, but no change in reproductive prognosis was observed (Chen et al., 2017). Before that, another group postulated that aspirin restored endometrial blood flow, preventing relapse of adhesions after surgery (Chen et al., 2016). Sildenafil citrate has also been tested. Several case studies show promising results using it in terms of endometrial thickness increase and reproductive outcomes in patients with EA (Sher & Fisch, 2000; 2002; Takasaki et al., 2010) and AS (Zinger et al., 2006). However, a randomized clinical trial (RCT) from 2013 reports that this drug can improve endometrial thickness but not reproductive outcomes (Dehghani et al., 2013). Another therapeutic option is pentoxifylline in combination with the antioxidant vitamin E. Synergy between these two drugs increases the endometrium thickness in several quasi-experimental studies (Acharya et al., 2009; Letur-Könirsch et al., 2002; Ledee-bataille et al., 2002). Nevertheless, an RCT in 2009 (Aleyasin et al., 2009) reported no difference in endometrial thickness.

Thus, the effectiveness of the different treatments is often discordant, preventing their routine adoption in clinical practice with AS and EA patients.

3.2 | Stem cell therapies, growth factors, and other molecules

3.2.1 | Stem cell-based therapy

Stem cell therapies may treat diseases or conditions for which few treatments exist due to the well-described regenerative potential of these types of cells (Rohban & Pieber, 2017). The success of stem cell therapy has been extensively demonstrated in other medical fields such as cardiology (Müller et al., 2018), neurology (Song et al., 2018), or orthopedics (Akpancar et al., 2016). In the reproductive field, stem cell treatments show promising results in animal models. Male mouse bone marrow–derived stem cells (BMDSCs) were transplanted into a female AS murine model (Alawadhi et al., 2014), and by detecting the Y chromosome, stem cells were shown to arrive at the damaged site in the endometrium and promote the recovery of endometrial function. This improvement was determined based on a decrease in fibrotic area and an increase in pregnancy rates in mice treated with BMDSCs. Mesenchymal BMDSCs (BMMSCs) also improved endometrial thickness in a rat model with uterine damage via migration kinetics toward the injury site and immunomodulatory properties. After treating rats with BMMSCs, an increase in endometrial thickness and higher expression of the endometrial markers, directly related to improved functionality and receptivity, vimentin, cytokeratin, integrin β3 (ITGβ3), leukemia inhibitory factor (LIF) was observed. Pro-inflammatory cytokines, such as tumor necrosis factor (TNF-α) and interleukin (IL) 1β, were downregulated while basic fibroblast growth factor (bFGF) and IL6, both anti-inflammatory cytokines, were upregulated, promoting an immunotolerant environment (Zhao et al., 2015). In 2018, (Gao et al., 2018) described the effectiveness of mesenchymal BMDSCs in an AS murine model. Improved cell proliferation, increased expression of LIF, and reduced fibrosis in the endometrium were observed. Another study by Cervelló et al. in 2015 reported similar results in murine models using human BMDSCs positive for the CD133 antigen ($CD133^+$BMDSCs) (Cervelló et al., 2015). These labeled stem cells were observed around endometrial blood vessels, inducing proliferation in surrounding cells and regulating the paracrine factors thrombospondin 1 and insulin-like growth factor 1 (IGF1). The $CD133^+$BMDSCs used by Cervelló et al. came from a human pilot study of 16 AS and EA patients where BMDSCs were mobilized by G-CSF, an enhancer of the production of progenitors and stem cells by the bone marrow and their subsequent release into the bloodstream, collected through peripheral blood aphaeresis and isolated based on $CD133^+$ expression (Santamaria et al., 2016).

Although bone marrow is the most common source of stem cells for the treatment of endometrial alterations, stem cells can be derived from other tissues. Indeed, administration of mesenchymal stem cells (MSCs) derived from the umbilical cord increased glandular count, reduced endometrial fibrosis, and promoted cell proliferation in an AS murine model (Tang et al., 2016; Zhang et al., 2018). Further, MSCs derived from the amnion restored endometrial injury by reducing fibrosis, improving endometrial morphology (Ouyang et al., 2020), and exerting immunomodulatory properties (Gan et al., 2017). MSCs obtained from adipose tissue also restored endometrial tissue via increased vascularization and decreased fibrotic area in a murine model (Kilic et al., 2014), as did those obtained from menstrual blood. Hu et al. reported that MSCs derived from menstrual blood could restore endometrial function via increasing the expression of vascular endothelial growth factor (VEGF), vimentin, and keratin, and also pregnancy rates (Hu et al., 2019).

All reviewed types of stem cells, such as BMDSCs (Nagori et al., 2011; Singh & Seth, 2014; Saldin et al., 2016) and those derived from the umbilical cord (Cao et al., 2018), adipose tissue (Sudoma et al., 2019), and menstrual blood (Tan et al., 2016) have also been used in human studies. These works have reported how stem cell therapy, obtained from a variety of sources, can increase endometrial thickness allowing embryo implantation and successful pregnancies in women with AS and EA.

3.2.2 | Stem cell paracrine properties

Injected BMDSCs likely act in a paracrine manner via secretion of biomolecules as final effectors (Gnecchi et al., 2008; Schinköthe et al., 2008). These molecules are as important, if not more so than differentiation and repopulation of the stem cells in modulating the composition of the local environment to evoke tissue repair. These biomolecules include cytokines, growth factors, and extracellular matrix (ECM) components (metalloproteinases, collagens) and are

involved in biological processes such as cell proliferation and migration, cytoprotection, angiogenesis, reducing fibrosis and apoptosis, ECM homeostasis, reducing inflammation, and immunosuppression (Baraniak & McDevitt, 2010; Gnecchi et al., 2008, 2016). This suggests that the application of the biomolecules secreted by stem cells, called the secretome, including lipids, free nucleic acids, soluble proteins, and extracellular vesicles, could be sufficient to activate regeneration or restoration of a specific tissue rather than necessitating the transplantation of stem cells (Beer et al., 2017). Indeed, the secretome derived from MSCs has anti-inflammatory, antiapoptotic, antimicrobial, and angiogenic properties and promotes wound healing and tissue repair (Vizoso et al., 2017). This secretome-based approach has been tested in animal models of different human diseases such as liver (Driscoll & Patel, 2019) or cerebrovascular (Maki et al., 2018) diseases and clinical trials (Konala et al., 2016), rather than in the gynecological field. The few published works using the stem cell secretome for endometrial repair are mentioned further in the text, in the Hydrogels section.

Besides, growth factors and cytokines are secreted by human stem cells including those derived from bone marrow (Baberg et al., 2019; Oskowitz et al., 2011), umbilical cord (An et al., 2017), and adipose tissue (Chang et al., 2017; Mussano et al., 2017). Deeper investigation and identification of these paracrine factors will aid the development of noninvasive therapies that could replace stem cell therapy in gynecological pathologies. Our group has taken the first steps in this field, reporting that $CD133^{+}$BMDSCs injected in an AS murine model (Cervelló et al., 2015) activated Serpine 1, which promotes cell migration and is involved in decidualization (Lumbers et al., 2015), and Jun proto-oncogene, which promotes endometrial epithelial cell proliferation while decreasing the expression of cyclin D1, a regulator of the cell cycle, through a paracrine mechanism to aid endometrial regeneration. This creates an immunomodulatory environment in endometrial tissue that promotes regenerative processes (De Miguel-Gómez et al., 2020). Paracrine molecules can be also delivered from exosomes, nano-sized extracellular vesicles that release active paracrine molecules (Yu et al., 2014). Exosomes were successfully used to promote endometrial regeneration and restore fertility rates in a murine model (Zhao et al., 2020), implicating this delivery method as a promising treatment tool. Indeed, exosomes derived from adipose-derived MSCs may restore endometrium to normal morphology, decrease fibrosis, and increase the expression of pro-regenerative factors such as ITGβ3, LIF, and VEGF, thus supporting an improvement in implantation and pregnancy rate (Zhao et al., 2020).

3.2.3 | Growth factors and other molecules

Growth factors and other molecules have therapeutic effects both individually and in combination, though most studies are in vitro or animal models and the clinical translation to human treatment is undetermined. Hepatocyte growth factor, an enhancer of in vitro proliferation and migration of human endometrial epithelial cells (Sugawara et al., 1997) and transforming growth factor β (TGF-β) isoforms promotes in vitro endometrial remodeling (Nasu et al., 2005) along with platelet-derived growth factor (PDGF) isoforms, which stimulate proliferation and migration among cultured human endometrial stem cells for endometrial tissue repair and support endometrial tissue contraction and remodeling (Matsumoto et al., 2005). Besides, epidermal growth factor (EGF), PDGF-BB, and basic fibroblast growth factor promote in vitro endometrial stromal and epithelial colony-forming units, an intrinsic characteristic of somatic stem cells (Gargett et al., 2008).

