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"Identification of Specific Somatic Stem Cell Markers in the Human Endometrium and Mechanisms of the Bone Marrow for Endometrial Regeneration"

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CERTIFICA:

Que el trabajo de investigación titulado: **"Identification of Specific Somatic Stem Cell Markers in the Human Endometrium and Mechanisms of the Bone Marrow for Endometrial Regeneration"** ha sido realizado íntegramente por Dña. Nuria López Pérez 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 2 de mayo de 2018.

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ABBREVIATIONS

A

ABCG2: ATP-binding cassette sub-family G member 2
ACTB: actin beta
AGPT1: angiopoietin 1
Akt: protein kinase B
APC: allophycocyanin
AS: Asherman's syndrome

B

B2M: beta-2-microglobulin
BCRP1: breast cancer resistance protein 1
BDNF1: brain-derived neurotrophic factor
BMDSCs: bone marrow-derived stem cells
BMPX: bone morphogenetic protein X
BSA: bovine serum albumin

C

CCL2: C-C motif chemokine ligand 2 (also MCP-1)
cDNA: complementary DNA
CK18: cytokeratin 18
c-KIT: tyrosine-protein kinase Kit
ColIA: type IA collagenase
CSC: cancer stem cell
CSF-1: colony stimulating factor 1
CSPG5: chondroitin sulfate proteoglycan 5
CT: threshold cycle
CXCL8: C-X-C motif chemokine ligand 8

D

DAB: 3,3'-diaminobenzidine
DAPI: 4',6-diamidino-2-phenylIndole
DMEM: Dulbecco's modified eagle's medium
DNA: deoxyribonucleic acid
DNase: deoxyribonuclease
dNTPs: deoxynucleoside triphosphate

E

E_2: estradiol
EA: endometrial atrophy
ECM: extracellular matrix
EDTA: ethylenediaminetetraacetic acid
EGF: epidermal growth factor
EPCAM: epithelial cell adhesion molecule
ESC: embryonic stem cell

F

FACS: fluorescence-activated cell sorting
FBS: fetal bovine serum
FC: clow cytometry
FCS: fetal calf serum
FFPE: formalin-fixed paraffin-embedded
FGFX: fibroblast growth factor X
FSH: follicle stimulating hormone

G

g: gravity (centrifugation measurement)
GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GDNF: glial cell-derived neurotrophic factor
G-CSF: granulocyte-colony stimulating factor
GnRH: gonadotropin-releasing hormone
GRP: gastrin-releasing peptide

H

HBSS: Hank's buffered salt solution
HCG: human chorionic gonadotropin
H&E: hematoxylin-eosin.
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF: hepatocyte growth factor
HK: housekeeping gene
HPRT1: hypoxanthine phosphoribosyltransferase 1
HRP: horseradish peroxidase

I

ICAM1: intercellular adhesion molecule 1
ICC: immunocitochemistry
ICE6: epithelial SP-derived cell line
ICE7: stromal SP-derived cell line
IF: immunofluorescence
IGF-1: insulin-like growth factor 1
IHC: immunohistochemistry
IL-X: interleukin X (X=number)
iPSC: induced pluripotent stem cell
IVF: in vitro fertilization

K

KEGG: Kyoto encyclopedia of genes and genomes
KITLG: KIT ligand (also SCF)

L

LEFTY1: left-right determination factor 1
LEFTY2: left-right determination factor 2
LGR5: leucine-rich repeat–containing heterotrimeric guanine nucleotide–binding protein–coupled receptor 5
LH: luteinizing hormone
LIF: LIF interleukin 6 family cytokine

M

MAPK: mitogen-activated protein kinase
MK: Midkine (or MDK)
mL: milliliter
mM: millimolar
MS1: Musashi-1
MSC: mesenchymal stem cell

N

$NaHCO_3$: sodium bicarbonate
NGS: normal goat serum
NK: natural killer
NOD-SCID: non-obese diabetic/severe combined immunodeficiency
NSP: non-Side Population

O

o/n: overnight
O_4: oxygen
Oct-4: octamer-binding transcription factor 4
OSGIN1: oxidative stress induced growth inhibitor
OVX: ovariectomy

P

P/S: penicillin/streptomycin

P_4: progesterone

PBS: phosphate buffered saline

PDGF: platelet-derived growth factor

PDGF-Rβ: platelet-derived growth factor-receptor beta

PE: phycoerythrin

PFA: paraformaldehyde

PGF: placental growth factor

PGC: primordial germ cell

PI: propidium iodide

PI3k: phosphoinositide-3-kinase

POST-TT: 3 months post-treatment

PR: progesterone receptor

PRE-TT: pre-treatment

PTN: pleiotrophin

R

rASRM: revised American Society for Reproductive Medicine (score)

RNA: ribonucleic acid

RNase: ribonuclease

RPS6K: ribosomal protein S6 kinase B1 (also p70)

RPLP0: ribosomal protein lateral stalk subunit P0

RR: reconstitution rate

RT: room temperature

RT-qPCR: real time quantitative polymerase chain reaction

S

SDS: sodium dodecyl sulfate

Sox-2: sex determining region Y-box 2 (SRY)

SP: Side Population

SPF: specified pathogen-free

SSC: somatic stem cell

SSEA1: stage-specific embryonic antigen 1

SUSD2: Sushi domain containing 2

T

TA cells**:** transit amplifying cells

TF: total endometrial fraction (disaggregated endometrial cells)

TGFα: transforming growth factor alpha

TGFβ: transforming growth factor beta

THBS1: thrombospondin 1

TIMP-X: TIMP metallopeptidase inhibitor X

TNFα: tumor necrosis factor alpha

Tween 20: polysorbate 20

U

UKM: Universitätsklinikum Münster

V

VEGF: vascular endothelial growth factor

VIM: vimentin

Latin letters

α-SMA: alpha-smooth muscle actin

μL: microliter (= 10^{-3} mL)

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RESUMEN

INTRODUCCIÓN

El endometrio humano

El endometrio es la pared mucosa que recubre el interior del útero. Es probablemente uno de los tejidos más complejos del cuerpo humano ya que se encuentra altamente vascularizado y en constante renovación. Estos cambios se producen como respuesta a oscilaciones en los niveles hormonales de estrógenos y progesterona durante el ciclo menstrual. El grosor del endometrio aumenta debido a estos cambios hormonales, variando desde los 0,5 mm en la fase proliferativa hasta los 7 mm en la secretora. El ciclo menstrual es un proceso cíclico de degeneración y regeneración endometrial que sufren mensualmente las mujeres en edad reproductiva, unas 400-500 veces en su vida, y la duración promedio es de 28 días. El hipotálamo secreta la hormona liberadora de gonadotropina (GnRH), que estimula la pituitaria anterior para que libere la hormona folículoestimulante (FSH) y la luteinizante (LH). Estas últimas estimulan la producción de hormonas esteroideas, estrógenos y progesterona por parte del ovario. Todas estas hormonas interactúan y afectan a la foliculogénesis, la ovulación, la fase lútea y el crecimiento endometrial, disminuyendo sus niveles progresivamente conforme avanza el ciclo menstrual.

Las dos **fases del ciclo menstrual** se denominan proliferativa y secretora, ambas separadas por el momento de la ovulación y acabando con la fase menstrual o menstruación.

- La fase proliferativa es una fase de duración variable que coincide con la maduración folicular en el ovario. Comienza tras la menstruación debido a los bajos niveles de estrógenos, y comprende el periodo hasta la ovulación. Cuando comienza esta fase, el endometrio es más fino al principio y se regenera posteriormente en la fase proliferativa tardía debido al aumento en los niveles de estrógenos.
- La fase secretora se produce tras la ovulación, coincide con la actividad funcional del cuerpo lúteo en el ovario, y suele tener una duración fija en cada mujer, alrededor de 14 días. Los niveles de progesterona aumentan tras la ovulación, decidualizándose el endometrio en mitad de esta fase, preparándose de esta forma para la implantación ante una posible fecundación. Al final de la fase secretora, si no se ha producido fecundación, los niveles de estrógenos y progesterona

disminuyen, se produce vasoconstricción de las arterias espirales endometriales, y el sangrado menstrual.

- Tras la caída de los niveles de estrógenos y progesterona al degenerarse el cuerpo lúteo, se produce de nuevo la menstruación y la consiguiente descamación y regeneración tisular (duración de 3 a 7 días). En esta fase menstrual el cuerpo lúteo permanece activo secretando hormonas durante 10 días aproximadamente si no se ha producido fecundación. Si se hubiera producido, los niveles de progesterona se mantendrían altos y no se produciría la menstruación.

En cuanto a la **composición celular**, el endometrio está compuesto por células estromales, epiteliales, una densa red vascular y células inmunes residentes.

- El estroma es un tejido conectivo compuesto por matriz extracelular y células, en su mayoría fibroblastos. Estos últimos sufren cambios morfológicos durante la remodelación de la matriz extracelular en el proceso de decidualización en la fase secretora del ciclo menstrual. Este proceso, producido a los 6 o 7 días tras la subida de progesterona, consta de cambios morfológicos, bioquímicos y genéticos producidos por los cambios hormonales de estrógenos y progesterona.
- El epitelio se compone de células epiteliales que se encuentran formando una monocapa de células cuboides polarizadas, en los compartimentos luminal y glandular. Se encuentran reguladas por las hormonas esteroideas ováricas de forma directa o indirecta a través del estroma, y su principal función es permitir el diálogo entre el embrión y el endometrio, además de servir como barrera contra patógenos.
- La vasculatura uterina comienza en el miometrio. La angiogénesis en este tejido tiene lugar durante el desarrollo folicular hasta la formación del cuerpo lúteo, además de durante el desarrollo endometrial, la implantación y la formación de la placenta en la gestación.
- Las células inmunes también forman parte del tejido endometrial, siendo importantes para la protección del tracto genital frente a enfermedades, así como para evitar el rechazo inmunológico del embrión en el proceso de implantación.

Todos los tipos celulares anteriormente descritos se organizan en dos capas que conforman la **morfología del endometrio**: la funcional (dos terceras partes) y la basal (tercio más interno del tejido, próximo al miometrio).

- La capa funcional es la más gruesa y más externa, la que responde a los cambios hormonales y la encargada de albergar al embrión. Por lo tanto, es la que sufre los

procesos de proliferación y degeneración a lo largo del ciclo menstrual. También es la capa que se descama casi completamente durante cada menstruación.

- La capa basal es la más fina, y permanece constante a lo largo de todo el ciclo menstrual, con cambios leves, cuya función es proporcionar la regeneración de la capa funcional cuando se descama en la menstruación.

Patologías endometriales

Una desregulación en la proliferación de las células endometriales puede hacer que se creen diferentes patologías, como son la endometriosis, el síndrome de Asherman (AS) o la atrofia endometrial (EA).

La endometriosis es una enfermedad dependiente de estrógenos que consiste en el crecimiento de tejido endometrial fuera de la cavidad uterina, que es su nicho fisiológico, y se asocia con dolor e infertilidad. Hay diferentes teorías para explicar su origen, pero ninguna de ellas se relaciona con los diferentes grados de la enfermedad ni explica todas las posibles localizaciones que puede presentar. Los diferentes grados se organizan de acuerdo a: severidad, localización, profundidad y tamaño del tejido endometrial formado. La teoría más aceptada para el desarrollo de la endometriosis es la menstruación retrógrada, depositándose de esta forma fragmentos y células endometriales fuera de la cavidad uterina, sobre todo en el peritoneo y en los órganos abdominales. Estas células proliferarían como consecuencia de las oscilaciones hormonales durante el ciclo menstrual, causando inflamación crónica y la formación de adhesiones. Sin embargo, también es posible la formación de tejido endometriósico fuera de la cavidad abdominal, que podría encontrar explicación en la teoría de las células madre. Células derivadas de la médula ósea podrían diferenciarse a tejido endometrial, dando lugar a endometrio ectópico en cualquier lugar. Otras teorías son: la metaplasia celómica, anormalidades del sistema inmune, causas genéticas, ambientales, estilo de vida o debido a la presencia de células madre germinales remanentes del crecimiento embrionario durante la formación de los conductos de Müller. Es posible por tanto que la patogénesis de esta enfermedad se deba a una combinación de factores genéticos, hormonales, inmunológicos y ambientales. En cuanto a tratamiento, la terapia médica es ineficaz frente a esta patología, mientras que la eliminación de las lesiones endometriósicas por laparoscopia mejoran las probabilidades de embarazo, pero de forma moderada.

El síndrome de Asherman (AS) es una patología endometrial caracterizada por la presencia de adhesiones en el útero o en el cérvix, que pueden desembocar en una falta parcial o total de endometrio funcional. Las posibles causas de este síndrome

pueden ser desde un legrado o un aborto voluntario, hasta lesiones uterinas producidas por procedimientos quirúrgicos menos agresivos, o infecciones. Hay distintos grados de la enfermedad dependiendo de la extensión uterina afectada por las adhesiones, pudiendo ser leve, moderada o severa. Esta patología principalmente produce problemas reproductivos como desajustes menstruales, fallo de implantación, restricción del crecimiento intrauterino, placentación anormal o aborto recurrente. No se conoce ningún tratamiento que haya sido exitoso en preservar el útero libre de adhesiones y proporcione una recuperación de la fertilidad. No obstante, sí se han realizado algunos abordajes experimentales exitosos mediante trasplante de células madre derivadas de médula ósea (BMDSCs), concretamente progenitores endoteliales $CD133^{+}$, en pacientes con AS y EA.

La atrofia endometrial (EA) es una patología infrecuente en la población también conocida como endometrio atrófico, fino o refractario, y se caracteriza por una falta de crecimiento endometrial. Conlleva problemas reproductivos porque un correcto grosor endometrial es esencial para la implantación y el éxito reproductivo. La falta de crecimiento endometrial puede ser producida por varios factores de riesgo, como son las causas inflamatorias, iatrogénicas o idiopáticas. No se conoce ningún tratamiento efectivo, habiéndose desarrollado algunos estudios novedosos con células madre como el explicado en el párrafo anterior.

Células madre

Las células madre son células no especializadas y relativamente indiferenciadas que puede continuar dividiéndose indefinidamente, dando lugar a células hijas que pueden permanecer indiferenciadas como célula madre (auto-renovación), o bien pueden comprometerse y diferenciarse terminalmente, dando lugar a tipos celulares específicos. De esta forma, las células madre se caracterizan por la multipotencialidad, la auto-renovación, y el mantenimiento del balance entre ambos procesos. De mayor a menor grado de indiferenciación, las células madre pueden ser: totipotentes, pluripotentes, multipotentes, oligopotentes o unipotentes. Del mismo modo, dependiendo de la fuente de obtención de dichas células madre, éstas pueden ser: células madre embrionarias, germinales primordiales, de origen fetal, de líquido amniótico, de cordón umbilical, somáticas (o adultas), pluripotentes inducidas o cancerígenas. Las células madre suponen una herramienta muy útil para el estudio de enfermedades, de mecanismos de regeneración tisular o de desarrollo y testado de fármacos.

Las células madre se encuentran en compartimentos anatómicos definidos denominados nichos, los cuales permiten el mantenimiento de las propiedades de las

células madre y la renovación del tejido. El nicho está formado por estas células, matriz extracelular y las células adyacentes del nicho, todas ellas interaccionando entre sí. Por lo tanto, el nicho es un sistema dinámico, en constante cambio debido a las señales que se intercambian entre las células madre y las células adyacentes, las cuales determinan la tasa y el patrón de división de las primeras. La división puede ser simétrica, con el fin de perpetuarse como célula madre, o asimétrica para dar lugar a células diferenciadas. En el nicho existe una jerarquía celular, donde coexisten células madre, células intermedias (más diferenciadas) de amplificación transitoria y rápida división (o células TA), y células diferenciadas. Se cree que hay nichos de células madre en los órganos adultos que sufren un elevado recambio, habiendo identificado este nicho en algunos tejidos y órganos (como el folículo piloso o la médula ósea), y siendo las regiones perivasculares potenciales nichos de células madre.

La comunicación dentro del nicho se mantiene mediante factores secretados, así como mediante las uniones célula-célula. Hasta ahora se ha descrito un gran número de moléculas solubles y factores paracrinos que intercambian las células del nicho y las células madre somáticas para regular la función de estas últimas. Estudios recientes basados en técnicas novedosas en el campo de la medicina regenerativa han demostrado no solo la diferenciación directa de células madre para la regeneración tisular, sino también el efecto terapéutico de factores secretados por las mismas, que pueden actuar de forma autocrina y paracrina. Estos factores pueden estimular el proceso regenerativo mediante diferentes vías: induciendo neovascularización, mediante soporte trófico para permitir la supervivencia celular y la reparación de tejidos dañados o enfermos, a través de propiedades antiinflamatorias e inmunomoduladoras mediante la creación de un microambiente específico, o mediante el reclutamiento y activación de células madre somáticas endógenas. El papel que pueden desempeñar los factores liberados por las BMDSCs en la regeneración endometrial cuando hay alguna patología o daño tisular también ha sido descrito, más concretamente en AS y EA.

Células madre en el endometrio humano

La presencia de células madre somáticas es clave para entender la capacidad regenerativa que conservan muchos tejidos adultos. El endometrio humano es un tejido único en el cual la capa funcional sufre durante cada ciclo menstrual procesos de crecimiento, descamación y regeneración a lo largo de toda la vida reproductiva de la mujer, mientras que la basal permanece intacta. Este proceso cíclico parece indicar que existe una población de células madre en este tejido, sobre todo en la capa basal,

ya que es la que permanece relativamente constante, albergando por tanto las células madre endometriales que regenerarían la capa funcional.

El alto potencial proliferativo de las células endometriales, la diferenciación a otros tipos celulares del linaje mesodérmico (como miocitos o adipocitos), la expresión de marcadores de pluripotencia o la capacidad clonogénica son algunas características que se han usado para justificar la pluripotencialidad celular *in vitro*. Por el contrario, la formación de tejido en modelos animales a partir de células madre es la prueba más importante *in vivo* para demostrar la capacidad de regeneración tisular. La regeneración endometrial en modelos murinos se ha conseguido mediante la inyección de células endometriales totales, con células pertenecientes a la *Side Population* (SP), con éstas suplementadas con fracción endometrial para aumentar la eficiencia de reconstrucción, y con células aisladas para el marcador W5C5.

Pese a la gran cantidad de evidencias que apoyan la existencia de una población de células madre en el endometrio humano, no se conoce la localización de las mismas dentro del tejido. Para poder identificarlas y aislarlas es necesario conocer marcadores específicos, y hasta ahora diversas moléculas se han postulado como específicas de células madre endometriales: células positivas para los marcadores CD146/PDGF-Rβ, W5C5 (con capacidad probada de reconstrucción endometrial en modelos animales), LGR5, EPCAM, SSEA1, Musashi-1 (MS1), y Sox-2. Además, también se cree que la SP alberga una población de células madre en el endometrio, ya que el fenotipo SP hace referencia a una población heterogénea que parece contener células madre en multitud de tejidos humanos adultos. El fundamento de esta técnica se basa en la capacidad que tienen las células pertenecientes a la población SP de expulsar el colorante Hoechst 33342 (de unión al ADN) por canales de membrana de tipo ABCG2, denominados BCRP1. Poseer estos canales de membrana es una característica de célula madre.

En el tejido endometrial estas células SP representan un porcentaje bajo que oscila entre el 1,68 y el 2,7% de la fracción epitelial, y entre el 0,4 y el 3,1% de la estromal. Se ha visto que están en mayor proporción en la fase proliferativa temprana que en el resto del ciclo, se encuentran tanto en la capa basal como en la funcional, y se localizan principalmente en regiones perivasculares, lugar que se ha descrito anteriormente como potencial albergue de células madre en otros tejidos. Como se ha mencionado anteriormente, esta población ha demostrado tener capacidad de creación de tejido endometrial *in vivo*, aumentándose la eficiencia de reconstrucción cuando se inyectan células endometriales totales como soporte.

La falta de marcadores específicos en algunos tejidos que permitan la identificación de células madre, como es el caso del endometrio, hace que sea difícil saber si hay un solo tipo de célula madre en el nicho tisular (células madre endógenas específicas del tejido, residentes en el nicho), o si existe también un aporte exógeno con otro tipo celular procedente de un tejido diferente. Nos referimos a las células madre procedentes de la médula ósea, BMDSCs, las cuales se ha visto que son capaces no solo de regenerar las células sanguíneas, sino también de migrar a otros tejidos y participar en su homeostasis.

Las células madre endógenas, residentes en el endometrio, podrían ser remanentes de origen fetal, células epiteliales y mesenquimales fetales que habrían permanecido indiferenciadas en el endometrio humano. Estas células se encontrarían en el nicho y participarían en la regeneración endometrial durante cada ciclo menstrual, o darían lugar a enfermedades endometriales como la endometriosis cuando reciben ciertos estímulos hormonales.

La fuente exógena vendría dada por la contribución de la médula ósea, viajando las células BMDSCs a la sangre y llegando a tejidos lejanos espacialmente, manteniéndose indiferenciadas o diferenciándose al tipo celular del tejido de destino. Estas BMDSCs están compuestas por células de diferente origen: células madre hematopoyéticas, estromales, células de la SP y células somáticas progenitoras (multipotentes). Esta contribución se ha mostrado en varios tejidos tanto en modelos animales como en humano. Más concretamente, se ha estudiado la contribución endometrial de estas células tras un trasplante de médula ósea, tanto en humanos como en ratón. Del mismo modo, hay diversos trabajos que han demostrado una contribución de las BMDSCs que migran hasta el endometrio cuando hay una situación de daño tisular en humano, específicamente en EA y AS. Tras el trasplante intrauterino de BMDSCs, sobre todo progenitores endoteliales $CD133^{+}$, se produjo una neovascularización en el endometrio, un aumento del grosor endometrial y una mejora del resultado reproductivo en estas mujeres con las patologías endometriales mencionadas. En paralelo se hizo la misma aproximación, pero en un modelo animal, mediante terapia con BMDSCs derivadas de las pacientes anteriores, las cuales fueron marcadas previamente a la inyección. Se obtuvo que las células trasplantadas permanecían en bajo número en el endometrio y se localizaban alrededor de los vasos sanguíneos, donde se encontrarían probablemente induciendo neovascularización en este tejido. De esta forma, a pesar del bajo número de células trasplantadas remanentes en el endometrio, éste aumentó su proliferación. Consecuentemente, las células BMDSCs inyectadas probablemente se encontrarían involucradas en la

proliferación de células adyacentes mediante mecanismos paracrinos, promoviendo la reparación tisular.

Finalmente, se concluye que coexisten dos poblaciones de células madre que contribuirían a la regeneración endometrial, de manera que la población de células madre que podemos encontrar en el endometrio esté formada por un conjunto de tipos celulares, no solo por células madre endometriales residentes en el nicho endometrial. Habría de esta forma dos fuentes: una endógena, con células madre que residen de forma permanente en el nicho endometrial, y una exógena, con células madre derivadas de la médula ósea que migrarían al endometrio cuando se produce un daño.

OBJETIVOS

El objetivo principal de esta tesis es el estudio de las fuentes endógena y exógena de células madre somáticas endometriales en relación con el potencial de regeneración y reparación sobre el endometrio humano.

Objetivos específicos

- Identificar marcadores específicos de células madre somáticas endometriales y aislar las células de acuerdo a los marcadores seleccionados W5C5 e ICAM1, así como la *Side Population* endometrial.
- Evaluar el potencial de reconstrucción endometrial de los marcadores descritos anteriormente mediante un ensayo de xenotrasplante en ratones inmunodeprimidos.
- Estudiar las propiedades de células madre de las distintas poblaciones celulares inyectadas en el modelo animal mediante la presencia del marcador de células madre Musashi-1 en el tejido endometrial formado.
- Correlacionar la presencia de células positivas para W5C5, ICAM1 y *Side Population* con el grado de endometriosis en muestras de endometrio eutópico de pacientes con esta patología.
- Identificar factores solubles secretados tras la terapia génica con células madre autólogas derivadas de médula ósea, en pacientes con síndrome de Asherman y/o atrofia endometrial.

METODOLOGÍA

Identificación y aislamiento de células madre somáticas en el endometrio humano mediante marcadores específicos

Este estudio fue aprobado por el comité ético del Instituto Universitario-IVI (Universidad de Valencia) (1203-C-098-IC-F). Los procedimientos realizados en animales también fueron aprobados por los comités éticos de la Universidad de Valencia (Comité Ético Animal 2015/VSC/PEA/00073). Todas las donantes de biopsia endometrial, tanto en España como en Alemania, firmaron consentimiento previamente a la intervención.

Tras la obtención de las biopsias endometriales se separaron los tipos celulares de interés, estroma y epitelio, en condiciones de esterilidad mediante un procedimiento de digestión mecánico y enzimático.

Análisis y aislamiento de células endometriales para marcadores específicos de células madre mediante citometría de flujo – Modelo animal

En un primer momento para la selección de posibles marcadores específicos de células madre, se realizó inmunocitoquímica y citometría de flujo para fracciones celulares estromales y epiteliales obtenidas a partir de biopsias endometriales humanas. Se analizó el porcentaje de diversos marcadores en endometrio humano: EPCAM, ICAM1, SSEA1, W5C5, ABCG2, TNFα y THBS1, por ser descritos en la bibliografía algunos de ellos, y otros por ser los marcadores de membrana más expresados en la SP. Debido a su porcentaje en endometrio humano (consistente en ambas fracciones celulares y compatible con lo que cabría esperar de una población de células madre en un tejido), se seleccionaron W5C5 e ICAM1. Se aislaron células de acuerdo a estos marcadores por citometría de flujo activada por fluorescencia (FACS) que, además de detectar y cuantificar fluorescencia, separa las poblaciones celulares de interés. Se obtuvieron así células positivas y negativas para los marcadores de interés, así como células endometriales totales sin marcar.

Para la realización del modelo animal, además del aislamiento de células endometriales totales (TF), y positivas y negativas para W5C5 e ICAM1, se cultivaron líneas celulares derivadas de SP, denominadas ICE6 (línea derivada de SP epitelial) e ICE7 (línea derivada de SP estromal). Ambas líneas fueron derivadas por el grupo de la Dra. Irene Cervelló en 2011, y se cultivaron durante 9-11 pases en hipoxia hasta llegar a confluencia, momento en el que se recogieron las células y se prepararon para su inyección a partes iguales. En todos los casos se prepararon suspensiones de 500.000 células, y fueron inyectadas en los modelos animales en un volumen de

inyección de 30 μL. Para el modelo de xenotrasplante se usaron 28 ratonas NOD-SCID ovariectomizadas e inmunodeprimidas, a las cuales se les inyectaron las suspensiones celulares bajo la cápsula suprarrenal de los riñones derechos. Los controles negativos fueron los riñones izquierdos no inyectados. Se usaron 3 ratonas por condición, siendo los diferentes grupos experimentales: fracción total endometrial (TF), células $W5C5^+$, $W5C5^-$, $W5C5^+$+TF, $ICAM1^+$, $ICAM1^-$, $ICAM1^+$+TF y ICE6/7.

Análisis del tejido endometrial formado en los modelos murinos

Las ratonas xenotrasplantadas se sacrificaron a los 60 días tras la inyección (a excepción de las inyectadas con células marcadas con rodamina, las cuales fueron sacrificadas a distintos tiempos como control de la técnica de inyección) y se extrajeron los riñones para valorar la formación de tejido endometrial. Todos los órganos extraídos se fijaron en paraformaldehído y se incluyeron en bloques de parafina, siendo posteriormente cortados en sucesivas secciones de 4 μm de grosor para su estudio mediante tinción con hematoxilina-eosina (H&E) para ver el grosor de la cápsula, o mediante inmunohistoquímica o inmunofluorescencia para estudiar la expresión de diferentes marcadores de endometrio o de células madre.

Con el fin de poder detectar marcadores endometriales en el tejido formado bajo la cápsula suprarrenal, se estudió la expresión de vimentina (VIM), receptor de progesterona (PR) y citoqueratina 18 (CK18) mediante inmunofluorescencia. Se usó la eficiencia de reconstitución o RR para expresar estos resultados cualitativamente, siendo un porcentaje que hace referencia al número de riñones que expresan los tres marcadores endometriales (VIM, CK18 y PR) en relación al número total de riñones inyectados por condición.

Además de marcadores endometriales, se estudió la presencia de un marcador de células madre endometriales previamente descrito por el grupo del Dr. Götte, Musahi-1 o MS1, en el tejido formado mediante inmunohistoquímica. Para la interpretación de los resultados, se contaron las células positivas para el marcador de células madre MS1, y el resultado se representó en función del área (100.000 μm^2). Se siguió esta aproximación para unificar resultados y poder comparar las distintas condiciones, siendo una manera objetiva teniendo en cuenta la mayor o menor generación de tejido endometrial. Esta parte se realizó en la Universidad de Münster (UKM), en el laboratorio del Dr. Martin Götte, con una beca de la Generalitat Valenciana para estancias predoctorales fuera de la Comunidad Valenciana.

También se estudió la presencia de células proliferativas en el tejido formado mediante inmunohistoquímica para el marcador de proliferación Ki67.

Estudio de células madre y factores paracrinos en endometrios patológicos: endometriosis, síndrome de Asherman y atrofia endometrial

Estudio de los marcadores W5C5, ICAM1 y *Side Population* en endometriosis

Se usaron biopsias endometriales procedentes de mujeres sanas y con endometriosis, así como de fase proliferativa y secretora, recogidas en el Hospital Universitario de Münster. Tras el aislamiento de las fracciones epiteliales y estromales, las células se marcaron con anticuerpos para la detección y cuantificación de células positivas para los marcadores W5C5 e ICAM1. Además, se marcó otra fracción celular con Hoechst 33342 para la cuantificación de la población SP en esas pacientes.

Identificación de factores paracrinos secretados por células madre derivadas de médula ósea involucrados en regeneración endometrial

Las muestras de partida fueron muestras incluidas en parafina del estudio del Dr. Xavier Santamaría en 2016, publicado en *Human Reproduction*. Partimos de muestras antes y 3 meses después del tratamiento con BMDSCs $CD133^+$ de 4 mujeres con AS y/o EA, las cuales mejoraron la neovascularización, el grosor endometrial, la histología del tejido y el resultado reproductivo tras el tratamiento.

Se obtuvieron secciones de estos tejidos incluidos en parafina, se extrajo el ARN total, se convirtió en ADN y se pre-amplificó para las rutas de interés. Posteriormente se realizaron microchips de expresión génica (*arrays*) de PCR cuantitativa a tiempo real (RT-qPCR) para estudiar la expresión génica diferencial de ambas condiciones (antes y después de tratamiento), para las rutas de señalización EDG/PDGF, para factores de crecimiento humanos y para factores de crecimiento angiogénicos

Una vez que se obtuvieron los genes diferencialmente expresados con los 3 *arrays* de estudio, se seleccionaron aquellos más sobreexpresados (upregulados) y aquellos más regulados a la baja (downregulados) tras el tratamiento con BMDSCs $CD133^+$. Estos genes serían posibles candidatos a factores secretados por estas células que podrían estar implicados en la regeneración endometrial observada en estas pacientes. Finalmente se validó uno de los más upregulados mediante inmunofluorescencia (Midkina, MK), viendo la expresión antes y 3 meses después del tratamiento con estas células madre. También se realizó una anotación funcional para

ver qué rutas estaban más representadas con los genes incluidos en los *arrays,* mediante la herramienta KEGG.

RESULTADOS

Inmunocaracterización, identificación y aislamiento de candidatas a células madre en endometrio humano

La identificación y aislamiento de células madre somáticas endometriales se basó en la expresión de los marcadores EPCAM, ICAM1, SSEA1, W5C5, BCRP1, TNFα y THBS1. El porcentaje de las células $ICAM1^+$ fue 3,5 ± 2,14% en la fracción epitelial, y un 1,62 ± 0,97% en la estromal. De la misma forma, el porcentaje de células $W5C5^+$ fue 5,42 ± 2.97% y 2,95 ± 0.56% en las fracciones epitelial y estromal respectivamente. Los demás marcadores fueron excluidos por mostrar unos porcentajes no consistentes en ambas fracciones, o demasiado altos o bajos para ser compatibles con una población de células madre en un tejido. Una vez seleccionados estos dos marcadores, W5C5 e ICAM1, se realizaron los modelos animales, añadiendo además ratonas inyectadas con fracción total y con las líneas celulares ICE6/7 derivadas de SP.

En cuanto a la eficiencia de reconstitución, o RR, en las fracciones negativa y positiva pura para W5C5, se observó expresión de los 3 anticuerpos (RR) en el 66% y el 33% de los casos, respectivamente. La fracción positiva suplementada con TF por el contrario alcanzó un RR del 100%, observándose expresión de los 3 anticuerpos en todos los riñones de esta condición. Igualmente, tras el xenotrasplante con células $ICAM1^-$ y $ICAM1^+$ se formó menos tejido endometrial (66% y el 33% respectivamente) que con respecto al grupo de células positivas suplementadas con TF (100%). Por lo tanto, la reconstitución endometrial fue mayor en las fracciones negativas que en las positivas puras para ambos marcadores, siendo máxima con la adición de fracción endometrial total. Por otra parte, la inyección de líneas ICE dio como resultado una reconstitución del 100%, como en el caso de la TF o las fracciones positivas suplementadas.

La formación de tejido endometrial se estudió también mediante la tinción H&E, para ver si a primera vista se encontraban diferencias entre las condiciones experimentales en cuanto al engrosamiento de la cápsula suprarrenal. Los resultados no permitieron ver diferencias, ya que todos los modelos murinos engrosaban la cápsula tras la administración de las suspensiones celulares, a excepción del control negativo (riñones no inyectados).

En cuanto a la presencia de células positivas para MS1 en las cápsulas suprarrenales de los riñones inyectados, lo que pretendemos estudiar son las células madre endometriales contenidas en las fracciones celulares inyectadas, las cuales podrían permanecer indiferenciadas en el tejido endometrial formado en la cápsula suprarrenal a los 60 días tras la inyección (momento del sacrificio de las ratonas). De esta forma, podríamos comparar las características de células madre de cada condición experimental y cada marcador (W5C5, ICAM1 y SP). Debido a la alta heterogeneidad de las muestras, el error es muy alto, por lo que no se obtuvieron diferencias significativas, pero estos resultados nos sirven para ver una tendencia en función de la expresión de MS1 por condición. Se obtuvo que el grupo con más expresión de MS1 fue aquel inyectado con las líneas derivadas de SP. Tras este grupo, los riñones inyectados con las fracciones positivas para los marcadores suplementados con TF fueron los que más expresión mostraron (W5C5$^+$+TF y ICAM1$^+$+TF), seguidos por aquellos inyectados con fracción endometrial total. Los grupos con células negativas para los marcadores de estudio fueron los que mostraron menos señal que los grupos anteriores, pero más que los grupos solo con células positivas aisladas para los marcadores, habiendo más expresión con células W5C5$^+$ que con ICAM1$^+$.

Estudio de poblaciones candidatas a células madre en endometriosis

Con este estudio pretendemos caracterizar el porcentaje de células positivas para los marcadores W5C5 e ICAM1, así como la población SP, en endometrio eutópico procedente de pacientes sanas y pacientes con endometriosis, en fase proliferativa y secretora. Debido a la limitación en la obtención de muestras, así como la alta variabilidad, las comparaciones que hicimos fueron las siguientes: entre las fases proliferativa y secretora de pacientes con endometriosis tipo I (rASRM I), y entre pacientes sanas y pacientes rASRM I en fase secretora. En el primer caso, las poblaciones celulares positivas para los marcadores de estudio W5C5 e ICAM1, así como la población SP, fue mayor en el caso de la fase proliferativa. En la segunda comparación en fase secretora, se obtuvo que el porcentaje de células ICAM1$^+$ y SP era mayor en el caso de rASRM I, y similar (incluso un poco menor) en el caso de células W5C5$^+$. Sin embargo, el error es alto y no podemos establecer ningún resultado claro al no haber diferencias significativas.

Identificación de factores paracrinos secretados por células madre derivadas de médula ósea involucrados en regeneración endometrial bajo condiciones patológicas: síndrome de Asherman y atrofia endometrial

Tras el análisis histológico de 4 pacientes con AS y/o EA, se observó una mejora histológica en todas ellas, con la presencia de tejido endometrial más organizado y más glándulas. Además, había un aumento significativo en cuanto al número de vasos sanguíneos maduros tras el tratamiento.

El análisis de expresión génica diferencial permitió identificar los 5 genes más upregulados: CSPG5, LEFTY1, MK, GRP y FGF1. Estos genes están relacionados con factores de crecimiento, diferenciación, proliferación y migración celular, así como con reparación tisular y angiogénesis. Por el contrario, los 5 más downregulados fueron: CXCL8, CCL2, LEFTY2, IL12A y OSGIN1, involucrados en procesos inflamatorios, inmunes, inmunomodulatorios y apoptosis.

Se validó MK por inmunofluorescencia debido a que es un factor de crecimiento que ha sido previamente relacionado con reparación tisular, migración, angiogénesis, supervivencia celular, además de haber sido postulado como una diana terapéutica para el tratamiento de diversas enfermedades. Tras su validación se observó que antes del tratamiento solo una paciente expresaba esta proteína (no demasiada cantidad de señal en el tejido endometrial), pero 3 meses tras el tratamiento con células $CD133^+$ todas las pacientes expresaban MK con una gran intensidad, en células estromales con un patrón periglandular.

Tras la anotación funcional de los genes, se vio que la ruta más representada era PI3K-Akt, relacionada con el mantenimiento de la multipotencia en células madre somáticas, estando incluidos en esta ruta un 35,3% de los genes analizados.

DISCUSIÓN

Tras el modelo de xenotrasplante con suspensiones celulares aisladas de acuerdo a los marcadores W5C5, ICAM1 y SP, se ha demostrado la formación de tejido endometrial bajo la cápsula suprarrenal de ratonas inmunodeprimidas y ovariectomizadas. Los resultados obtenidos parecen apoyar la necesidad de fracción endometrial como suplemento de las células positivas para los marcadores W5C5 e ICAM1, que actúen como células de soporte o nicho para aumentar la eficiencia de reconstrucción endometrial en modelos animales. Puede ser que las células positivas aisladas para ambos marcadores necesiten células de soporte para imitar su nicho fisiológico y mantenerse como células madre, diferenciándose en caso contrario y teniendo una menor capacidad de regeneración tisular. Esto se ha corroborado mediante el estudio de expresión de MS1 (marcador descrito de células madre endometriales) en el tejido endometrial formado, pudiendo retener la expresión de este marcador, y por lo tanto las

características de células madre, aquellas células positivas para los marcadores de estudio que estén suplementados con células endometriales totales. En cualquier caso, la población que tiene mayor capacidad de regeneración endometrial es la SP, una población heterogénea que parece albergar una población de células madre en multitud de tejidos, entre ellos el endometrio. El nicho endometrial se encontraría en regiones perivasculares, demostrado por la presencia de células humanas trasplantadas y células proliferativas.