In vivo, stromal cell-derived factor 1 (SDF1α) improved stem cell engraftment in an AS mouse model receiving BMDSCs therapy (Ersoy et al., 2017). Later, the synergic effect of SDF1α and BMDSCs in a murine model of AS was also reported and endometrial regeneration levels after a single application of SDF1α were similar to those from stem cells alone (Yi et al., 2019). Similarly, BMDSC therapy improved endometrial thickness and reproductive outcomes by transfecting stem cells with VEGF in a mouse model with injured endometrium (Jing et al., 2018). Molecules such as icariin and ligustrazine, common plant derivatives used in traditional Chinese medicine, were tested in rat models of uterine damage. Le et al., (2017) reported the positive effect of icariin on endometrial thickness and the expression of several pro-regenerative factors. Ye et al. (2019) reported similar results with ligustrazine.

The use of these factors in treating endometrial pathologies in humans has not been reported. Just the use of the G-CSF for endometrial regeneration has been described, but results are controversial. Two independent clinical trials (Barad et al., 2014; Eftekhar et al., 2014) reported that G-CSF treatment does not significantly increase endometrial thickness. However, a later meta-analysis analyzing 11 different studies concluded that intrauterine perfusion of G-CSF can improve endometrial thickness along with clinical pregnancy and implantation rates in patients with a thin endometrium (Xie et al., 2017).

3.3 | Emerging therapeutic alternatives

As discussed above, stem cell therapy is effective in inducing endometrial regeneration in animal models. However, stem cell therapy is costly, invasive, and painful, and therefore not an ideal intervention. Additionally, depending on the type of stem cells, other issues such as ethical and moral questions, risk of teratoma formation, and low retention of cells may arise (Kim & Park, 2017). The use of single molecules has not been deeply explored, as we previously detailed, and most studies use them as enhancers of stem cell action. However, the lack of consensus and the weaknesses of the published studies have promoted the emergence of other therapeutic alternatives, such as platelet-rich plasma (PRP).

3.3.1 | Platelet-rich plasma

PRP is a plasma fraction with a supra-physiologic platelet concentration consisting of biologically active biomolecules like growth factors, such as PDGF, TGFβ, or SDF1α; cytokines; and other proteins, inside α-granules platelets, which are key components for tissue repair (Anitua et al., 2012; Mussano

et al., 2016). These molecules can only be released after breaking the plasma membrane of platelets (i.e., using calcium chloride), a process called activation or degranulation (Fréchette et al., 2005). Further, PRP can be easily obtained via centrifugation to create a gradient in which the lower part of the plasma fraction is enriched in platelets from a peripheral blood sample (Dohan Ehrenfest et al., 2018). This methodology is a minimally invasive procedure appropriate for autologous treatment in AS and EA patients (Pietrzak & Eppley, 2005).

In vitro experiments based on human endometrial cell processes such as cell migration or proliferation (Aghajanova et al., 2018; Wang et al., 2018) describe a positive effect of PRP on regeneration mechanisms. Overexpression of genes and proteins related to a healthy endometrium and regeneration processes, such as estrogen (ERα) and progesterone (PR) receptors (Marini et al., 2016), VEGF, and procollagen type I (Anitua et al., 2016), are also reported.

In vivo rodent models with injured endometrium also show promising results after intrauterine administration of autologous PRP, decreasing fibrosis and increasing expression of several markers of proliferation (Ki-67), angiogenesis (VEGF), and normal endometrial function (cytokeratin, homeobox A10 -HOXA10-) (Jang et al., 2017). Further, PRP administration improves endometrial morphology, reduces the degree of fibrosis, and produces a higher number of implantation sites and live-births (Kim et al., 2020). PRP was also described as a promotor of the regenerative action of BMDSCs (Zhou et al., 2020). These authors suggested that PRP enhances stem cell differentiation through the nuclear factor κB pathway. They proposed that PRP regeneration activity was based on the activation of the NF-κB p50 subunit, which induces the upregulation of the anti-inflammatory cytokine IL-10, described to be involved in endometrial regeneration after injury (Xue et al., 2019). In addition, we recently corroborated that PRP promotes in vitro endometrial cell proliferation and migration and regenerates endometrial tissue after damage in a murine model. This effect is strengthened when blood is obtained from the umbilical cord, the most undifferentiated blood source (De Miguel-Gómez et al., 2021). These results suggest that the umbilical cord could be a good source of plasma for treating endometrial pathologies and other regenerative medicine applications, as demonstrated by other groups (Castellano et al., 2017; Ehrhart et al., 2018). PRP has also been tested in women with either thin endometrium (Chang et al., 2019; Eftekhar et al., 2018; Kim, Shin et al., 2019; Molina et al., 2018; Nazari et al., 2016; Tandulwadkar et al., 2017) or AS (Javaheri et al., 2020; Zadehmodarres et al., 2017). However, not all these human works presented a robust study design. From the results obtained by those conducted as clinical trials (Chang et al., 2019; Eftekhar et al., 2018; Javaheri et al., 2020; Tandulwadkar et al., 2017), it could be concluded that PRP is an effective therapeutic option for treating patients with thin endometrium in which the cause is not IUA (this was an exclusion criterion in the majority of existing studies).

3.3.2 | Tissue engineering solutions and bioengineering approaches

In 1988, tissue engineering was defined as the "application of engineering and life science basis toward the development of biological substitutes for improving, maintaining, or restoring tissue natural functions" (Skalak & Fox, 1988). Bioengineering is a fundamental pillar for tissue engineering based on the use of biomaterials to support tissue regeneration (Brien, 2011). Biomaterials used to model the human endometrium are typically collagen (Gentleman et al., 2003), proteoglycans (formed of glycosaminoglycans, such as heparin or keratin sulfates, covalently attached to a core protein) (Rnjak-kovacina et al., 2017), alginate (Nayak et al., 2020), and chitosan (Choi et al., 2016). They can be used alone or in combination with stem or fully differentiated cells, growth factors, or other biomolecules that work synergistically with the biomaterial (Brien, 2011).

Endometrial regeneration can occur in murine models using collagen scaffolds in combination with other approaches. Collagen and growth factors, such as bFGF (Sun et al., 2011) or VEGF (Lin et al., 2012), and stem cells, derived either from the bone marrow (Ding et al., 2014) or embryonic tissues (Song et al., 2015), have a synergic effect on endometrial regeneration in a rat model with an excised portion of one of the uterine horns. Endometrial morphology (H&E staining, endometrial thickness, and the number of endometrial glands), regeneration of muscular cells (αSMA quantification), blood vessel density (vWF quantification), and pregnancy outcomes improved after the combined treatments.

Several ongoing clinical trials are also evaluating collagen scaffolds loaded in combination with umbilical cord MSCs (National Library of Medicine US) or autologous BMDSCs (National Library of Medicine US), to treat intrauterine adhesions in women. These studies have not reported results yet. However, the good results of the previously mentioned animal models using either the secretome or the stem cells themselves, and in the addition of the well-known biocompatibility of the collagen scaffolds with humans, make the expected results promising.

Decellularized scaffolds

Scaffolds made from ECM after decellularization (removal of all cellular components of a biological scaffold while retaining the ECM structure) of tissues or whole organs have also been evaluated. Scaffolds are a relatively new concept first applied in the assisted reproduction field in 2014 in a rat uterus (Santoso et al., 2014). A longitudinal segment of a rat uterus was decellularized using two different methods, sodium dodecyl sulfate (SDS) and high hydrostatic pressure, and both supported regular pregnancies. Later, other groups reseeded the decellularized scaffolds before replanting them in animal models. Successful endometrial regeneration was noted in rat models using decellularized uterine scaffolds, obtained using SDS or Triton-X 100, grafted into an injured uterus after reseeding with rat primary uterine cells (Miyazaki & Maruyama, 2014) and mesenchymal BMDSCs (Hellström et al., 2016). Both studies reported comparable reproductive outcomes in the groups with cell-seeded

scaffold transplant and control groups. Maruyama's group also described the importance of scaffold orientation and reported that if the uterine patches were reverse oriented (luminal part in the outside while the serosal side remained in the inside, or lumen), the regenerated uterine tissue was aberrant (Miki et al., 2019).