En el estudio de correlación de endometriosis con las distintas poblaciones candidatas a ser células madre (W5C5, ICAM1 y SP) no se obtuvieron diferencias significativas debido a la heterogeneidad celular, así como a la alta variabilidad en el perfil de las pacientes de partida. Sin embargo, se pudo ver una tendencia entre el porcentaje de las poblaciones anteriores con los distintos grados de la enfermedad que parece indicar que, en aquellas pacientes con endometriosis en comparación con pacientes sanas, así como en fase proliferativa frente a secretora, hay una mayor población de células positivas para estos marcadores.

En cuanto a la fuente exógena de células madre endometriales, se obtuvo el perfil génico diferencial de aquellas mujeres antes y después del tratamiento con células $CD133^+$ derivadas de médula ósea, que permitirían identificar los factores secretados. Tras el análisis de expresión génica con los genes incluidos en las rutas de señalización EGF/PDGF, factores de crecimiento humanos y factores de crecimiento angiogénicos, se obtuvo que los genes más upregulados estaban relacionados con funciones de diferenciación, proliferación y migración celular, así como con reparación tisular y angiogénesis. Por el contrario, los downregulados estaban implicados en procesos inflamatorios, inmunes, inmunomodulatorios y apoptosis. Por lo tanto, el tratamiento con BMDSCs $CD133^+$ podría estar causando una inmunomodulación que disminuiría la respuesta inmune e inflamatoria, disminuyendo a su vez la probabilidad de rechazo de las células trasplantadas y favorecer la regeneración tisular al aumentar la neoangiogénesis, el crecimiento celular y la diferenciación. La validación de uno de los genes más upregulados, MK, mostró un gran aumento de expresión tras el tratamiento, así como un patrón periglandular. Esto podría indicar que la regeneración endometrial podría deberse a una regeneración de las glándulas epiteliales.

Además, tras la expresión diferencial de los genes contenidos en los *arrays*, la anotación funcional de los mismos mostró que la ruta más representada con los genes estudiados era la ruta de señalización PI3K-Akt, involucrada en el mantenimiento de pluripotencia

de células madre mesenquimales. Las BMDSCs podrían estar regulando esta vía de señalización, induciendo de esta forma la regeneración endometrial.

La terapia con células madre presenta un amplio abanico de posibilidades en el ámbito de la medicina regenerativa. La investigación en este campo permitiría conocer mejor la fisiología de un tejido y su funcionamiento, pudiendo de esta forma tratar diversas enfermedades, así como desarrollar órganos personalizados mediante la ingeniería de tejidos.

CONCLUSIONES

1. El modelo de xenotrasplante demostró que “poblaciones de células madre” puras de endometrio humano, como $W5C5^+$ y $ICAM1^+$, no tienen la habilidad de reconstruir endometrio de forma eficiente.
2. El suplemento con fracción endometrial total debe incluir células pertenecientes al nicho que aumentarían el potencial regenerativo de estas posibles poblaciones de células madre.
3. La *Side Population* es una población heterogénea que albergaría una población de células madre en el endometrio humano, apoyado por la alta capacidad de regeneración endometrial mostrada en el modelo animal.
4. La identificación de marcadores específicos de células madre somáticas endometriales, como fuente endógena, es esencial para el estudio de la biología endometrial, el aislamiento de células madre, y el entendimiento de las patologías endometriales.
5. Las células madre exógenas secretan múltiples factores que pueden ejercer un efecto terapéutico mediante acciones paracrinas, como hemos observado tras el tratamiento con células madre de médula ósea en pacientes con patologías endometriales.
6. Los factores secretados por las células madre podrían promover de forma exógena la regulación de la ruta de señalización PI3K-Akt, asociada con el mantenimiento de la multipotencia en células madre mesenquimales.
7. Los genes downregulados tras la terapia celular podrían promover un escenario inmunomodulatorio favoreciendo de esta forma la remodelación tisular. Por el contrario, los upregulados (incluyendo MK) están involucrados en crecimiento celular, angiogénesis, migración, diferenciación y reparación tisular.
8. Estos factores secretados por células trasplantadas pueden influenciar el microambiente del nicho o activar células madre somáticas endógenas para la regeneración del tejido.

I. INTRODUCTION

"Sólo hay una cosa que hace que un sueño sea imposible de alcanzar: el miedo al fracaso"

Paulo Coelho

I. INTRODUCTION

1. The human endometrium

1.1. The anatomy of the reproductive system

The female reproductive system consists of ovaries, fallopian tubes (also called oviducts), the uterus, the vagina, the external genitalia and mammary glands. These structures are involved in ovulation, fertilization, support of the embryo and fetus and the birth and care of an infant. Female primary sexual characteristics include the internal genitalia, which are located within the pelvis: vagina, uterus, cervix, fallopian tubes, and ovaries (Figure 1), as well as the external genitalia found outside the pelvis: perineum, mons pubis, clitoris, urethral (urinary) meatus, labia majora and minora, vestibule, greater vestibular (Bartholin's) glands, Skene's glands, and the periurethral area. Female secondary sexual characteristics include external features (except external genitalia) that differentiate the adult female from the adult male, such as enlarged breasts and the distribution of fat in the torso (Jones and Lopez, 2014). The main organs responsible for the reproductive process along the female reproductive system are the ovaries and the uterus.

The ovaries, or female gonads, are oval-shaped organs located in the abdominal cavity and are responsible for the production of female gametes, oocytes, and the secretion of several hormones including estrogens, progesterone and inhibin. They are dynamic organs where the type and level of hormones as well as the stages of oocyte development vary throughout the cycle. This production of gametes is called oogenesis and takes place in cavities called follicles, which harbor cells recovering the inner surface that protect and feed the developing oocytes. Similarly, the process of preparing a single oocyte for ovulation is called the folliculogenesis. Each follicle harbors only one oocyte, which needs approximately 28 days to mature. Ovaries are linked to the uterus through the fallopian tubes that transport the oocyte from the ovaries to the uterus, making the communication between both gametes (spermatozoa and oocyte) possible, which is needed for fecundation (this mainly takes place in this tract). Additionally, the uterus is the biggest organ in the female reproductive system: it is an inverted pear-shaped and muscular organ located in the intraperitoneal region, in the pelvis. It is the organ commissioned to harbor the embryo/blastocyst/fetus during the pregnancy period once the fecundation occurred. The endometrium is a mucosal layer covering the inner surface of the uterus, and is considered essential in the reproductive process, supporting implantation and harboring the embryo after

fecundation. It is probably one of the most complex tissues in the human body because of its high vascularization and it is in continuous change and renovation influenced by hormonal oscillations in estrogen and progesterone levels during the menstrual cycle in humans and higher primates (Speroff and Fritz, 2005; Simón *et al.*, 2009). The endometrium is one of the main tissues in the uterus that is most sensitive to ovarian steroid hormones. The endometrial lining thickens throughout the menstrual cycle from 0,5 mm in the proliferative phase to 7 mm (and even up to 10 mm) in the secretory phase (McLennan and Rydell, 1965).

The main structure following these two previously mentioned internal organs is the vagina. This organ is linked to the uterus through the cervix and connects the reproductive system with the outside. The cervix is responsible for the delivery when the pregnancy ends, being dilated at the end of the pregnancy period, producing contractions in the uterus and expelling the fetus through the birth canal, the vagina.

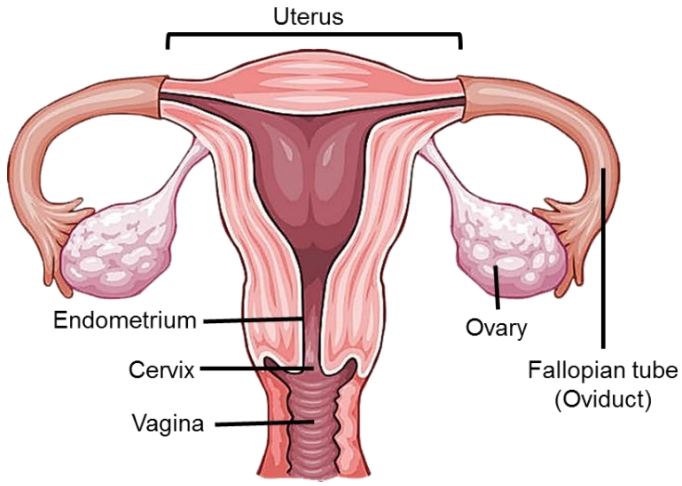

Figure 1. Organs belonging to the female reproductive system. *The reproductive organs consist out of the ovaries and uterus, both communicated to each other through the fallopian tubes and connected with the outside through the cervix and the vagina.*

1.2. The menstrual cycle

The menstrual cycle is a cyclical process of endometrial degeneration and regeneration regulated by hormonal changes. This cycle has an approximated monthly periodicity and is repeated on average 400 to 500 times during the women's reproductive life. The mean duration of the cycle is 28 days, but can last between 24 and 36 days in 90% of women, where the endometrial thickness will fluctuate between 0.5 mm and 7 mm (McLennan and Rydell, 1965) (Figure 2).

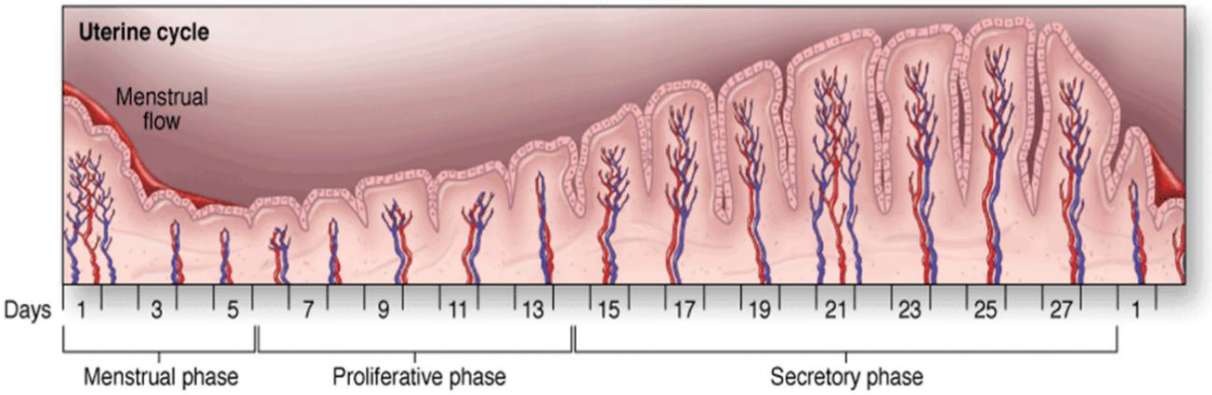

Figure 2. Phases of the endometrium along the menstrual cycle. *The endometrial thickness can vary from 0.5 mm in the early proliferative phase up to 7 mm in the secretory phase. The endometrial lining thickens in response to estrogen in the proliferative phase (from the first day after menstruation until day 14 more or less, when the ovulation occurs). After ovulation, in the secretory phase the endometrium decidualizes. The secretory phase lasts from day 14 until the menstrual phase (day 1 of the cycle). The window of implantation would be situated around days 19-21 (7-10 days after the luteinizing hormone surge) of the menstrual cycle. After the cycle is finished menstruation occurs and the cycle repeats. Image modified from http://www.accessmedicine.com.*

The cycles of growth and shedding as part of each reproductive period are a consequence of the hormonal action affecting this tissue. A coordinated response to estrogen and progesterone leads to these variations in the human endometrium. The hypothalamus secretes the gonadotropin-releasing hormone (GnRH), which stimulates the anterior pituitary to release both follicle stimulating hormone (FSH) and luteinizing hormone (LH) in turn, which ultimately leads to ovulation. These hormones stimulate the production of steroid hormones, estrogens and progesterone from the ovary, as well as other peptides released in an autocrine, paracrine and endocrine manner. All the mentioned hormones interact among each other and affect the folliculogenesis, the ovulation, the luteal phase and the endometrial growth, diminishing the hormonal levels according the menstrual cycle (Hawkins and Matzuk, 2008).

The menstrual cycle can be divided into three phases: the proliferative phase followed by the secretory phase (coinciding with the follicular and the luteal phase in the ovary respectively), separated by the moment of ovulation and ending with the menstrual phase (Figure 3):

- **Proliferative phase:** this phase has a variable length that concurs with the ovarian follicular maturation and is defined as the period following the menstrual phase, triggered by the low levels of estrogens, until the ovulation. When this phase starts, the functional layer of the endometrium is already shed, with less thickness at the beginning. As estrogen levels begin to rise, the endometrial lining thickens later in

the late proliferative phase due to glandular hyperplasia and an increase in the extracellular matrix (ECM) in stromal cells. Glandular epithelial cells increase their height and become pseudo-stratified when ovulation approaches. With the ovulation this phase ends and the secretory phase starts (Yen, Jaffe and Barbieri, 2001; Hawkins and Matzuk, 2008).

- **Secretory phase**: this phase coincides with the functional activity of the corpus luteum in the ovary, with a fixed duration of around 14 days. Progesterone levels rise after ovulation, and the endometrium decidualizes at mid-secretory phase, getting ready for the implantation process after a possible fecundation. In the late secretory phase, in absence of fecundation, spiral arteries vasoconstrict and menstrual bleeding occurs in response to the drop in both estrogen and progesterone levels (Hawkins and Matzuk, 2008).

- **Menstrual phase**: after the decline in hormonal levels caused by the degeneration of the corpus luteum, menstruation occurs once again (3-7 days length), as well as the subsequent involution of the endometrium and tissue regeneration. In this phase, corpus luteum remains secreting hormones actively for 10 days approximately, if fecundation did not occur. Otherwise, if fecundation occurred, progesterone levels remain high and menstruation would not come out (Hawkins and Matzuk, 2008).

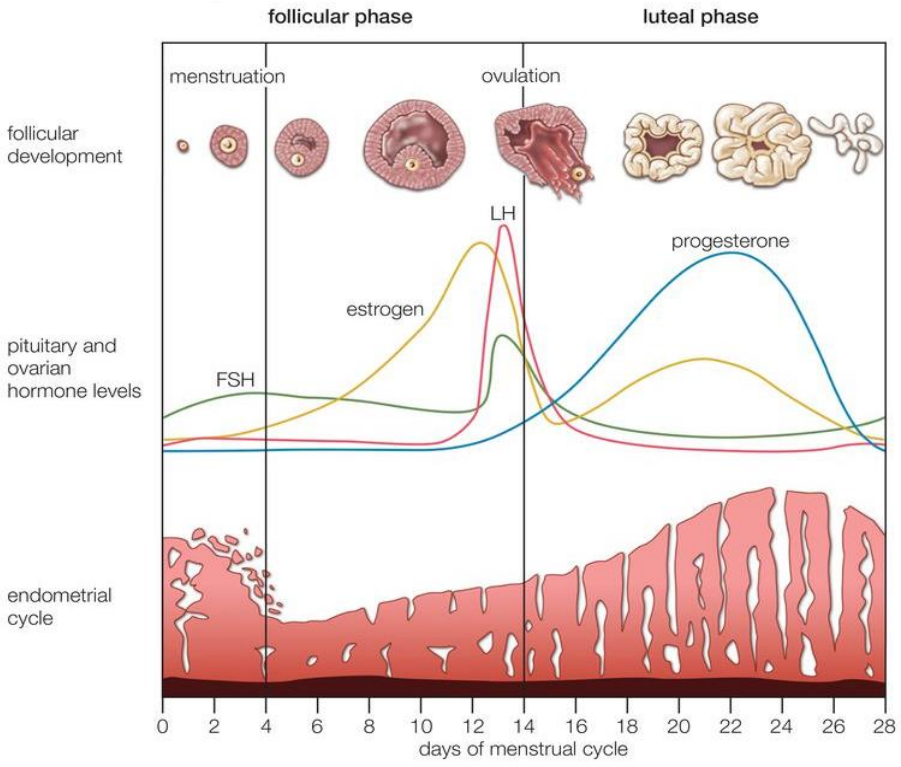

Figure 3. Dynamics of the menstrual cycle relating to endometrial growth, hormone levels and follicular development. *Ovarian and endometrial cycles are shown throughout the menstrual cycle. The ovarian follicular phase is correlated with both the menstrual and*

proliferative phases (endometrial cycle), and the luteal phase corresponds to the endometrial secretory phase. The endometrium is a hormonally responsive organ, and is influenced by several hormonal oscillations during the menstrual cycle, with an increase of estrogen and a peak of LH and FSH triggering the ovulation, followed by an increase of progesterone. In absence of pregnancy, estrogen and progesterone levels drop, the endometrium is shed and the cycle repeats. LH: luteinizing hormone; FSH: follicle stimulating hormone. Image from www.verywell.com

1.3. Cellular composition of the human endometrium

Moving up to the cellular composition, the human endometrium is mainly composed by stromal and epithelial cells, a dense vascular net and resident immune cells.

- **Stroma:** this connective tissue is composed by ECM and cells, mostly fibroblast. These last ones undergo morphological changes during the ECM remodeling in the decidualization period in the secretory phase of the menstrual cycle. This process is characterized by morphological, biochemical and genetic changes produced by the changes in estrogens and progesterone levels. The decidualization takes place 6-7 days after the progesterone peak, when fibroblasts increase their size and shape, becoming bigger and polygonal, secreting hormones and ECM proteins (Simón *et al.*, 2009).

- **Epithelium**: epithelial cells exist as a monolayer of polarized and cuboid cells in the luminal and glandular compartments. They are regulated by ovarian steroid hormones directly or indirectly through stromal cells, and their main function is allowing the embryo-endometrial crosstalk, as well as acting as a barrier against pathogens (Simón *et al.*, 2009).

    - Luminal epithelium: it regulates the adhesion process between the embryo and the endometrium, and the morphology of these cells vary throughout the menstrual cycle by modifications in the plasma membrane and the cytoskeleton. In presence of estrogens, these cells develop long microvilli and tight junctions in the apical lateral membrane. In the secretory phase, when estrogen levels diminish, microvilli also reduce and apical bulges become more prominent in the uterine lumen (Simón *et al.*, 2009).

    - Endometrial glands are composed by glandular epithelium, with cells responsible for nourishing the embryo in its first hours after implantation. These glands become longer and more complex during the secretory phase, secreting necessary molecules for the implantation and the nourishment of the blastocyst (Simón *et al.*, 2009).

- **Vascular compartment**: the uterine vasculature starts in the myometrium, where arcuate arteries become radial arteries in the same layer and become basal arteries where the endometrium starts. From here, spiral arteries emerge, which maintain the deepest layer of the endometrium (basal layer / basalis). In the most superficial layer (functional layer / functionalis), branches emerge from these spiral arteries, irrigating a surface of 4-8 mm^2 each. Angiogenesis in this tissue occurs during the follicular development until the formation of the corpus luteum, as well as during the endometrial regeneration, implantation and formation of the placenta during the pregnancy (Simón *et al.*, 2009).

- **Immune cells**: immune cells also form part of the endometrial cell population. The stroma has a cell population enriched in lymphoid and myeloid cells in the basal layer (10-15% of endometrial cells are lymphocytes, mainly natural killer cells, NK) and during the late and premenstrual secretory phases. These NK cells are a type of lymphocyte that kills cells infected with virus or tumor cells by lysis. There is also a huge amount of suppressor and helper T cells, and few B and plasmatic cells, giving a characteristic lymphoid cell pattern to the endometrium. T and B cells are lymphocytes that trigger a specific immune response: while B cells detect an antigen (free or cell-presented), immune response does not take place until they are activated by helper T lymphocytes. When the latter identify the presented molecule as foreign, they release cytokines that activate B lymphocytes. On the other hand, helper T lymphocytes are responsible for stopping the subsequent immune response. This ample population of T lymphocytes in the endometrium can act on stromal and epithelial cells by releasing cytokines (Yen, Jaffe and Barbieri, 2001). The immune populations existing in the endometrium are essential for the protection of the genital tract against diseases, as well as to avoid the immune rejection from the embryo in the implantation process (Simón *et al.*, 2009).

1.4. The structure of the endometrial tissue

The mentioned above cell types are organized in two layers that compose the morphology of the endometrium: the functional layer or functionalis (two-third parts) and the basal layer or basalis (the inner third part if the tissue, bordering with the myometrium) (Figure 4).

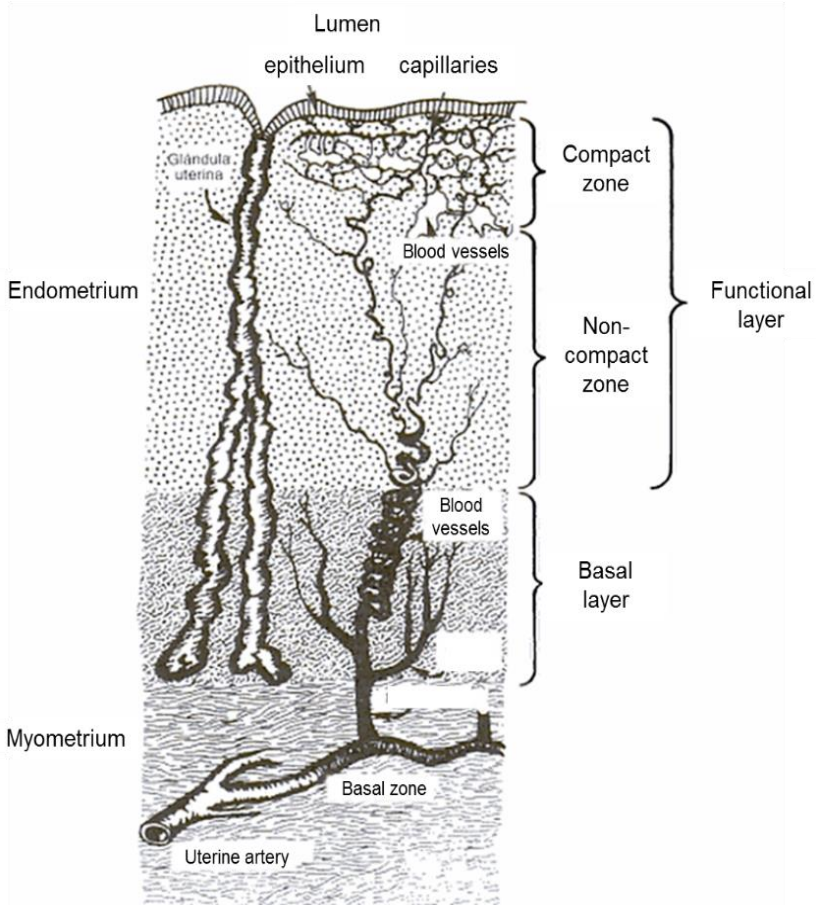

Figure 4. Histological organization of the human endometrium. *Depiction of different layers within the human endometrium with their vasculature. The basal layer is adjacent to the myometrium with the basement of the glands from the functional layer, where there is a denser stroma and the supportive vasculature of this tissue. Towards the uterine lumen there is the functional layer, first less dense and then denser with stromal cells and a dense capillary network. Finally, there is the luminal epithelium that is in contact with the uterine cavity. Image modified from Yen, Jaffe and Barbieri, 2001.*

Both layers are essential for the endometrial proliferation all over the menstrual cycle, and are as follows:

- **Functional layer**: this is the thickest and the most external/luminal layer, which responds to hormone oscillations and is responsible for harboring the embryo during the pregnancy. This layer suffers/undergoes proliferation, secretion and shedding each menstrual cycle (Speroff and Fritz, 2005; Hawkins and Matzuk, 2008). It is a transient and compact layer composed by stroma underlying the luminal epithelium and a looser mid part (non-compact) with glands (Yen, Jaffe and Barbieri, 2001).

- **Basal layer**: this is the thinnest layer that remains without any important change during the menstrual cycle, with the role of providing the foundation for the regeneration of the functional layer when it sheds during menstruation (Speroff and Fritz, 2005; Hawkins and Matzuk, 2008). It is located on top of the myometrium, under the loose functional layer, and it contains the base of the glands that span both layers, a looser stroma and the supportive vasculature for the regeneration of the functionalis (Yen, Jaffe and Barbieri, 2001).

Both described layers can be organized according to the polarized phenotype of the cells forming part of this tissue, with a progressively higher cell proliferation from the functional to the basal layer (Yen, Jaffe and Barbieri, 2001).

1.5. Endometrial pathologies affecting the reproductive outcome

The endometrium is a tissue with a high renovation potential that harbors and nourishes the embryo during its growth, thus several endometrial pathologies might affect the reproductive outcome of the woman affected. These pathologies are usually related to aberrant cell growth, either by excess (cancer or endometriosis) or by defect (like Asherman's syndrome or endometrial atrophy).

1.5.1. *Endometriosis*

Endometriosis is defined as the presence of functional endometrial glands and stroma outside the uterine cavity, which is its physiological niche. This pathology is present in 5-15% of women in their reproductive age (peaking with 25 to 35-year-old women), reaching a prevalence of 30% in women with infertility and up to 45% in those ones with chronic pelvic pain (Mehedintu *et al.*, 2014; Vercellini *et al.*, 2014). This chronic inflammatory condition, which is estrogen-dependent, shows up in the first years of menstruation and is associated with pelvic pain (dyspareunia and dysmenorrhea) and infertility (Vercellini *et al.*, 2014). The biology of this condition is unclear, the etiology remains indeterminated and there are currently different theories that explain endometriosis. However, none of these adequately explains the different possible types nor the variable possible locations of these endometriotic implants. Different types of endometriosis are related to severity, amount, location, depth (>5 mm under the peritoneum is considered deep endometriosis) and size of the endometriotic implants. The revised American Society for Reproductive Medicine score (rASRM) is currently the best-known classification of endometriosis and is one of the most used around the world. It is important to take into account that the severity grades are not related to the symptoms, but with the alteration level (due to the pathology) in relation to the normal woman anatomy, which is directly related to the fertility. Laparoscopy allows to evaluate the grade of rASRM: rASRM stage I is minimal, rASRM stage II is mild, rASRM stage III is moderate and rASRM stage IV is severe (Mehedintu *et al.*, 2014).

The most widely accepted mechanism for the development of endometriosis is via retrograde menstruation (Figure 5), proposed by Sampson in the 20's (Sampson, 1927). During the retrograde menstruation, fragments and endometrial cells could be

placed outside the endometrial cavity, especially in the peritoneum and abdominal organs, establishing ectopic endometrial implants. As a result of the menstrual cycles, these implanted cells would proliferate causing chronic inflammation and the formation of adhesions (Vercellini *et al.*, 2014). This theory is the most convincing model due to the histological similarity between the eutopic normal endometrium and the ectopic implants outside the uterine cavity, as well as the frequent localization of endometriotic implants in the pelvic cavity (Figure 5). Retrograde menstruation is very common in normal menstruated women; in fact, normal menstrual debris is present in up to 90% of women at reproductive age. However, not all of them develop endometriosis, which only affects 1 in 10 of these women. Therefore, other factors might explain the difference of prevalence of this condition that could also explain the existence of endometriosis in extra pelvic locations, such as the lung or the brain (very strange but possible cases not explained by retrograde menstruation) (Mehedintu *et al.*, 2014). Another explanation for endometriosis in locations outside of the uterine cavity could be the "stem cell hypothesis". In this theory, bone marrow-derived stem cells (BMDSCs) could differentiate into endometrial tissue resulting in the formation of ectopic endometriotic implants in any location (Macer and Taylor, 2012). Other theories are coelomic metaplasia, immune system abnormalities, genetic causes, environmental/lifestyle factors or due to the presence of remnant germ stem cells from the embryonic development during the formation of Müllerian ducts (Mehedintu *et al.*, 2014; Vercellini *et al.*, 2014; Makiyan, 2017). Therefore, the pathogenesis of endometriosis may involve a combination of genetic, hormonal, immune and environmental factors, all of which are nowadays under study to assess the real causes of this condition, which is still far from being understood completely.

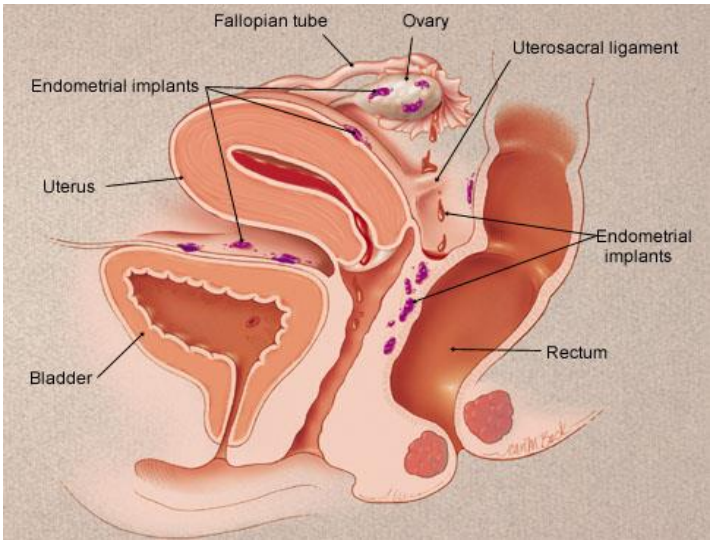

Figure 5. Retrograde menstruation process and ectopic implantation sites. *Basic anatomy of the retrograde menstruation with the usual implantation sites of endometriotic tissue, such as the ovary and the bladder. Image from Wellbery, 1999.*

The diagnostic tools for endometriosis are laparoscopy, laparotomy or hysteroscopy, and subsequent histological analysis of the lesions, as well as transvaginal ultrasound, computerized tomography and nuclear magnetic resonance in some cases. Medical treatment can be used to relief the pain (contraceptives or progestin), or surgical treatment for advanced endometriosis, with the latter method there is a lower risk of recurrence (Wellbery, 1999). Consequently, medical treatment is probably ineffective for this pathology, while the laparoscopic removal of the endometriotic lesions offers a moderate improvement in the possibility of achieving pregnancy. So, assisted reproduction seems to be a promising alternative to the surgery for those patients seeing that 30% of infertile women suffer from endometriosis (Vercellini *et al.*, 2014). Moreover, several studies over the years have demonstrated that the oocyte quality is impaired in women with ovarian endometriosis, necessitating assisted reproduction techniques to even oocyte donation in some cases (Sanchez *et al.*, 2017).

1.5.2. *Asherman's syndrome*

Asherman's syndrome (AS) is an endometrial pathology first described by Joseph G. Asherman in 1948 (Asherman, 1948), characterized by the presence of adhesions inside the uterus (Figure 6) or the cervix, leading to a total or partial loss of functional endometrium. The estimated prevalence is around 1.5% in women of reproductive age, up to 39% in those with recurrent pregnancy loss, and up to 40% in those with curettage after delivery or abortion (http://www.ashermans.org, International Asherman´s Association).

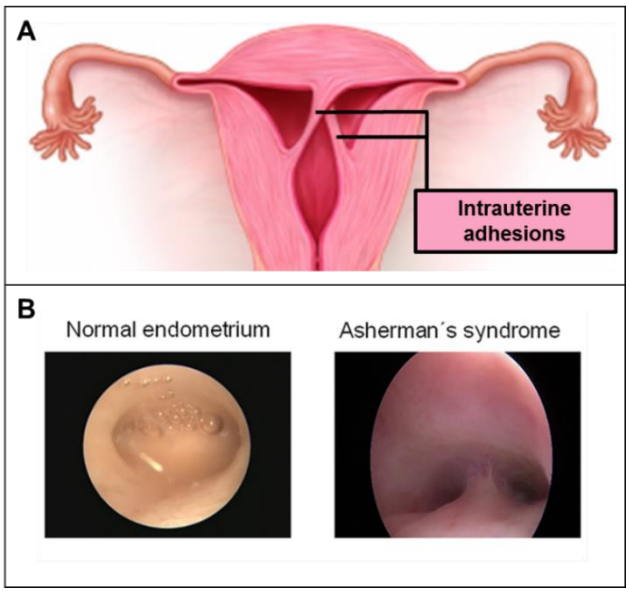

Figure 6. Endometrial adhesions produced by Asherman's syndrome. (A) *Uterus with the characteristic adhesions of Asherman's syndrome patients. Image adapted from*

http://www.drsinghals.com/adhesiolysis-ashermanns-syndrome/ ***(B)*** *Comparison by laparoscopy between a normal endometrium and one with Asherman's syndrome. Images from the work of Santamaria et al., 2016.*

The possible causes for this disorder can range from curettage (90% of the cases of AS) or volunteered interruption of pregnancy, to uterine lesions caused by less invasive surgery procedures or infections like genital tuberculosis. There are different ways to classify AS patients, and one way to establish the different stages was described by the American Fertility Society in 1988, which depends on the extensiveness of the adhesions, as well as their morphology and the menstrual pattern. This classification is therefore: mild (type I), moderate (type II) or severe (type III), and the adhesions can be filmy or dense, and located in one spot or confluent, not affecting the vasculature in most cases, which is easier for the treatment. This pathology can be related to pelvic pain and retrograde menstruation, as well as amenorrhea in some cases or hypomenorrhea, but mainly entails reproductive problems such as menstrual dysregulation, implantation failure, intrauterine growth restriction, abnormal placentation or recurrent miscarriage (Conforti *et al.*, 2013).

An accurate diagnosis is possible with imaging of the uterine cavity, so the best diagnostic tool is the hysteroscopy, but also sonohysterography or hysterosalpingography. The most extensive treatment is the removal of the adhesions with the aim of recovering the uterine cavity by hysteroscopy, sometimes assisted by laparoscopy when the adhesions are dense and the entrance via the cervix is difficult (Kilic *et al.*, 2014). However, most of the times the adhesions reappear, especially in severe cases. For now there has not been any successful treatment discovered to keep the uterus free of adhesions and therefore to recover the fertility. However, some novel experimental approaches have been carried out which could lead to a future treatment for those women who want to be pregnant. In 2011, one patient with severe AS became pregnant after transplantation of BMDSCs, specifically angiogenic endometrial somatic stem cells (SSC), in the uterine cavity guided by ultrasound after uterine curettage. After the subsequent hormonal treatment and checking endometrial thickness (8 mm), *in vitro* treatment was carried out and pregnancy was achieved (Nagori, Panchal and Patel, 2011). This improvement in the reproductive outcome thanks to autologous cell therapy was also demonstrated in the study of Dr. Santamaría *et al.* in 2016, where $CD133^+$ BMDSCs were introduced in women with AS and/or endometrial atrophy (EA). Following this, uterine adhesions diminished and both endometrial thickness and neovascularization increased, but in a transient manner. Moreover, several spontaneous pregnancies were achieved, as well as after

in vitro fertilization, giving rise to a childbirth (Santamaria *et al.*, 2016). These advances in cell therapy indicate that the field of regenerative medicine could suppose an effective therapy for women with endometrial pathologies.

1.5.3. *Endometrial atrophy*

Endometrial atrophy (EA) is an infrequent pathology among the population, also known as atrophic, thin or refractory endometrium, and is characterized by a lack of endometrial growth. The right endometrial thickness is essential for implantation and reproductive success (Figure 7). However, there is no consensus regarding to which endometrial thickness should be considered normal or thin, but several studies postulate that a thin endometrium would be that of ≤6-8 mm. This pathology has a prevalence of 2.4% among patients undergoing cycles of assisted reproduction and it increases up to 5% among women younger than 40 years old, reaching a percentage of 25% in those older than 40 with non-stimulated cycles.

Lacking the correct endometrial thickness can result from several risk factors such as inflammatory or iatrogenic causes (surgical interventions or repetitive/aggressive curettages), as well as idiopathic causes. The triggers for the development of EA could be: (a) poor glandular epithelium growth and (b) poor releasing of vascular endothelial growth factor (VEGF), which is related to decreased blood irrigation and an insufficient vascular development. Same as in AS, women with EA usually have lower implantation rates, more frequently miscarriage and, in summary, lower pregnancy rates (Mahajan and Sharma, 2016).

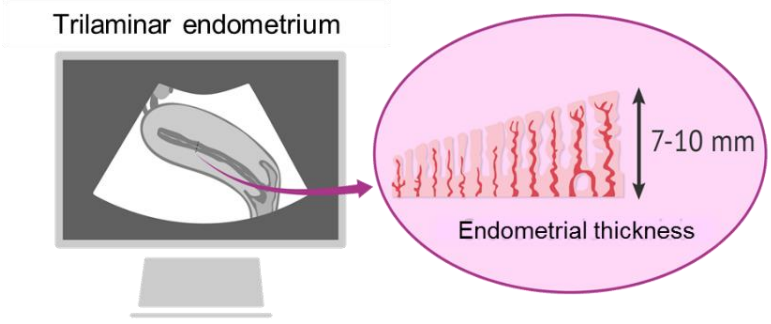

Figure 7. Evaluation of endometrial thickness and endometrial pattern by transvaginal echography. *Endometrial lining can be measured by ultrasound, which can vary up to 7-10 mm in the secretory phase as well as having a trilaminar pattern to achieve pregnancy. Image adapted from https://www.reproduccionasistida.org.*

The lack of endometrial proliferation is not necessarily accompanied by the formation of intrauterine adhesions. Even though the endometrium reaches a normal thickness, the reproductive outcome can be still poor (Shufaro *et al.*, 2008). Several treatments

have been applied to the patients but none of them has been validated due to its limited success. The lysis of the uterine adhesions (adhesiolisis) by hysteroscopy is the most invasive treatment. The intrauterine injection of granulocyte-colony stimulating factor (G-CSF) showed an increase in the endometrial thickness and even an improvement in the pregnancy rates (Gleicher, Vidali and Barad, 2011). Other treatments are the maintenance of estrogen administration for 14-82 days, the injection of human chorionic gonadotropin (HCG) during the follicular phase of the menstrual cycle, as well as the use of drugs to increase the blood flow like the aspirin (Mahajan and Sharma, 2016). As it has been described at the end of the section *"1.5.2. Asherman's syndrome",* the study of Dr. Santamaría *et al*. in 2016 achieved a reproductive improvement in women with AS and/or EA by the use of cell therapy with $CD133^+$ BMDSCs (Santamaria *et al.*, 2016).