Not only single fragments but also the decellularization of the whole uterus has been achieved in different animal models. The decellularization of an entire rat uterus using several protocols, of which sodium deoxycholate was the best for preserving ECM, was reported (Hellström et al., 2014). Similar results were also reported in a later study using a whole sheep uterus. In this study, rings from a bioengineered uterus were recellularized using sheep fetal BMDSCs and the decellularized uterus fragments maintained the reseeded cells in vitro for 2 weeks (Tiemann et al., 2020). The same recellularizing procedure using human endometrial stem cells was also applied to decellularized scaffolds obtained from a whole porcine uterus, which successfully maintained ECM and vascular network integrity (Campo et al., 2017). Further, an ovine acellular uterus scaffold was harvested in rats and became recellularized with endometrial tissue and vascular cells (Daryabari et al., 2019). Finally, decellularized endometrial scaffolds recellularized again with endometrial cells, both obtained from human samples, responded to 28-day hormone treatment in vitro, complete with the secretion of decidual markers (Olalekan et al., 2017).

Hydrogels

Another bioengineering approach is the use of hydrogels, which are three-dimensional hydrophilic polymer networks (Hoffman, 2002) derived from hyaluronic acid (Liu et al., 2019; Kim, Park et al., 2019) or different poloxamers (Yang et al., 2017; Zhang et al., 2020), among other biomaterials. To enhance the synergies of this novel approach and other classical therapies, artificial hydrogels have been applied in several murine models together with other factors or cells. These combinations have been performed using estradiol embedded in an aloe-poloxamer (Yao et al., 2020) and a heparin-poloxamer hydrogel (Zhang et al., 2020) for restoring endometrium in rat models of intrauterine adhesions. Both studies reported improvement in the endometrial morphology status and a reduction in fibrosis, as well as the overexpression of cell proliferation and endometrial regeneration factors. Hydrogels in combination with the chemokine SDF1α restored the endometrium and improved endometrial thickness, fibrotic area, number of glands, and embryo implantation rate (Wenbo et al., 2020). Different types of cells or their derivatives, such as endometrial stromal cells (Kim, Park et al., 2019), BMDSCs (Yang et al., 2017), and the stem cell secretome (Liu et al., 2019) are reported to improve therapeutic effects when combined with hyaluronic acid or poloxamer-based hydrogels in murine models of endometrial damage.

Hydrogels could be directly related to decellularized scaffolds because they can also derive from decellularized ECM (Saldin et al., 2016). A recent study reported the in vitro effectiveness of a hydrogel obtained from a decellularized amniotic ECM and combined with estradiol loaded in microspheres (Chen et al., 2020) with an increased cell proliferation rate and a higher expression of EGF and IGF-1 and its receptors after culturing Ishikawa cells (a human endometrial adenocarcinoma cell line) with this hydrogel. Further, we recently developed a biocompatible and stable hydrogel derived from decellularized porcine endometrium that supports in vitro culture of human endometrial cells, either epithelial and/or stromal, enhancing cell proliferation. This tissue-specific hydrogel may improve endometrial regeneration and pregnancy rates in a murine model by remodeling the original tissue (López-Martínez et al., 2021). Thus, hydrogels and decellularized scaffolds could be the most promising technique for regenerating the endometrium and improving classical cell culture techniques used in assisted reproduction.

Microfluidics

Microfluidic technology has emerged as a method to model reproductive organs in vitro and to assist in evaluating therapeutic solutions for endometrial pathologies (Campo et al., 2020). A microfluidic device included a coculture of primary human stromal and endothelial cells in which the hormonal changes occurring during the menstrual cycle were simulated (Gnecco et al., 2017); this approach allowed the study of the implication of the vascular endothelium during the decidualization process (Gnecco et al., 2017). A microfluidic platform termed EVATAR, containing reproductive tract tissues and peripheral organs mimicking a 28-day human menstrual cycle was also described (Xiao et al., 2017). These technologies will promote the in vitro study of new therapeutic options not only for the endometrium but also for the rest of the reproductive organs.

3.4 | Endometrial organoids

In the last years, organoids, defined as genetically stable in vitro–cultured 3D structures that encompass key features of in vivo organs (Schutgens & Clevers, 2020), have emerged as an alternative to conventional in vitro cell culture systems. These 3D biological structures have been revealed as key models for several diseases, drug screenings, testing, and benchmarking for novel therapeutic approaches, as well as a potential tool of personalized medicine (Clevers, 2016). Thus, for endometrial management, not only in AS/EA patients but also in endometriosis, organoids could be a promising instrument either to better understand the pathogenesis of AS/EA or screen incoming untested therapies. This novel approach could complement or even reduce the studies performed in animal models before the clinical translation to humans.

In the last decade, organoids have been derived from different human tissues such as the liver (Huch et al., 2015) or prostate (Karthaus et al., 2014). More recently, several groups have obtained them from human endometrial tissue. These organoids exhibited the characteristics of uterine glands in vivo, expressing specific epithelial, such as epithelial cell adhesion molecule (EPCAM), and secretory, such as mucin-1, markers. These organ-like structures also responded to hormonal stimulation (estrogen and progesterone) by the overexpression of ERα and PR or the secretion of the progesterone-associated endometrial protein (PAEP) that

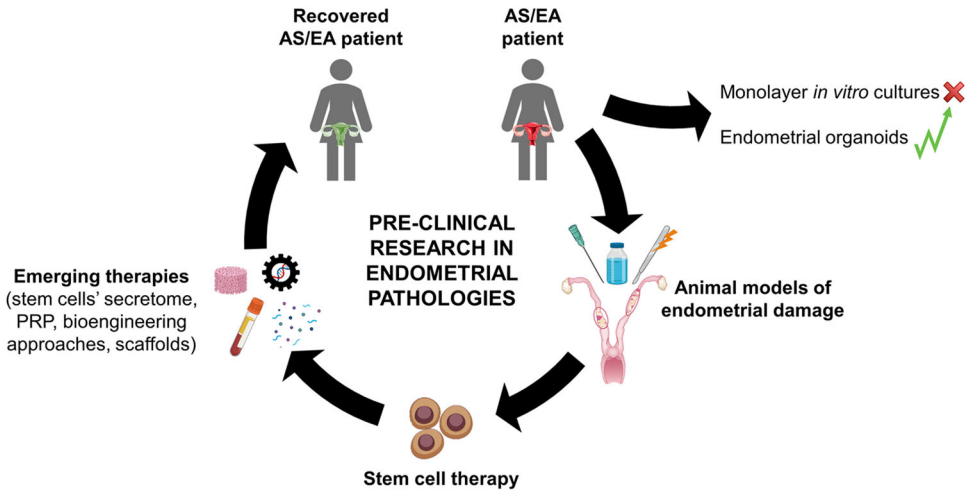

FIGURE 1 Overview of the research and preclinical process in the management of endometrial pathologies. Patients with damaged endometrium (Asherman's syndrome (AS) or Endometrial atrophy (EA)) still lack completely effective treatments. The animal models are essential in the pursuit of therapy for these patients. In those animals (mainly mice and rats), mechanical (needles, electrical scalpels) and chemical (ethanol) methods can generate endometrial damage. Over the years, stem cell therapy has been revealed as the most promising option, however other approaches such as the use of the stem cells' secretome, platelet-rich plasma (PRP), and bioengineering techniques (hydrogels, collagen scaffolds, microfluidics), or even the combination of all of them, have emerged as good alternatives in the field. Moreover, the in vitro systems are also very useful but have many limitations for being able to extrapolate results. The development of endometrial organoids, which simulate more closely an in vivo tissue or organ, are emerging as promising research tools. Figure created with Biorender. com and adapted from De Miguel-Gómez et al., 2020

reveals decidualization, among other features (Turco et al., 2017). Similarly, in 2018, another group isolated organoids not only from the human endometrium but also from murine samples (Boretto et al., 2017). They reported the endometrial epithelium-like phenotype by expression of E-cadherin, ERα, and cytokeratin, by mucin-1 secretion, and by the response to ovarian hormones. This group also published organoids directly derived from human patients, opening then the door to disease modeling and personalized medicine for endometrial-associated pathologies (Boretto et al., 2019).

After the revision of all works cited along with this review and despite those describing human studies, we want to remark the importance of basic science and standard animal models in the study of novel treatments for specific endometrial disorders (AS/EA) before the clinical translation. The generally smaller size of the animal models together with the bigger litter size, short generation times, and more availability of tissue (endometrium in this case) for molecular studies are the main advantages of using animals prior testing in humans (Carter, 2020). Besides, while promising therapies and study platforms are emerging, they still need to be further explored.