2. Stem cells

2.1. Definition and classification of human stem cell types

According to Alberts *et al*., a stem cell is a relatively undifferentiated cell that can continue dividing itself indefinitely, yielding daughter cells which can remain undifferentiated as stem cell (self-renewal), or they can commit and differentiate irreversibly giving rise to specialized cell types (Alberts *et al.*, 2002). In this way, stem cells are characterized by multipotency, self-renewal and maintenance of the balance between both processes. That they can divide unlimitedly does not mean that the division rate is fast; in fact, stem cells tend to divide slowly (Alberts *et al.*, 2002).

There are several stem cell types depending on their potency, i.e., depending on the number of cells they are able to differentiate to. The potency is indirectly proportional to the differentiation capability, and the basic classification (Figure 8) from more to less differentiation potential is as follows: totipotent, pluripotent, multipotent, oligopotent and unipotent.

- **Totipotent**: stem cells that can differentiate to any cell type and give rise to a complete organism, with embryonic and extraembryonic (placenta) components. They have the maximum grade of undifferentiation and are the zygote cells present after the fertilization in the first divisions.

- **Pluripotent**: these are more differentiated than the previous ones, so they can create a complete organism but not produce the extraembryonic components; they have the capability to differentiate to almost any cell type (endoderm, mesoderm

and ectoderm). They are the embryonic stem cells and those derived from the three layers resultant from the early stages of differentiation of these embryonic stem cells.

- **Multipotent**: they can differentiate to cell types of the same lineage or embryonic layer; for example, hematopoietic stem cells can differentiate to leucocytes, erythrocytes and platelets. They are broadly used in transplants of damage tissues.

- **Oligopotent**: these cells are able to differentiate in only few cell types, such as myeloid or lymphoid stem cells.

- **Unipotent**: these cells are able to divide without differentiating in a different cell type, but this self-renewal ability makes them stem cells, like those of a tissue, i.e., the muscle.

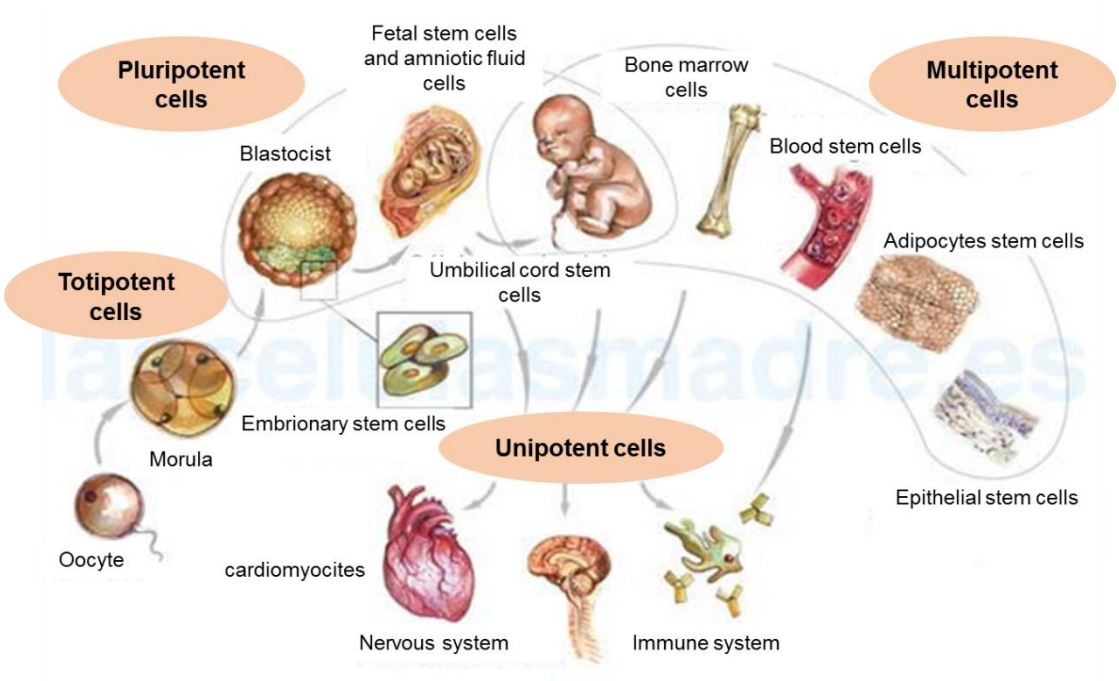

Figure 8. Stem cell classification according to their potency. *Stem cells can be totipotent if they come from an early embryo, pluripotent if they come from a blastocyst or from any of the embryonic layers, multipotent if they come from adult tissues and unipotent if they have division potential but not differentiation potential to other cell types. There are also fetal, umbilical cord and amniotic fluid stem cells, with characteristics varying depending on the stage of gestation. Image adapted from http://delatandoalaciencia2.blogspot.de/p/definicion-y-tipos.html*

Another possible classification is based on their origin, i.e., the source of collection:

- **Embryonic stem cells (ESCs):** ESCs are stem cells obtained from an embryo with up to five days of embryonic development, before implantation. These cells can be derived from the inner cell mass of a blastocyst or from the early embryo (morula or single blastomeres), and are totipotent or pluripotent and potentially immortal cells,

being able to give rise to a complete organism because they can differentiate in the three embryonic layers and in the germline. They were first isolated from a blastocyst-stage mouse embryo in 1981 (Evans and Kaufman, 1981) and from a human embryo in 1998 (Thomson and Marshall, 1998).

- **Primordial germ cells (PGCs):** they originate from the separation of a group of pluripotent cells from the rest of the embryo at the beginning of the embryogenesis. They are pluripotent with characteristics similar to those of ESCs and are the precursors of female gametes responsible for fertilization and, consequently, for the formation of a new organism.

- **Fetal stem cells:** they are a primitive type of cells obtained from fetal organs (up to 10-week-embryos) as well as from fetal blood and bone marrow. These cells allow the formation of all fetal tissues and organs because they are multi- or pluripotent.

- **Amniotic fluid stem cells:** they are derived from the amniotic fluid, which contains cells from embryonic and extraembryonic tissues, in addition to differentiated and undifferentiated cells derived from the ectoderm, endoderm and mesoderm. Their characteristics vary depending on the moment of gestation and the existence or absence of fetal abnormalities. They are very active, although with a lower differentiation potential compared to ESCs.

- **Umbilical cord stem cells:** they are multipotent stem cells obtained from umbilical cord blood (hematopoietic, also present in the bone marrow) or from the cord itself (mesenchymal). These tissue-specific stem cells have characteristics similar to embryonic and hematopoietic cells, who can differentiate to blood cells and immune system cells. They can be used in the regeneration and repair of every type of tissue, which is why they are the most popular type used in investigation. It is also possible to obtain stem cells derived from **placenta.**

- **Somatic/adult stem cells (SSCs):** they are pluripotent or multipotent (differentiation in one or several lineages) and in some cases unipotent cells, with slow division rate and obtained after embryo development. Their origin is not known in most of cases but they are present in almost every adult tissue and organ, where they are in charge of maintaining homeostasis and repair, residing in specific microenvironments called niches. The most studied SSCs are BMDSCs, which can be hematopoietic stem cells with the ability to differentiate to every blood cell type, or stromal/mesenchymal stem cells that can give rise to non-hematopoietic stem cells such as adipocytes, chondrocytes, osteocytes or connective tissue.

- **Induced pluripotent stem cells (iPSCs):** these pluripotent stem cells were first created in 2006 by Yamanaka's investigation group by modifying (cell reprogramming) four genes from mouse SSCs, obtaining iPSCs cells with properties similar to those of ESCs (cells equally therapeutic but without the necessity to have an embryo to derive them). The most interesting point of this work was to discover that the four genes (Oct3/4, Klf4, Sox2, and c-Myc) with the role of maintaining the pluripotency of ESCs were the same that can result in the dedifferentiation of them to obtain iPSCs. Thanks to this discovery Dr. Yamanaka received the Nobel Prize in Physiology and Medicine in 2012 with John Gurdon.

- **Cancer stem cells (CSCs):** they are similar to normal stem cells due to their self-renewal and differentiation potential, but the mechanisms that usually tightly control these processes are altered. This dysregulation makes them resistant to chemotherapy and with a high capability to repair DNA damage; a constant expansion and an aberrant daughter cell production is caused, leading to uncontrolled growth. They comprise a small cell number inside the tumors from where they can be isolated, for example from breast, brain or liver cancer.

Stem cells open a wide range of possibilities in science, such as the study of pathologies, tissue repair, mechanisms of development or drug testing. Additionally, each type of stem cell has its advantages and disadvantages, being essential in the development, repair, maintenance and repair of tissues and organs. For example, hematopoietic stem cells (derived from bone marrow, umbilical cord or peripheral blood) are useful in the treatment of patients with metabolic, immune or blood diseases.

2.2. Stem cell niche

2.2.1. Concept of "niche" as stem cells "home"

The concept of a stem cell niche was first proposed by Schofield in 1978 who described it as an anatomically defined compartment wherein SSCs reside, where they can maintain their stem cell properties and assure the renovation in the tissue (Schofield, 1978). The niche is a specific anatomic location composed of SSCs, ECM and adjacent niche cells, all of them interacting with each other. Niche cells are inhibiting the differentiation and proliferation of SSCs in an intrinsic way, with cells dividing very slowly (Fuchs, Tumbar and Guasch, 2004). This microenvironment is crucial for the self-renewal, the maintenance and the survival of these SSCs because of various factors: (a) stem cells need a suitable environment to survive, for example to minimize the accumulation of damage because of their immortality and the high

number of divisions they carry out. This makes the existence of specific metabolic requirements and the support of adjacent cells necessary. This “nutritive” function makes the niche essential. (b) The regulatory role or feedback. The niches not always have the same size inside the tissue, they expand or contract or they have stochastic variations; they are not static in function or number, they are dynamic systems. This control can be provided by growth factors or by cell surface molecules produced by niche cells. Consequently, some niche cells would not be only supportive or nutritive cells, rather they would provide information about the tissue to assure its maintenance, repair and a correct balance between division and quiescence. (c) The SSC niche is a coordination tool between tissue compartments, like occurs in the hair follicle niche (Lander *et al.*, 2012). Finally, the SSC niche would provide structural and trophic support, topographic information and physiological signals for the regulation of SSC functions inside the tissue or organ.

As mentioned above, the niche is a dynamic system necessary for sustaining tissues and is in constant change due to the interaction of signals between stem cells and niche cells, which determine the rate and pattern of division of these SSCs and the ability to generate more mature offspring. The characteristic quiescent state or low division rate of SSCs are controlled epigenetically, transcriptionally and post-translationally (Tweedell, 2017), and are taken out of it when the niche provides the adequate signals. These signals control both the proliferation of SSCs through symmetric divisions (controlling the stem cell pool as undifferentiated SSCs, self-renewal) and the differentiation through asymmetric division (multipotency), as well as provide protection against cell death or uncontrolled division (which would lead to tumor formation) (Moore and Lemischka, 2006). The asymmetric division, and the subsequent differentiation of the SSCs towards other cell types, can be caused by their polarization because of the tight relation between them and niche cells (Figure 9). The ratio between symmetric and asymmetric divisions is essential for the niche survival and the creation of surrounding committed cells, directing the flow of differentiated progeny (Fuchs, Tumbar and Guasch, 2004). Quiescent and active SSCs can coexist at the same time in the same niche, as well as the bidirectional conversion between them (Tweedell, 2017). The differentiation of committed daughter cells to undifferentiated stem cells is also possible when damage occurs (Visvader and Clevers, 2016).

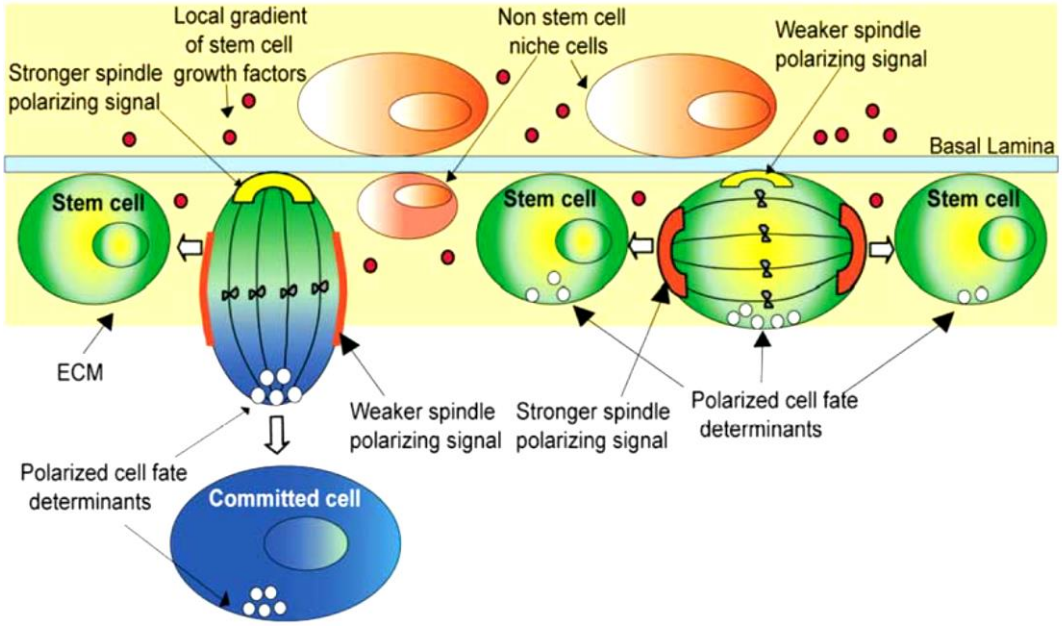

Figure 9. Comparison between symmetric and asymmetric divisions of stem cells inside the niche. *The division pattern is based on the cellular polarity, regulating the stem cell fate. Asymmetric division happens when polarization is enhanced in the junction with the basal lamina, and symmetric division takes place when the polarization is enhanced in the contact zone between cells. The mitotic spindle also influences the pattern during cell divisions, as well as the distribution of differentiation signals or stemness-determining molecules. A descendant daughter cell continues as SSC if it retains signals to inhibit the differentiation and maintain self-renewal, whilst the progeny will differentiate if it receives few stemness factors or many differentiation factors. ECM: extracellular matrix. Image from Fuchs, Tumbar and Guasch, 2004.*

Due to the dynamism of this cell system, the niche allows a regulation in the division rate between quiescence and division in any moment, responding to a hierarchical distribution and activating SSCs in a periodic way to create progenitor or transit amplifying (TA) cells. These TA cells have intermediate characteristics between SSCs and differentiated cells, created by asymmetric division of stem cells. They have the ability to divide very fast in a limited way in last instance giving rise to differentiated cells (without division capability) and adult/mature cell lineages (Moore and Lemischka, 2006) (Figure 10). Last, perivascular regions seem to be potential SSC niches such is the case with hematopoietic or mesenchymal stem cells (Scadden, 2006; Oh and Nör, 2015).

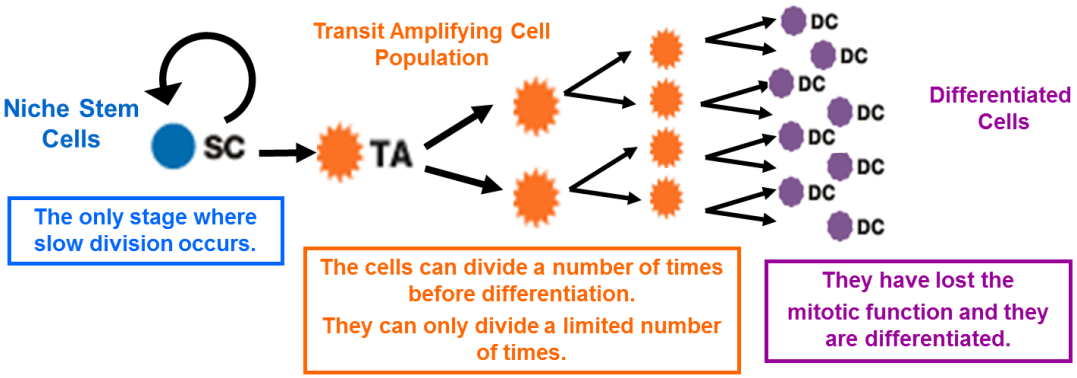

Figure 10. Hierarchical distribution of SSCs inside a niche within a tissue*. Diagram with different stem cell types present inside an SSC niche. The stem cell (SC) can divide through symmetric division to perpetuate itself as stem cell and then to increase the stem cell reserve, or it can divide itself in an asymmetric way to create TA cells (fast division). TA cells would give rise to a committed progeny with cells differentiated in specific cell types (DC). DC: differentiated cell; SC: stem cell; TA: transit amplifying.*

A very interesting property of the niches is that they retain their activity even in absence of endogenous SSCs; i.e., if we add exogenous SSCs inside a niche they can act as SSCs of this tissue. This can be due to both the occupation of empty, partially filled or ectopic niches, and the displacement and replacement of endogenous SSCs. In any case, the niche must be previously depleted of any endogenous stem cell. However, to be able to restock a SSC niche and to study possible therapeutic options, it's necessary to locate it inside the tissue or organ and to know how it functions, something unknown in most cases (Spradling, Drummond-Barbosa and Kai, 2001). Niches can be as diverse as the cells inside them, with each niche specialized in the particular tissue in which they are found. The function of the cells inside the niche and their dysregulation are so important that it is linked with cancer processes or ageing.

Stem cells have been identified in several tissues so far, such as bone marrow (the most characterized and studied), brain, hair follicle, small intestine, testis and ovary (Moore and Lemischka, 2006).

2.2.2. Mechanisms of stem cell regulation

The communication by secreting factors, affecting surrounding or distant cells, inside the SSC niche is essential for its homeostasis, cell division and differentiation. The Wnt signaling pathway seems to be very important in maintaining the stem cell self-renewal in intestine and blood, and in the differentiation of cells forming part of the hair follicle. Secreted factors by niche cells, cell-cell adherence junctions (with transmembrane proteins called cadherins) between niche cells and SSCs are

essential as well for cell communication, maintaining SSCs in tight proximity and preserving their stem cell conditions. This tight communication between SSCs and their environment is also preserved by adhesion molecules called integrins at the basal lamina inside the niche. The loss of both integrins and adherence junctions affects the spatial organization, the adhesiveness and the proliferation of SSCs (Fuchs, Tumbar and Guasch, 2004)

Many soluble molecules and paracrine factors that regulate the stem cell functions between niche cells and SSCs have been described, especially in *Drosophila melanogaster;* this was reviewed by Dr. Scadden in 2006 (Scadden, 2006). Despite the amount of information already available, we are still far from understanding the mechanisms directing the spatial organization of this microenvironment.

Recent studies based on novel techniques in the field of regenerative medicine demonstrated the therapeutic effect of numerous stem cell secreted factors, affecting in an autocrine and paracrine manner, which seem to be essential for the role of these SSCs (Gnecchi *et al.*, 2005, 2006, 2008, 2016; Baraniak and McDevitt, 2010; Burdon *et al.*, 2011; Hodgkinson *et al.*, 2016).

Figure 11 shows the important effect of soluble factors in cardiac function studied by Dr. Gnecchi in 2008, who described the presence of only few transplanted cells in relation to the huge cardiac improvement observed and therefore postulating the paracrine effect of these stem cells (Gnecchi *et al.*, 2008). It has also been confirmed that these factors stimulate the regenerative process via: (a) neovascularization, (b) trophic support for the cell survival and repair in diseased or injured tissues, (c) anti-inflammatory or immunomodulatory properties creating a specific microenvironment, or (d) recruitment and activation of endogenous SSCs (Burdon *et al.*, 2011; Murphy, Moncivais and Caplan, 2013).

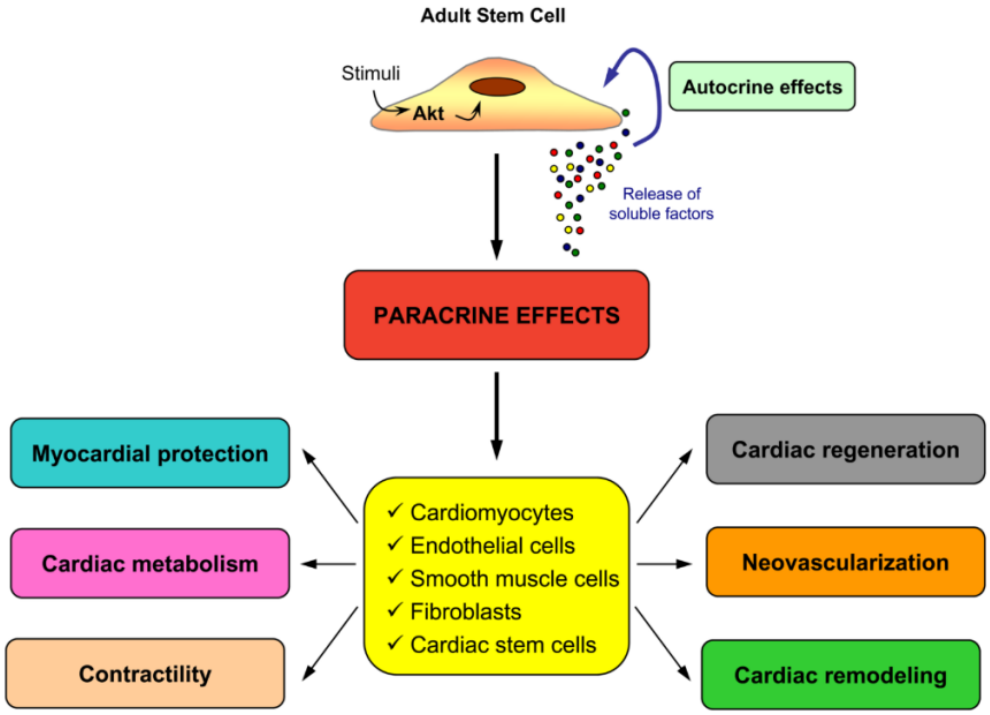

Figure 11. Autocrine and paracrine effects of SSCs-secreted soluble molecules in cardiac repair. *Molecules secreted by SSCs can act in an autocrine manner on the stem cells themselves or in a paracrine manner on different cell types present in the cardiac tissue, such as cardiomyocytes or fibroblasts. These soluble molecules modulate several functions like cardiac metabolism, cell contractility, tissue regeneration, neovascularization or cardiac remodeling. Image from Gnecchi et al., 2008.*

Multiple studies over many years have attempted to assess not only the direct differentiation of stem cells in tissue regeneration, but also the role of secreted factors (autocrine and paracrine) by mesenchymal stem cells, such as in renal injury (Tsuji and Kitamura, 2015), in cardiac repair (Gnecchi *et al.*, 2005, 2006; Henning, 2012, 2016; Khanabdali *et al.*, 2016), in brain damage (Rhee *et al.*, 2016) or in liver regeneration (Adas *et al.*, 2016). The potential role of BMDSCs in endometrial regeneration related to endometrial disorders was also described (Morelli, Rameshwar and Goldsmith, 2013; J. Zhao *et al.*, 2014, 2015; Cervelló *et al.*, 2015; Gil-Sanchis *et al.*, 2015; Santamaria *et al.*, 2016). In 2015 a study with mice models was developed, in which SA was artificially induced by uterine curettage, and BMDSCs derived from AS and/or EA patients were later injected (by tail or intrauterine injection) (Cervelló *et al.*, 2015). To be able to track the cells in the mouse endometrium they were labelled with superparamagnetic iron oxide nanoparticles before injection. After cell therapy with BMDSCs in the animal model, a small number of engrafted stem cells were observed in the endometrial tissue, which could not directly generate sufficient endometrial cells to justify the therapeutic benefits and the histological improvements observed. Consequently, the improvement in endometrial

quality after cell therapy was postulated to be caused mainly by secreted factors from SSCs more than from the stem cell division itself. However, the identification of these regenerative factors and their therapeutic use for the treatment of endometrial pathologies are still under study in different lines of investigation. In Reproductive Medicine, the future identification of these factors would allow the creation of a non-invasive therapy to treat endometrial diseases with a cocktail of commercially available factors, without the necessity of extracting the patients' own cells.

3. Presence of somatic stem cells in the human endometrium

3.1. Evidences for the existence of endometrial somatic stem cells

A key for understanding the regenerative capability of many adult tissues is to assess the presence of SSCs within them, as has been done for the hair follicle, brain, muscle, retina, blood or intestine (Cotsarelis, Sun and Lavker, 1990; Doetsch *et al.*, 1999; Jackson, Mi and Goodell, 1999; Tropepe *et al.*, 2000; Zhang *et al.*, 2003; Barker *et al.*, 2007). The human endometrium is a unique tissue where the functional layer suffers growth, breakdown and regenerative processes during menses throughout the female reproductive life (around 400-500 times) while the basal layer remains constant (Prianishnikov, 1978; Padykula *et al.*, 1984), as described in section "*1. The human endometrium*", more concretely in "*1.2. The menstrual cycle*". This outstanding regenerative capacity, which can even be recuperated during menopause with hormonal therapy, seems to suggest the presence of a SSCs population in this tissue (Figure 12). This is most likely in the basal layer, it remains relatively constant each menstrual cycle and is from where the endometrium regenerates, giving rise to the new upper functionalis layer.

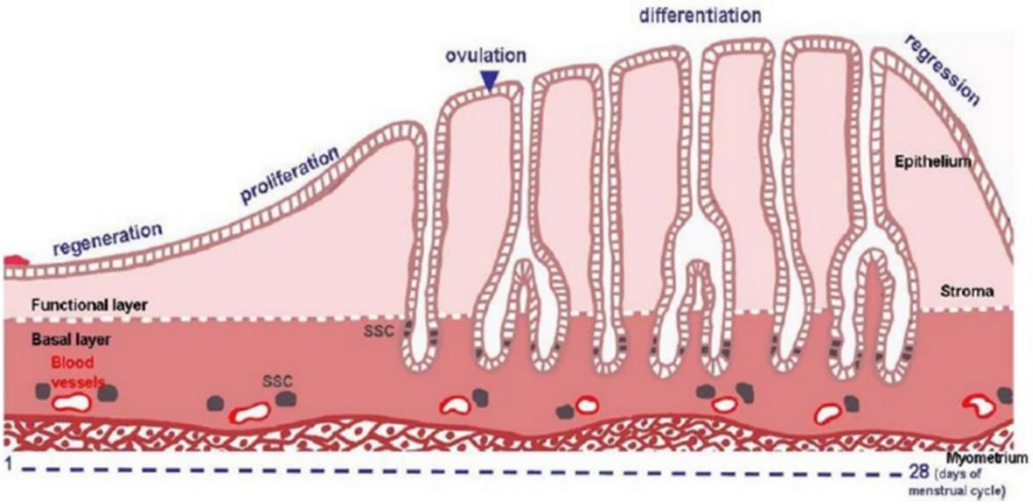

Figure 12. The menstrual cyclic processes of the endometrium and the postulated location of SSCs. *Different phases that the endometrium cycles through are specified: regeneration, proliferation, differentiation and regression, with ovulation occurring in the middle*

of these 4 phases. The postulated location of endometrial SSCs is also represented in the epithelial and stromal compartments, with a putative perivascular location in this last cell type. SSC: somatic stem cell. Image from Cervelló, Mirantes, et al., 2011.

In murine models, the presence of SSCs was demonstrated with the label-retaining cells (LRCs) approach, which permits to identify these cells in the mouse. The staining of cells with 5-bromo-2'-deoxyuridine (BrdU) has also permitted to study the localization of this endometrial SSC population in the mouse endometrium by the detection of cells retaining the dye, LRCs. BrdU is a synthetic nucleoside analog to thymidine that is incorporated into the genomic DNA during the replication phase of the mitotic cell cycle, replacing thymidines almost entirely. This labelling technique is based on the slow cycle rate of SSCs (division only occurs when tissue must be repaired, Figure 10) so when cells are labelled with BrdU dye during the animal growth, the ones retaining the dye in adulthood will be those with a slow division rate. Thus, labelled cells can be identified and quantified, making possible to locate the potential stem cell niche housing these endometrial SSCs by differentiating them from actively dividing cells that will have lost the dye. Along 2006 and 2007, several studies were conducted to confirm the presence of these stem cells in the mouse endometrium. LRCs were observed in a variable percentage depending on the cell type and the animal age, at perivascular locations (the most probably location for the endometrial SSC niche), both in epithelium and in stroma (in higher proportion) (Chan and Gargett, 2006; Cervelló *et al.*, 2007; Szotek *et al.*, 2007).

In humans, the high proliferative potential of the endometrial cells, the differentiation into other cell types, the expression of markers of pluripotency and the clonogenic activity are some of the properties that can be used to justify cell pluripotency *in vitro*. The clonogenicity (or clonal activity) is defined as the capability of a stem cell to create clones or colonies when seeded at low cell densities. In 2004 the clonogenicity of endometrial SSCs was first demonstrated, with a higher clonogenicity in epithelial compared to stromal cells, obtaining two colonies of different size and density (Chan, Schwab and Gargett, 2004). The same group showed in 2005 the existence of endometrial SSCs in the endometrium of menopausal women, which do not have a functional layer, this seems to indicate that SSCs are located in the basal layer as previously mentioned (Schwab, Chan and Gargett, 2005). In 2007 the clonogenicity of a population positive for the markers CD146 and PDGF-Rβ was demonstrated, in 2012 in a population positive for the marker W5C5 and in 2012 for epithelial and stromal Side Population (SP) in comparison with non-Side Population (NSP) (Cervelló *et al.*, 2010). The SP is a population that harbors cells with the property of extrude the DNA-binding

dye Hoechst 33342 through membrane channels named BCRP1 (breast cancer resistance protein 1), which is a stem cell feature. The SP phenotype is associated with the presence of a heterogeneous population including SSCs in many adult tissues, i.e., a stem-cell-enriched population, explained in detail in section "*2.1.1. Side Population approach*".

The capability of postulated SSC populations to differentiate into other cell types of a different mesodermal lineage, such as myocytes, chondrocytes, osteocytes or adipocytes has also been studied (Schwab and Gargett, 2007; Cervelló *et al.*, 2010; Cervelló, Mas, *et al.*, 2011; Masuda *et al.*, 2012; Shoae-Hassani *et al.*, 2013), which is another assay to test the stemness features of these cells *in vitro*.

The formation of new tissue in animal models starting from stem cells is the most important assay to demonstrate their tissue regeneration ability *in vivo*. The animal assays are conducted in mice due to their similar estrous cycle, so human endometrial SSCs have been studied *in vivo* in xenotransplanted murine models. In 2007 the endometrial reconstitution from endometrial disaggregated cells was tested by injecting them under the mouse kidney capsule, testing the stemness features of the total endometrial fraction (Masuda *et al.*, 2007). Later, the group of Dr. Masuda accomplished the endometrial reconstitution again, but this time by injecting SP cells (Masuda *et al.*, 2010). This had low efficiency probably due to the necessity of cells acting as niche or support cells. In 2011 they wanted to improve the animal model by also introducing the total endometrial fraction as support of these endometrial SSCs, reaching an improvement of the transplant efficiency (Miyazaki *et al.*, 2012). In 2010 the group of Dr. Cervelló achieved endometrial tissue reconstitution using SP cells as well, but in this case with a subcutaneous injection in mice, and one year later also in kidney capsule with SP-derived cell lines (Cervelló, Mas, *et al.*, 2011) (Figure 13). In 2012 the stromal tissue reconstitution using $W5C5^+$ cells was also successful (Masuda *et al.*, 2012).

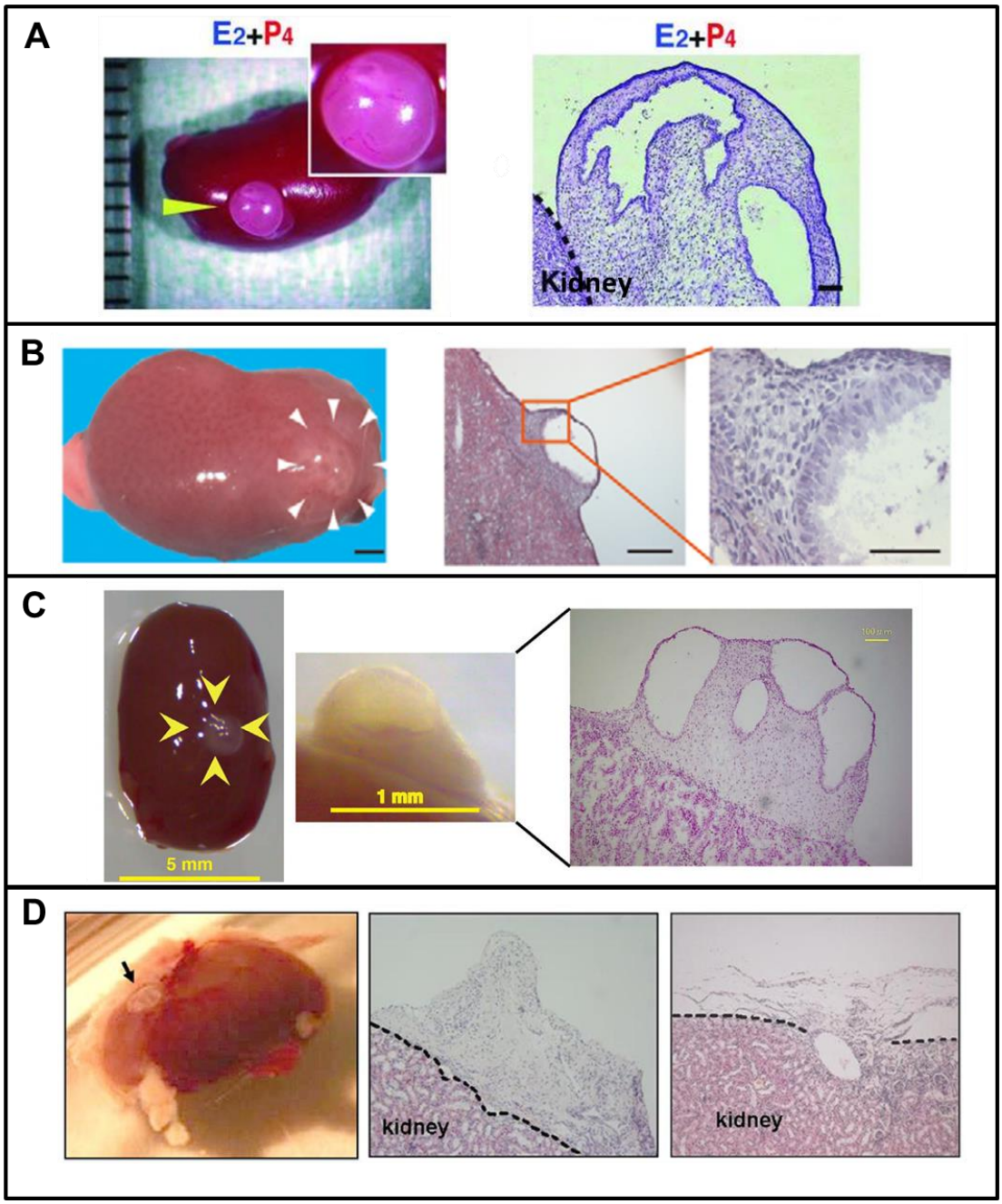

Figure 13. Endometrial reconstruction after the injection of endometrial SSCs in mouse kidneys. ***(A)*** *Endometrial reconstitution obtained by Dr. Masuda et al. in 2007 after injecting disaggregated endometrial cells.* ***(B)*** *Endometrial reconstitution obtained by Dr. Masuda et al. in 2010 using SP cells.* ***(C)*** *Endometrial reconstitution obtained by Dr. Miyazaki et al. in 2012 after the injection of SP cells supplemented with disaggregated endometrial cells.* ***(D)*** *Endometrial reconstitution obtained by Dr. Cervelló et al. in 2011 after the injection of SP-derived cell lines. In every case, the injection was performed under the kidney capsule of immunedeficient mice, and all the structures were visible both macroscopically and microscopically. E_2: estradiol; P_4: progesterone. Figures from Masuda et al., 2007, 2010; Cervelló et al., 2011; Miyazaki et al., 2012.*

In summary, the existence of endometrial SSCs, both in humans and in mice, has been tested *in vivo* and *in vitro* in several studies (Table 1).

Date	Test	Objectives	Relevant results	Reference
2004	Clonogenicity of stromal and epithelial cells in human endometrium.	To assess the clonogenicity of epithelial and stromal SSCs in human endometrium, as well as their phenotype and the growth factors involved.	Few big and dense colonies (corresponding to SSCs) and other smaller but more numerous, with non-dense and big cells (associated to TA cells), more abundant in epithelial cells. Three growth factors in charge of maintaining this clonogenicity in both cell types: TGFα, EGF and PDGF-BB.	(Chan, Schwab and Gargett, 2004)
2005		To assess if the clonogenicity of SSCs differs according to the menstrual phase or the endometrial state (active or inactive).	The clonogenicity of epithelial and stromal SSCs does not change between secretory and proliferative phase, neither among active, cyclic or inactive (menopause) endometrium. This is the first evidence of stromal and epithelial clonogenic cells within inactive endometrium.	(Schwab, Chan and Gargett, 2005)
2006	LRC approach in mouse endometrium.	Identification of LRCs in mouse endometrium and inside the endometrial SSC niche.	Localization of LRCs, mobilized after treatment with estrogen, in the junction between myometrium and endometrium for the first time, and around blood vessels and glands.	(Chan and Gargett, 2006)
2007			Presence of LRCs in mouse endometrium in decreasing concentration with the animal age. Location in the lower part of the stroma and co-localization with markers of undifferentiation: c-KIT and Oct-4.	(Cervelló *et al.*, 2007)
		Identification of LRCs in mouse endometrium and myometrium.	Existence of LRCs in endometrium and myometrium in a progressive way. Postulated common origin of endometrial and myometrial SSCs from Müllerian ducts (respond to hormonal stimulus).	(Szotek *et al.*, 2007)
	Endometrial reconstruction in an animal model.	Endometrial reconstitution capability of total endometrial cells under kidney capsule in a mouse xenotransplantation model.	Formation of vascularized endometrial tissue that responds to hormonal oscillations (proliferation, differentiation and menstruation).	(Masuda *et al.*, 2007)
	Clonogenicity and differentiation	To assess the clonogenicity, differentiation potential and	Higher clonogenic activity of $CD146^{+}PDGF\text{-}R\beta^{+}$ cells in comparison to $CD146^{-}PDGF\text{-}R\beta^{-}$ cells. Differentiation into	(Schwab and Gargett, 2007)

	of human stromal SSCs.	location of $CD146^{+}PDGF\text{-}R\beta^{+}$ stromal cells.	adipogenic, chondrogenic, osteogenic and myogenic lineages, expression of SSC markers and perivascular location.	
2010	Clonogenicity and endometrial reconstruction in animal model.	Clonogenicity and endometrial reconstitution potential of endometrial SP cells under kidney capsule in a mouse xenotransplantation model.	Endometrial-like tissue formation with organized glands and vasculature after transplantation of SP cells (more in the proliferative phase of the menstrual cycle) in comparison to NSP cells (specially resulting in stromal cells *in vivo*). Low efficiency of endometrial reconstruction (8%) probably due to the need of a niche or niche-like cells. SP needs NSP support to have clonal capacity, so it seems to indicate the important interaction among different cell types. Perivascular location of these endometrial SSCs present in both layers: basalis and functionalis.	(Masuda *et al.*, 2010)
	Clonogenicity, differentiation and endometrial reconstruction of endometrial SP (SSCs) in animal model.	Clonogenicity, differentiation potential towards osteogenic and adipogenic lineages of SP cells, and endometrial reconstitution potential in a xenotransplantation mouse model.	Stromal and epithelial SP populations have clonogenic capability under hypoxic conditions in comparison to NSP cells (not significant in the epithelial fraction). Successful differentiation to adipocytes and preosteoblasts, as well as endometrial reconstruction after the injection of stromal SP cells subcutaneously.	(Cervelló *et al.*, 2010)
2011	Differentiation and endometrial reconstruction in animal model.	SP cell lines derivation, differentiation to other cell types and endometrial reconstitution under kidney capsule in a mouse xenotransplantation model.	Derivation of cell lines from epithelial and stromal SP cells under hypoxic conditions by cloning efficiency, genetically stable up to 15 passages and with expression of undifferentiation and mesenchymal-origin markers. Differentiation potential to adipocytes, osteocytes, and formation of endometrial-like tissue when transplanted under kidney capsule.	(Cervelló, Mas, *et al.*, 2011)
2012	Endometrial reconstruction in animal model.	To stablish an accurate animal xenotransplantation model for endometrial reconstruction	An optimal endometrial reconstitution (100%) was achieved with the supplementation of SP cells with endometrial total cells, with the formation of epithelial, stromal and endothelial cells. The	(Miyazaki *et al.*, 2012)

		under kidney capsule.	percentage of endothelial cells was higher in SP than in NSP cells.	
	Clonogenicity, differentiation and endometrial reconstruction in animal model.	To assess the purification potential of W5C5 marker for endometrial SSCs.	$W5C5^+$ cells have more clonal activity and more differentiation potential to adipocytes, chondrocytes, osteocytes, myocytes and endothelial cells. Ability to create stromal tissue *in vivo.*	(Masuda *et al.*, 2012)
2013	Differentiation of endometrial SSCs to myocytes.	Obtaining of smooth muscle cells starting from endometrial SSCs.	Differentiation of endometrial SSCs (positive for CD146, CD105 and CD90) to smooth muscle cells in hydrogel matrix, with medium supplemented with myogenic growth factors.	(Shoae-Hassani *et al.*, 2013)

Table 1. Summary of important studies that support the existence of endometrial SSCs. *Year of publication, type of evidence that shows the presence of SSCs in endometrium, objectives, relevant results and references. CD146, CD105 y CD90: cluster of differentiation 146, 105 and 90; c-KIT: tyrosine-protein kinase Kit or CD117; EGF: epidermal growth factor; NSP: non-Side Population; Oct-4: octamer-binding transcription factor 4; PDGF-BB: platelet-derived growth factor bb; SP: Side Population; SSC: somatic stem cell; TGFα: transforming growth factor alpha.*

3.2. Human endometrial stem cell markers

As mentioned above, endometrial SSCs were first suggested in humans in 2004 (Chan, Schwab and Gargett, 2004; Taylor, 2004), and are probably located in the basal layer of the endometrium (Schwab, Chan y Gargett, 2005; Gargett, 2007; Cervelló and Simón, 2009; Cervelló *et al.*, 2010; Maruyama *et al.*, 2010; Gargett, Schwab and Deane, 2016). However, their characterization, identification and isolation remain difficult due to the lack of validated, specific markers. Indeed, based on the expression of different markers there are discrepancies in the identification of endometrial SSCs. Nevertheless, markers defining the properties of bona fide stem cells could only be identified by *in vivo* assays that functionally define stem cells.

3.2.1. *Specific stem cell markers*

Initial studies indicated that human endometrial cells positive for both **CD146** and platelet-derived growth factor-receptor β (**PDGF-Rβ,** also called CD140b) show high clonogenicity in relation to double-negative cells, having the potential to differentiate into adipogenic, chondrogenic, osteogenic and myogenic lineages, expressing SSC markers as well as having a perivascular location. CD146 is the cluster of differentiation 146, a member of the immunoglobulin superfamily and a cell adhesion

molecule used as a marker for endothelial cells because it is a laminin receptor located in the vascular wall. CD146 is also expressed in human activated T cells, endothelial progenitors, mesenchymal stem cells (MSCs), endothelium of blood vessels and smooth muscle. PDGF-Rβ is essential for the development of the vasculature stabilizing the newly formed vessels by recruiting pericytes. While both markers are also expressed in basalis and functionalis, CD146 is located in endothelial cells around blood vessels and PDGF-Rβ is only present in stromal cells (Schwab and Gargett, 2007). In addition, $CD146^+$ cells also positive for **W5C5** (sushi domain containing 2 or SUSD2) have greater differentiation potential than $CD146^+PDGF\text{-}R\beta^+$ cells. This marker is a type I transmembrane protein that takes part in cell migration and adhesion. Moreover, $W5C5^+$ cells have more clonogenicity than the total endometrial fraction or the negative fraction for this marker, a similar multipotency than $PDGF\text{-}R\beta^+CD146^+$ cells and have the potential to generate stromal tissue *in vivo* when isolated, cultured and transplanted in an animal model (Masuda *et al.*, 2012) (Figure 14). The mentioned properties suggest that $W5C5^+$ cells might contribute to stromal vascular regeneration (Ulrich *et al.*, 2014) supporting the idea that this marker could be considered as a possible endometrial stem cell marker.

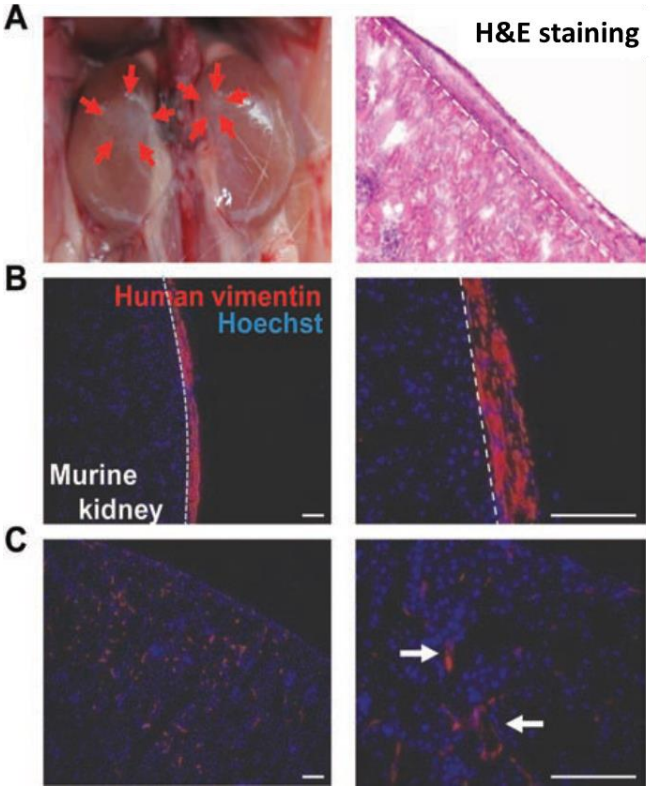

Figure 14. Reconstruction of stromal tissue after transplantation of W5C5⁺ cells. *Isolated cells were cultured and injected under the kidney capsule of mice.* ***(A)*** *Visible structures in the kidneys and hematoxilin-eosin (H&E) staining of the thickened region are represented, as well as* ***(B)*** *human vimentin-expressing cells in the capsule (stromal type), with cells dyed with Hoechst (blue), and in* ***(C)*** *other cells located inside the kidney (invasive type). Figure adapted from Masuda et al., 2012.*

Recently, the group of Dr. Cervelló confirmed the presence of the universal stem cell marker leucine-rich repeat–containing heterotrimeric guanine nucleotide–binding protein–coupled receptor 5 (**LGR5**) positive cells at perivascular regions of the lower

functionalis in both epithelial and stromal compartments in the human endometrium (Cervelló *et al.*, 2017). LGR5 is a membrane receptor that uses the Wnt signaling pathway when activated, and it is expressed in actively dividing and long living cells with multipotent features. Moreover, $LGR5^+$ cells are present in the SSC niche of adult tissues such as the small intestine, colon (Barker *et al.*, 2007, 2008), hair follicles (Morris *et al.*, 2004; Barker *et al.*, 2008), kidney (Barker *et al.*, 2012) or mammary glands (De Visser *et al.*, 2012) and in cancer as well (Wang, Jiang and Fan, 2015). Therefore, $LGR5^+$ cells are thought to be tissue-resident cells or transient recruited macrophages contributing to components of the endometrial stem cell niche in a transient manner (Gil-Sanchis *et al.*, 2013; Cervelló *et al.*, 2017). In another study, stromal LGR5-expressing cells were also identified in the endometrium from women with endometriosis, which co-localized with epithelial markers and the expression pattern was different according to the grade of the disease (Vallvé-Juanico *et al.*, 2017).

Another proposed stem cell marker is the epithelial cell adhesion molecule (**EPCAM**, or CD326). Positive cells for this marker are involved in cell signaling, migration, proliferation and differentiation. Moreover, they have oncogenic potential, and are also expressed in epithelial tissues and several types of epithelial-derived cancers (such as colon cancer), progenitor cells and stem cells. It has been identified as an epithelial marker (Fayazi, Salehnia and Ziaei, 2016) associated with endometrial progenitor cells due to their clonal efficiency and their capability to create gland-like structures in three dimensional cultures (Gargett *et al.*, 2009).

The stage-specific embryonic antigen 1 (**SSEA1**, or CD15) is an adhesion molecule involved in phagocytosis and chemotaxis processes, associated with cell adhesion, migration and differentiation, and is expressed in pluripotent stem cells. This molecule has been postulated as another surface marker due to the fact that the SSEA1-expressing population is higher in epithelial cells in the basalis layer from human endometrium, which gives rise to the functionalis layer (Valentijn *et al.*, 2013).

Musashi-1 and Sox-2 markers have also been related to endometrial SSCs. **Musashi-1** (MS1) is an RNA-binding protein associated with maintenance and asymmetric cell division of stem cells in the nervous system, playing an essential role in cell self-renewal because of the increase in Notch signaling (Okano *et al.*, 2005). It has been postulated as a novel SSC marker both in mouse and in humans in several tissues because of the functions in which it is involved (balance between self-renewal and differentiation). MS1-positive cells have been localized in the mouse central nervous

system (Kaneko *et al.*, 2000), in mouse and human hair follicles (known for its long-standing stem cell niche) (Sugiyama-Nakagiri *et al.*, 2006) and in other human organs and tissues, such as the stomach (Akasaka *et al.*, 2005) and mammary glands (Clarke *et al.*, 2005). These MS1-positive cells also have a high proliferation rate similar those of stem cells', and co-localize with the stem cell markers Notch-1 and telomerase within the human endometrium (Götte *et al.*, 2008). In 2008, the expression of MS1 was observed for the first time in human endometrium in isolated stromal and epithelial cells and in stromal cell aggregates. The small MS1-positive population is enriched at the basalis layer and also during the proliferative phase of the menstrual cycle, which seems to point to their implication during endometrial regeneration (Götte *et al.*, 2008). Additionally, the proportion of MS1-expressing cells is higher in endometrial carcinoma, endometriosis and endometrial hyperplasia compared to healthy endometrium (Götte *et al.*, 2008; Lu *et al.*, 2011), and MS1 was also described as a cancer stem cell marker (Götte *et al.*, 2008; Katz *et al.*, 2014; Ma *et al.*, 2015; Wang, Jiang and Fan, 2015).

Sox-2 is a transcription factor also called "sex determining region Y-box 2" (SRY) and is implied in the maintenance of pluripotency in SSCs and neural stem cells. Sox-2 positive cells regulate both proliferation and differentiation of SSCs, specially endometrial stem cells, and is one of the factors implied in cell reprogramming to create iPSCs (as well as c-Myc, Oct-4 and Klf-4) (Takahashi *et al.*, 2007). The presence of Sox-2-positive cells in endometrium was first described in 2011 at perivascular location and, although their number in the endometrium is low, it does increase in the proliferative menstrual phase. The presence of Sox-2 in endometriosis was also reported, with a high expression in the diseased endometrium in comparison to a healthy one, which may support the theory of origin of endometriosis based on stem cells (Götte *et al.*, 2011).

Table 2 is a summary of all the markers described above, along with the evidence presented to postulate their possible nature as endometrial stem cell markers.

Marker	Evidence	Reference
CD146 - PDGF-Rβ	Higher clonal activity of double positive cells, differentiation to several lineages, perivascular localization and expression in both endometrial layers.	(Schwab and Gargett, 2007)
W5C5	Higher differentiation capability than $CD146^+PDGF\text{-}R\beta^+$ cells, formation of endometrial tissue *in vivo* in animal model.	(Masuda *et al.*, 2012)
LGR5	Expression in both cell types at perivascular regions in the functional layer. $LGR5^+$ cells in several SSC niches and in endometriosis.	(Morris *et al.*, 2004; Barker *et al.*, 2007, 2008, 2012; De Visser *et al.*, 2012; Gil-Sanchis *et al.*, 2013; Wang, Jiang and Fan, 2015; Cervelló *et al.*, 2017; Vallvé-Juanico *et al.*, 2017)
EPCAM	Expression in epithelial tissues, progenitor cells and stem cells.	(Gargett *et al.*, 2009; Fayazi, Salehnia and Ziaei, 2016)
SSEA1	Expression in pluripotent stem cells and in the epithelium of the endometrial basal layer.	(Valentijn *et al.*, 2013)
Musashi-1	Expression in several adult tissues and endometrium. High proliferation and co-localization with SSC markers in human endometrium (epithelium and stroma), especially in the basal layer and in the proliferative menstrual phase. Higher expression in endometrial pathologies.	(Kaneko *et al.*, 2000; Akasaka *et al.*, 2005; Clarke *et al.*, 2005; Okano *et al.*, 2005; Sugiyama-Nakagiri *et al.*, 2006; Götte *et al.*, 2008; Lu *et al.*, 2011; Katz *et al.*, 2014; Ma *et al.*, 2015; Wang, Jiang and Fan, 2015)
Sox-2	Presence of $Sox\text{-}2^+$ cells in endometriosis and around blood vessels in healthy endometrium, especially in the proliferative phase.	(Takahashi *et al.*, 2007; Götte *et al.*, 2011)

Table 2. Summary of surface markers investigated in relation to endometrial SSCs. *The evidence that leads to postulate the nature of stem cells of the cells expressing the markers CD146, PDGF-Rβ, W5C5, LGR5, EPCAM, SSEA1, Musashi-1 and Sox-2 is shown, as well as the reference in each case.*

3.2.2. Side Population approach

In the absence of specific stem cell markers that allows for the identification and isolation of endometrial SSCs, the Side Population or SP method has been used to study a population that appears to harbor a SSC population in several tissues (Zhou *et al.*, 2001). Dr. Goodell provided the first evidence for the existence of a stem cell-enriched population in mouse hematopoietic cells in 1996 (Goodell *et al.*, 1996). The SP is characterized by its low level of red and blue fluorescence when cells are studied in a flow cytometer after being incubated with the DNA-binding dye Hoechst 33342 (Figure 15). The SP approach (explained in detail in the section "*1.2. Side Population in eutopic endometrium from healthy women and women with endometriosis using flow cytometry"* from the B project in materials and methods section) is based on the capability of SP cells to extrude the Hoechst dye through an ABCG2 channel called BCRP1, which is a property of stem cells. Verapamil is a drug used to block the ABC-family channels, so it is used as the negative control of this technique because cells with verapamil will close their channels (if there were) and they will not be able to extrude the Hoechst, resulting in a lack of fluorescence (and the tail signature of SP cells).

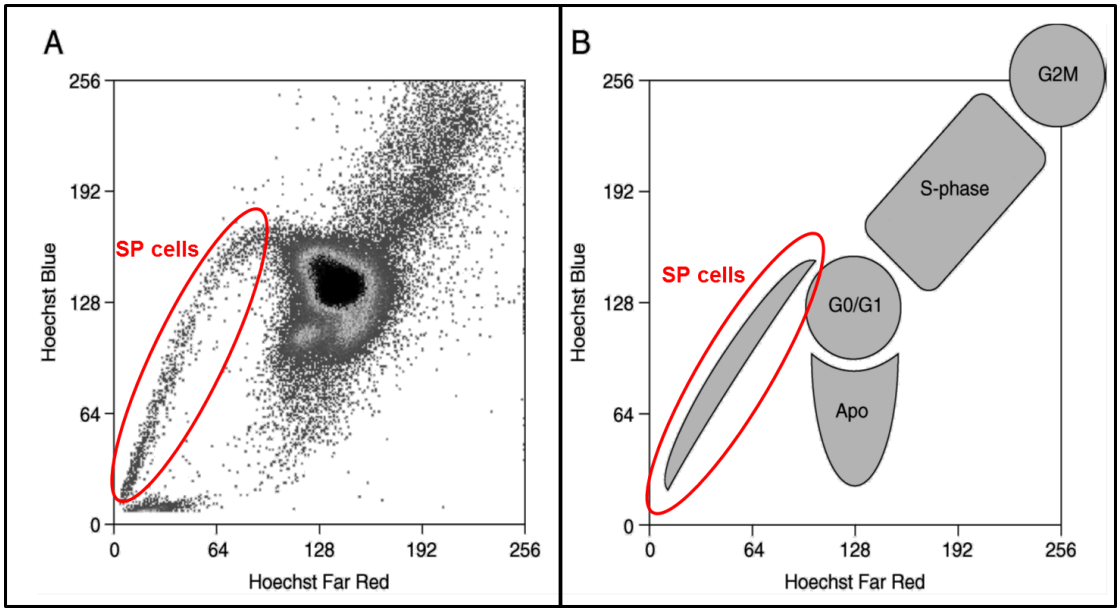

Figure 15. Side Population cells visualized by flow cytometry. (A) *Flow cytometry plot for a cell population after treatment with Hoechst 33342.* ***(B)*** *Diagram of the cell populations obtained in the A plot, with the typical tail shape characteristic for the Side Population (SP). Apo: apoptotic cells; G0/G1: no-division phase, S-Phase: DNA synthesis phase; G2M: cell division phase. Figure adapted from Petriz, 2007.*

The SP phenotype associates a heterogeneous SSCs-containing population present in many adult tissues, such as skin (Larderet *et al.*, 2006), myometrium (Ono *et al.*,

2007), lung (Martin, 2008), dental pulp (Iohara *et al.*, 2008) and heart (Unno, Jain and Liao, 2012), as well as the endometrium (Kato *et al.*, 2007; Cervelló *et al.*, 2008, 2010, 2012; Tsuji *et al.*, 2008; Maruyama *et al.*, 2010; Masuda *et al.*, 2010; Cervelló, Mas, *et al.*, 2011). In the endometrium the SP represents a small percentage of the total population that fluctuates between 1.68-2.7% in the epithelial fraction and 0.4-3.1% in the stromal fraction (Cervelló *et al.*, 2010; Masuda *et al.*, 2010). The SP is enriched in the early proliferative phase, both in the basal and in the functional layers of the endometrium, and located mainly at perivascular regions, which have been previously described as a possible SSC residence in other tissues. Moreover, SP are positive for the epithelial marker PECAM1 (platelet endothelial cell adhesion molecule 1) that seems to point to a possible role for them as endothelial progenitor cells with an important function in angiogenesis (Masuda *et al.*, 2010). The SP population has been studied by several experts over many years (Kato *et al.*, 2007) (Table 1). The investigation from Dr. Cervelló identified and isolated endometrial SSCs (Cervelló and Simón, 2009; Cervelló *et al.*, 2010) and derived several SP cell lines based on cloning efficiency under hypoxic conditions and with Hoechst 33342 methodology (Cervelló, Mas, *et al.*, 2011). More importantly, these cells (fresh or cultured) can reconstitute endometrial tissue *in vivo* when transplanted under the kidney capsule or subcutaneously in immunocompromised mice (Cervelló *et al.*, 2010; Masuda *et al.*, 2010; Cervelló, Mas, *et al.*, 2011) (Figure 13). However, endometrial reconstruction capability remains low, likely due to the lack of an appropriate niche or supporting cells, which is essential for the development of stem cells (Cervelló *et al.*, 2010; Masuda *et al.*, 2010). This hypothesis was confirmed by injecting these putative endometrial SSCs along with endometrial cells to mimic the uterine microenvironment (Miyazaki *et al.*, 2012), increasing the transplant efficiency that way. However, this SP is an enrichment of SSCs, and probably TA cells too, which makes a specific purification of endometrial SSCs (only possible with defined markers) necessary.

3.3. The origin of endometrial somatic stem cells

As previously described, SSCs have been identified in many human adult tissues and in several ways: by the SP technique, by clonogenicity, by the expression of undifferentiation markers, by the differentiation potential to different cell types and by assessing their self-renewal capability. This made, in some cases, defining the stem cell niche and even surface markers possible, which allowed their study in detail as happened with hematopoietic stem cells for example. However, the lack of validated and specific stem cell markers in some tissues, like the endometrium, complicated the determination of the nature of these SSCs and their identification. There may only be

one type of SSC present in the stem cell niche (tissue-specific endogenous stem cells, residing inside the niche) (Figure 16A), or come from an exogenous contribution with another cell type from a different tissue. Here we mainly refer to BMDSCs, which are able to regenerate not only blood cells, but also to migrate to a different organ and participate in its homeostasis, as is the case in muscle, brain, liver, heart and vascular endothelium (Figure 16B) (Blau, Brazelton and Weimann, 2001). So, it is possible that there are two different sources of stem cells that contribute to the endometrial regeneration, and therefore the SSC population that we can find in the endometrium is formed by a set of cell types and not only by endometrial resident SSCs in the endometrial stem cell niche.

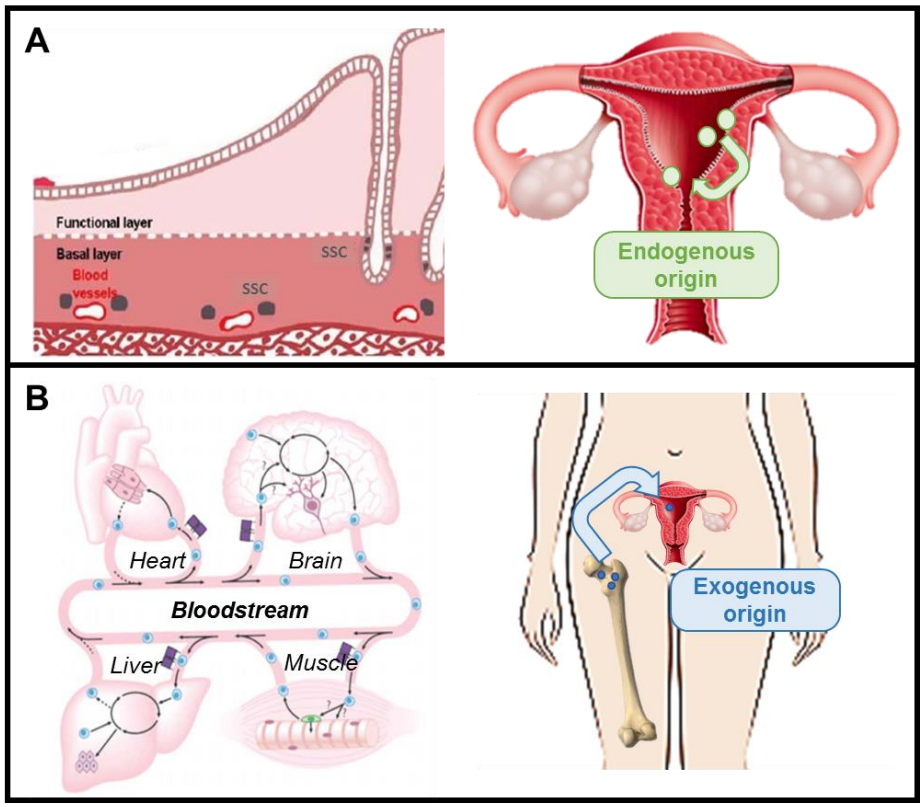

Figure 16. Possible contributions to the endometrial niche: sources of SSCs. (A) *Resident stem cells inside the niche would constitute the endogenous source. The putative location is represented in the left picture, adapted from Cervelló, Mirantes, et al., 2011.* ***(B)*** *The exogenous source would harbor cells migrating from the bone marrow towards the endometrium when there is damage or injury, as it happens in other tissues such as heart, brain, liver or muscle. Left image adapted from Blau, Brazelton and Weimann, 2001. SSC: somatic stem cell.*

3.3.1. *Endogenous source of stem cells: resident stem cells in the human endometrium*

It is thought that the endogenous source of stem cells in the endometrial niche has a mesenchymal origin, this is because endometrial SSCs are positive for CD90 mesenchymal stem cell marker and negative for CD34 and CD45 hematopoietic

markers (Cervelló *et al.*, 2010). These SSCs are able to differentiate *in vitro* into different cell types of the mesodermal lineage too, such as adipocytes, osteocytes (Cervelló *et al.*, 2010; Cervelló, Mas, *et al.*, 2011) or myocytes (Shoae-Hassani *et al.*, 2013). Some experts think that endometrial SSCs could be fetal remnants, residual fetal epithelial or mesenchymal cells inside the human endometrium that are still undifferentiated in adulthood (Snyder and Loring, 2005). During the embryo development, the uterus is derived from the Müllerian duct (that also gives rise to the fallopian tubes, cervix and vagina) and is created from the coelom epithelium (mesoderm origin). In that way, cells belonging to the Müllerian ducts have a great differentiation potential to create mesoderm-derived cell types, like epithelial, connective and muscular tissue. Some cells from the adult human endometrium could retain these differentiation capabilities giving rise to these endometrial stem cells residing in the human niche. These niche-resident SSCs would regenerate the endometrium each menstrual cycle, but would also have the potential to create some endometrial diseases such as endometriosis when they receive certain hormonal stimuli (Lu *et al.*, 2011). These cells could be identified by specific markers as described along the previous section "3.2.1. *Specific stem cell markers"* as well as by the SP technique introduced in *"3.2.2. Side Population approach".* The above-mentioned markers and populations would allow to identify resident stem cells inside the niche as well as to locate the endometrial niche, which is not detected as of yet.

3.3.2. *Exogenous source: bone marrow-derived stem cells*

Another possible explanation involves the contribution of the bone marrow, with BMDSCs migrating and reaching spatially distant tissues via blood, remaining undifferentiated or differentiating into the cell type of the target tissue or organ. These BMDSCs are composed by cells of different origins: hematopoietic stem cells, stromal cells, SP cells or progenitor somatic cells (multipotent). This contribution has been demonstrated in several tissues in both animal and humans models, for example in smooth muscle (Ferrari *et al.*, 1998), central nervous system (Brazelton *et al.*, 2000; Mezey *et al.*, 2000), heart (Jackson *et al.*, 2001; Orlic *et al.*, 2001), and liver (Petersen *et al.*, 1999; Theise *et al.*, 2000) (Figure 16C). The opposite migration of SSCs, for example with muscle stem cells migrating back to the bloodstream, has also been observed. This fact indicates a greater potential than expected because SSCs could live outside their niche and contribute to other tissues. Once arrived at the new location, they can remain undifferentiated as stem cells or differentiate themselves to another cell type both in presence and in absence of tissue damage (Blau, Brazelton and Weimann, 2001).

In addition, the expression of the hematopoietic marker CD34 was observed in many endometrial stromal SSCs (Blau, Brazelton and Weimann, 2001), and Dr. Taylor assessed the endometrial contribution of BMDSCs in 2004 after a bone marrow transplantation both in humans (Taylor, 2004) and mice (Du and Taylor, 2007). Afterwards, several groups studied the endometrial regenerative potential of BMDSCs after the same procedure in humans (Mints *et al.*, 2008; Ikoma *et al.*, 2009; Cervelló *et al.*, 2012) and in an animal model of Asherman's syndrome (Alawadhi *et al.*, 2014). Similarly, the contribution of BMDSCs moving to the endometrium when there is tissue damage has been tested in humans (Cervelló *et al.*, 2012; Gil-Sanchis *et al.*, 2015).

Recently, novel studies were developed to show the regenerative effects of BMDSCs in murine models and in patients with endometrial pathologies (AS and EA) (Cervelló *et al.*, 2015; Santamaria *et al.*, 2016). In humans, after the intrauterine transplantation of BMDSCs, mainly $CD133^+$ hematopoietic progenitors, endometrial neovascularization was observed and four patients even got pregnant (Santamaria *et al.*, 2016). CD133 is a marker expressed in immature hematopoietic and progenitor cells with high proliferative activity, and in circulating cells with endothelial regenerative capacity as well. It has been demonstrated that these cells have regenerative features with not only hematological applications, and that it is safe to use them in human diseases (Cervelló *et al.*, 2015). In parallel, the same approach was carried out in an animal model, by cell therapy using $CD133^+$ BMDSCs from the previous patients (with AS and/or EA) labelled before injection (Cervelló *et al.*, 2015). Engrafted cells remained at low number in the endometrium and were located around blood vessels, possibly inducing neoangiogenesis. The endometrial quality improved in spite of the low amount of engrafted cells in the endometrium (similar results were obtained by Dr. Gnecchi in cardiac regeneration (Gnecchi *et al.*, 2008)). Consequently, injected BMDSCs would be probably involved in the proliferation of neighbor cells through paracrine mechanisms promoting tissue repair (Cervelló *et al.*, 2015). The release mechanism of cytokines or molecules that act in a paracrine manner has been previously studied in various works (explained in detail in the section "2.2.2. *Mechanisms of stem cell regulation".* In these studies, it was shown that cell therapy with BMDSCs promotes the neovascularization, inhibits apoptosis, has anti-inflammatory effects and enhances the proliferation and differentiation of endogenous cells. Not only the direct differentiation of transplanted cells would lead to a therapeutic improvement, but also the release of soluble molecules from stem cells (Burdon *et al.*, 2011).

To conclude, regarding the origin of SSCs, there are two main sources coexisting to contribute to endometrial regeneration: an endogenous source, with stem cells permanently residing in the endometrial niche, and an exogenous source with BMDSCs that would migrate to the endometrium when the tissue is damaged. To be able to differentiate both populations of stem cells, screening of specific SSC markers would allow us to locate the endogenous stem cell pool inside the tissue and, therefore, to locate the endometrial stem cell niche.

II. HYPOTHESIS

"No debemos dejar de explorar, y al final de nuestras exploraciones llegaremos al lugar del que partimos, y lo conoceremos por primera vez"

Thomas Stearns Eliot

II. HYPOTHESIS

The hypothesis of this study is the existence of two stem cell sources (endogenous and exogenous) in the human endometrium.

For the validation of this statement, we aimed to test (I) postulated human somatic stem cell specific markers contributing to endometrial reconstruction in a murine model as endogenous reservoir; and (II) the role of regenerative factors released by bone marrow-derived stem cells as exogenous origin in women with endometrial pathologies.

III. OBJECTIVES

"La ciencia no es sino una perversión de sí misma a menos que tenga como objetivo final el mejoramiento de la humanidad"

Nikola Tesla

III. OBJECTIVES

1. Main objective

The main objective of this thesis is the study of endogenous and exogenous sources of endometrial somatic stem cells in relation to their potential to regenerate and repair the human endometrial tissue.

2. Specific objectives

2.1. To identify specific endometrial somatic stem cell markers and to isolate cells for selected markers W5C5 and ICAM1, as well as endometrial Side Population.

2.2. To assess the endometrial reconstitution potential of previously described markers using a xenotransplantation assay in immunodeficient NOD-SCID mice.

2.3. To study the stem cell properties of injected human cells by assessing the presence of stem cell marker Musashi-1 in the human endometrial-like tissue formed in the animal model.

2.4. To correlate the presence of W5C5, ICAM1 and Side Population cells with the stage of endometriosis in eutopic endometrium.

2.5. To identify secreted factors, released after autologous bone marrow-derived stem cell therapy, in patients with Asherman's syndrome and/or endometrial atrophy.

IV. EXPERIMENTAL DESIGN

"Tengo una gran fe en los tontos, autoconfianza le llaman mis amigos"

Edgar Allan Poe

IV. EXPERIMENTAL DESIGN

Identification and isolation of somatic stem cells in human endometrium with potential stem cell markers. Proof of concept in animal models

To achieve the formation of endometrial-like tissue, 500,000 cells of several cell subsets were injected under the kidney capsule of ovariectomized and immunodefficient female mice. Human endometrial cells positive for W5C5 and ICAM1, as well as SP cell lines, were tested in the animal models. During the experiment, these mice were hormonally treated with estrogens and progesterone until sacrificing 60 days after the xenotransplantation. Finally, the analysis of the newly formed tissue was carried out by immunofluorescence for the following three endometrial markers: vimentin, progesterone receptor and cytokeratin. In addition, immunohistochemistry for the well-established endometrial stem cell marker Mushashi-1 and for the proliferative marker Ki67 were carried out.

Analysis of somatic stem cell markers in human eutopic endometrium from endometriosis samples

To analyze the possible stem cell-origin of endometriosis, and as another proof of stemness of described markers, samples from healthy women and from women with endometriosis were collected and analyzed by flow cytometry. The percentages of positive populations for W5C5, ICAM1 and SP were obtained and compared in order to stablish a relation between stem cell markers and the stage of the disease.

Paracrine factors secreted by bone marrow-derived stem cells involved in endometrial regeneration

To identify factors secreted by bone marrow-derived stem cells, we started with paraffinized samples from a previous study. We used human biopsies from patients with AS and/or EA treated with $CD133^{+}$ BMDSCs, which were chosen because of the neovascularization and the tissue improvement observed after the cell therapy. The differential gene expression before and after cell therapy was conducted using RT-qPCR arrays. After the analysis, upregulated and downregulated genes were identified, as well as the pathway most represented by the studied genes. Finally, one of the most upregulated genes was validated by immunofluorescence in the studied human tissues.

V. MATERIALS AND METHODS

"La inacción engendra la duda y el miedo. La acción genera confianza y coraje. Si quieres vencer el miedo, no te sientes en casa y pienses en ello. Sal y ponte a trabajar"

Dale Carnegie

V. MATERIALS AND METHODS

A. Identification and isolation of somatic stem cells in human endometrium with different markers. Proof of concept in animal models

1. Identification of specific somatic stem cell markers in human endometrium

1.1. Ethical statements

This study was approved by Instituto Universitario-IVI Institutional review board and ethics committee (University of Valencia, Spain) (1203-C-098-IC-F). Procedures performed on animals were also approved by the review boards of the University of Valencia (animal ethical committee 2015/VSC/PEA/00073, Annexe I). All the women, both in Spain and Germany, provided written informed consent before the endometrial biopsy collection.

1.2. Collection of human endometrial samples

Human endometrial cells were obtained from endometrial biopsies using a biopsy catheter (Pipelle; Gynetics Medical Products, Hamont Achel, Berlgium) under sterile conditions. Biopsies obtained in Spain (approximately 50 biopsies) were collected from fertile oocytes donors in the IVI Valencia clinic. The extraction was carried out during the proliferative phase of the stimulated menstrual cycle few hours before ovulation, when oocytes were retrieved. Samples obtained in Germany (17 biopsies) were obtained in the IVF laboratory from the University Hospital of Münster (Universitätsklinikum Münster, UKM) during the proliferative or secretory phase of the menstrual cycle. Only those patients with previous laparoscopy were used for the project, having therefore confirmed the presence or absence of endometriosis as well as the stage of the disease (if present).

All the samples were placed immediately in Dulbecco's modified eagle medium (DMEM, Sigma-Aldrich, Spain) with antibiotics and antimycotics (0.1% penicillin/streptomycin (P/S, Thermo Fisher Scientifc, Spain) and 0.1% Amphotericin B (Thermo Fisher Scientifc, Spain)) after their collection under sterile conditions. After transport and storage at 4ºC, the sample was processed and all the procedures were conducted in aseptic conditions in a laminar flow hood inside the cell culture room. The sterility of all the procedures was assured by using sterile material and the appropriate clothing for an aseptic cell culture room (lab coat, gloves, lab cap, shoe covers and even mask if necessary) to avoid contamination.

1.3. Epithelial and stromal cell isolation from human endometrium

The biopsies were subjected to an epithelial and stromal cell separation protocol in order to obtain viable single cell suspensions. For this purpose, biopsies were first washed with phosphate buffered saline (PBS, Sigma-Aldrich, Spain) in order to remove mucus and blood traces (which is typical for this type of samples), and stromal and epithelial fragments were isolated according to a stablished protocol described by Dr. Simón in 1993. Briefly described, endometrial biopsies were disaggregated by mechanical and enzymatic procedures to obtain suitable cell suspensions for our protocol. After receiving the tissue specimens they were mechanically dissected with scalpels (fragments smaller than 1 mm^3) (Figure 17), followed by type IA collagenase (ColIA, Sigma-Aldrich, Spain) treatment overnight (o/n) in DMEM at a final concentration of 10 mg/mL. This enzyme disaggregates the tissue by hydrolyzing/breaking the collagen that keeps cells together (intercellular junctions), releasing that way small fragments and isolated cells after incubation.