4 | CONCLUSION

Classical management of AS and EA is lacking effectiveness, so new approaches have emerged for endometrial regeneration to increase fertility options when this tissue is damaged (Figure 1). Stem cell therapy is the most widely explored and different sources of stem cells have shown promising results in animal models. However, there are disadvantages to stem cells, and new alternatives that can enhance or even replace the regenerative mechanisms of stem cells are changing the field of endometrial regeneration. Adjuvants or promotors of stem cells have been proposed as treatment methods and the emergence of high-tech solutions, such as platelet-rich plasma or bioengineering-based techniques, are likely the best alternatives. Due to the relatively recent emergence of these therapeutic options, robust clinical trials are needed to corroborate the promising findings in experimental models.

Clinical translation of these new approaches will rely on the generation of a well-established animal model of endometrial injury in which to evaluate treatment options. In this context, recent studies based on the successes and limitations of these animal models have evaluated different methods for simulating AS/EA, concluding that ethanol is a better induction of endometrial damage than only mechanical curettage (Kim et al., 2018). Defining the optimal animal model for translational research could strengthen the reproducibility and globalization of these kinds of approaches. Additional studies with stronger designs (including higher population sizes or more robust control groups, to enumerate a few features to improve) dealing with important yet-unresolved questions, mainly regarding the pathogenesis of AS/EA, are needed to corroborate the use of emerging options for treating endometrial pathologies and fulfill not only a successful treatment but also a complete understanding of both pathologies. Lastly, the emergence of new research tools, like organoids, can also change and improve the current methods to study AS and EA and to screen different therapies.

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CONFLICT OF INTEREST

L.D.m.-G., M.R., I.C. and A.P. report no conflicts of interest.

AUTHOR CONTRIBUTIONS

Lucía de Miguel Gómez and Mónica Romeu: literature research, manuscript drafting, and critical discussion. Irene Cervelló and Antonio Pellicer: review design, manuscript drafting, and critical discussion.

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5. ANNEX V. SCIENTIFIC PRODUCTION

5.1. Works submitted to international conferences directly related with the present Ph.D. thesis

Oral communication. "COVID19-free endometrium: undetectable viral RNA in endometrial biopsies from positive symptomatic SARS-CoV-2 women". Authors: **de Miguel-Gómez L**, Romeu M, Pellicer N, Faus A, Pellicer A, Cervelló I. 36th congress of European Society of Human Reproduction and Embriology (ESHRE). Online congress.2021. *Related with the appendix.*

Poster communication. "Human platelet-rich plasma from adult peripheral and umbilical cord blood: endometrial regeneration and proteomic profiles in a murine model of Asherman Syndrome". Authors: **de Miguel-Gómez L**, López-Martínez S, Faus A, Campo H, Mollá-Robles G, Pellicer A, Domínguez F, Cervelló I. 67th congress of Society for Reproductive Investigation (SRI). Vancouver (Canada). 2020. *The congress was cancelled due to COVID-19 pandemic.*

Poster communication. "Stem cell therapy induces a shift from an inflammatory environment towards an immune tolerant scenario promoting endometrial tissue tegeneration". Authors: **de Miguel-Gómez L**, López-Martínez S, Faus A, Hervás D, Ferrero H, Cervelló I. 35th congress of ESHRE. Vienna (Austria). 2019.

Poster communication. "Human platelet-rich plasma from patients with uterine pathologies contributes to endometrial regeneration: a current therapeutic approach?" Authors: **de Miguel-Gómez L**, Faus A, López-Martínez S, López-Pérez N, Castillón G, Pellicer A, Cervelló I. 66th SRI. Paris (France). 2019.

5.2. Works submitted to international conferences not related with the present Ph.D. thesis

Poster communication. López-Martínez S*, Rodríguez-Eguren A*, **de Miguel-Gómez L**, Faus A, Francés-Herrero E, Pellicer A, Ferrero H, Cervelló I. "Extracellular matrix hydrogels from decellularized endometrium promote tissue regeneration and fertility restoration in a murine model of endometrial damage." 68th congress of SRI. Boston (USA). 2021. *Co-author-ship.

Poster communication. López-Martínez S*, Campo H*, **de Miguel-Gómez L,** Faus A, Ferrero H, Cervelló I. "Bioengineering endometrial extracellular matrix hydrogels: a new option to improve human three-dimensional endometrial cell culture and organoid development." 67th congress of SRI. Vancouver (Canada). 2020. *The congress was cancelled due to COVID-19 pandemic*. *Co-author-ship.

Oral communication. López-Martínez S, Campo H, **de Miguel-Gómez L**, Faus A, Ferrero H, Cervelló I. "Improving three-dimensional in vitro culture methods of human endometrial stem cells: bioengineering tissue-specific hydrogels." 35th congress of ESHRE. Vienna (Austria). 2019.

5.3. Scientific articles not related with the present Ph.D. thesis

López-Martínez S*, Rodríguez-Eguren A*, **de Miguel-Gómez L**, Francés-Herrero E, Faus A, Díaz A, Pellicer A, Ferrero H, Cervelló I. 2021. Bioengineered endometrial hydrogels with growth factors promote tissue regeneration and restore fertility in murine models. *Acta Biomat* 2021; S1742-7061(21)00551-1. *Co-author-ship.

Francés-Herrero E*, Juárez-Barber E*, Campo H, López-Martínez S, **de Miguel-Gómez L**, Faus A, Pellicer A, Ferrero H, Cervelló I. Improved models of human endometrial organoids based on hydrogels from decellularized endometrium. *J Pers Med* 2021;**11**:504. *Co-author-ship.

López-Martínez S*, Campo H*, **de Miguel-Gómez L**, Faus A, Navarro AT, Díaz A, Pellicer A, Ferrero H, Cervelló I. A natural xenogeneic endometrial extracellular matrix hydrogel toward Improving current human in vitro models and future in vivo applications. *Front Bioeng Biotechnol* 2021;**9**:639688. *Co-author-ship.

Buigues A*, Marchante M*, **de Miguel-Gómez L**, Martinez J, Cervelló I, Pellicer A, Herraiz S. Stem cell-secreted factor therapy regenerates ovarian niche and rescues follicles. *Am J Obstet Gynecol* 2021;**225**:65.e1-65.e14. *Co-author-ship.

Francés-Herrero E, **de Miguel-Gómez L**, López-Martínez S, Campo H, Garcia-Dominguez X, Diretto G, Faus A, Vicente JS, Marco-Jiménez F, Cervelló I. Development of decellularized oviductal hydrogels as a support for rabbit embryo culture. *Reprod Sci* 2021;**28**:1644-1658.

APPENDIX:

EVALUATION OF THE POTENTIAL IMPACT OF SARS-COV-2 ON THE HUMAN ENDOMETRIUM: A PRELIMINARY STUDY

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ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ACE2	Angiotensin-converting enzyme 2
ARDS	Acute respiratory distress syndrome
AT1R	Angiotensin II type 1 receptor
AT2R	Angiotensin II type 2 receptor
CDC	Centers for disease control and prevention
COVID-19	Coronavirus disease 2019
C_T	Cycle threshold
DNA	Deoxyribonucleic acid
E	Envelope protein
FR	Respiratory frequency
HUiP	Hospital Universitari i Politècnic
ICU	Intensive unit care
IL	Interleukin
M	Membrane protein
MASR	Mas receptor
mRNA	Messenger ribonucleic acid
N	Nucleocapsid protein
n	Population size
PaO_2/FiO_2	Ratio of partial pressure arterial oxygen (PaO_2) and fraction of inspired oxygen (FiO_2)
PHEIC	Public health emergency of international concern
PRR	Pro-renin receptor
qRT-PCR	Quantitative real-time polymerase chain reaction
RAS	Renin-angiotensin system
RNA	Ribonucleic acid
S	Spike protein
SD	Standard deviation

SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
TMPRSS	Transmembrane protease serine 2
TNFα	Tumor necrosis factor
WHO	World Health Organization

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1. INTRODUCTION

1.1. 2020 global pandemic: coronavirus disease 2019 (COVID-19)

The novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causal agent for coronavirus disease 2019 (COVID-19). This epidemic was declared a public health emergency of international concern (PHEIC) on January 30, 2020, the highest level of alarm of the World Health Organization (WHO) (World Health Organization, 2021). Since the first cases of unexplained pneumonia reported in Wuhan (China), SARS-CoV-2 has infected more than 190 million people and caused more than 4 million deaths worldwide at the time of writing (July 29, 2020; World Health Organization, 2021). These data vastly exceed those from SARS-CoV infections in 2002 (Sun *et al.*, 2020).