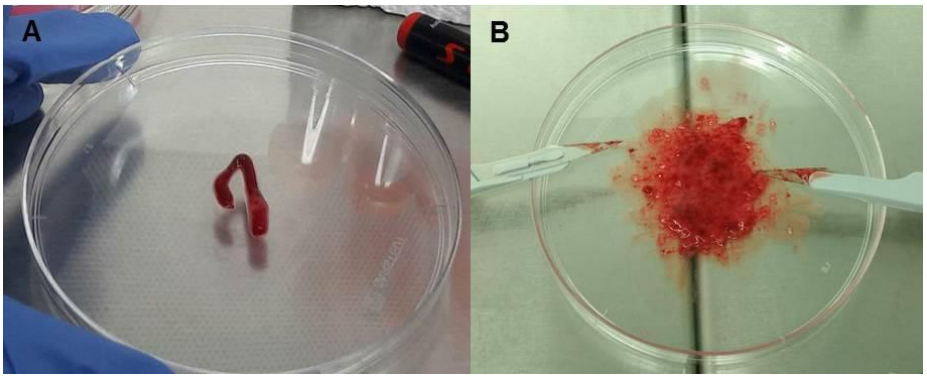

Figure 17. First steps for isolation of single cells from human endometrial biopsy. (A) *Biopsy obtained with a catheter after removing the blood and mucus.* ***(B)*** *Very small fragments obtained after mechanical digestion with scalpels.*

The following day, stromal and epithelial cells, as well as glands, were separated based on a size-basis protocol using gravity sedimentation, and both cell types were filtrated in order to eliminate mucus and to obtain a clean cell fraction (Simón *et al.*, 1993) (Figure 18). To separate both cell types, the sample was first vigorously agitated after being incubated o/n with ColIA, and stored vertically for 10 min to allow the glands and epithelial cells to precipitate, leaving the stromal cells in suspension. The procedure was repeated twice with fresh DMEM, filtrating the stromal suspension in 50μm–pore diameter strainers (Partec, Celltrics, Germany) to remove all the mucus and non-disaggregated tissue and to obtain a clean stromal cell fraction. The precipitated fraction (epithelial cells and glands) was treated with Tryple Select (Thermo Fisher Scientific, Spain), which is similar to trypsin, with the aim to disaggregate the glands

(trypsinization process). After the disaggregation, a volume of 5 mL DMEM was added and the epithelial cell suspension was filtered in another tube as done with the stromal suspension. After that, stromal and epithelial fractions were centrifuged for 6 min at 600 g and pellets were treated with DNase (Sigma-Aldrich, Spain) to eliminate the DNA released from cell lysis. The DNase activity was stopped by adding more DMEM after a minute more or less. The cells were then treated with erythrocytes lysis buffer (1.5M NH_4Cl, 100 mM $NaHCO_3$ and 1 mM Ethylenediaminetetraacetic (EDTA) pH 7.4) for 5 min to remove red blood cells by hypotonic shock.

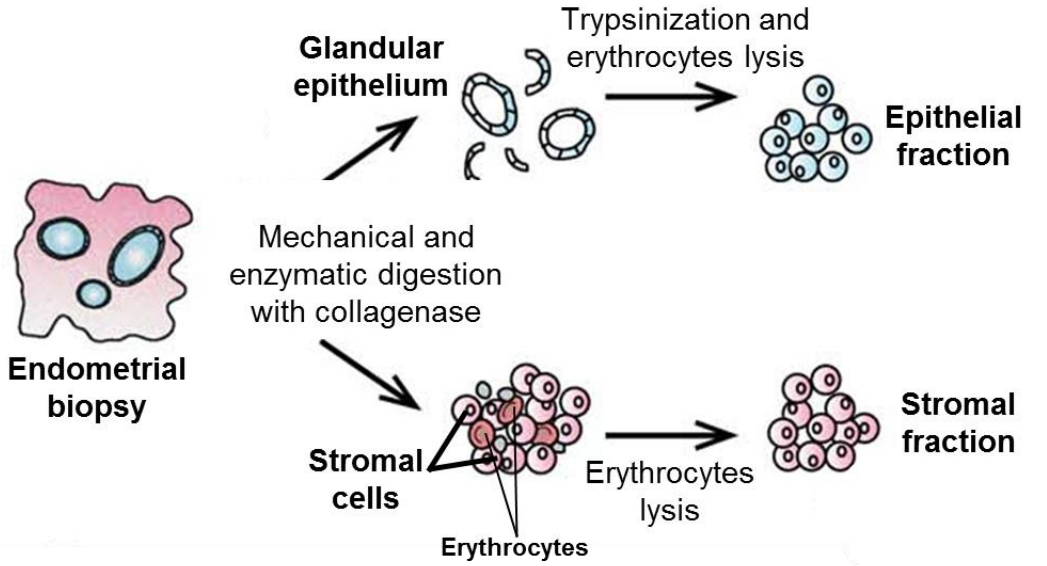

Figure 18. Diagram showing the isolation of stromal and epithelial cells from an endometrial biopsy. *Mechanical digestion was used to obtain very small tissue fragments, and enzymatic digestion and filtration allowed the separation of both cell types (epithelium and stroma), to achieve clean cell fractions after osmotic shock to remove the remaining erythrocytes. Figure modified from Miyazaki et al., 2012.*

After digestion with DNase and the elimination of erythrocytes, both cell suspensions were centrifuged again for 6 min at 600 g. Finally, cells were resuspended in DMEM and counted with Trypan blue staining (Sigma-Aldrich, Spain). Trypan blue is a vital dye used for exclusion staining methods based on the permeabilization of the cell membrane of dead cells. This dye can enter dead cells due to its damaged membrane, staining dead cells in blue while leaving live cells unaffected, which are counted in the Neubauer chamber and taken into account for the cell counting.

1.4. Analysis and subsequent isolation of endometrial cells for specific markers by flow cytometry

1.4.1. Analysis of stem cell markers in epithelial and stromal fractions

To select specific markers for the study, immunocytochemistry (ICC) and flow cytometry (FC) were performed in epithelial (n=4) and stromal (n=6) cell fractions from human endometrium for SSEA1, W5C5, EPCAM, ICAM1, TNFα (tumor necrosis factor alpha), THBS1 (thrombospondin 1) and BCRP1.

ICC is an immunological technique that consists in labelling cells in suspension with an antibody (linked to a fluorochrome) with affinity for one specific surface antigen, resulting in fluorescently labelled positive cells. Afterwards, these cells are analyzed using a flow cytometer. FC is a technology that allows the multiparametric analysis of individual cells in suspension, allowing the study of several characteristics like size and cell complexity, or the expression of surface markers simultaneously. This technique is based on the use of a laser beam that impacts over aligned particles in suspension passing in front of it within the liquid stream, and are classified subsequently in function of the scattered and transmitted light (information regarding duration, intensity and light spectrum) (Figure 19). A system of mirrors and optical filters route these signals to their detectors, which collect the information and converted it to digital signals. There are various types of detectors that can observe different aspects of the cell population:

- When light traverses a cell suspension it will be diffracted, and this diffraction will be detected as a reduction in incident light by a photodetector situated behind the cell stream and in front of the light beam: this is the forward scatter detector. The measurement made from this detector allows the discrimination of cells by size, and its intensity is proportional to the diameter of the cell.

- To study the complexity (granularity) of cells there is another detector located at an angle of 90º respective to the light source: this is the side scatter detector. This detector has the function of collecting the information from the dispersed light (with the same wavelength of the laser) by the cells. The amount of dispersed light will vary in function of the internal complexity and the intracellular structures of cells.

- There are other detectors for wavelengths emitted by the fluorochromes (different wavelengths than emitted by the laser), which detect the fluorescence discerning among different fluorochromes in function of their emission wavelengths.

Forward and side scattered light, as well as fluorescence from labelled cells, is divided into defined wavelengths and organized by different filters and mirrors within the flow cytometer. Fluorochromes are responsible for the fluorescence measured by the cytometer, because they are molecules that emit visible light (longer wavelength, of emission) when a shorter wavelength is applied (for excitation). This data is collected as analog electrical signals that are converted in digital signals and processed by a computer to generate histograms that allows us to observe the results in a representative and easy way.

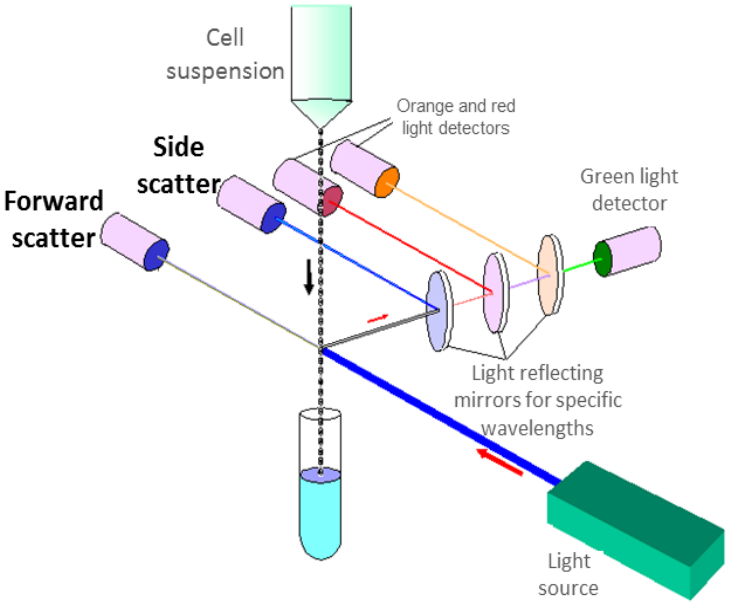

Figure 19. Schematic diagram showing a flow cytometer. *Diagram showing the different detectors present in a flow cytometer, with the goal of collecting the information relative to scattered and transmitted light by the cells. Cells are in a cell suspension (flow indicated with the black arrow) and are exposed (one cell at a time) to the laser beam (direction indicated by red arrows). Cell characteristics are studied through a detector in front of the light source (forward scatter), another one located at an angle of 90º with regard to the light source (side scatter) and a last one that collects the different wavelengths emitted by fluorochromes (after passing through different mirrors). Figure adapted from http://www.cnb.csic.es/index.php/es/.*

For the ICC, after cell separation of both fractions from endometrial biopsies as described in the section "*1.3. Epithelial and stromal cell isolation from human endometrium"*, cells were treated with PBS supplemented with 5% bovine serum albumin (BSA) (50 μL/million cells) for 30 minutes at room temperature (RT) to avoid non-specific binding of primary antibodies to antigens. Cells were then centrifuged for 6 minutes at 600 g and an aliquot was reserved for isotype labelling of each antibody used. Isotype was used as a control to confirm the specific binding of the antibody to the antigen present on the cell surface, allowing the normalization of the results and showing the background signal inherent to the antigen-antibody binding in this assay.

After centrifugation, cell pellets (precipitated cells) were resuspended in PBS-BSA 1% (100 μL/million cells) and antibodies were added and incubated for 45 minutes at 4ºC

in darkness. Antibodies and isotypes used in FC to study the percentage of different markers in human endometrium are detailed in Table 3.

Marker	Antibody	Reference	Volume
EPCAM (CD326)	Alexa Fluor 488 mouse IgG2bk anti-human CD326	324210, BioLegend	5 μL
ICAM1 (CD54)	PE mouse IgG1k anti-human CD54	555511, BD Pharmingen	10 μL
SSEA1 (CD15)	Alexa Fluor 488 mouse IgMk anti-mouse/human CD15	125610, BioLegend	5 μL
W5C5 (SUSD2)	PE mouse IgG1k anti-human SUSD2	327406, BioLegend	5 μL
ABCG2 (CD338)	APC mouse IgG2bk anti-human CD338	332020, BioLegend	10 μL
TNFα	PE mouse IgG1k anti-human TNFα	502909, BioLegend	10 μL
THBS1	FITC rabbit IgG anti-THBS1	ABIN749565, Bioss	1 μg/μL
IgG2bk Isotype	Alexa Fluor 488 mouse IgG2bk isotype	400329, BioLegend	2,5 μL
IgG2bk - APC Isotype	APC mouse IgG2bk isotype	401210, BioLegend	10 μL
IgG1k - PE Isotype	PE mouse IgG1k isotype	556650, BD Pharmingen	10 μL
IgG1k - PE Isotype	PE mouse IgG1k isotype	400114, BioLegend	5 μL
IgMk Isotype	Alexa Fluor 488 mouse IgMk isotype	401617, BioLegend	1 μL

Table 3. Antibodies and isotypes used for the analysis of the selected stem cell markers in human endometrial biopsies by flow cytometry. *The name of the marker, the antibody used, the commercial reference as well as the volume per million cells are provided.*

After labelling, cells were centrifuged for 6 minutes at 600 g and washed with PBS to remove the unbound antibodies, followed by another centrifugation step. Cells were also treated with propidium iodide (PI) to exclude dead cells during the analysis in the flow cytometer. PI is used in FC to assess the cellular viability or the DNA content in a cell cycle analysis, emitting at a wavelength of 620 nm. The principle of this staining is

the same as with Trypan blue: dead cells have an injured membrane that allows the entrance of these dyes while live cells do not, resulting in a different signal in the flow cytometer diagram (cells excluded for cell analysis). For this purpose, cells were treated with 5 µg/mL PI in darkness during 20 minutes or more, and they were then analyzed using a Cytomics FC500 flow cytometer (Beckman-Coulter, CA, USA).

The markers mentioned above were tested in human endometrium for different reasons. Some markers (SSEA1, W5C5 and EPCAM) were selected because of the evidence published by several authors (as described in the introduction, more specifically in section "*3.2.1. Specific stem cell markers"*). Others (ICAM1, TNFα and THBS1) were selected because they were the most highly expressed membrane markers in the SP, these results were obtained with SP arrays in the work of Dr. Cervelló et al. in 2010 (Cervelló *et al.*, 2010). In addition, BCRP1 was selected for being characteristic of stem cells derived from the SP technique (Tsuji *et al.*, 2008) (channel to extrude drugs) described previously in sections "*3.1. Evidences for the existence of endometrial somatic stem cells"* and in "*3.2.2. Side Population approach"*, in the introduction*)*.

We performed a statistical analysis to express the results for the markers studied in this analysis; a graph with error bars (standard deviation) was represented with Microsoft Excel.

1.4.2. *Isolation of W5C5 and ICAM1 positive and negative cells in epithelial and stromal fractions*

After the flow cytometry assay, W5C5 and ICAM1 were selected since the percentage of positive cells for both markers in both endometrial fractions (stromal and epithelial) were consistent with the expected percentage of stem cells in one tissue. Moreover, W5C5 has been previously described as an endometrial stem cell marker (Masuda *et al.*, 2012), and ICAM1 is one of the most expressed membrane markers from the SP (Cervelló *et al.*, 2010).

For isolating positive and negative cell suspensions for both markers, after cell counting, epithelial and stromal cells were joined and non-specific binding sites were blocked as described in the previous section (PBS-BSA 5% (50 µL/million cells) for 30 minutes at RT). Cells were treated with antibodies and isotypes as specified in Table 4, as well as with PI to exclude dead cells.

Marker	Antibody	Reference	Volume
ICAM1 (CD54)	PE mouse IgG1k anti-human CD54	555511, BD Pharmingen	10 µL
W5C5 (SUSD2)	APC mouse IgG1k anti-human SUSD2	327408, Biolegend	2,5 µL
IgG1k – PE Isotype	PE mouse IgG1k isotype	556650, BD Pharmingen	10 µL
IgG1k – APC Isotype	APC mouse IgG1k anti-human CD338	400119, Biolegend	20 µL

Table 4. Antibodies and isotypes used for the isolation of cells for W5C5 and ICAM1 from human endometrial biopsies by FACS. *The name of the marker, the antibody used, the commercial reference as well as the volume per million cells are provided.*

Cell isolation by fluorescence-activated cell sorting (FACS) was performed for selected markers (ICAM1/CD54 and W5C5/SUSD2), from both epithelial and stromal cells mixed before cell sorting (n=37 biopsies), with a MoFlo® (Dako, Denmark, http://www.dako.com) jet-in-air high-speed sorter for final cell separation. FACS is a specific type of flow cytometry based on fluorescence labelling, also being called cell sorter. This type of flow cytometry detects and quantifies fluorescence, and isolates cell populations of interest. Each cell population (according to the fluorochrome) will have a different charge and two electromagnetically charged plates divert these to be separated into one tube or another (Figure 20).

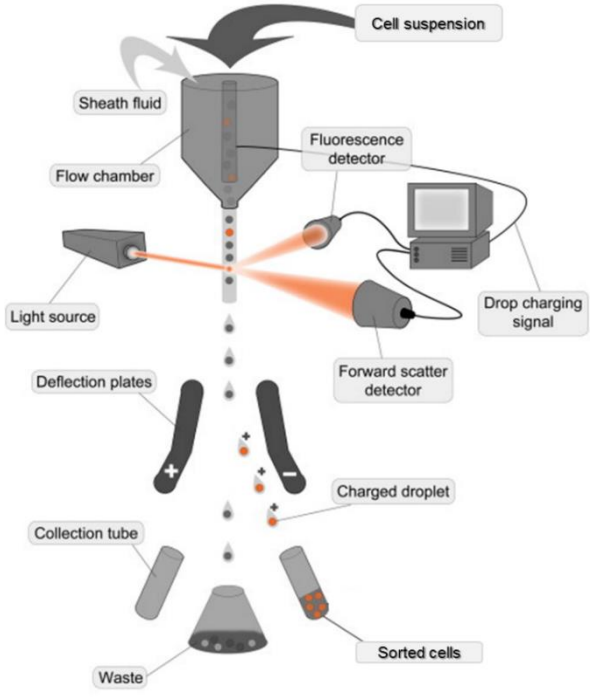

Figure 20. Diagram of a flow cytometer with cell separation capability (FACS). *Each individual cell enters as a single droplet, and this drop is given an electronic charge depending on the fluorescent labelling. Cells are then positively or negatively charged and subsequently separated as independent samples by a magnetic field with deviation plates. For example, a single FITC-stained cell would be given a positive charge, while a single PE-stained cell would be given a negative charge. Different cell populations can be separated at the same time, removing non-labelled cells. Image from* ***Doležel et al.****, 2014.*

After cells were isolated, they were prepared for the animal model as described in the section "*2.1. Experimental design*" (Table 5) with 500,000 cells per condition in a final volume of 30 μL Hank's buffered salt solution (HBSS, Sigma-Aldrich, Spain) supplemented with 2% fetal bovine serum (FBS, Sigma-Aldrich, Spain) and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Thermo Fisher Scientifc, Spain).

1.5. Cell culture of human endometrial Side Population cell lines (ICE6/7)

For this part of the project we attempted to identify and isolate SSCs in human endometrium using different markers, SP-derived cell lines were also tested. The latter are called ICE6 and ICE7, epithelial and stromal stem cell lines respectively, were obtained using Hoechst methodology and cloning efficiency (Cervelló, Mas, *et al.*, 2011) and deposited in the repository Applied Biological Materials Inc. (Clone ICE6&Clone ICE7, Richmond, B.C.. V6V 2G2, Canada) as well as in Fundación IVI facilities. Passages 9-11 for both cell lines were cultured and maintained under hypoxic conditions (2% O_2) until confluence. Then, cells were trypsinized for 7 minutes at 37ºC for each cell line and resuspended in clonal medium (DMEM-F12 (supplemented with glutamine, Thermo Fisher Scientifc, Spain) + 0.1% P/S + 0.1% Amphotericin B + 10% FBS). Finally they were mixed in equal proportions to a final concentration of 500,000 cells to be injected in the animal model in a final volume of 30 μL HBSS supplemented with 2% FBS and 10 mM HEPES.

2. Animal model.

The institutional review board and ethics committees approved all procedures involving animals (animal ethical committee 2015/VSC/PEA/00073), and their manipulation was carried out by veterinarians of the Animal Department of the Medicine Faculty at the University of Valencia.

The demonstration of the endometrial-like tissue formation *in vivo* is the most powerful evidence of the functional features of SSCs, for these female NOD-SCID (non-obese diabetic/severe combined-immunodeficiency) mice were used for the xenotransplantation model in this study. This strain was chosen for having severe deficiencies in the production and function of T cells, B lymphocytes, and in some cases also NK cells, and for being immunologically unable to treat infection or reject transplanted cells or tissues. Because of these characteristics, this model is widely used in science for proof-of-concept experiments.

2.1. Experimental design

Twenty-eight female NOD-SCID mice (strain code 394; NOD.CB17-Prkdc^{scid}/ NCrCrl from Charles River Laboratories, Spain) were ovariectomized at 5-6 weeks of age, and then used for xenotransplantation experiments following the guidelines and hormonal treatment previously described by Dr. Cervelló et al. in 2011 and summarized in Figure 21 (Cervelló, Mas, *et al.*, 2011). Then, mice were anesthetized by isofluorane inhalation at 5% at first, which was maintained at 2% during the surgical intervention. They were also treated with analgesic (buprenorphine 0,08mg/kg each 12h) and anti-inflammatory drugs (meloxicam 0,3mg/kg of mouse each 24h) subcutaneously until 3 days after the intervention. For injection, candidate cells were resuspended in 30 μL HBSS supplemented with 2% FBS and 10 mM HEPES. Veterinarians made a dorsal-horizontal incision looking for the right kidney of each mouse to enable cell injection. Once localized, the kidney was minimally externalized and the single cell suspension was injected (500,000 per transplant in every case) under the kidney capsule with a 27G needle. During the intervention, an 0.36 mg pellet releasing estradiol for a period of 60 days (E2; SE121, 17β-estradiol 0.36 mg/60 days; Innovative Research of America) was administered subcutaneously to each mouse in the back of the neck. Progesterone doses (P4; Dr. Carreras, Hospital 14, Barcelona, Spain) were also administered in two periods of 12 days (1 mg/day) until the sacrifice. During the experiment mice stayed in the specified pathogen-free (SPF) room. 60 days after injection, mice were sacrificed and organs were removed and stored in paraformaldehyde (PFA) for inclusion as described below in *"2.3.1. Murine organs collection and processing of formalin-fixed paraffin-embedded tissue".*

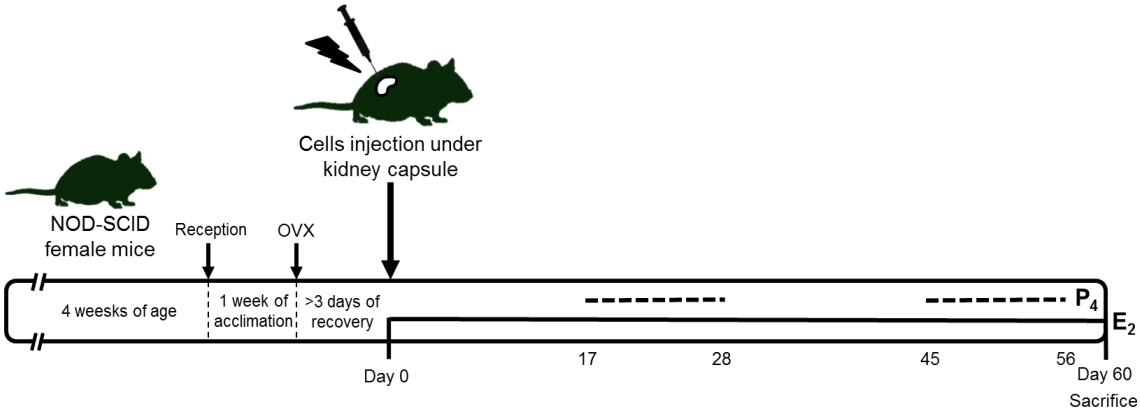

Figure 21. Workflow and experimental design of the animal model. *Receiving of 4-week-old mice, 1 week of acclimation (period allowing animals to stabilize in a new environment and promoting animal welfare), and then the ovariectomy (OVX). The animals need some recovery time (around 3 days) to be injected with cell suspensions under the kidney capsule. Hormonal guidelines of progesterone (P_4) and estradiol (E_2) are specified in the diagram, as well as the sacrifice of animals 60 days after the cell injection. Figure adapted from López-Pérez et al., 2018.*

Cell suspensions (500,000 cells for every condition) were prepared according to W5C5 and ICAM1 markers, with pure positive and pure negative fractions, as well as positive cells supplemented with disaggregated endometrial cells (TF, total endometrial fraction). SP-derived epithelial (ICE6) and stromal (ICE7) cell lines were also used in equal proportion. Control mice with only TF cells and mice injected with rhodamine-labelled cells (discussed in next section "*2.2. Labelling of endometrial human cells using Molday ION Rhodamine"*) were also tested. Three mice were used for each condition, except for the W5C5 pure positive fraction (one mice died during the experiment) and for rhodamine-labelled cells (with 5 mice to test the retention of injected cells at different time points). All the conditions are detailed in Table 5.

Total endometrial fraction (TF)	**ICAM1**	**W5C5**	**SP cell lines**
• 500,000 disaggregated cells from endometrial biopsy (n=3) • 500,000 disaggregated cells from endometrial biopsy labelled with rhodamine B (n=5)	• 500.000 ICAM1^{+} (n=3) • 100.000 ICAM1^{+} + 400.000 TF (n=3) • 500.000 ICAM1^{-} (n=3)	• 500.000 W5C5^{+} (n=3) • 100.000 W5C5^{+} + 400.000 TF (n=2) * • 500.000 W5C5^{-} (n=3)	250,000 ICE6 + 250,000 ICE7 (n=3)

Table 5. Experimental design. *Cell populations injected throughout the experiment, according to W5C5, ICAM1 and Side Population (SP).* ***n*** *is the number of animals per condition. *: one of the animals died during the experiment. TF: total endometrial fraction, disaggregated endometrial cells from human biopsies.*

2.2. Labelling of endometrial human cells using Molday ION Rhodamine.

To check the injection protocol and to trace human endometrial cells injected under the kidney capsule, five animals were treated with TF (epithelial and stromal cells) labelled with Molday ION Rhodamine B using a published protocol (Cervelló *et al.*, 2015). This is a non-transfection-based method to label and track cells in an *in vivo* model.

Rhodamine B is a xanthene-based and fluorescein-derived fluorescent chemical compound from the family of rhodamines used to track the cell flow. This is because it creates iron deposits that can be detected by Prussian blue staining. Cells were therefore labelled with 50 µg/mL of Molday ION Rhodamine B (CL-50Q02-6A-50, [2mg Fe/ml], BioPAL, Malaysia) in a suspension with medium containing 10% FBS and 0.1% antibiotics and antimycotics, during 17h (o/n) at 37°C. After the incubation, cells were centrifuged, supernatant was removed and cells were resuspended in 30 µL HBSS

supplemented with 2% FBS and 10 mM HEPES before proceeding to the cell injection under right kidney capsules.

The five mice injected with labelled cells were euthanized at different time points after injection (13, 32, 47, 50 and 60 days) and injected kidneys were extracted to track these human cells, as well as spleens and lungs (controls). The spleen was used as positive control due to the presence of iron deposits in the tissue, and lung as the negative control. The cell engraftment efficiency was assessed by the visualization of intracellular iron deposits in mice kidneys with the Prussian blue staining and a counterstain with eosin, explained in detail in the *"2.3.3. Detection of iron deposits by Prussian blue staining"*.

2.3. Histological and immunohistochemical characterization of candidate endometrial-like tissue

After the collection of murine organs, histological analysis and immunostainings were performed in order to assess the engraftment of the injected cell populations (endometrial and stem cells), as well as Prussian blue staining to study the location of injected cells.

2.3.1. Murine organ collection and processing of formalin-fixed paraffin-embedded tissue

Xenotransplanted mice, excluding the ones injected with rhodamine-labelled cells that were sacrificed at different time points, were euthanized 60 days after the cell injection experiments and their kidneys were analyzed to assess the presence of endometrial cells.

All harvested organs were fixed in 4% PFA for 24 hours at 4ºC, and transferred to 70% ethanol o/n at 4ºC. The next day the tissues were dehydrated in successive ethanol (70%, 80%, 96% and 100%) and xylene washes (twice for 30 minutes), to be finally embedded in paraffin using two passages of 90 minutes at 60ºC, and then left to solidify on ice. When organs were finally formalin-fixed paraffin-embedded (FFPE), they were serially sectioned at 4 μm with a microtome (HM 310, Microm), stretched in a water bath at 37ºC, mounted onto glass slides (Superfrost plus, Thermo Scientific) and incubated o/n at 37ºC to assure the tissue adhesion to the slide.

For the later study of the tissue sections, FFPE endometrial sections were deparaffinized. This was done by leaving the sections firstly o/n at 37°C, followed by 1 hour at 60°C and then with three xylol washes for 10 minutes. After that, sections

were left in decreasing concentrations of ethanol for 5 minutes each to rehydrate the tissue (100%, 96%, 80% and 70%), and ending with a final wash in distilled water.

2.3.2. Histological analysis

Several serial sections (each 5 cuts) were stained with hematoxylin-eosin (H&E) to macroscopically study the kidney capsule thickening, indicative for the formation of endometrial-like tissue (in normal conditions the kidney capsule is a not thickened and a hardly observable tissue layer). For this purpose, filtered Harris hematoxylin and eosin were used for 6 minutes each. After that, slides were washed with running and distilled water. Finally, slides were dried at RT and mounted with Eukitt mounting medium (Sigma-Aldrich, Spain) for observation under the microscope.

2.3.3. Detection of iron deposits by Prussian blue staining

Prussian blue staining was used to visualize the presence of iron deposits found inside the cells after treatment with Molday ION Rhodamine (Molday particles are contained within vacuoles or lysosomes in the cytoplasm). The ionic iron reacts with acidic ferrocyanide producing a blue color that can be observed under the microscope. The reagents used belong to the ACCUSTAIN IRON STAIN kit (HT20; Sigma-Aldrich, Spain), and in addition we had to prepare the Pararosaniline Working Solution (1mL of pararosaniline solution with 50 mL of water) and the Working Iron solution (potassium ferrocyanide solution with hydrochloric acid solution in equal volumes) before starting the protocol. After deparaffinization, slides were placed in Working Iron Solution for 10 minutes, rinsed in distilled water and followed by for 4 minutes in Working Pararosaniline Solution. Then, slides were dried at RT and mounted with Eukitt solution. The controls used were mouse spleen as positive control and mouse lung as negative one.

2.3.4. Immunofluorescence for endometrial tissue markers: vimentin, cytokeratin 18 and progesterone receptor

We performed immunofluorescence (IF), which is an immunostaining method that allows the detection of specific antigens in tissues using antibodies. In this technique, the primary antibody recognizes and binds with a certain antigen, this primary antibody is then recognized by a secondary fluorescently labelled antibody (because of its specificity for the specie where the primary antibody has been created). The fluorochrome bound to the secondary antibody will fluoresce when it is excited by light with a specific wavelength, resulting in the emission of light of a longer wavelength that is detectable with a fluorescent microscope.

With the purpose of characterizing any formed endometrial-like tissue present in the kidney capsule, IF for vimentin (VIM), cytokeratin 18 (CK18) and progesterone receptor (PR) were performed. Deparaffinization was the first step carried out for the accomplishment of this assay as described at the end of the section "*2.3.1. Murine organs collection and biological samples processing"* after cutting samples with the microtome (4 μm-thick) from FFPE tissue blocks. Briefly, the first incubation was at 37ºC o/n to assure the adhesion of the tissue to the slide, and then 1 hour at 60ºC, followed by 3 washes with xylol for 10 minutes, and several washes with ethanol (100%, 96%, 80% and 70%) and distilled water (5 minutes each) to rehydrate the tissue.

After tissue fixation, crosslinks are produced with several structural proteins, masking tissue antigens. To liberate these antigens within the tissue and assuring the success of the technique, a process called antigen retrieval is necessary. For that reason, a heat-induced antigen retrieval was performed by using citrate buffer 10 mM 0.05% Tween-20 pH6, for 20 minutes at 95ºC. Non-specific binding sites of antibodies also have to be blocked by using BSA, detergent (Tween-20 or Triton X-100) and normal goat serum (NGS, only if the secondary antibodies were made in goat). For the cases of intracellular or transmembrane antigens, a permeabilization step with PBS supplemented with detergent is also needed. Finally, slides were incubated with primary antibodies against human VIM, CK18 and PR, and with fluorescently labelled secondary antibodies. Primary antibodies used (in PBS supplemented with 1% BSA) were: *mouse monoclonal anti-vimentin* (ab8069, Abcam) diluted at 1:200 for 45 minutes at RT, *rabbit polyclonal anti-human cytokeratin 18* (ab52948, Abcam) diluted at 1:500 for 30 minutes at RT and *rabbit* p*olyclonal anti-human progesterone receptor* (A0098, DAKO) diluted at 1:500 for 30 minutes at RT. After this incubation, slides were washed with PBS to remove the excess of unbound antibodies and specific fluorescent secondary antibodies were added in solution with PBS to be observed by fluorescence. Secondary antibodies used were *goat anti-mouse Alexa 488 Fluor* (Invitrogen, A-21121) for VIM, *goat anti-rabbit Alexa 488 Fluor* (Invitrogen, A-11034) for CK18 and *goat anti-rabbit Alexa 555 Fluor* (Invitrogen, A-21429) for PR; all of them diluted at 1:500 for 30 minutes at RT. After washing again with PBS and distilled water to remove the unbound secondary antibody, slides were counterstained with 6-diamidino-2-phenylindole (DAPI) (Prolong Gold Antifade reagent with DAPI. Invitrogen, P-36931) for the nuclei staining (Invitrogen, CA, EEUU), mounting immediately after the addition with an aqueous mounting medium.

Conditions, specifications and concentration for each antibody are detailed in Table 6, as well as the controls. Immunolocalization for VIM, CK18 and PR was visualized, and pictures were acquired with several magnifications under the fluorescent Nikon Eclipse 80i microscope.

	Antigen retrieval	**Cellular permeabilization**	**Blocking solution**	**Primary antibody**	**Secondary antibody**	**External positive controls**	**External negative controls**
Vimentin (VIM)	Citrate buffer 10mM 0.05% Tween-20 pH 6 20min, 95ºC	PBS + Triton X-100 0.05% 10min, RT	PBS-BSA 15% + Triton X-100 0.05% + NGS 20% 90min, 37ºC	Mouse anti-Vimentin (ab8069, Abcam) Dilution 1:200 45min, RT RRID:AB_306239	Goat anti-mouse Alexa 488 Fluor (Invitrogen, A-21121) Dilution 1:500 30min, RT RRID:AB_141514	Human endometrium	Mouse small intestine
Cytokeratin 18 (CK18)	Citrate buffer 10mM 0.05% Tween-20 pH 6 20min, 95ºC	PBS + Triton X-100 0.1% 10min, RT	PBS-BSA 5% + Tween-20 0.05% + NGS 4% 30min, RT	Rabbit polyclonal Anti-Cytokeratin 18 (ab52948, Abcam) Dilution 1:250 30min, RT RRID:AB_869873	Goat anti-rabbit Alexa 488 Fluor (Invitrogen, A-11034) Dilution 1:500 30min, RT	Human endometrium	Mouse kidney
Progesterone receptor (PR)	Citrate buffer 10mM 0.05% Tween-20 pH 6 20min, 95ºC	PBS + Triton X-100 0.05% 10min, RT	PBS-BSA 15% + Triton X-100 0.05% + NGS 20% 60min, RT	Polyclonal Rabbit anti-human Progesterone Receptor (A0098, DAKO) Dilution 1:50 1h, RT RRID:AB_2315192	Goat anti-rabbit Alexa 555 Fluor (Invitrogen, A-21429) Dilution 1:500 30min, RT RRID:AB_141761	Human endometrium	Mouse kidney

Table 6. Antibodies and experimental conditions used for the study of vimentin (VIM), cytokeratin 18 (CK18) and progesterone receptor (PR) by immunofluorescence*. Table summarizing the immunostainings for Vim, CK18 and PR, with the different steps implied: antigen retrieval, cellular permeabilization, blocking for non-specific bindings, primary and secondary antibodies and controls. BSA: bovine serum albumin; NGS: normal goat serum; PBS: phosphate buffered saline.*

2.3.5. Immunohistochemistry for the stem cell marker Musashi-1

Immunohistochemistry (IHC) is an indirect immunostaining method using the same principles as IF, but here the secondary antibody is not conjugated with a fluorochrome but with a biotin molecule. After the binding of the antibodies, 3,3'-diaminobenzidine (DAB) chromogen is added, this reacts with the peroxidase forming a avidin-biotin-peroxidase complex that gives rise to a brown reaction product that can be observed with normal light microscope.

With the purpose of checking the presence of the stem cell marker MS1 in the newly formed tissue within the kidney capsule, IHC for this marker was performed. The technique described in this section was performed within the facilities of University Hospital of Münster, in the laboratory of Prof. Martin Götte during my PhD stay in Germany. This stay was possible thanks to the grant for PhD stays obtained from the Consellería d'Educació, Investigació, Cultura i Esport (Generalitat Valenciana). This group has experience with this antibody and set up a protocol to use in FFPE tissue (Götte *et al.*, 2008).

For this technique, sections were deparaffinized as previously-described, antigen retrieval was performed with Target Retrieval Solution (S1699, DAKO), for 35 minutes at 95°C, to break down formalin networks and facilitate antibody binding. A first blocking was carried out for the endogenous peroxidase activity with the reagent of Envision HRP system (DAKO, Germany) for 5 minutes at RT, and then a second blocking with Aurion BSA-c 1% (900.099, AURION) for 30 minutes at RT to avoid non-specific reactions. After these incubations, primary antibody *mouse monoclonal anti-human Musashi-1* (MAB2628, R&D Systems) was added at a dilution of 1:100 in antibody diluent (S0809, DAKO) and incubated o/n at 4ºC. Then, the biotinylated secondary antibody (labelled polymer-HRP from Envision HRP system, DAKO) was added and sections were treated with the DAB chromogen after several washings. The reaction in negative controls (primary antibody omitted) was stopped simultaneously with the samples and positive control (human endometrium). After stopping the reaction with distilled water, slides were counterstained with Mayer's Hemalaum reagent for few seconds and washed with running and distilled water, to be finally mounted with aqueous mounting medium.

In the table below (Table 7) the details regarding the solutions used, specifications, as well as antibodies and concentrations of this section are summarized.

	Antigen retrieval	Blocking solution	Primary antibody	Secondary antibody	External positive control	External negative control
Musahi-1 (MS1)	DAKO Target Retrieval Solution 1X (S1699, DAKO) 35min, 95°C	Aurion BSA-c, 1% (900.099 AURION) 30min, RT	Monoclonal Mouse anti-human Musashi-1 (282613, R&D Systems) Dilution 1:100 o/n, 4°C RRID:AB_2235632	Envision HRP system (DAKO) 30min, RT	Human endometrium	Primary antibody omitted

Table 7. Antibodies and experimental conditions used for the study of Musashi-1 (MS1) by immunohistochemistry. *Table summarizing the different steps implied: antigen retrieval, cellular permebilization, blocking for non-specific bindings, primary and secondary antibodies and controls. BSA: bovine serum albumin.*

MS1-positive cells were counted and represented respective to the density found in a certain area. As kidney capsule thickness can vary a great deal, even within the same kidney, MS1-positive cells were represented per 100,000 μm². This approximation was adopted to unify results and to be able to compare them, as an objective manner taking into account the extent of generation of endometrial-like tissue. So the signal for MS1 was depicted with a graph with error bars (standard deviation) represented with Microsoft Excel tool.

2.4. Endometrial reconstitution efficiency

The reconstitution rate (RR) is the measure we adopted to compare the engraftment efficiency after cell transplantation, using a concept modified from the work of Dr. Masuda (Masuda *et al.*, 2010). The RR in the present work is expressed as a percentage, resulting from the number of kidneys expressing all three antibodies (VIM, CK18 and PR) in relation to the number of kidneys transplanted per condition. As a result, each condition has a RR calculated using the following formula:

$$\boldsymbol{RR}\ (\%) = \frac{\boldsymbol{Number\ of\ kidneys\ expressing\ VIM, CK18\ and\ PR}}{\boldsymbol{Number\ of\ kidneys\ per\ condition}}\ \boldsymbol{x\ 100}$$

RR is therefore a qualitative manner to represent the results. There are many positive cells within the kidney capsule in the case of PR which made it very difficult to quantify, whilst with VIM the pattern is diffuse in some cases and perinuclear in other, what made quantifying numerically even more problematic. To unify the detection of the three antibodies, we adopted a qualitative manner using the presence or absence of a signal, previously used by other experts.

2.5. Proliferation analysis by Ki67 assay

With the aim to study the cell proliferation in the newly formed endometrial-like tissue within the kidney capsule, IHC for the proliferation marker Ki67 was performed. Deparaffinization was followed by antigen retrieval with citrate buffer, permeabilization, and two blockings: one for the endogenous peroxidase activity with the reagent of Envision HRP system, and another with NGS for non-specific binding. Slides were then incubated with the primary antibody *rabbit polyclonal anti-Ki67* (AB9260, Millipore) at a dilution of 1:100 for 10 minutes at RT, washed, and incubated with the biotinylated secondary antibody (labelled polymer-HRP) from the Envision HRP kit (DAKO). Finally, DAB chromogen was added to observe the result. The reaction in negative controls (primary antibody omitted, as well as mouse small intestine as external control) was stopped at the same time as the samples and positive control (mouse brain). After stopping the reaction with distilled water, slides were counterstained with Harris hematoxylin diluted 1/5 for few seconds and washed with running and distilled water, to be finally dried at RT and mounted with Eukitt mounting medium.

In the table below (Table 8) the details regarding the solutions used, specifications, as well as antibodies and concentrations of this section are summarized.

	Antigen retrieval	**Cellular permeabilization**	**Blocking solution**	**Primary antibody**	**Secondary antibody**	**External positive control**	**External negative control**
Ki67	Citrate buffer 10mM 0.05% Tween-20 pH 6. 20min, 95ºC	PBS + NGS 1% + Triton X-100 0.4% 2 X 10min, RT	PBS + Triton X-100 0.4% + NGS 10%. 90min, 37ºC	Rabbit polyclonal anti-Ki67 (AB9260, Millipore) Dilution 1:100 10min, RT RRID:AB_2142366	Envision HRP system (DAKO) 30min, RT	Mouse small intestine	Mouse brain

Table 8. Antibodies and experimental conditions used for the study of Ki67 by immunohistochemistry*. Table summarizing the different steps implied: antigen retrieval, cellular permeabilization, blocking for non-specific bindings, primary and secondary antibodies and controls. BSA: bovine serum albumin; NGS: normal goat serum; PBS: phosphate buffered saline.*

B. Stem cells and paracrine factors in pathological endometrium: endometriosis, Asherman's syndrome and endometrial atrophy

1. Study of W5C5$^+$, ICAM1$^+$ and Side Population cells in endometriosis

1.1. W5C5$^+$ and ICAM1$^+$ cells of eutopic endometrium from healthy women and women with endometriosis using flow cytometry

The technique described in this section was performed within the facilities of University Hospital of Münster, in the laboratory of Prof. Martin Götte during my PhD stay in Germany. This stay was possible thanks to the grant for PhD stays obtained from the Conselleria d'Educació, Investigació, Cultura i Esport (Generalitat Valenciana).

The protocol was the same as described in the sections "*1.3. Epithelial and stromal cell isolation from human endometrium" and "1.4. Analysis and subsequent isolation of endometrial cells for specific markers by flow cytometry"* using the antibodies listed in Table 4.

The flow cytometer used in this case was the Cyflow Space from Sysmex/Partec (Görlitz, Germany), equipped with a 488nm Argon Laser, a 635nm Red Laser Diode and a 375 nm UV-Laser. The data caption was made with the FloMax Software from Quantum Analysis (Münster, Germany).

1.2. Side Population of eutopic endometrium from healthy women and women with endometriosis using flow cytometry

1.2.1. Hoechst 33342 staining protocol

The SP approach consists of passing a cell suspension through a flow cytometer and detecting (and also isolating by FACS) this specific population. The basis is the capability of SP cells to extrude the vital dye Hoechst 33342 through BCRP1 channels, which is a stem cell property. This efflux results in a specific fluorescence pattern, giving rise to a lateral region of low fluorescence (total SP cells in this sample) as it is shown in Figure 15 (introduction section). Apart from staining with Hoechst, cells were also stained with PI to exclude dead cells.

With respect to the incubation with Hoechst, almost every cell will incorporate it but those with BCRP1 channels will extrude it, emitting light at both in the far-red spectrum (>675nm) and in blue (450nm), which is detected by the flow cytometer. In this technique, we use a blocker for these channels, verapamil, which is a drug used to block ABC-type transporters. Those cells with verapamil will close their channels (if

there were any) and will not extrude Hoechst, losing fluorescence and results in the disappearing the characteristic lateral region of this population. The diagram obtained after the addition of verapamil shows the background signal of this technique.

After the isolation of endometrial cells as described in "*1.3. Epithelial and stromal cell isolation from human endometrium",* cells were resuspended in SP medium (DMEM + 2% fetal calf serum (FCS) + 1% P/S + 10 mM HEPES) at a million cells/mL and incubated with Hoechst for the study of SP cells. Cells were divided equally in two tubes, one of them for the SP quantification and another one for the negative control with Hoechst plus verapamil. Each million cells (0.5-2 millions) is resuspended in 1 mL SP medium with Hoechst at a final concentration of 2.5 μg/mL and verapamil at 0.05 mM. Cells were incubated for 90 minutes at 37ºC (in a water bath) and agitated each for 15 minutes to avoid cell precipitation. After this incubation, PI was added at 2 μg/mL for a period from 20 minutes up to 3 hours until the flow cytometer analysis.

1.2.2. *Analysis of Side Population cells*

The basis of the SP technique has been explained in detail in the section *"1.2.1. Hoechst 33342 staining protocol"* and the basis for ICC and FACS in "*1.5.1. Analysis of stem cell markers in epithelial and stromal fractions*" and "*1.4.2. Isolation of endometrial positive and negative cells for W5C5 and ICAM1 in epithelial and stromal fractions*" respectively.

After the incubation with Hoechst, verapamil and PI, cell suspensions were studied using a Cyflow Space from Sysmex/Partec (Görlitz, Germany) with the software FloMax from Quantum Analysis (Münster, Germany). Red and blue fluorescence from the incubation with Hoechst were detected with filters 405/30 nm y 670/20 nm respectively, and PI with one of 613/20 nm. SP cells were only quantified, not isolated, obtaining a biparametric plot as shown in Figure 15 (introduction section), where blue and red fluorescence can be observed, giving rise to a percentage belonging to the Side Population (as it is depicted in the introduction, more specifically in "*3.2.2. Side Population approach"*).

We performed a statistical analysis to express the results for the markers studied (W5C5, ICAM1 and SP) in this analysis; a graph with error bars (standard deviation) was represented with Microsoft Excel.

1.3. Flow cytometric data analysis

Data from FC of endometrial samples were studied establishing two comparisons. One comparison was between both menstrual phases (proliferative versus secretory phase) in patients with rASRM stage I endometriosis (low grade, mild endometriosis), and the other one was with the different cell populations (positive cells for W5C5, ICAM1 and SP) between healthy and rASRM stage I endometriosis patients during the secretory phase.

2. Identification of paracrine factors secreted by bone marrow-derived stem cells involved in endometrial regeneration

2.1. Biological samples and inclusion criteria

Endometrial tissue samples were taken from several patients enrolled in the study of Dr. Santamaria *et al*. in 2016 (NCT02144987) to study their neovascularization development and their histological and reproductive improvement. The patients numbered as 5, 7, 12 and 13 in the original study were named here as A, B, C and D (Table 9).

Patient	Age	Pathology	Prior repair attempts	Maximum pre-treatment endometrial thickness (mm)	Maximum post-treatment endometrial thickness (mm)	Reproductive outcome
A	42	EA with intramural myoma	3 hysteroscopy	5	6,8	No pregnancy
B	34	EA + AS Stage II with myoma and 80% fibrotic adhesions	9 hysteroscopy	3,5	N.S.	Spontaneous pregnancy. Premature rupture of membranes (17 weeks)
C	35	EA	2 hysteroscopy	4,3	5,7	Baby born
D	43	EA + AS Stage II	None	3	8	Ectopic pregnancy

Table 9. Characteristics of patients from the study of paracrine factors involved in endometrial regeneration. *Features of selected patients, as well as their age and condition (Asherman's syndrome, AS, and/or endometrial atrophy, EA), prior repair attempts, maximum pre- and post- operative (3 months later) thickness and the reproductive outcome after the autologous treatment with CD133^{+} bone marrow-derived stem cells (BMDSCs). N.S.: not specified. Patients named as A, B, C and D correspond to patients 5, 7, 12 and 13 from the study of Santamaria et al., 2016, respectively.*

The cell therapy method carried out in these patients is described in the work of Dr. Santamaria in 2016 (Santamaria *et al.*, 2016) and summarized in Figure 22. Patients had endometrial pathologies, AS and/or EA, and they improved their neoangiogenesis 3 months after the cell therapy with autologous BMDSCs. The BMDSCs used in this study are $CD133^+$ cells and are a bone marrow-derived pool of cells with characteristics similar to progenitor cells, with the ability to travel to the bloodstream and to improve neoangiogenesis starting from the existing endothelium in several diseases, such as myocardial infarction or arteriosclerosis. Besides, this marker is used for isolating stem cells in brain, kidney, prostate and liver, with a very safe use in regenerative medicine. The uses and characteristics of these progenitor cells are summarized in the work of Santamaria in 2016 (Santamaria *et al.*, 2016). They can be mobilized to pass into the bloodstream by G-CSF, this is a cytokine used for this purpose in autologous and allogenic transplants. In the study of Santamaria *et al.*, 5 days after the injection of G-CSF, mononuclear cells were isolated through peripheral blood apheresis to use them in the autologous transplants into de spiral arterioles by catheterization.

Once patients were selected from the original study, the tissue histology was assessed from biopsies taken before (PRE-TT) and 3 months after the treatment (POST-TT) with $CD133^+$ BMDSCs by H&E staining. Several cuts were made with the microtome from FFPE tissue from the patients for both conditions and they were incubated with filtered Harrys hematoxylin first and eosin then, during 6 minutes each at RT, washing later the slides with running water and finally with distilled water. They were dried at RT and mounted with Eukitt to observe them under the normal light microscope.

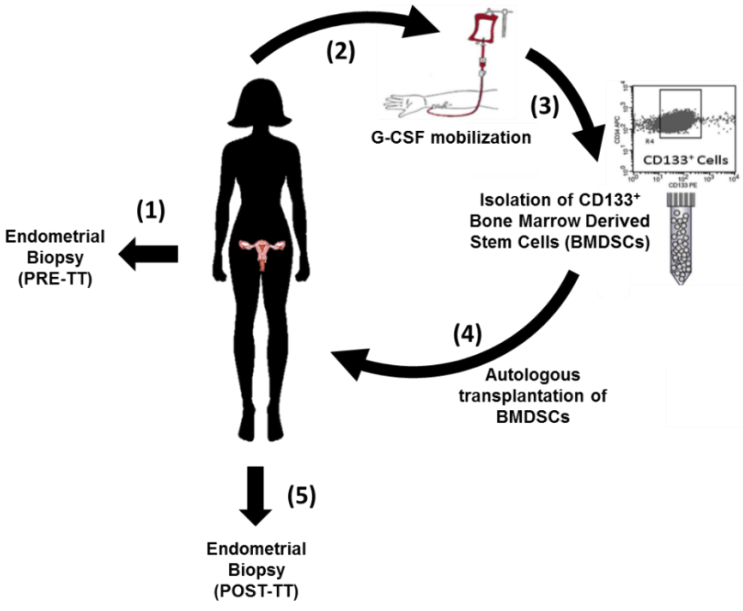

Figure 22. Study design carried out by Santamaria et al. in 2016. *(1) A biopsy was taken before the intervention from patients with AS and/or EA (PRE-TT). (2) After that, $CD133^+$ BMDSCs were mobilized with G-CSF and (3) they were isolated by FACS. (4) These autologous stem cells were transplanted in the same women and (5) another biopsy was taken 3 months after the intervention (POST-TT). BMDSCs: bone marrow-derived stem cells; G-CSF: granulocyte-colony stimulating factor; POST-TT: post-treatment (3 months); PRE-TT: pre-treatment. (Santamaria et al., 2016)*

2.2. Neoangiogenesis assay

We studied in this thesis the short-term effect (3 months) of the treatment, as Figure 23 shows. The average number of mature blood vessels (neoangiogenesis) increased in the patients (A-D, Table 9), studied in the original work with the markers α-SMA and CD31. Results are provided as mean of mature blood vessels per condition (PRE-TT and POST-TT, with error bars included) in Figure 26A, and number of mature blood vessels per patient and condition in Figure 26B.

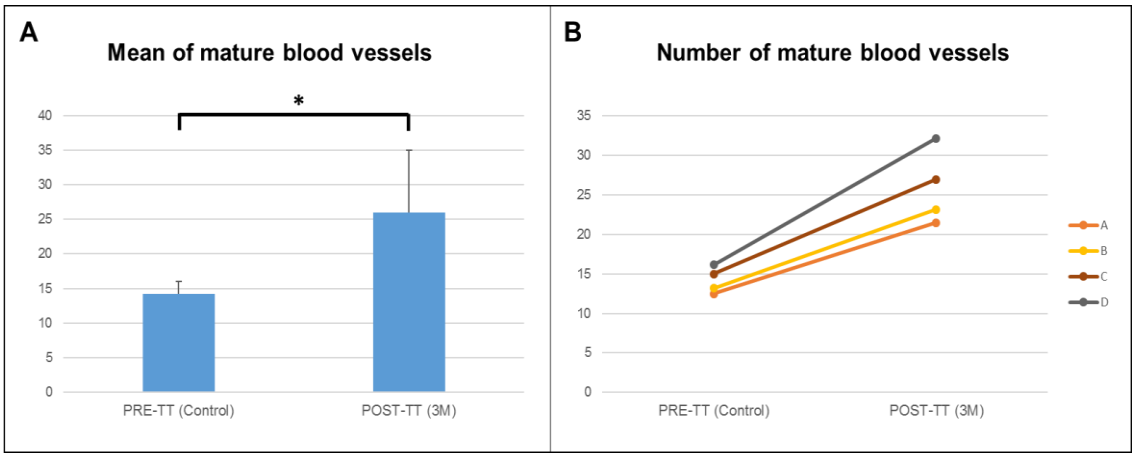

Figure 23. Neovascularization after therapy with CD133⁺ BMDSCs. (A) *Mean of mature blood vessels per condition: before (control) and 3 months (3M) after treatment.* ***(B)*** *Number of mature blood vessels per patient and per condition. *: The difference is statistically significant between both conditions, with a p-value<0.05. Data taken from the study of Santamaria et al., 2016.*

Statistical analysis of the results was conducted with the SPSS 17.0 program (IBM, MD, USA). A paired sample t-test analysis was carried out to study the differences observed in the number of blood vessels among patients at both times. A p-value less than 0.05 is considered significant.

2.3. RNA extraction and conversion into cDNA from endometrial biopsies

FFPE biopsies from patients were cut in 5 µm sections with a microtome (HM 310, Microm); one cut per patient and condition was made, resulting in four sections per condition in total (four PRE-TT and four POST-TT cuts). Sections had to be between 5 and 20 µm thick according to the protocol below. The workflow is summarized in Figure 24. The entire procedure was carried out very carefully and with nuclease-free materials and reagents, cleaning everything with RNase-Zap (cleaning agent for removing RNase, R2020, Sigma-Aldrich, Spain) to prevent ribonuclease activity.

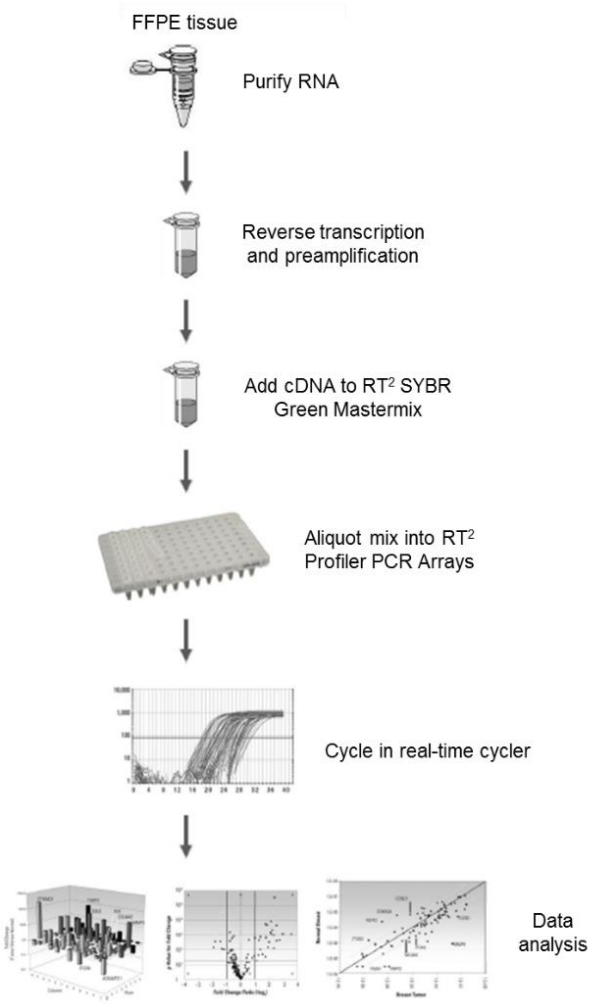

Figure 24. Workflow starting from FFPE tissue until the data analysis after RT² Profiler PCR Arrays. *Total RNA was extracted from experimental samples (FFPE tissue) and then reverse-transcribed to cDNA and preamplified for the studied pathways. RT-qPCR was finally made, and data was analyzed obtaining representative plots with the QIAGEN software. Image adapted from RT² Profiler PCR Array Handbook.*

2.3.1. *RNA extraction from paraffin-embedded tissue*

Once tissue was cut with the microtome from FFPE blocks, total RNA was purified with the RNeasy FFPE kit (QIAGEN, Spain) according to the manufacturer's instructions, with the maximum volume of choice (specified by the protocol) because we had more than 2 tissue sections per condition (PRE-TT and POST-TT). All the centrifugations were carried out at RT. Before starting the protocol, RNase-free DNase I was reconstituted with RNase-free water, as well as the RPE buffer with absolute ethanol, keeping all the reagents at RT.

First, tissues were placed in a 2 mL tube and deparaffinization solution was added, incubating for 3 minutes at 56ºC. PKD buffer was consecutively added and the tube was centrifuged at 11,000 g for a minute before adding proteinase K for tissue digestion. After incubating at 56ºC for 15 minutes with proteinase K, temperature was increased until 80ºC for 15 minutes with agitation. This high temperature has the function of revert at least part of the modifications created in the nucleic acids after

the use of PFA. A resulting lower transparent part was transferred to a new 2 mL tube and incubated on ice for 3 minutes, and then centrifuged for 15 minutes at 20,000 g. The supernatant was carefully transferred to a new tube without touching the pellet (containing DNA and cell debris). To remove the remaining DNA, DNase Booster Buffer and DNase were added and incubated for 15 minutes at RT, followed by RBC buffer and then absolute ethanol. The solution was put in a RNeasy MinElute column (maximum 700 µL each time) and centrifuged for 15 seconds at 8,000 g, repeating the procedure with the entire volume (including the precipitation created after adding the ethanol). After the centrifugations, RPE buffer was added to the column and centrifuged again in the same conditions, this last centrifugation was repeated for 2 minutes without adding any washing medium. The material of interest was retained in the membrane, the RNA was finally eluted in 12 µL of RNase-free water.

2.3.2. *Reverse transcription and preamplification*

After RNA extraction, a reverse transcription of this RNA to its complementary DNA (cDNA) was done; they were at the same time preamplified for human specific pathways related to growth factors and angiogenesis. The procedure was conducted according to the guidelines described in RT² PreAMP cDNA Synthesis Handbook (QIAGEN, Spain) by using the RT² PreAMP cDNA Synthesis Kit. Each array, compatible with our thermal real-time StepOne Plus Real-Time PCR (Applied Biosistems, Thermo Fisher Scientific, Spain), was made for both samples PRE-TT and POST-TT.

For the reverse transcription, the genomic DNA elimination mix for each sample was prepared using the First-Strand cDNA Synthesis protocol from FFPE sample, by mixing 500 ng RNA, 2µL GE buffer and RNase-free water up to 10 µL per reaction, incubating it for 5 minutes at 42ºC and leaving it on ice for another minute. The reverse-transcription mix was prepared with the reagents of the kit, by mixing 5X buffer BC3, control P2, cDNA synthesis enzyme mix, RNase inhibitor and RNase-free water until a volume of 10 µL per reaction. Once this mix was completed, these 10µL were added to 10µL of genomic DNA elimination mix, mixed well and incubated in a thermocycler for 30 minutes at 42ºC before stopping the reaction (and de DNase activity) at a high temperature of 95ºC for 5 minutes. This mix has DNA free of genomic DNA.

A preamplification step was required because the initial amount of material was too small to be able to detect changes in genes of interest. For this reason, the preamplification was done only for cDNA related to the pathways below: Human

EGF/PDGF Signaling Pathway (PAHS-040ZC), Human Growth Factors (PAHS-041ZC) and Human Angiogenic Growth Factors (PAHS-072ZC).

The two necessary solutions were mixed: 12.5 µL of RT² PreAMP PCR Mastermix and 7.5 µL of RT² PreAMP Pathway primer mix (specific for each pathway). Thereafter, 5 µL of cDNA synthesis reaction was pipetted with the previously mentioned preamplification mix, and preamplification was carried out in a real-time cycler with the following program: 1 cycle at 95ºC for 10 minutes, followed by 8 cycles at a) 95ºC for 15 seconds and then b) at 60ºC for 2 minutes, and was kept on ice after a total of 9 cycles. 2 µL of Side Reaction Reducer was subsequently added to the samples, incubating first at 37ºC for 15 minutes, followed heat-inactivation of the reaction by incubating at 95ºC for 5 minutes. Nuclease-free water was added to the preamplified samples until reaching 111 µL.

2.4. Differential gene expression profiling

To test the gene differences between both conditions, RT-qPCR was performed (RT^2 Profiler PCR Arrays, format C, QIAGEN, Spain) for each condition and for the three above-mentioned pathways: Human EGF/PDGF Signaling Pathway (PAHS-040ZC), Human Growth Factors (PAHS-041ZC) and Human Angiogenic Growth Factors (PAHS-072ZC). These arrays measure the expression of different genes to compare diverse experimental conditions.

2.4.1. *Basis of the polymerase chain reaction (PCR)*

Polymerase chain reaction (PCR) is a widely used technique in basic science and molecular biology (Figure 25A). This method relies on creating a large number of copies *in vitro* from a particular DNA fragment or a specific DNA sequence of interest. To be able to amplify, two elements are necessary: a DNA template and a pair of specific primers to start copying from this template. These primers are short single-stranded DNA fragments (around 20 nucleotides, enriched in guanine and cytosine to stabilize the sequence) complementary to the target region and flanking it (called forward and reverse), leading to the initiation of the elongation. A heat-stable enzyme called DNA polymerase (Taq polymerase is the most used, isolated from the thermophilic bacterium *Thermus aquaticus*) is responsible for the synthesis of the new DNA fragments by hybridizing and adding nucleotides (dNTPs) complementary to the DNA template, using the primers as starting points. This newly synthetized DNA fragment is used as a new template in the following cycles, exponentially generating thousands to millions of copies of a particular DNA sequence (Figure 25B). This

synthesis needs repeated cycles of heating and cooling, resulting in different temperature-dependent reactions around 25-40 times. Each cycle involves 3 phases:

- The **denaturation** or separation phase of the DNA double helix, at a temperature of 95ºC. This temperature can vary a little depending on the guanine-cytosine content or the length of the strands.
- The second phase is the **annealing** or primer hybridization phase to the single stranded DNA template, at a temperature around 50-60ºC
- The third phase is the **synthesis** of the DNA strand complementary to each template by DNA polymerase that adds nucleotides starting from primers, at a temperature around 68-72ºC.

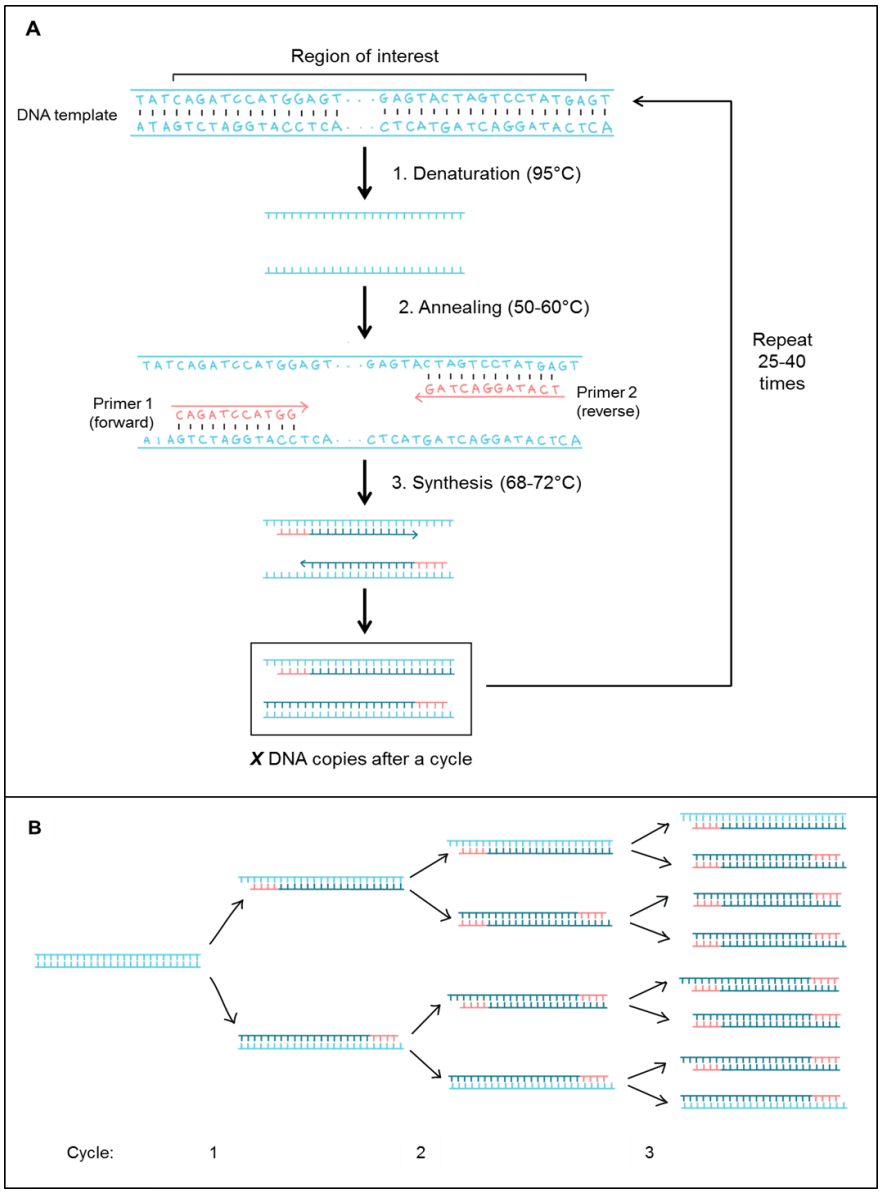

Figure 25. Basis of the polymerase chain reaction (PCR). (A) *Outline of a classic PCR with the three different phases: denaturation of the DNA double helix, primer hybridization or*

annealing, and synthesis of the new DNA strand. These phases are repeated around 25-40 times until obtaining millions of copies of the starting DNA fragment of sequence of interest. ***(B)*** *Exponential increase of DNA strands throughout several PCR cycles. Figure adapted from https://es.khanacademy.org/*

The real-time quantitative polymerase chain reaction, or RT-qPCR, is a technique that studies the gene expression where in addition to amplifying a DNA template it also quantifies it, giving information regarding the amount of DNA in the sample. For this purpose, a fluorescently labelled probe that is later displaced and degraded as the new DNA strand is synthetized is added to the reaction. This probe is then excited and the real-time cycler is able to detect this fluorescent signal, measuring the amount of synthetized product in each amplification cycle (Figure 26A). This is possible because the fluorescence is proportional to the quantity of product. The use of a fluorochrome enables the detection of specific DNA fragments by their melting temperature (Tm), which is specific of each amplified sequence. With the parameters showed in the resulting diagram, we can study the result of the RT-qPCR (Figure 26B). The baseline is defined as the amount of PCR cycles in which a reporter fluorescent signal is accumulating but remains under the detection limits. The threshold is the level where the fluorescence becomes measurable and the point where the exponential phase starts. The exponential phase defines the threshold cycle (CT) as the first cycle of DNA exponential synthesis because fluorescence is greater than the threshold, so a lower CT indicates a higher initial DNA amount (amplified and detected in early cycles).

This type of PCR is normalized with housekeeping genes (HK), which have a constant expression because of their constitutive expression.

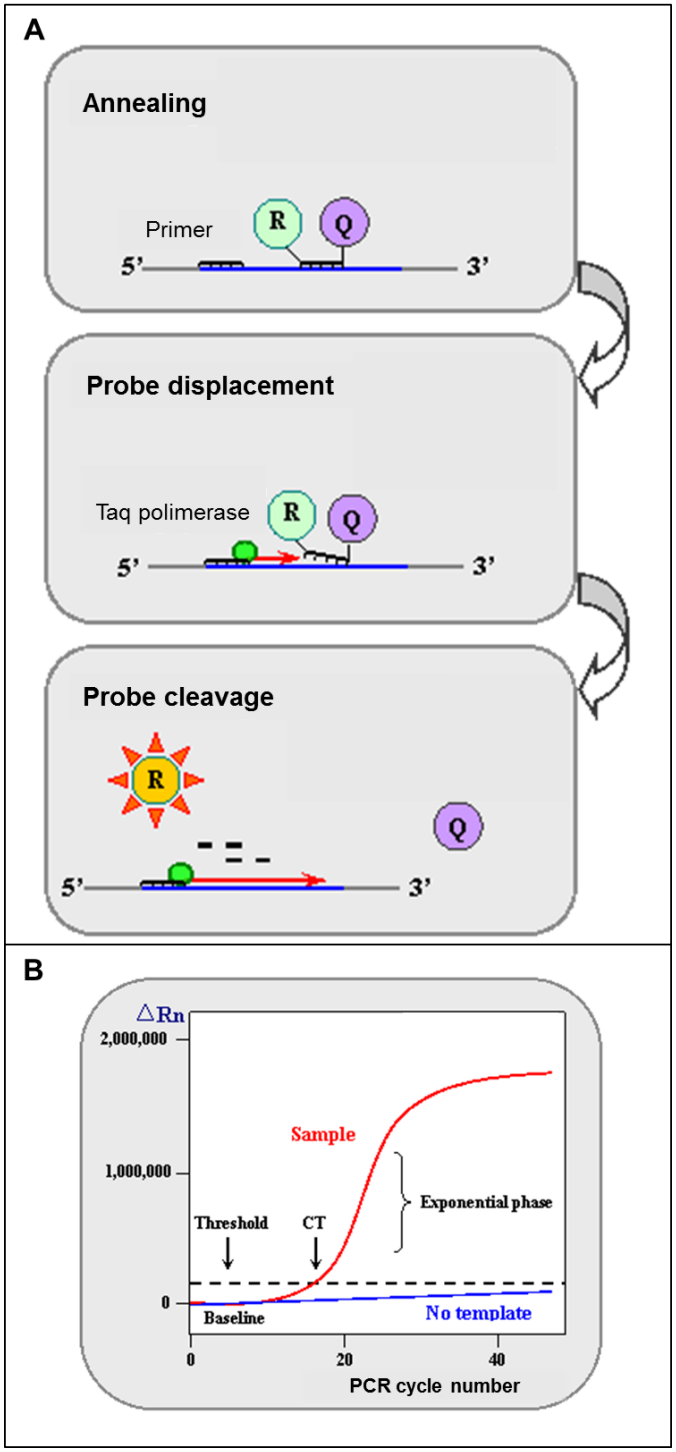

Figure 26. Basis of the real-time quantitative polymerase chain reaction (RT-qPCR). (A) *Fluorescently labelled probes bound to the DNA template (DNA strands separated with the primers bound). R is the reporter fluorophore that emits at a wavelength absorbed by the quencher fluorophore Q. When the template is being copied, the probe is degraded by the newly synthetized strand, resulting in the separation of the R and Q molecules. This results in the R fluorophore emitting fluorescence that is quantified by the real-time cycler.* ***(B)*** *Graph obtained after the RT-qPCR. ΔRn is the increase in fluorescence in each time point represented according to the PCR cycle. Figure adapted from https://www.ncbi.nlm.nih.gov/probe/docs/techqpcr/.*

2.4.2. *Gene expression analysis by RT-qPCR Arrays*

In our study, the probes used during the RT-qPCR by using RT² PCR Profiler Arrays are linked to the fluorophore SYBR Green, which emits at a wavelength of 522nm (green) when excited at 488nm (blue).

After the preamplification of the pathways of interest, RT² Profiler PCR Arrays (96-well plates, format C, Figure 27) were performed with the samples following the manufacturer's instructions: RT² SYBR Green Mastermix 2X, 102 µL of preamplified cDNA and nuclease-free water until 2,550 µL. The volume was precisely dispensed by pipetting 25 µL per well in this 96-well plate. Plates were then sealed and centrifuged for a minute at 1,000 g to assure the absence of bubbles and the presence of the reagents at the bottom of the plate, this plate was stored then at 4°C until used.

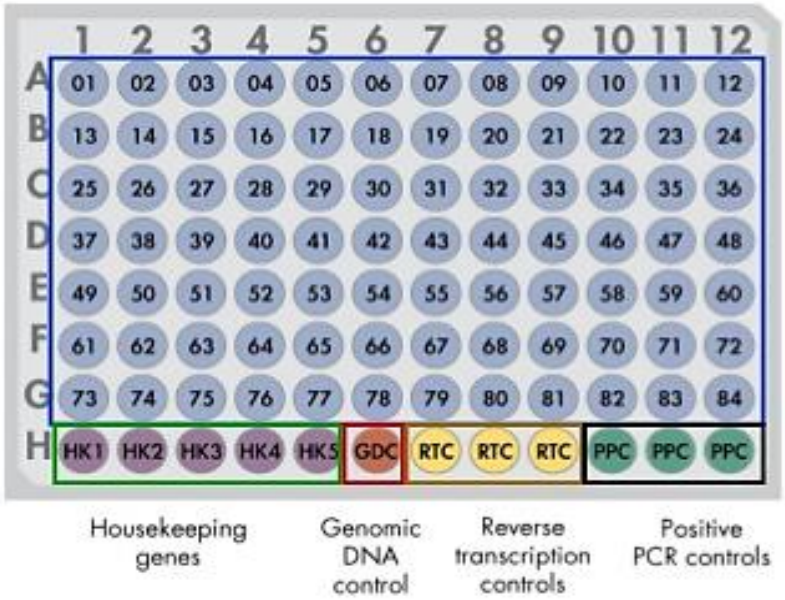

Figure 27. Structure of RT² PCR Profiler Arrays (96 well-plate, format C). *There are 84 wells with probes for different genes related to the same signaling pathway in positions A01-G12. The last row is composed by controls. Housekeeping genes (HK) 1, 2, 3, 4, and 5 are, respectively: beta-actin (ACTB), beta-2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyl transferase 1 (HPRT1) and ribosomal protein lateral stalk subunit P0 (RPLP0). There are controls for genomic DNA, for reverse transcription and for PCR as well. Image from RT² Profiler PCR Array Handbook.*

StepOnePlus Real-Time PCR (Applied Biosystems, Thermo Fisher Scientific, Spain) real-time cycler was programmed to conduct the program: 1 cycle of 10 minutes at 95ºC and 40 cycles of a) 15 seconds at 95ºC and b) 1 minute at 60ºC, registering the fluorescent signal at the end of each cycle.

Genes included in the arrays are summarized in Table 10.