SARS-CoV-2 infection is usually suspected when dry cough and fever appear. However, COVID-19 patients have reported a wide range of clinical manifestations, ranging from mild symptoms to severe illness, classifying the disease into three levels: mild, severe, and critical (Table I; Wang *et al.*, 2020). Aging and underlying diseases have been revealed to worsen the prognosis (Deng and Peng, 2020). Although most of the symptoms correlate to the respiratory system, cases of neurological complications, liver injury, blood disorders, or cardiac injury have also been reported (Wang *et al.*, 2020).

Clinical manifestations of COVID-19		
Fever, dry cough, fatigue, shortness of breath, muscle ache, confusion, headache, sore throat, rhinorrhea, chest pain, nausea, diarrhea, vomiting, chills, sputum production, hemoptysis, dyspnea, bilateral pneumonia, anorexia, chest pain, leucopenia, lymphopenia, olfactory and taste disorders, higher levels of plasma cytokines.		
Severity levels of COVID-19		
Mild	**Severe**	**Critical**
- Fever - Cough - Fatigue - Ground-glass opacities - Mild pneumonia	- Dyspnea - Hypoxemia - F_R ≥30/min - PaO_2/FiO_2 < 300 - Lung infiltrates >50% - ICU needed	- ARDS - Respiratory failure - Septic shock - Multiple organ failure - Severe metabolic acidosis - Coagulation dysfunction

Table I. Clinical manifestations and severity levels of COVID-19. *ARDS: acute respiratory distress syndrome; FR: respiratory frequency; ICU: intensive unit care; PaO_2/FiO_2 ratio of partial pressure arterial oxygen (PaO_2) and fraction of inspired oxygen (FiO_2). Table adapted with permission (Wang et al., 2020).*

The novelty of the virus and its associated symptoms have generated many important questions about its effects on human health. Among them, there are unknown factors by which tissues may be affected. Although SARS-CoV-2 predominantly infects the respiratory tract, infection of other organs may be possible (Synowiec *et al.*, 2021). Different studies have reported viral ribonucleic acid (RNA) detection (by quantitative nucleic acid amplification techniques) not only from blood, fecal, and, rarely, urine samples (Young *et al.*, 2020; Zheng *et al.*, 2020) but also from heart, liver, kidney, and sometimes even brain tissues (Puelles *et al.*, 2020; Wichmann *et al.*, 2020) obtained after autopsies. Although, SARS-CoV-2 could be spreading through the entire human body, the viral load and, consequently, infectiousness in these extrapulmonary organs seems to be too low to infect *in vitro* cell cultures (Hopfer *et al.*, 2021).

1.2. Main characteristics of SARS-CoV-2

SARS-CoV-2 belongs to the *Coronaviridae* family. Thus, it is a positive-sense single-stranded enveloped RNA virus that incorporates three types of transmembrane proteins: spike (S), envelope (E), and membrane (M), in addition

to the structural protein nucleocapsid (N; Figure 1; Neuman and Buchmeier, 2016; Ke *et al.*, 2020).

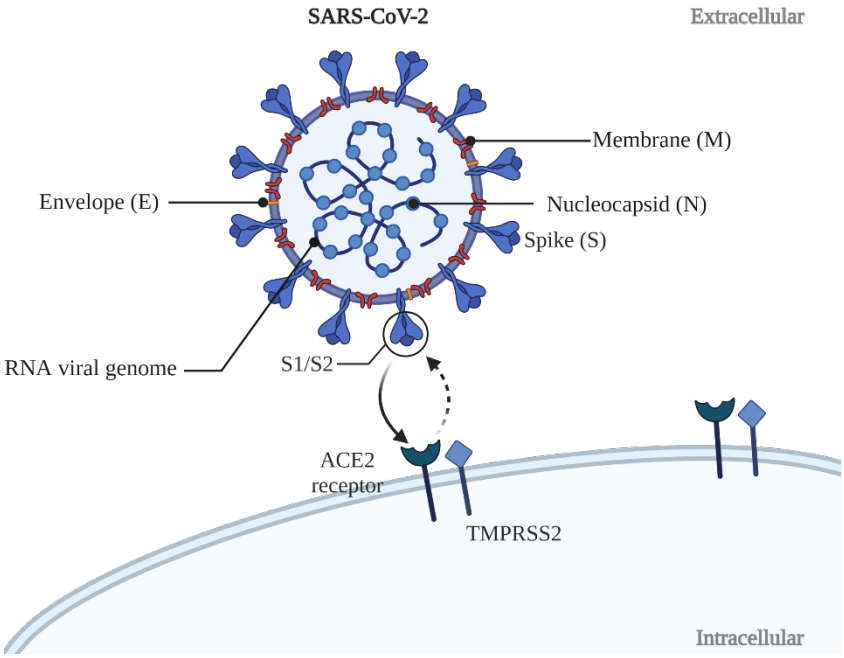

Figure 1. Schematic diagram of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). *This virus is composed of envelope (E), nucleocapsid (N), spike (S), and membrane (M) proteins. SARS-CoV-2 viral entry is mediated by S proteins (containing S1 and S2 subunits) on the surface of SARS-CoV-2 binding with the host cell receptor ACE2. ACE2: angiotensin-converting enzyme 2; SARS-CoV-2: severe acute respiratory syndrome coronavirus-2; TMPRSS2: transmembrane protease serine 2. Created with www.BioRender.com.*

The S proteins promote invasion of the virus in target cells through specific binding to cell-surface receptors that enable the entry. Effective viral entry also requires S protein priming by the host cell' serine protease TMPRSS2, which exposes the S2 domain of the spike, responsible for virus-host membrane fusion (Figure 1; Shang *et al.*, 2020; Scudellari, 2021). Once the host cell has internalized the virus, viral RNA is quickly translated, suppressing the translation of the host messenger RNAs (mRNA) in favor of the virus' survival and spread (Scudellari, 2021). In this context, the angiotensin-converting enzyme 2 (ACE2) has been proposed and widely described as the primary cell entry receptor for SARS-CoV-2 (Hoffmann *et al.*, 2020; Scudellari, 2021).

1.3. ACE2: expression and function

The gene encoding the ACE2 receptor is expressed in the lungs (Pinto *et al.*, 2020), which are likely the most affected organs during COVID-19, in addition to nasal and oral mucosae (Xu *et al.*, 2020), the target tissues for diagnosis of SARS-CoV-2 infection. Besides its role as a viral receptor, ACE2 plays a key role in maintaining tissue homeostasis. ACE2 is a member of the renin-angiotensin system (RAS) pathway which has crucial roles in regulating blood pressure, inflammation, sodium reabsorption, fibrosis, and ultimately tissue remodeling processes (Ingraham *et al.*, 2020). Notably, ACE2 expression is not limited to the respiratory tract tissues. This gene has been detected in the intestines (Zhang *et al.*, 2020), testis (Douglas *et al.*, 2004), kidneys (Maksimowski *et al.*, 2020), and pancreas (Müller *et al.*, 2021).

1.3.1. ACE2 in the human body: importance of the RAS system

Remarkably, there are two antagonistic pathways co-existing in the RAS pathway. The first involves the conversion of angiotensin I led by renin, the consequent transformation to angiotensin II led by the angiotensin-converting enzyme (ACE) and finally the attachment to the angiotensin II type 1 receptor (AT1R; Figure 2A). The binding to AT1R favours inflammation by inducing the expression of pro-inflammatory cytokines (e.g., tumor necrosis factor alpha (TNFα) and interleukin 6 (IL6); Franco *et al.*, 2020). While the second path, led by ACE2, is responsible for attenuating inflammation. This receptor converts angiotensin I and II to their respective metabolites, angiotensin 1–9 and 1–7 (Figure 2A). In turn, angiotensin 1-7 attaches to the Mas receptor (MASR) while the remaining angiotensin II, binds to the angiotensin II type 2 receptor (AT2R). Both of MASR and AT2R antagonize the effects of AT1R by promoting anti-inflammatory events (Figure 2A; Franco *et al.*, 2020; Silhol *et al.*, 2020).