PAHS-040Z		PAHS-041Z		PAHS-072Z	
ACTR2	KRAS	AMH	IL10	AGGF1	IFNG
AKT1	LTA	BDNF	IL11	AMOT	IL10
AKT2	MAP2K1	BMP1	IL12B	ANG	IL12A
AKT3	MAP2K4	BMP10	IL18	ANGPT1	IL12B
ARAF	MAP2K7	BMP2	IL1A	ANGPT2	IL17F

ATF1	MAP3K2	BMP3	IL1B	ANGPTL1	IL6
ATF2	MAPK1	BMP4	IL2	ADGRB1	CXCL8
BAD	MAPK10	BMP5	IL3	BMP2	KITLG
BCAR1	MAPK3	BMP6	IL4	BTG1	KLK3
BCL2	MAPK8	BMP7	INHA	CCL15	LEP
BRAF	MAPK9	BMP8B	INHBA	CCL2	MDK
CASP3	MKNK1	CECR1	INHBB	CD55	NPPB
CASP9	MMP7	CLC	JAG1	CD59	NPR1
CBL	NCK2	CSF1	JAG2	CHGA	PDGFB
CCND1	NFATC3	CSF2	LEFTY1	COL18A1	PDGFD
CHUK	NFKB1	CSF3	LEFTY2	COL4A3	PF4
COL1A1	NRAS	CSPG5	LIF	CSF3	PGF
CREB1	NUP62	CXCL1	LTBP4	CXCL10	PLG
CSNK2A1	PDGFA	DKK1	MDK	CXCL11	PPBP
CSNK2B	PDGFB	ERAP1	MSTN	CXCL12	PRL
DUSP1	PDGFRA	EREG	NDP	CXCL13	PROK1
DUSP6	PDPK1	FGF1	NGF	CXCL14	PTN
EGF	PIK3CA	FGF11	NODAL	CXCL2	RHOB
EGFR	PIK3R1	FGF13	NRG1	CXCL3	RNH1
EGR1	PIK3R2	FGF14	NRG2	CXCL5	RUNX1
EIF4E	PLAT	FGF17	NRG3	CXCL6	SERPINC1
ELK1	PLCG1	FGF19	NRTN	CXCL9	SERPINE1
EPS8	PPP2CA	FGF2	NTF3	EDIL3	SERPINF1
FASLG	PRKCA	FGF22	OSGIN1	EREG	SPINK5
FN1	PTEN	FGF23	PDGFC	FGF1	STAB1
FOS	RAF1	FGF5	PGF	FGF13	TGFA
FOXO3	RAP1A	FGF6	PSPN	FGF2	TGFB1
GAB1	RASA1	FGF7	PTN	FGFBP1	THBS1
GRB2	RHOA	FGF9	SLCO1A2	FIGF	TIE1
GSK3A	RPS6KA5	FIGF	SPP1	FN1	TIMP1
GSK3B	RPS6KB1	GDF10	TDGF1	FOXO4	TIMP2
HBEGF	SHC1	GDF11	TGFB1	FST	TIMP3
HRAS	SRC	GDNF	THPO	GRN	TNF
IKBKB	STAT1	GPI	TNNT1	GRP	TNNI2
IL2	STAT3	HBEGF	TYMP	HGF	TNNI3
JAK1	STAT5A	IGF1	VEGFA	IFNA1	TYMP
JUN	TP53	IGF2	VEGFC	IFNB1	VEGFA

Table 10. Genes included in the RT-qPCR arrays (RT^2 Profiler PCR Arrays, format C, QIAGEN). *PAHS-040ZC: Human EGF/PDGF Signaling Pathway, PAHS-041ZC: Human Growth Factors, and PAHS-072ZC: Human Angiogenic Growth Factors.*

2.5. Bioinformatics analysis

The data obtained from the RT-qPCR was analyzed using StepOne Software v2.3, establishing the same threshold value for the same array of both conditions (PRE-TT and POST-TT) to be able to compare them correctly. Results were subsequently normalized with HK genes with the analysis tool supplied by the QIAGEN webpage. The differences in CTs of the 5 HK genes were studied between both conditions, because HK genes are useful to normalize results and the amount of starting DNA, because they theoretically do not change their expression among conditions. We chose the manual normalization with geometric mean using the 4 HK genes that had a similar variation in both conditions, excluding the HK with more variation (because it would distort the results). Geometric mean was chosen because, although it did not vary a lot with respect to the arithmetic mean, the investigation of Dr Vandesompele *et al.* demonstrated in 2002 that it was more precise (Vandesompele *et al.*, 2002). Chosen HK genes were the same for all the arrays: beta-actin (ACTB), beta-2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein lateral stalk subunit P0 (RPLP0).

2.5.1. *Filtering low quality data*

The previous analysis resulted in a fold change for each gene and each array, which is the difference between the gene expression in both conditions we are analyzing. Genes with a low quality value were deleted from the subsequent analysis. QIAGEN report classifies the obtained fold change's comments in four groups:

- No commented: CTs are between the reliable intervals.

- Comment A: This gene's average CT is relatively high (> 30) in either the control or the test sample and is reasonably low in the other sample (< 30). This fold-change result may also have greater variations if p value > 0.05; therefore, it is important to have a sufficient number of biological replicates to validate the result for this gene.

- Comment B: This gene's average CT is relatively high (> 30), meaning that its relative expression level is low, in both control and test samples, and the p-value for the fold-change is either unavailable or relatively high (p > 0.05). This fold-change result may also have greater variations; therefore, it is important to have a sufficient number of biological replicates to validate the result for this gene.

- Comment C: This gene's average threshold cycle is either not determined or greater than the defined cut-off value (default 35) in both samples meaning that its

expression was undetected, making this fold-change result erroneous and un-interpretable

Hence, genes with B and C comments in QIAGEN report were not considered in the analysis because their fold change is not reliable. After filtering, all genes were taken together, because some genes are repeated along the three arrays analyzed.

2.5.2. Functional annotation of genes

The 184 genes contained in the three arrays were joined and uploaded to ”KEGG marker - Search pathway” web tool included in KEGG database (R Core Team, 2016) in order to see in which pathways they are involved. From the 184 genes, 41 of them (16.7%) were not included in KEGG database. The remaining 143 genes were annotated to KEGG pathways. The KEGG annotation was analyzed and plotted using R programming language (R Core Team, 2016). General results for this annotation were represented using a bar plot with the number of up- and down- regulated genes belonging to each pathway. The pathway most represented in our genes list was colored according the fold change value of each gene by using ”KEGG marker - Color pathway” web tool included in KEGG database. Color gradient depends on the fold change value, from the highest in red to the lowest in blue, using the white color to mark the baseline.

2.6. Validation by immunofluorescence of one of the most upregulated genes: Midkine

After obtaining the highest up- and down- regulated genes, some sections were cut from FFPE blocks from each patient and each condition (PRE-TT and POST-TT), and IF was conducted for one of the most upregulated genes: Midkine (MK, or MDK), using human ileum as external positive control and human kidney as external negative control. MK was selected for being a secreted growth factor involved in promoting cell growth, differentiation (Muramatsu, 1993), migration, angiogenesis, regeneration (Ochiai *et al.*, 2004; Ikutomo *et al.*, 2014; Liedert *et al.*, 2014), cell survival (S.-L. Zhao *et al.*, 2014), etc… Additionally, it has been postulated as a therapeutic target for several disorders (Muramatsu and Kadomatsu, 2014). Due to its functions, we postulated it as a good candidate to validate.

Briefly, tissues were deparaffinized by heat, xylol, alcohol and water as previously described. The antigen retrieval mas carried out with citrate buffer and membranes were permeabilized with detergent to facilitate the entrance of the antibody inside the cells. Non-specific binding was blocked in a solution containing BSA, detergent and

NGS for 1 hour at RT. Antibodies used were: *monoclonal mouse anti-human MK* (sc-46701, Santa Cruz Biotechnology) as primary antibody (dilution 1:250, 4ºC, o/n) and goat anti-mouse IgG1 488 (A-21121, Invitrogen) as secondary antibody (dilution 1:500, RT, 30 minutes). Sections were finally stained with DAPI and immunolocalization were observed under the fluorescent Nikon Eclipse 80i microscope.

All the conditions for this IF are specified in Table 11.

	Antigen retrieval	**Cellular permeabilization**	**Blocking solution**	**Primary antibody**	**Secondary antibody**	**External positive control**	**External negative control**
Midkine (MK)	Citrate buffer 10mM 0.05% Tween-20 pH 6. 20min, 95ºC	PBS + Triton X-100 0.2% 10min, RT	PBS-BSA 5% + Tween-20 0.05% + NGS 5%. 30min, RT	Monoclonal Mouse anti-human MK (sc-46701, Santa Cruz Biotechnology) Dilution 1:250 o/n, 4°C RRID:AB_627949	Goat anti-mouse Alexa 488 Fluor (Invitrogen, A-21121) Dilution 1:500 30min, RT RRID:AB_141514	Human ileum	Human kidney

Table 11. Antibodies and experimental conditions used for the study of Midkine (MK) by immunoflurorescence*. Table summarizing the different steps implied: antigen retrieval, cellular permeabilization, blocking for non-specific bindings, primary and secondary antibodies and controls. BSA: bovine serum albumin; NGS: normal goat serum; PBS: phosphate buffered saline.*

VI. RESULTS

"Cuando llegues al final de lo que debes saber, estarás al principio de lo que debes sentir"

Khalil Gibran

VI. RESULTS

A. Identification and isolation of somatic stem cells in human endometrium with potential stem cell markers. Proof of concept in animal models

1. Immunocharacterization, identification and isolation of candidate somatic stem cells in human endometrium

1.1. Immunocharacterization of human endometrium and identification of specific somatic stem cell markers

In order to identify specific SSC markers, the immunocharacterization and identification of endometrial SSCs from human endometrium was based on the expression of the following markers: EPCAM, ICAM1, SSEA1, W5C5, BCRP1, TNFα and THBS1. As described in "*1.4.1. Analysis of stem cell markers in epithelial and stromal fractions*" in the materials and methods section, the selection of the studied markers was based on a study of the literature (SSEA1 (Valentijn *et al.*, 2013), W5C5 (Masuda *et al.*, 2012), EPCAM (Gargett *et al.*, 2009), and BCRP1 (Tsuji *et al.*, 2008)), and including the most highly expressed membrane markers in the SP (ICAM1, TNFα, and THBS1).

Figure 28 represents two histograms showing the immunophenotype of all tested markers after studying the epithelial and stromal fractions from human endometrial biopsies by FC assay.

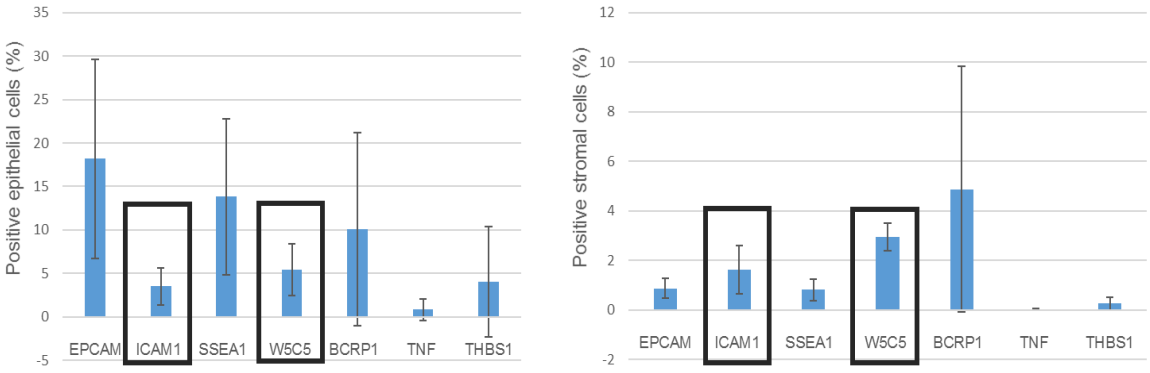

Figure 28. Immunophenotype of human endometrium by flow cytometry. *Flow cytometric histograms showing the percentage of positive cells for EPCAM, ICAM1, SSEA1, W5C5, BCRP1, TNFα and THBS1 in dissociated human endometrial biopsies (n=4 epithelium and n=6 stroma). Data is shown as mean with error bars (standard deviation). The two endometrial SSC markers selected for subsequent isolation and animal models are indicated by a black rectangle. Figure from López-Pérez et al., 2018.*

The percentages of ICAM1$^+$ cells were 3.5 ± 2.14% in the epithelial and 1.62 ± 0.97% in the stromal fractions. Similarly, W5C5$^+$ cells were found in 5.42 ± 2.97% and 2.95 ±

0.56% of the epithelial and stromal fractions, respectively. In contrast, TNFα and THBS1 exhibited limited and inconsistent expression; we therefore excluded these markers from further experiments. Highly variable expression of EPCAM, SSEA1 and BCRP1 was detected in all analyzed samples, resulting in their elimination for further experiments as well.

1.2. Isolation of candidate somatic stem cells in human endometrium

For animal models, we used W5C5 and ICAM1 positive and negative cells (markers selected from the previous immunocharacterization assay) isolated from total endometrium, as well as SP-derived cell lines (ICE6/7) due to their proved reconstitution potential in other studies (Cervelló *et al.*, 2010; Masuda *et al.*, 2010; Cervelló, Mas, *et al.*, 2011; Miyazaki *et al.*, 2012). Throughout the experiments by FACS, we observed that the percentages of W5C5$^+$ cells were 3.89% in human endometrium (without separating both cell fractions), 0.169% in ICE6 and 0.305% in ICE7 cell lines (Figure 29A). For ICAM1$^+$ cells, the percentages were 2.1% in human endometrium (without separating both cell fractions), and 96.2% and 97.2% in the same populations (Figure 29B). The high proportion of ICAM1$^+$ cells in SP cell lines was to be expected, this marker was selected due to its high expression in SP cells.

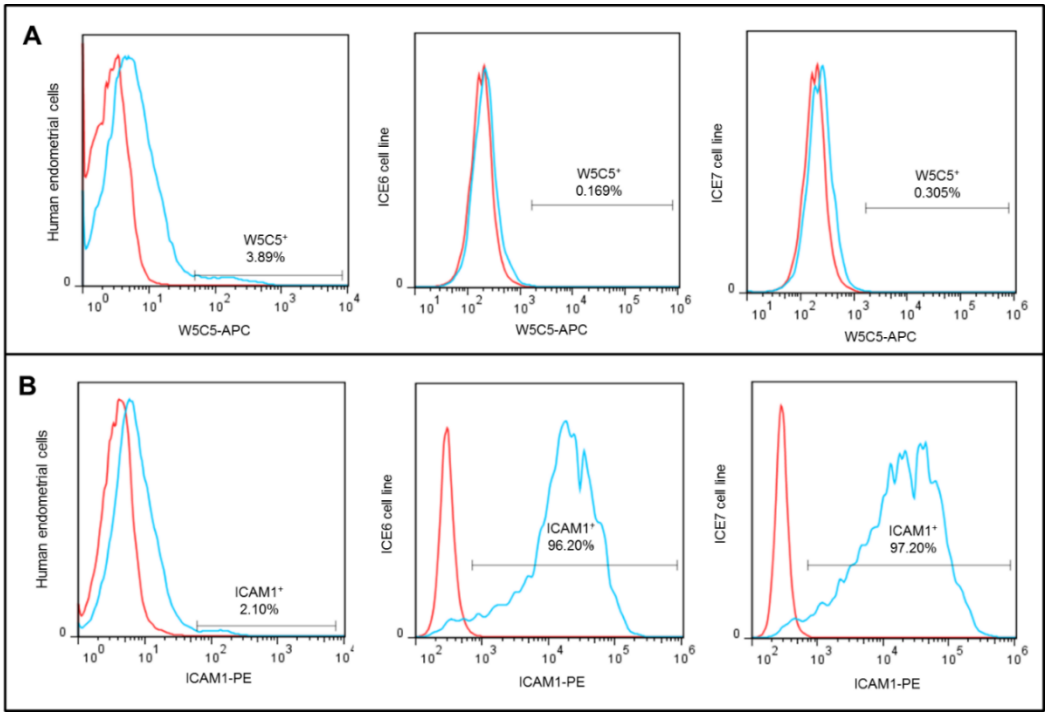

Figure 29. Inmunophenotype for W5C5 and ICAM1 of human endometrium and Side Population cell lines. *Blue lines in cell sorter plots indicate positive staining for each marker compared with isotype control samples (red line).* ***(A)*** *Representative FACS plots showing endometrial staining for W5C5-APC in total human endometrium as well as SP-derived cell lines are shown (ICE6 and ICE7).* ***(B)*** *Representative FACS plots showing endometrial staining for ICAM1-PE in the same cell populations than Figure 29A. Figure from López-Pérez et al., 2018.*

2. <u>In vivo model: endometrial reconstitution after xenotransplantation</u>

Positive and negative cells for W5C5 and ICAM1, as well as total endometrial cells, were freshly sorted for xenotransplantation assays. In addition, ICE6 and ICE7 cell lines were cultured in hypoxia and these endometrial cells were also used for xenotransplantation. Single cell suspensions were used for the injection (500,000 cells in any case, as summarized in Table 5, materials and methods section) under the kidney capsule in the right kidneys of immunodefficient and ovariectomized NOD-SCID female mice. Subsequently, the assessment of the formation of new endometrial-like tissue, as well as the location of proliferative cells, were carried out. Moreover, a control of the injection technique was performed with 5 mice to demonstrate the presence of human engrafted cells during 60 days at different time points. In the rest of experimental groups (TF, $W5C5^{+}$+TF, $W5C5^{+}$, $W5C5^{-}$, $ICAM1^{+}$+TF, $ICAM1^{+}$, $ICAM1^{-}$, ICA6/7), three mice per condition were injected. Unfortunately, one mouse died during the experiment in $W5C5^{+}$ condition (López-Pérez *et al.*, 2018).

2.1. Perivascular location of engrafted and proliferative cells

To verify the injection technique, we administered the total human endometrial fraction labelled with Molday ION Rhodamine B under the kidney capsule of some mice (n=5), and we tracked them using Prussian blue staining. Labelled engrafted cells were identified in these animals up to 60 days after cell injection. For this purpose, after sacrificing the animals, including the organs and preparing the slides, the tissue was stained with Prussian blue, a dye that permits the localization of rhodamine-labelled cells creating a blue precipitate that can be observed under the microscope. Engrafted cells under the kidney capsule were then localized at different time points after cell injection, as illustrated in Figure 30.

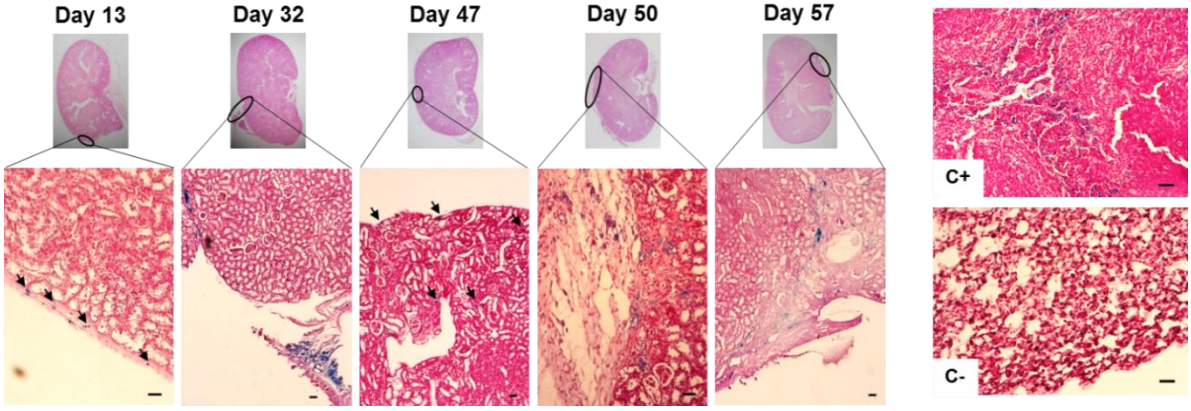

Figure 30. Verification of injection technique: Prussian blue staining of rhodamine-labelled cells in the kidney capsule at different time points. *Assay to test the retainment of injected cells at 13, 32, 47, 50 and 57 days after the injection under the kidney capsule of NOD-SCID mice. Regions with iron deposits are magnified from the kidney pictures. Arrows point out*

some labelled cells. In the images without arrows, human blue cells are clearly visible in blue cell clusters. Spleen and lung were used as positive (C+) and negative (C-) controls respectively. Scale bars: 20 µm. Figure from López-Pérez et al., 2018.

Concerning the location of these human cells injected in the kidneys, Prussian blue-positive cells with human origin were located around blood vessels in the kidney capsule (Figure 31A). Similarly, Ki67 expression identified human endometrial proliferating and active cells under the kidney capsule after 60 days near the same location as the engrafted cells in all xenotransplanted kidneys tested (Figure 31B).

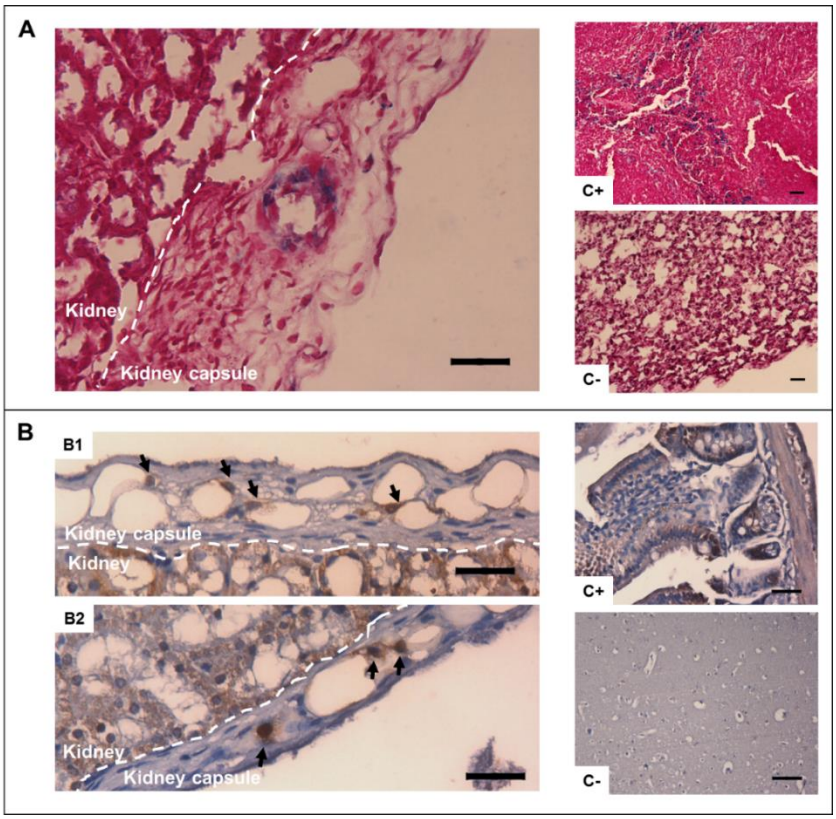

***Figure 31. Localization of human engrafted and proliferative cells in endometrial-like tissue within the kidney capsule**. **(A)** Prussian blue staining of xenotransplanted cells at perivascular location. Negative (C-) and positive (C+) controls used here are mouse lung and mouse spleen, respectively. **(B)** Proliferation assay by Ki67 showed the presence of dividing cells (indicated with arrows) around blood vessels in two representative regions within the kidney capsule (B1 and B2). Negative (C-) and positive (C+) controls used here are mouse brain and mouse small intestine, respectively. In the images (A, B1 and B2), the dotted lines indicate the border between the human reconstructed tissue and the mouse kidney. Scale bars: 20 µm. Figure from López-Pérez et al., 2018.*

2.2. Evaluation of the endometrial reconstitution efficiency: human endometrial makers' analysis

After confirming the engraftment using Prussian blue staining, we assessed the characterization of the newly formed tissue in order to test if the *de novo* tissue present in the experimental mouse kidney capsules corresponded to endometrial-like tissue. This was done by studying the reconstruction efficiency, a concept based on Masuda's study (Masuda *et al.*, 2010), in the different experimental groups by localizing three human endometrial markers by immunofluorescence: VIM, CK18 and PR. The reconstitution rate (RR, Table 12) was calculated for each group using the expression of these three markers. In the experimental design, we injected total human endometrial cells as control and also used these cells as a support for $W5C5^+$ and $ICAM1^+$ cell fractions to mimic the uterine microenvironment and to attempt to enhance the reconstitution efficiency. All the conditions showed *de novo* tissue formation within the kidney capsule to a different extent.

Antibodies / Endometrial cells	VIM	CK18	PR	RR
Total Fraction (TF)	+	+	+	100%
	+	+	+	
	+	+	+	
Non-injected kidney	-	-	-	0%
	-	-	-	
	-	-	-	
$W5C5^+$	-	+	+	33%
	+	-	-	
	+	+	+	
$W5C5^+$ +TF	+	+	+	100%
	+	+	+	
$W5C5^-$	+	+	+	66%
	+	+	+	
	-	-	-	
$ICAM1^+$	+	+	-	33%
	+	+	+	
	-	+	+	

ICAM1$^+$ +TF	+	+	+	100%
	+	+	+	
	+	+	+	
ICAM1$^-$	+	+	+	66%
	+	+	+	
	-	-	+	
SP cell lines	+	+	+	100%
	+	+	+	
	+	+	+	

Table 12. Table summarizing the expression of human vimentin (VIM), cytokeratin 18 (CK18) and progesterone receptor (PR) under the kidney capsule to evaluate the endometrial reconstitution after xenotransplantation in the murine model. *Endometrial reconstitution was evaluated by the expression of three human endometrial markers (VIM, CK18 and PR) for each condition: total fraction (TF), W5C5$^+$, W5C5$^+$+TF, W5C5$^-$, ICAM1$^+$, ICAM1$^+$+TF, ICAM1$^-$ and SP cell lines, as well as non-injected kidneys. The reconstitution rate (RR), represented as percentage, is indicated in the last row. Table from López-Pérez et al., 2018.*

The signal for VIM was observed to have two different patterns: perinuclear or diffuse within the endometrial-like tissue in the kidney capsule. The signal was nuclear for PR, and cytoplasmic for CK18. Due to the different expression patterns of these markers, it was very difficult to quantify the signal, so RR assesses the reconstruction efficiency in a qualitative manner (Figure 32).

Figure 32A shows TF injection and non-injected kidneys as controls. For W5C5 cells, in both negative and pure positive fractions, endometrial-like tissues were formed at different RRs (from 33% to 66%). Interestingly, W5C5$^+$ cells supplemented with TF had the largest RR (100%, Figure 32B), expressing all three antibodies in all of the kidneys of this condition. Similarly, xenotransplantation with ICAM1$^-$ and ICAM1$^+$ cells gave rise to lower endometrial reconstruction (RRs of 66 and 33% respectively) compared to the regenerative potential of the supplemented ICAM1$^+$ fraction that achieves 100% RR (Figure 32C). Unexpectedly, the ability to form endometrial tissue was greater in ICAM1$^-$ and W5C5$^-$ transplants than in their positive isolated equivalents, with a maximum potential when positive cells are supplemented with total endometrial cells (TF).

On the other hand, the transplants with SP cell lines had the capacity to generate endometrium similar to TF and therefore more than cells identified by the markers described previously (Figure 32D). Furthermore, the new tissue generated in SP cells transplants showed much clearer protein expression patterns than the rest of cell groups.

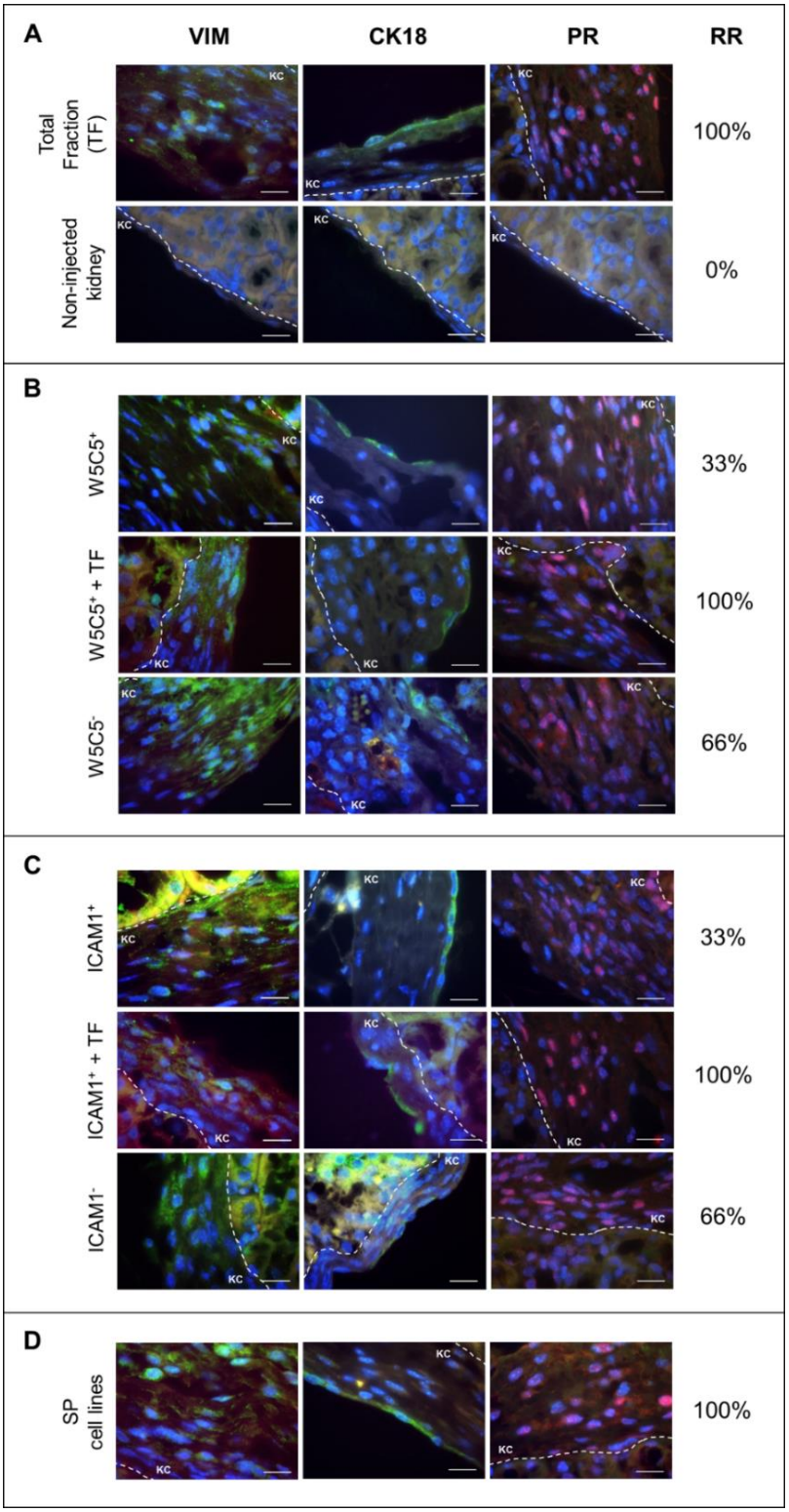

Figure 32. Reconstitution rate (RR) based on the presence of human vimentin (VIM), cytokeratin 18 (CK18) and progesterone receptor (PR). *Dotted lines indicate the border*

between the human reconstructed tissue in the kidney capsule (KC) and the mouse kidney. ***(A)*** *Kidneys injected with TF are the positive controls of the experimental design, and non-injected kidneys are the negative controls.* ***(B)*** *Panel corresponding to W5C5 marker, with positive (pure/not supplemented and supplemented) and negative fractions.* ***(C)*** *Panel corresponding to ICAM1 marker, with positive (pure/not supplemented and supplemented) and negative fractions.* ***(D)*** *Panel corresponding to ICE6 and ICE7 SP-derived cell lines. We considered 100% RR when the three markers (VIM, CK18 and PR) are expressed in all kidneys analyzed per condition. Scale bars: 10 µm. Figure from López-Pérez et al., 2018.*

Positive and negative external controls for specific antibodies are shown in Figure 33, these were human endometrium the positive control for VIM, CK18 and PR, and the negative ones were mouse small intestine for VIM, and mouse kidney (kidney capsule) for CK18 and PR.

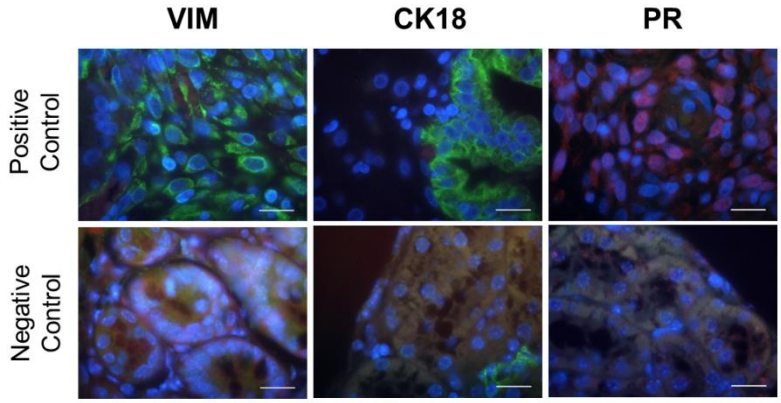

Figure 33. Positive and negative external controls for human vimentin (VIM), cytokeratin 18 (CK18) and progesterone receptor (PR) in immunofluorescence. *Human endometrium in the positive control for the three markers, and the negative control is mouse intestine for VIM and mouse kidney (capsule) for CK18 and PR. Scale bars: 10 µm. Figure from López-Pérez et al., 2018.*

The internal negative controls of the technique were tissue slides where primary antibody was omitted (Figure 34), because the absence of signal in this case is a proof that the signal is not the result of the unspecific binding of the secondary antibody and that it comes from the binding of the primary antibody.

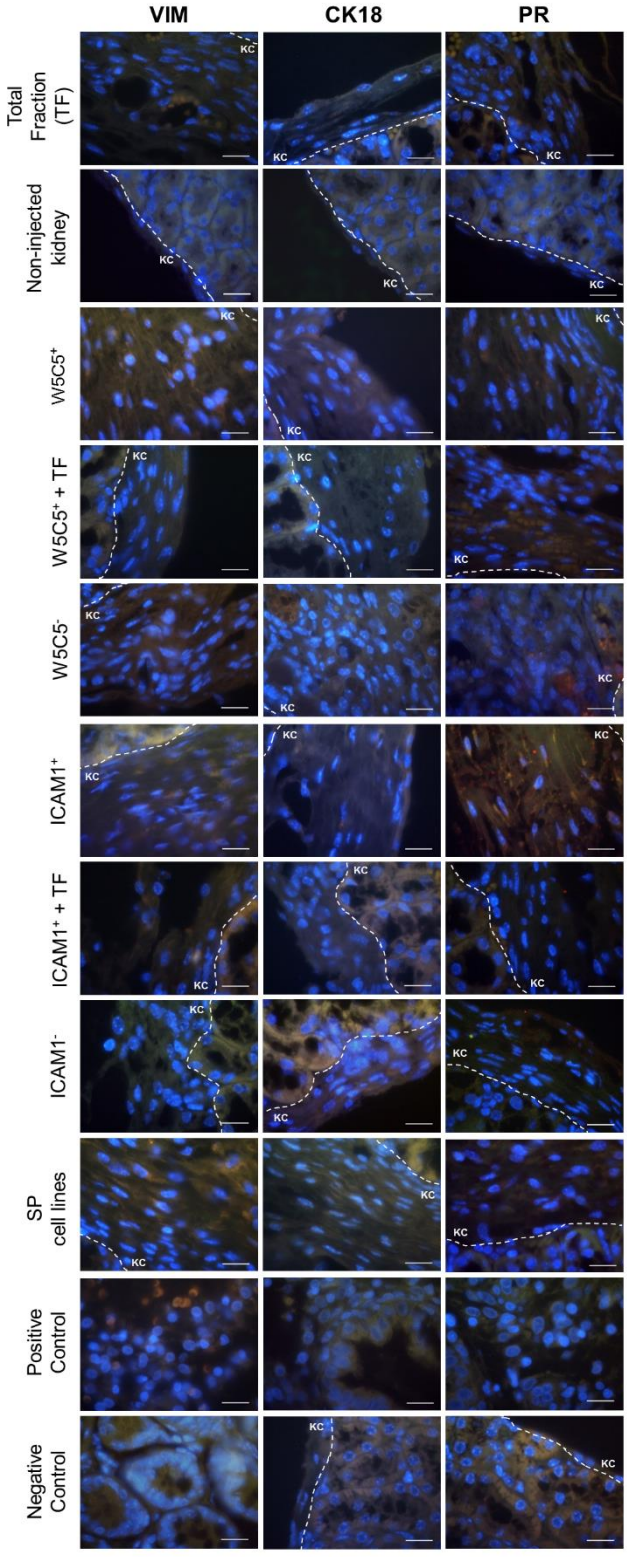

Figure 34. Negative reagent controls for human VIM, CK18 and PR. *Dotted lines indicate the border between the human reconstructed tissue in the kidney capsule (KC) and the mouse kidney. The absence of staining when the primary antibody is omitted is a control for the specific binding of the secondary antibody to the primary one. The last two rows belong to the external positive and negative controls (Figure 33). Scale bars: 10 µm. Figure from López-Pérez et al., 2018.*

2.3. Thickening of the kidney capsule after xenotransplantation

The characterization of the newly formed endometrial-like tissue was also performed by observing the kidney capsule thickness by H&E staining (Figure 35). This assay permitted to check the difference, if any, amongst kidney capsules along the different

conditions at first sight. However, results did not allow differentiating between the kidneys because all of them showed a thickened kidney capsule after the administration of cell suspensions (except for the negative controls, non-injected kidneys). A representative kidney per condition is depicted in Figure 35, as well as a magnification of representative results (results ordered according to their RR).

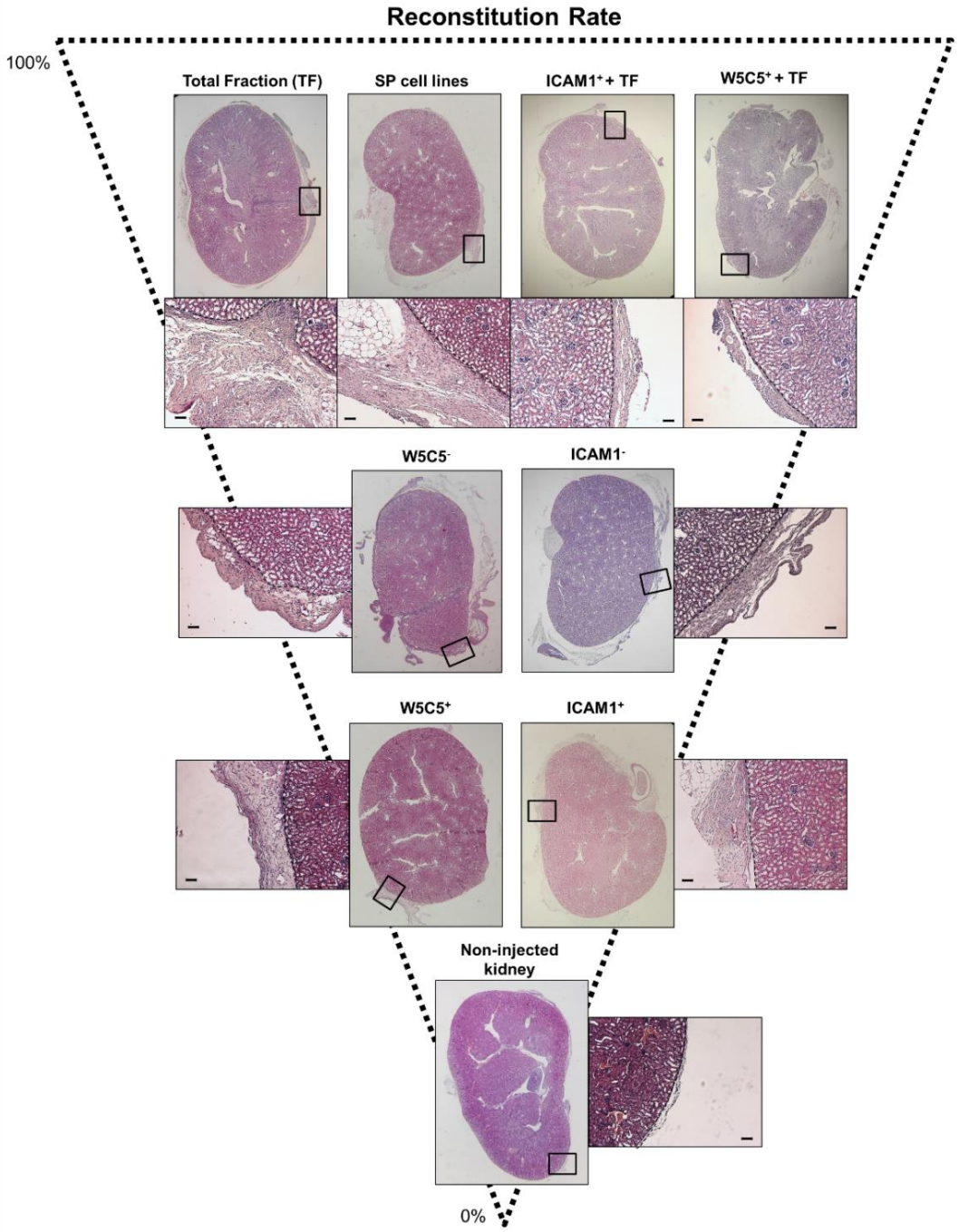

Figure 35. Kidney capsule thickness after xenotransplantation. *H&E staining of mouse kidneys corresponding to each experimental condition, including magnified regions of interest, are represented. Dotted lines in magnified regions indicate the border between the human reconstructed tissue and the mouse kidney. A decreasing trend was represented according to the reconstitution rate. Scale bars: 40 µm. Figure from López-Pérez et al., 2018.*

2.4. Presence of Musashi-1 expression to evaluate stem cell-properties of postulated stem cell populations

MS1 is a described stem cell marker in the human endometrium, so its expression in kidney capsules of xenotransplanted animals would be another proof of the stemness properties of the injected cell groups. Our aim was to study the endometrial SSCs contained in the injected fractions that could remain undifferentiated in the endometrial-like tissue formed under the kidney capsule 60 days after cell injection. This way, we were able to compare the stem cell properties of each experimental cell group and each marker (W5C5, ICAM1 and SP).