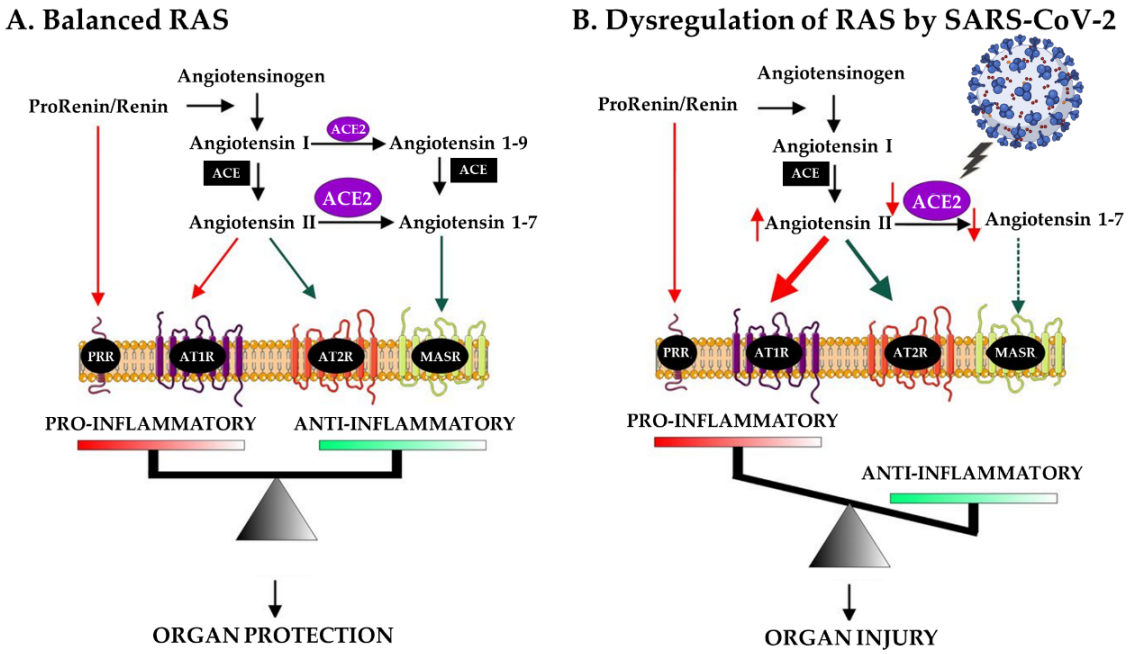

Figure 2. Renin-angiotensin system (RAS). *A. Schematic representation of the normal functioning of the RAS pathway. B. Schematic representation of the dysregulated RAS system, caused by SARS-CoV-2 infection, which results in a pro-inflammatory milieu potentially responsible for organ injury. ACE: angiotensin-converting enzyme; ACE2: angiotensin-converting enzyme 2; AT1R: angiotensin II type 1 receptor; AT2R: angiotensin II type 2 receptor; MASR: mas receptor; PRR: pro-renin receptor. Image adapted with permission (Franco et al., 2020); SARS-CoV-2 figure by www.BioRender.com.*

The balance achieved by the actions of ACE/ACE2, can be interrupted by different events, including the binding of SARS-CoV-2 to ACE2 (Figure 2B). The subsequent down-regulation of ACE expression would be promoting increased levels of angiotensin II, AT1R, and, consequently, the secretion of pro-inflammatory cytokines, which can ultimately cause lung and cardiovascular injury (Franco *et al.*, 2020). This hypothesis could also explain the increased levels of IL6 in patients with severe cases of COVID-19 (Franco *et al.*, 2020).

1.3.2. Role of ACE2 in the human endometrium

ACE2 expression is also described in human female reproductive organs and cells, including the vagina (Jing *et al.*, 2020), uterus (Xu *et al.*, 2020), placenta (Valdés *et al.*, 2006), ovaries (Reis *et al.*, 2011), and oocytes (Barragan *et al.*, 2020). More precisely, in the endometrium, it seems that angiotensin II promotes vascularization and growth of the endometrial stromal fraction due to the

described deficit of this molecule in patients with irregular bleeding (Li and Ahmed, 1996; Vaz-Silva *et al.*, 2009). Moreover, an imbalance in the RAS pathway has previously been correlated with endometrial cancer, endometriosis, and preeclampsia (Irani and Xia, 2008; Delforce *et al.*, 2017).

Regarding the specific location of this receptor in the endometrium, higher mRNA expression of *ACE2* has been found in epithelial (versus stromal cells) and during the secretory (compared to the proliferative phase) of the menstrual cycle (Vaz-Silva *et al.*, 2009). Protein expression analysis reaffirmed the higher expression of the receptor in the secretory phase; however, stromal cells during this phase showed a higher expression of ACE2 than their epithelial counterparts (Chadchan *et al.*, 2020). This finding was reinforced with an *in vitro* decidualization assay, suggesting the potential role of ACE2 in this crucial process arises during the secretory phase (Chadchan *et al.*, 2020). In parallel, the RAS pathway has been pointed out as dysregulated in endometrial cancer, revealing an overexpression of ACE2 in the endometrial carcinoma tissue (Delforce *et al.*, 2017).

2. RATIONALE AND OBJECTIVE OF THE STUDY

The review of the scientific literature presented in the Introduction brought up two crucial points, (1) the *ACE2* receptor has an essential role in SARS-CoV-2 infection, and (2) *ACE2* is expressed and actively functional in the endometrial tissue. These two facts raise the following question: could the endometrial *ACE2* receptor facilitate the effective entry of SARS-CoV-2 into the human endometrium?

The relevance of the answer to this question relies on the importance of the endometrium to reproduction, implying that infection of this tissue could influence reproductive outcomes, potentially even for future fertility treatments.

Thus, the objective of the present study was to study the presence of SARS-CoV-2 RNA and A*CE2* expression in the endometrium of symptomatic women who tested positive for the virus.

3. MATERIAL AND METHODS

3.1. Study design

Endometrial tissue samples were obtained from 15 symptomatic women (aged between 24 and 46 years) with COVID-19 and hospitalized in the Hospital Universitari i Politècnic (HUiP) La Fe (Valencia, Spain). The samples were analyzed for the presence of SARS-CoV-2 RNA and the expression of *ACE2* receptor using quantitative real-time polymerase chain reaction (qRT-PCR). The *ACE2* expression in the endometrium was also compared with nasopharyngeal epithelial swabs from a sample repository of women with COVID-19 (Figure 3). See Figure 3 for a schematic representation of the study design.

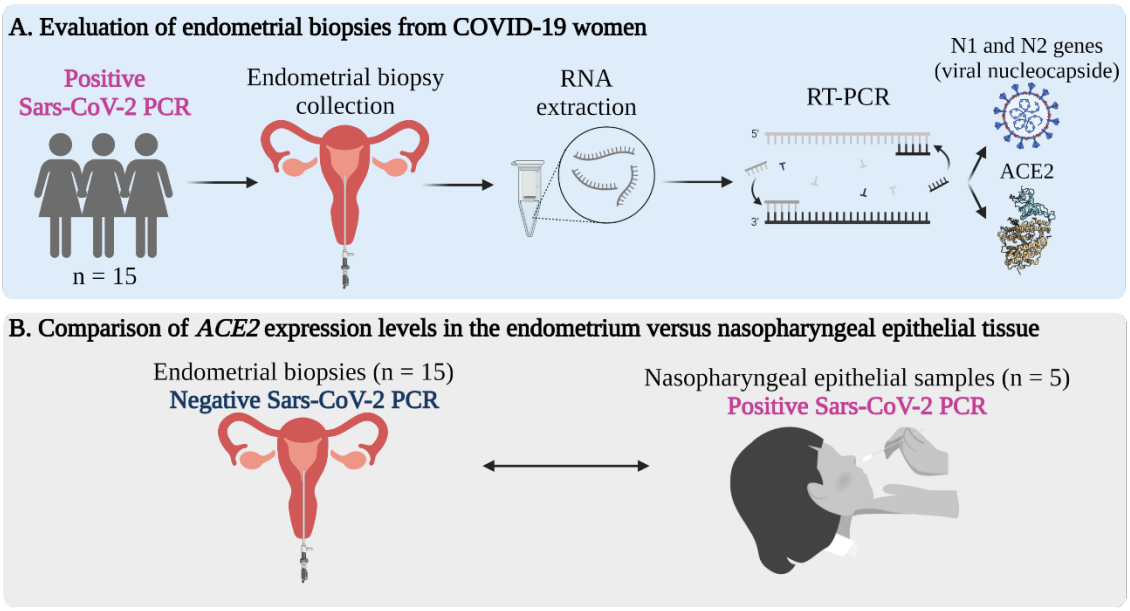

Figure 3. Study design. *A. Endometrial tissue samples were obtained from 15 hospitalized symptomatic women with COVID-19. All samples were analyzed for testing the presence of SARS-CoV-2 RNA and ACE2 receptor expression using qRT-PCR. B. Expression levels of ACE2 in the endometrial samples were compared with a repository of nasopharyngeal epithelial samples from women (n = 5) with COVID-19. Created with ww.BioRender.com.*

The research ethics committee of HUiP La Fe approved this study (2020-268-1) on May 12, 2020. All study participants received written and oral information on the study characteristics and consented verbally in the presence of at least one witness.

3.2. Sample collection and processing procedures

3.2.1. Study participants

Patients meeting the following criteria were included in the study: women aged 18 to 48 years with a positive result for SARS-CoV-2 infection indicated by qRT-PCR of nasopharyngeal swabs, hospitalization for COVID-19 in HUiP La Fe, and voluntary willingness to participate in this study. The exclusion criteria were: known infertility (ovulation disorder, severe endometriosis, Asherman's Syndrome, luteal phase deficiency, or fallopian tube obstruction); intrauterine contraceptive device use; severe COVID-19; or any disorder or condition that, in the point of view of the gynecologist, could be a risk for the patient.

A total of 47 women met the above criteria and were interviewed, but only 15 consented or were eligible to enroll in the study (from August to October 2020).

All endometrial biopsies from infected patients were taken by endometrial aspiration using an intrauterine cannula (Cornier pipelle) and preserved in RNA Later (Invitrogen, ref. AM7021) until cryopreservation. Tissue samples (median size of 3 mm^2) were freeze-dried at -80ºC in the biobank of the HUiP La Fe.

3.2.2. RNA extraction and reverse transcription

All RNA procedures were performed in the HUiP La Fe biobank facilities, which were authorized to manipulate these biological samples. RNA was extracted from thawed tissues using the miRNeasy mini kit (Qiagen, ref. 1038703). Briefly, endometrial tissue was first homogenized with QIAzol lysis reagent (Qiagen, ref. 79306) to induce phenol/guanidine-based lysis of samples. Then, RNA in the eluent was purified through silica-membrane–based spin columns, and concentration was measured using the spectrophotometer NANODROP 2000 (Thermo Scientific). Finally, RNA was reverse transcripted to its complementary DNA using the PrimeScript™ RT reagent kit (Takara, ref. RR037A).

3.3. Quantitative RT-PCR analyses of SARS-CoV-2 and *ACE2*

Bionos Biotech S.L. performed all qRT-PCR assays in their laboratories at the HUiP La Fe. Quantitative RT-PCR for SARS-CoV-2 detection was carried out using the U.S. Centers for Disease Control and Prevention (CDC)-approved 2019-nCoV RUO kit (IDT, ref. 10006713), including Taqman primers and probes for N1 and N2 regions of SARS-CoV-2 N gene (Table II). While qRT-PCR for human *ACE2* was performed using a predesigned assay (IDT, ref. Hs.PT.58.27645939), annealing to the DNA sequence of *ACE2* (exons 14 and 15).

Gene target	Primers and probes	Sequence, 5′→3′
N1	Forward primer	GAC CCC AAA ATC AGC GAA AT
	Reverse primer	TCT GGT TAC TGC CAG TTG AAT CTG
	Probe	ACC CCG CAT TAC GTT TGG TGG ACC
N2	Forward primer	TTA CAA ACA TTG GCC GCA AA
	Reverse primer	GCG CGA CAT TCC GAA GAA
	Probe	ACA ATT TGC CCC CAG CGC TTC AG
RPP30	Forward primer	AGA TTT GGA CCT GCG AGC G
	Reverse primer	GAG CGG CTG TCT CCA CAA GT
	Probe	TTC TGA CCT GAA GGC TCT GCG CG

Table II. 2019-nCoV CDC RUO kit: human sequences of primers and probes. *Primers for nucleocapsid genes N1, N2, and ribonuclease P/MRP Subunit P30 (RPP30; housekeeping gene). More information can be found at CDC website: https://wwwnc.cdc.gov/eid/article/26/8/20-1246-t1.*

In both cases, a cycle threshold (C_T) value < 37 cycles was defined as a positive result for viral detection (all measurements included technical triplicates, when possible). Results were normalized to the *RPP30* housekeeping gene (HSKG).

Additionally, *ACE2* expression in endometrial samples was compared to the average relative expression levels of a positive control group, including COVID-19 positive nasopharyngeal epithelial samples (n = 5) from a sample repository (women aged 27-48 years). Fold change in *ACE2* expression was calculated from three independent technical replicates for the HSK gene *RPP30*. For statistical significance, a *t*-test was applied to $\log2-\Delta\Delta C_T$.

4. RESULTS

4.1. Participants' characteristics

A total of 15 women, between 24 to 46 years old (mean years = 35.714, standard deviation (SD) = 7.485), were enrolled in the study. Notably, endometrial biopsies were collected at the different phases of the menstrual cycle (proliferative (n=3), early secretory (n=3), late secretory (n=7)) or from patients with amenorrhea (n=2). The number of days between the diagnosis of COVID-19 (based on a positive PCR result from a nasopharyngeal swab) and the collection of the endometrial biopsies ranged from 0 to 17 days (mean days = 5.000, SD = 4.440). Further of these patients are presented in Table III.

PATIENT	AGE	MENSTRUAL CYCLE PHASE	MAIN SYMPTOMS	DAYS FROM COVID-19 DIAGNOSIS TO EB COLLECTION
1	37	Proliferative	Cough, dyspnea, pneumonia Onset: 1 day before +PCR	9
2	25	Proliferative	General discomfort, pneumonia Onset: 4 days before +PCR	2
3	37	Proliferative	Pneumonia, hypoxemia Onset: 5 days before +PCR	10
4	36	Early secretory	Fever, dyspnea, arthralgia, bilateral pneumonia Onset: 4 days before +PCR	5
5	44	Early secretory	Cough, fever anosmia, cephalea, pneumonia Onset: 6 days before +PCR	4
6	40	Early secretory	Bilateral pneumonia Onset: 2 days before +PCR	1
7	46	Late secretory	Cough, fever, pneumonia Onset: 2 days before +PCR	5
8	24	Late secretory	Dyspnea, pneumonia Onset: 2 days before +PCR	3
9	44	Late secretory	Pneumonia Onset: 4 days before +PCR	17
10	39	Late secretory	Bilateral pneumonia Onset: 4 days before +PCR	5
11	28	Late secretory	Pneumonia Onset: 3 days before +PCR	0
12	32	Late secretory	Bilateral pneumonia Onset: 4 days before +PCR	1
13	40	Late secretory	Nasal congestion, cephalea, bilateral pneumonia Onset: 2 days before +PCR	7
14	41	Amenorrhea	Bilateral pneumonia Onset: 3 days before +PCR	5
15	24	Amenhorrea	Bilateral pneumonia Onset: Same day as +PCR	1

Table III. Patients' characteristics. *Age, phase of the menstrual cycle at endometrial biopsy collection, and main COVID-19 symptoms are indicated. Timing of symptom onset and endometrial biopsy collection are presented relative to the day of COVID-19 diagnosis confirmed by positive PCR (+PCR) of a nasopharyngeal sample. EB: endometrial biopsy; PCR: polymerase chain reaction.*

4.2. Quantitative RT-PCR results

In 14 of the 15 endometrial samples, neither N1 nor N2 genes were detected, revealing the absence of viral RNA in this tissue (Table IV). One endometrial

sample (from patient 7) was discarded for insufficient available RNA. HSKG, *RPP30*, was detected normally in the 14 samples (mean CT = 25.001, SD = 2.191), validating the process.

PATIENT	C_T VALUE N1	C_T VALUE N2	C_T VALUE *ACE2*	C_T VALUE *RPP30*	C_T *RPP30*/ C_T *ACE2*
1	ND	ND	ND	27.301±0.296	-
2	ND	ND	28.504±0.747	22.351±0.161	1.256±0.019
3	ND	ND	33.709±0.495	24.366±0.394	1.388±0.004
4	ND	ND	36.192	27.034	1.339
5	ND	ND	30.136±0.326	23.548±0.360	1.280±0.024
6	ND	ND	29.292±0.228	21.939±0.518	1.336±0.042
7	NA	NA	NA	NA	-
8	ND	ND	36.252±0.412	29.277±0.389	1.238±0.028
9	ND	ND	ND	24.758±0.157	-
10	ND	ND	35.595±0.849	26.746±0.324	1.331±0.020
11	ND	ND	34.446±0.590	24.366±0.503	1.414±0.029
12	ND	ND	28.649	24.182	1.185
13	ND	ND	29.839±0.389	21.971±0.441	1.359±0.044
14	ND	ND	ND	25.943±0.027	-
15	ND	ND	ND	26.282	-

Table IV. qRT-PCR results of SARS-CoV-2 and ACE2 detection. *Mean and standard deviation (SD) of the obtained cycle threshold (C_T) values for SARS-CoV-2, ACE2, RPP30 and ratio ACE2/RPP30 detection are indicated. The test was considered invalid when the housekeeping gene, RPP30, was not detected. NA: not available; ND: not detected.*

In 10 of the 14 (71.428 %) available endometrial samples, *ACE2* expression was detected. However, the CT values were quite high in some samples (mean CT = 32.261, SD = 3.261), indicating low expression. Nevertheless, these samples were validated with normal *RPP30* detection (mean CT = 24.998, SD = 2.195; Table IV). Remarkably, there was no significant difference in the *ACE2*/*RPP30* ratio throughout the different phases of the menstrual cycle.

In addition, the comparison of *ACE2* endometrial expression values with the positive control group (nasopharyngeal epithelial samples, n = 5, mean CT = 29.894, SD = 0.891) ratified the low expression in the endometrium, with *RPP30* detected normally (mean CT = 25.980, SD = 1.453). All endometrial samples, except those from patients 3 and 14 (due to insufficient material for three technical replicates), reported a statistically significant lower expression of ACE2 compared to the positive control group (Figure 4). Fold change values ranged from 0.014, in sample 12, to 0.682, in sample 14.

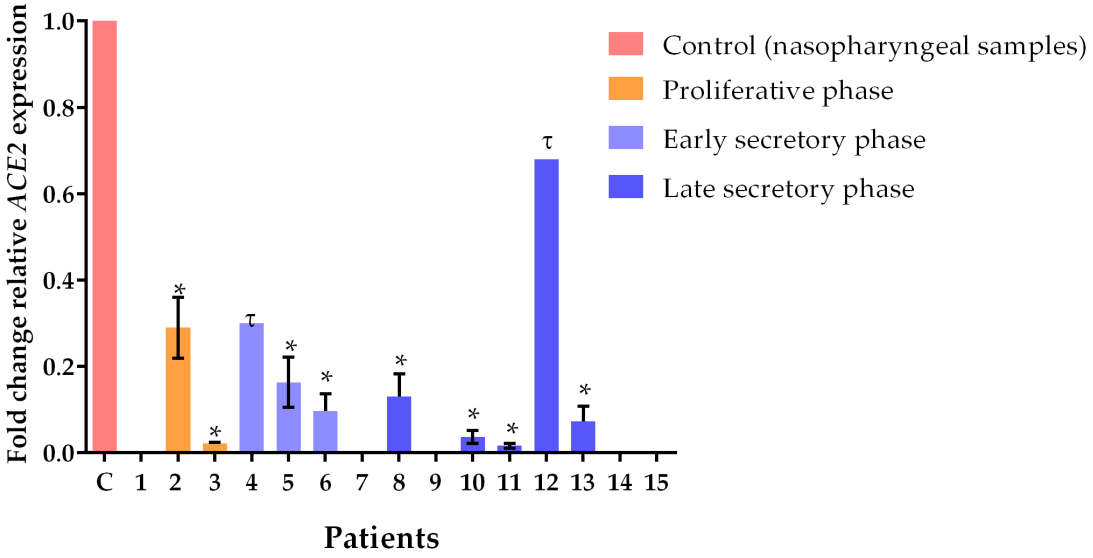

Figure 4. Analysis of the relative ACE2 mRNA levels in endometrial tissue of COVID-19 patients. *Graphical representation of the fold-change in ACE2 expression from all the endometrial samples compared to its averaged expression levels in a control group that included five nasopharyngeal epithelial samples derived from a COVID-19 positive sample repository, assessed under identical conditions. For statistical significance one-tailed t-test was applied on log2−ΔΔCT. Error bars represent the confidence interval. τ: significance not determined due to insufficient technical replicates, *P ≤ 0.0001.*

5. DISCUSSION AND CONCLUSIONS

Ongoing intensive research efforts are elucidating the characteristics and impacts of the novel SARS-CoV-2. Although the respiratory system is the most affected, SARS-CoV-2 viral infection can be systemic (Synowiec *et al.*, 2021). Due to the recent emergence of the pandemic, just a few studies in the field are available, and the potential implications in human reproduction are still unknown.

There have been some preliminary studies of the effects of SARS-CoV-2 on the female and male reproductive systems, but findings are controversial. While some studies indicate sperm samples from males in active and recovery phases of the infection have resulted as SARS-CoV-2-free (Song *et al.*, 2020; Guo *et al.*, 2021) others reported positive cases (Li *et al.*, 2020a; Gacci *et al.*, 2021). Similarly, a recent study related to the female reproductive system has shown how the oocytes from two women diagnosed with COVID-19 were resistant to the virus (Barragan *et al.*, 2020). However, several case reports or case series studying the vertical transmission of SARS-CoV-2 infection in pregnant women have described the potential risk of infection from mother to newborn (Caparros-Gonzalez *et al.*, 2020; Goh *et al.*, 2020). In fact, Fenizia *et al.* reported the birth of two children with COVID-19, one of which also tested positive for SARS-CoV-2 in their umbilical cord blood. A uterine transmission could explain this isolated case, but still, these results are inconclusive, and further studies are required (Fenizia *et al.*, 2020).

The need for invasive sample collection creates challenges in studying the effects of SARS-CoV-2 on female reproductive tissues, and to our knowledge, no study has assessed endometrial tissue from patients with COVID-19. In the present study, 14 endometrial biopsies from patients hospitalized with COVID-19 and confirmed as SARS-CoV-2–positive were analyzed. None of these endometrial tissues exhibited detectable viral RNA, independent of the time from COVID-19

diagnosis, the menstrual phase of the endometrial biopsy, or the age of the woman.

Despite the described expression of *ACE2*, as well as *TMPRSS2*, in the human endometrium, a recent *in silico* study has described that this tissue appears to be safe from SARS-CoV-2 infection in terms of key molecular effectors (Henarejos-Castillo *et al.*, 2020), at least through this mechanism of invasion. This group based their hypothesis on the low expression of the *ACE2* gene across the menstrual cycle, regardless of corroborating the higher expression of this gene in the early secretory phase (Vaz-Silva *et al.*, 2009). Henarejos-Castillo and colleagues also propose that the *ACE2* endometrial expression increases with age, and as such, so could the risk of SARS-CoV-2 entry into the endometrium. In agreement with this *in silico* work, the low mRNA expression of *ACE2* in the endometrium reported herein are reinforced with the significantly higher expression of *ACE2* detected in COVID-19–positive nasopharyngeal epithelial samples than in the endometrial biopsies. On the other hand, we cannot make any conclusions regarding the differential expression of *ACE2* between menstrual phases and/or the age of the patients, likely due to the small population size and the unequal representation of each menstrual cycle phase.

Considering that this study pioneers the analysis of endometrial biopsies from women with COVID-19, we are aware of several existing limitations, such as the difficulty to collect and/or process samples and the restricted population. Finally, due to current health measures and the infectious condition of the patients, only biopsies from symptomatic hospitalized patients were collected, resulting in asymptomatic women being excluded from the cohort. For these reasons, this work should be considered a preliminary step in the study of the impact of SARS-CoV-2 over the human endometrium.

Different big-data theorist analyses have studied the expression of *ACE2* gene along with other organs (Li *et al.*, 2020b). Notably, these analyses reveal the innately lower *ACE2* expression in the uterus compared with the kidneys (Batlle *et al.*, 2020) or heart (Shchendrygina *et al.*, 2021) also reported to be affected by SARS-CoV-2 infection. These recent studies strengthen our hypothesis that the low expression of endometrial *ACE2* would impede the virus from entering the tissue.

Based on the results of this study, the endometrium is likely safe from SARS-CoV-2 and, one possible explanation could be the low expression of the ACE2 receptor. However, the absence of the virus does not preclude the possibility of COVID-19 altering the endometrium due to its systemic affectation. Together with further studies in the field, these preliminary results could aid clinicians in managing female fertility, especially for patients wishing to conceive during this pandemic. Nevertheless, since *ACE2* expression has been reported to be higher in the placenta than in the uterus (Hikmet *et al.*, 2020), more profound studies are needed to correlate the present results with a potential vertical transmission to newborns.

Our findings indicate that SARS-CoV-2 is not present in the endometrium of women with COVID-19, at least in the short term. This conclusion is reinforced by the low expression of *ACE2*, encoding a viral entry receptor. Nonetheless, additional studies are needed to investigate the long-term impact of the virus on the endometrium and, consequently, on female reproductive function.

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