IHC of the kidneys belonging to each condition permitted the observation of MS1 expression within the endometrial-like tissue formed under the kidney capsule. In one of the positive controls, the endometrium (Figure 36A, upper image), only few cells organized in clusters showed expression for this marker (low percentage of cells, consistent with the low proportion of SSCs in one tissue). Moreover, MS1 expression was also observed around a blood vessel (Figure 36A, lower image), which is compatible with the postulated location of SSCs, described previously, and in endometriotic tissue (Figure 36B), described by Dr. Götte in 2008.

Results of MS1 expression were represented as the amount of positive cells per 100,000 μm^2. This was done because it is difficult to unify criteria from kidneys with different capsule thickness, and even inside the same kidney. Thus, an objective manner to show the results was by counting the total positive cells per kidney, measuring the total kidney capsule area and expressing the results in a certain area. Representative images of MS1 signal in the endometrial-like tissue formed in some injected kidneys are depicted in Figure 36C.

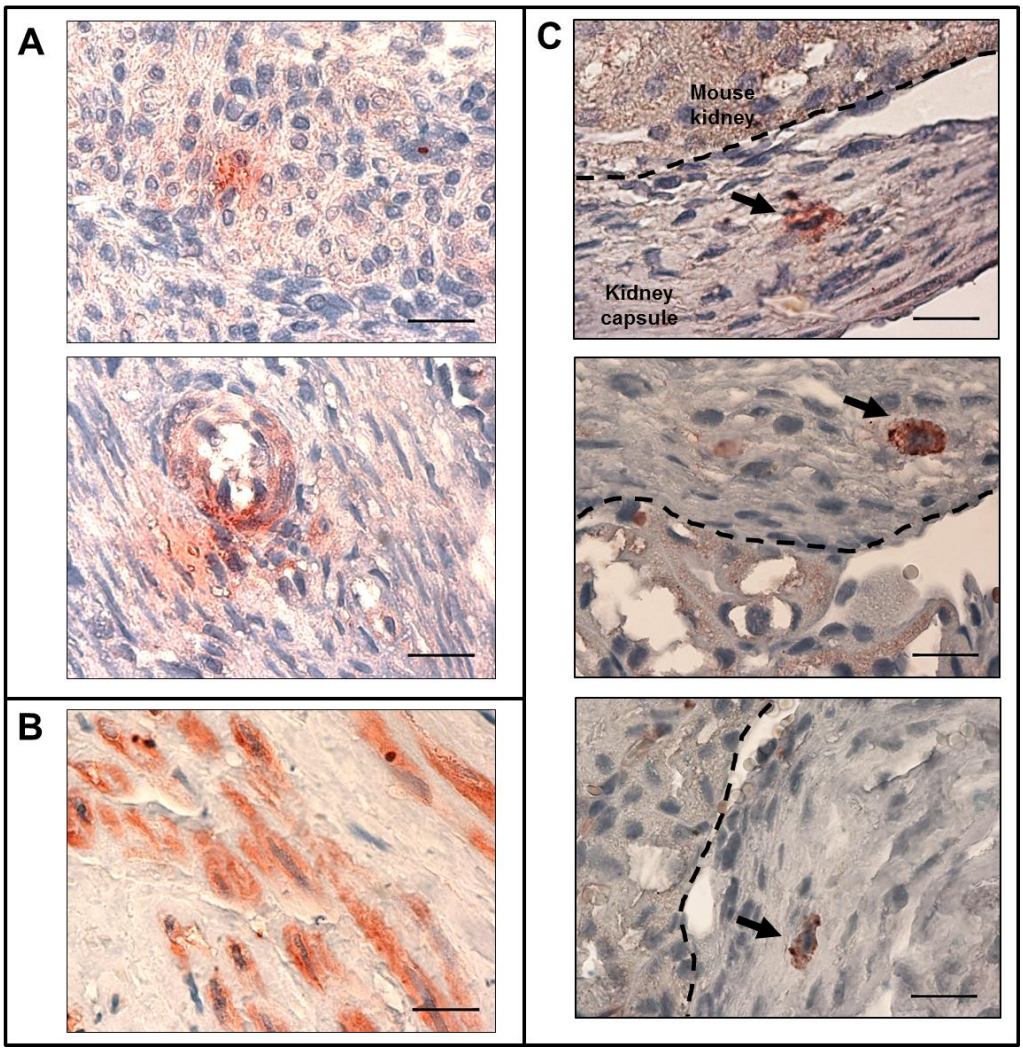

Figure 36. Musashi-1 (MS1) expression in studied samples. (A) *Positive cells in one of the positive controls (human endometrium). Two cell groups expressing MS1 inside the endometrial tissue, with one of them in a perivascular location (lower image).* ***(B)*** *Positive cells in another positive control (human endometriotic tissue). Abundant signal in isolated and grouped cells.* ***(C)*** *MS1 expression in representative regions of the endometrial-like tissue formed under the kidney capsule of xenotransplanted mice. Dotted lines indicate the border between the human reconstructed tissue in the kidney capsule and the mouse kidney. MS1⁺ cells are indicated with arrows. Scale bars: 10 μm.*

A graph summarizing the results is shown in Figure 37, where positive cells per condition (in an area of 100,000 μm^2) are expressed as arithmetic mean with error bars (standard deviation). Due to the high heterogeneity in the sample, the error is very high, resulting in no significant differences. However, the results are useful to detect a trend in relation to MS1 expression per condition.

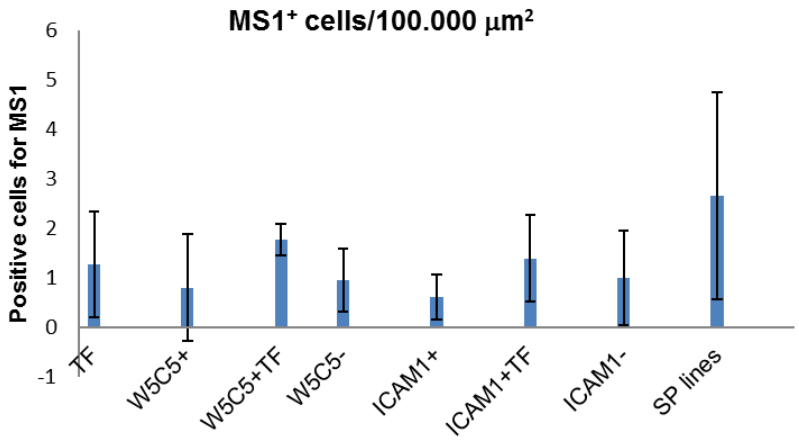

Figure 37. Graphic showing the positive cells for Musashi-1 (MS1) in relation to the area in each experimental condition. *MS1 positive cells are expressed in relation to 100,000 μm^2 for the experimental conditions: total fraction (TF), W5C5-positive cells supplemented with endometrial disaggregated cells (W5C5+TF), W5C5-negative cells (W5C5$^-$), positive cells isolated for W5C5 (W5C5$^+$), SP-derived cell lines (ICE6/7), and the same populations for ICAM1 (ICAM1$^+$, ICAM1+TF, ICAM1$^-$).*

As a result, MS1 expression appeared to be enhanced in SP cell lines. Following SP, positive fractions supplemented with total endometrial cells (W5C5$^+$+TF and ICAM1$^+$+TF) showed higher expression of MS1, followed by the TF group. Then, the negative pure fractions expressed less MS1 than the previous groups (in the same grade), but more than positive pure fractions. These results corroborate those obtained for endometrial-like tissue formation explained in section "*2.2. Evaluation of the endometrial reconstitution efficiency: human endometrial makers' analysis*".

B. Stem cells and paracrine factors in pathologic endometrium: endometriosis, Asherman's syndrome and endometrial atrophy

1. Study of candidate somatic stem cell populations in endometriosis

With this project, we aimed to study the percentage of positive and negative cells for ICAM1, W5C5 and SP in eutopic endometrium from healthy women and patients with endometriosis, both in proliferative and secretory phases. One problem that emerged was derived from using primary samples: on one side, after analyzing the studied populations, we found a great heterogeneity in patients with the same initial profile, healthy or suffering from endometriosis. On the other side, among patients with endometriosis, there was much variation regarding to the stage of the disease. This last observation allowed us to establish the following different groups for the comparison: healthy versus rASRM stage I endometriosis patients in secretory phase, and secretory versus proliferative phase in rASRM stage I endometriosis patients.

Analyzing the samples by FC resulted in graphs with four quadrants containing information for diverse cell subsets: Q1: $ICAM^+$, Q2: $W5C5^+$ $ICAM^+$, Q3: $W5C5^-$ $ICAM^-$, and Q4: $W5C5^+$.

Moreover, for each sample we performed the respective isotype control to remove the residual background created by non-specific bindings. Similarly, when human samples were analyzed to quantify the SP, the verapamil control was carried out to check that ABC channels were closed after its addition and therefore the signal of SP was specific.

In Figure 38A a representative flow cytometer plot is depicted that is very similar to those obtained by every sample analyzed for W5C5 and ICAM1, and a representative chart of the pertaining isotypes. In Figure 38B a graph resulting from the SP analysis is shown, as well as the control with verapamil.

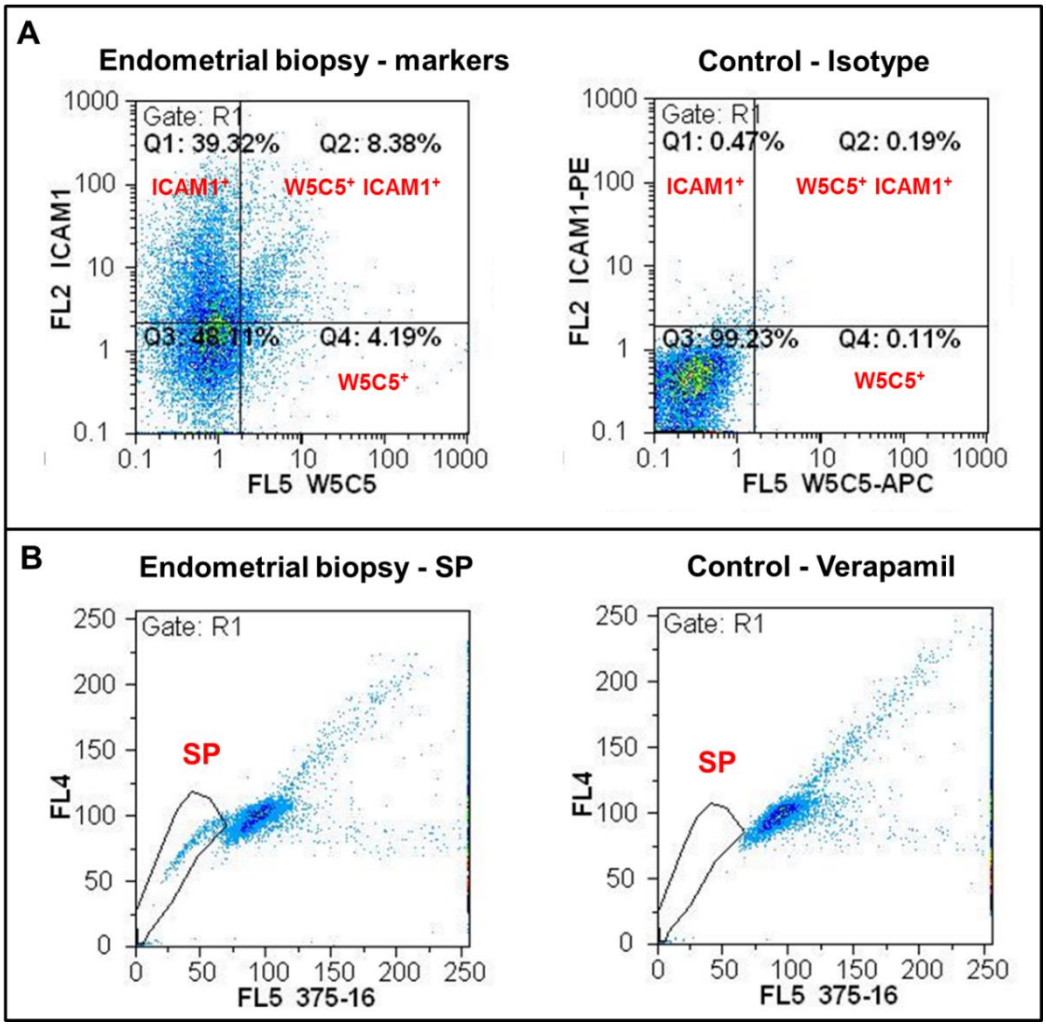

Figure 38. Representative flow cytometry plots for W5C5, ICAM1 and Side Population (SP) in human endometrium (healthy and endometriosis patients). (A) *Plots for W5C5 and ICAM1, as well as their isotypes as control. Q1: $ICAM1^+$, Q2: $ICAM1^+$ $W5C5^+$, Q3: $W5C5^-$ $ICAM^-$, Q4: $W5C5^+$.* ***(B)*** *Graphs to study the SP with verapamil chart as control of the technique. The tail corresponding to SP cells disappears when verapamil is added along with Hoechst. FLX refers to the excitation laser of the fluorochromes in each case.*

The initial plan was to compare healthy with high-grade endometriosis patients (rASRM stages III-IV) but working with human biopsies has its limitations. Sadly, there was very little control over the profile of the donor patients, we obtained a lot of low-grade endometriosis (rASRM stage I) but only a few healthy and high-grade endometriosis samples. With this small pool, it was difficult to compare populations because there were not enough controls (healthy) nor enough high-grade endometriosis patients, so it was impossible to perform a comparison between them. However, we were able to compare between proliferative and secretory phase in mild endometriosis patients (rASRM stage I) (Figure 39A), and healthy versus rASRM stage I endometriosis patients in secretory phase (Figure 39B). In the first case, cells expressing both markers (W5C5 e ICAM1), as well as SP, were enriched in the proliferative phase. In the second comparison, a higher expression of ICAM1$^+$ cells and SP in rASRM stage I endometriosis samples was observed, also having a similar percentage of W5C5$^+$ cells. However, biopsies are primary cells, what means that even though women have the same condition profile, there is still a high variablity between them, because of this we did not obtain significant results due to the high error and variability.

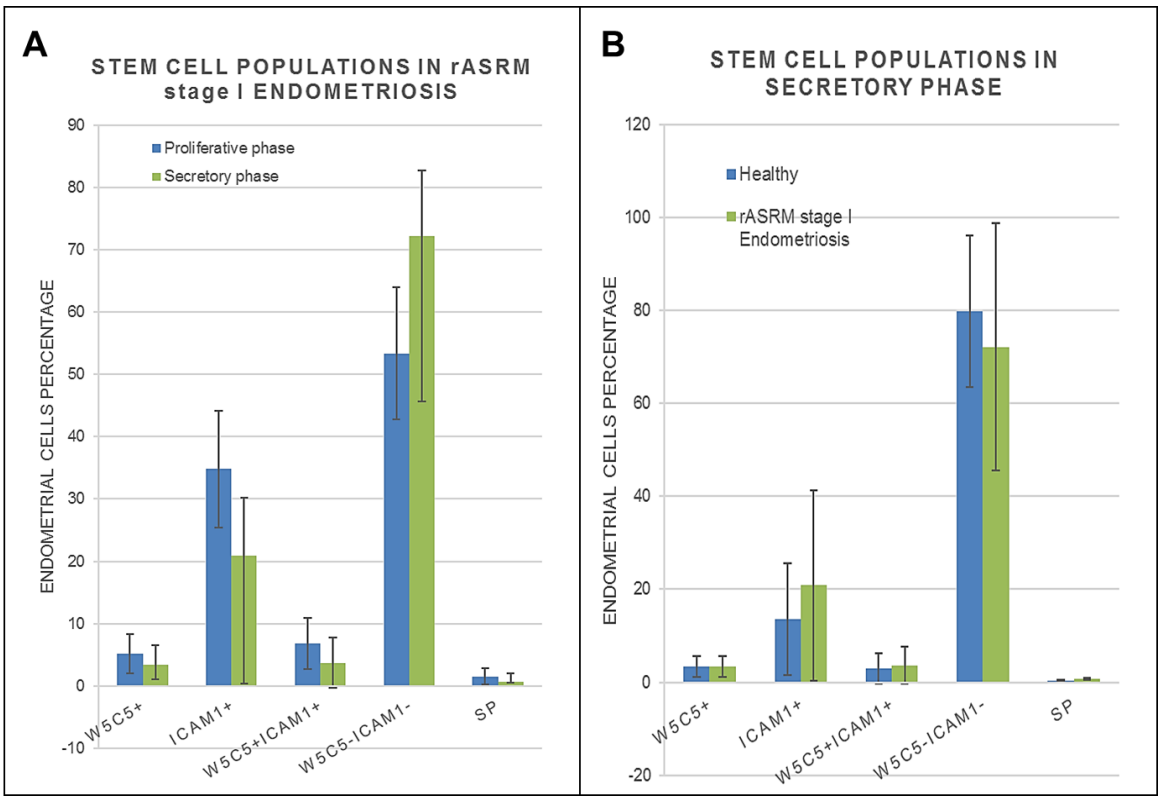

Figure 39. Comparison of stem cell populations based on W5C5, ICAM1 and Side Population (SP) in eutopic endometrium from healthy and endometriosis patients, in both proliferative and secretory phases. (A) *Comparison between proliferative and secretory phases of rASRM stage I endometriosis patients, with an increase of W5C5$^+$, ICAM1$^+$ and SP cells in proliferative phase.* ***(B)*** *Comparison of healthy and rASRM stage I endometriosis patients in secretory phase. ICAM1$^+$ and SP cells were increased in these last ones, with a similar population of W5C5$^+$ cells.*

2. Identification of the paracrine factors secreted by bone marrow-derived stem cells involved in endometrial regeneration under pathological conditions: Asherman's syndrome and endometrial atrophy

2.1. Histological improvement after cell therapy

H&E staining of tissue before and 3 months after $CD133^+$ BMDSCs injection (cell therapy) showed an improvement in endometrial histology in patients with AS and/or EA. In all cases, this improvement was noticeable (more in patients A and C, Figure 40) by the detection of more endometrial tissue in the samples, as well as better glands organization. Moreover, as we described in "*2.2. Neoangiogenesis assay*" in materials and methods section, they all increased the number of mature blood vessels after cell therapy.

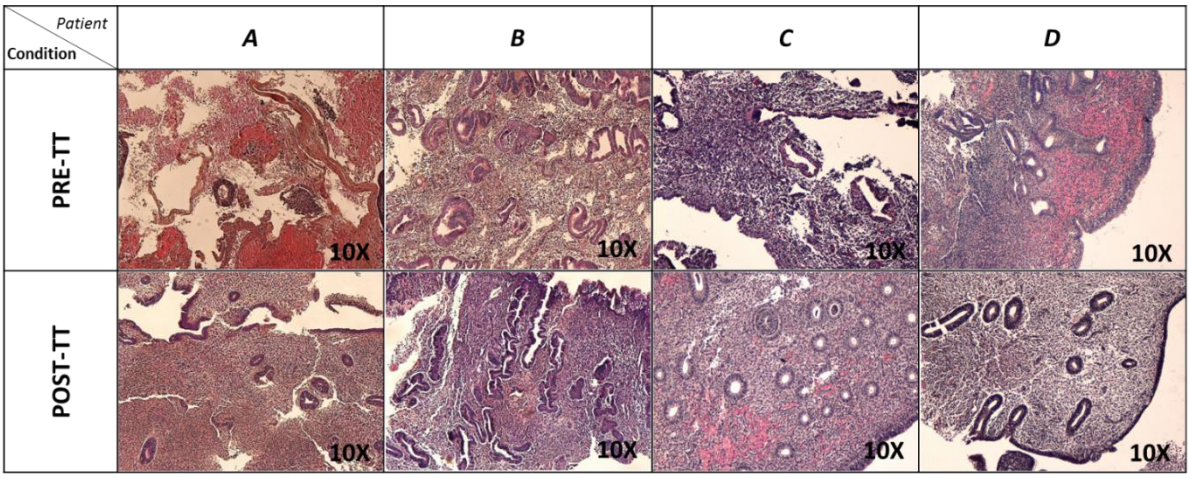

Figure 40. Histological study of samples before and after treatment with $CD133^+$ BMDSCs. *In all cases, an improvement in the histology was noticeable (more tissue and better-organized glands). Patients: A (5), B (7), C (12) and D (13).*

2.2. Description of the most differentially expressed genes

The starting material was endometrial FFPE biopsies from patients with Asherman's syndrome and/or endometrial atrophy, treated with $CD133^+$ BMDSCs. The samples were collected before (PRE-TT) and 3 months after (POST-TT) cell therapy. After collecting a pool of FFPE tissue sections per condition, RNA was extracted and reverse transcribed to cDNA, and this step was followed by preamplification for the three selected pathways of interest. RT-qPCR arrays were then performed for the following pathways: PAHS-040ZC: Human EGF/PDGF Signaling Pathway, PAHS-041ZC: Human Growth Factors, and PAHS-072ZC: Human Angiogenic Growth Factors.

After the first low quality data filtering, we kept 79 genes for the Human EGF/PDGF Signaling Pathway array (PAHS-040Z), 55 for the Human Growth Factors array (PAHS-041Z) and 64 for the Human Angiogenic Growth Factors array (PAHS-072Z). Some of these genes (184 genes in total) are shared among the arrays, as shown in the Venn diagram in Figure 41.

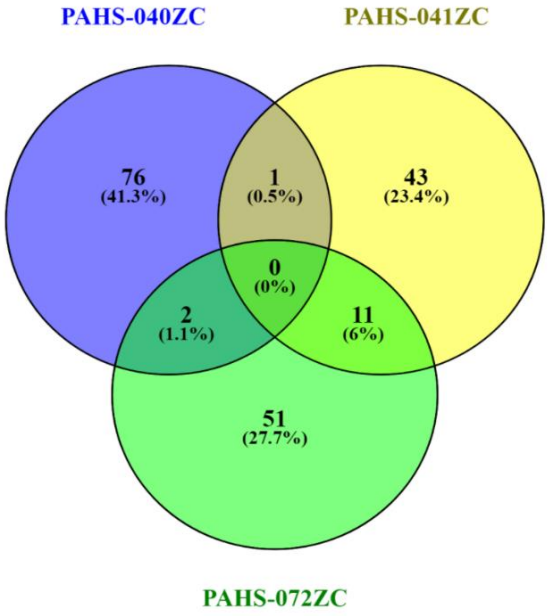

Figure 41. Venn diagram corresponding to genes included in the three arrays after the first low quality data filtering: Human EGF/PDGF Signaling Pathway (PAHS-040ZC), Human Growth Factors (PAHS-041ZC) and Human Angiogenic Growth Factors (PAHS-072ZC). *Some genes overlap among pathways, with percentages indicated after the filtering.*

To be able to obtain the most differentially expressed genes after the previous data processing, a normalization with the QIAGEN web was carried out with HK genes with least variation along the two conditions per array. The HK genes chosen in all the arrays were ACTB, B2M, GAPDH and RPLP0. Using another useful tool of QIAGEN web, charts and diagrams were made, which allowed studying the results in a visual manner. One type of diagram, the scatter plot, permitted us to detect the trend that both groups (PRE-TT and POST-T) followed at first sight. Figure 42 corresponds to the arrays where the most differentially expressed genes were included: PAHS-041ZC of Human Growth Factors (Figure 42A) and PAHS-072ZC of Human Angiogenic Growth Factors (Figure 42B). The Human EGF/PDGF Signaling Pathway array (PAHS-040ZC) was not represented in Figure 42 because it did not have differentially expressed genes in comparison with the other two; none of the most differentially expressed genes were taken from this array. The scatter plot compares the normalized expression (with HK genes) of each gen in the array between both conditions, and results are shown as dots (one for each gene) that permits the observation of expression changes (fold regulation, similar to fold change) in a fast and easy way.

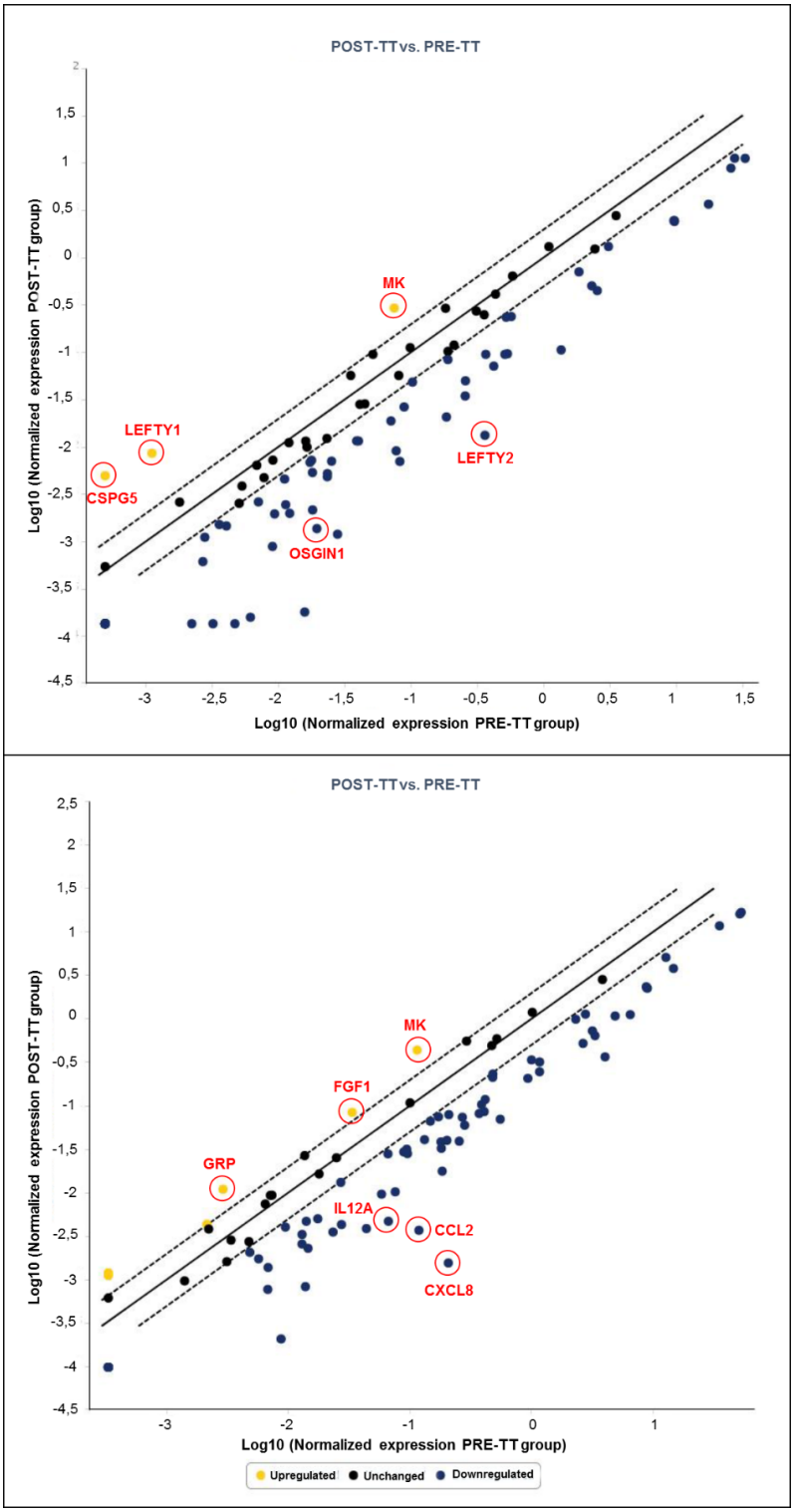

Figure 42. Differentially expressed genes after the treatment with CD133⁺ BMDSCs. *Scatter plots of both conditions PRE-TT and POST-TT, with yellow dots representing upregulated genes after treatment, blue ones correspond to downregulated ones and the black dots are the unchanged genes (inside the established threshold). The most differentially expressed genes are circled and named in red. The two arrays harboring the most differentially expressed genes are represented:* ***(A)*** *PAHS-041ZC and* ***(B)*** *PAHS-072ZC.*

After the analysis of the fold regulation, the most upregulated and downregulated genes were obtained, as we can see in Figure 42. The most upregulated genes were: CSPG5

(Chrondroitin sulfate proteoglycan 5), LEFTY1 (Left-right determination factor 1), MK (Midkine), GRP (Gastrin releasing peptide) and FGF1 (Fibroblast growth factor 1). The most downregulated ones were: CXCL8 (C-X-C motif chemokine ligand 8), CCL2 (C-C motif chemokine ligand 2), LEFTY2 (Left-right determination factor 2), IL12A (Interleukin 12A) and OSGIN1 (Oxidative stress induced growth inhibitor). The ten most differentially expressed genes after the treatment with $CD133^{+}$ BMDSCs are described in Table 13 and detailing the functions where they take part.

	Gene	Functions
Upregulated	**CSPG5** (Chrondroitin sulfate proteoglycan 5)	Neural growth and cell differentiation factor.
	LEFTY1 (Left-right determination factor 1)	This gene encodes a secreted ligand of the TFGβ (growth factor) superfamily of proteins. Cytokine activity, implicated in the recruitment and activation of transcription factors.
	MK (Midkine)	Growth factor that binds heparin and responds to retinoic acid. It promotes cell growth, migration, proliferation, tissue repair, angiogenesis and differentiation.
	GRP (Gastrin releasing peptide)	Involved in epithelial cell proliferation.
	FGF1 (Fibroblast growth factor 1)	Growth factor involved in embryonic development, cell growth, morphogenesis, tissue repair, cell survival, cell division, angiogenesis, cell differentiation and cell migration.
Downregulated	**CXCL8** (C-X-C motif chemokine ligand 8)	This chemokine is a potent angiogenic factor and is one of the major mediators of the inflammatory response, released in response to an inflammatory stimulus.
	CCL2 (C-C motif chemokine ligand 2)	Chemokine involved in immunomodulatory and inflammatory processes.
	LEFTY2 (Left-right determination factor 2)	This gene encodes a secreted ligand of the TFGβ superfamily of proteins. Cytokine activity, plays a role in endometrial bleeding and is associated with some types of infertility.
	IL12A (Interleukin 12A)	Cytokine that can act as a growth factor for activated T and NK cells, and for the differentiation of Th cells. Required for innate immunity.

	OSGIN1 (Oxidative stress induced growth inhibitor)	This molecule with growth factor activity induces apoptosis, controls both inflammatory and anti-inflammatory molecules, and regulates the differentiation and proliferation of normal cells through the regulation of cell death.

Table 13. Ten most differentially expressed genes in pathologic endometrium after cell treatment with CD133⁺ BMDSCs. *The 5 most upregulated genes obtained after the data analysis from RT-qPCR arrays are related to growth factors, cell proliferation, migration, differentiation, angiogenesis and tissue repair functions. On the other hand, the 5 most downregulated genes after the treatment are involved in inflammatory and immunomodulatory processes, apoptosis and cell death. Information from www.genecards.org.*

2.3. Identification of the most represented pathways

After describing the most differentially expressed genes, we performed a functional annotation of the genes included in the three arrays. From the 184 total analyzed genes (with good quality), we obtained that 41 of them (16.7%) were not included in KEGG database, so we annotated the remaining 143 genes to KEGG pathways with this tool. After that, the most represented pathways with their respective up- and down- regulated genes were obtained (Figure 43), but only the pathways with more than 5% of genes are shown (Figure 44). These pathways were obtained from the KEGG webpage, which is the Kyoto encyclopedia of genes and genomes, a database to study the functions and applications of biological systems at molecular level, showing molecular interactions.

Figure 43. Functional analysis of the effect of CD133⁺ cells in endometrial function: most represented KEGG pathways with upregulated and downregulated genes. *KEGG pathways represented with paracrine and growth factors' transcriptome with the genes included in the arrays. The number of genes upregulated (red) and downregulated (blue) are indicated for each pathway in the X-axis.*

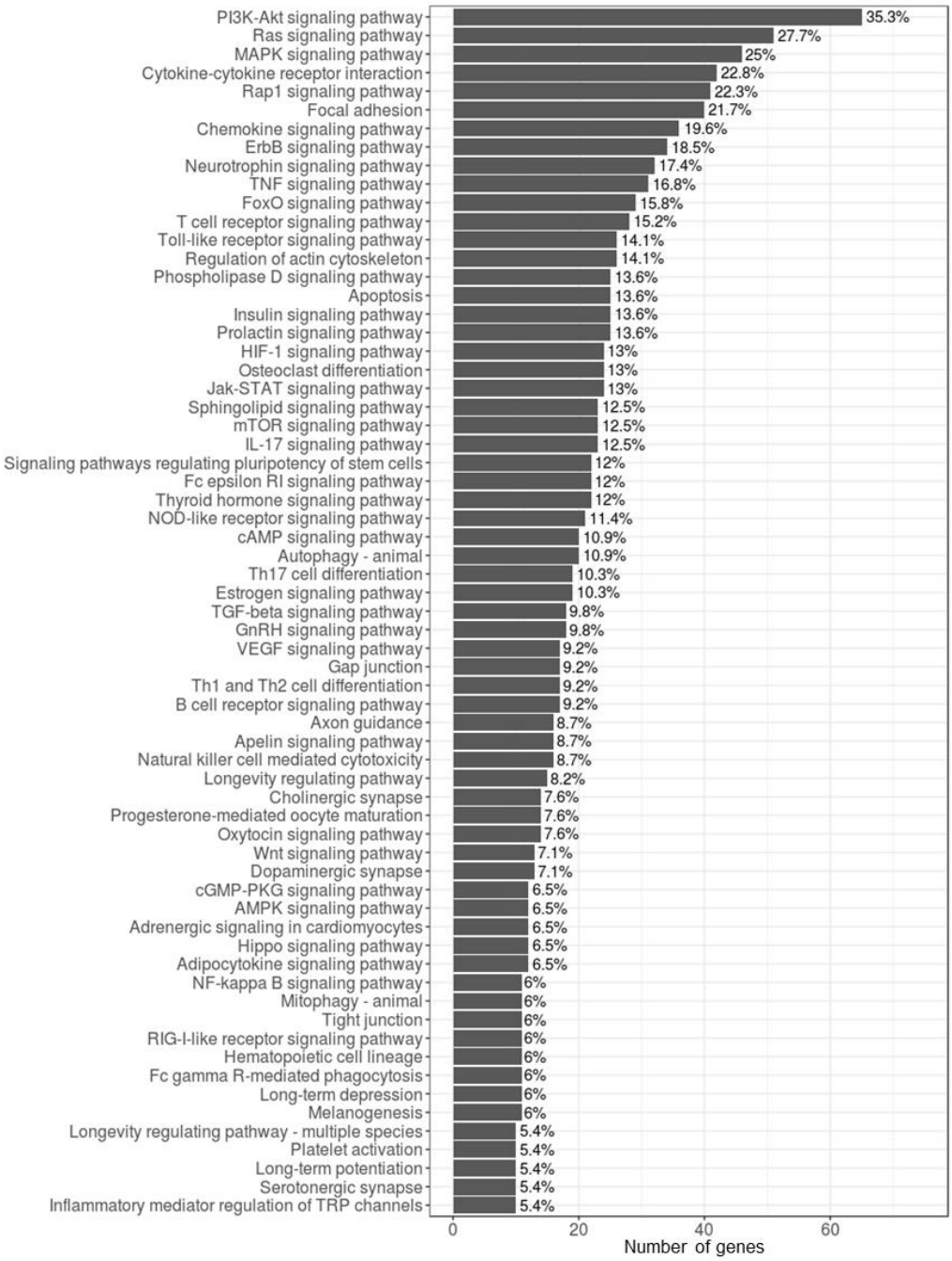

Figure 44. Functional analysis of the effect of CD133⁺ cells in endometrial function: most represented KEGG pathways with percentage of genes. *KEGG pathways represented with paracrine and growth factors' transcriptome with the genes included in the arrays. Only the pathways that have a representation higher than a 5% of genes included in the arrays are depicted, with the percentage of genes that belongs to each one. The number of genes are indicated for each pathway in the X-axis.*

Figure 45 represents the most represented pathway, PI3K-Akt (35.3% of the genes enclosed in the arrays belong to this pathway). Here, upregulated genes are coloured in red and the downregulated ones in blue, with different tone corresponding to the fold regulation. This pathway is related to the maintenance of multipotency in MSCs.

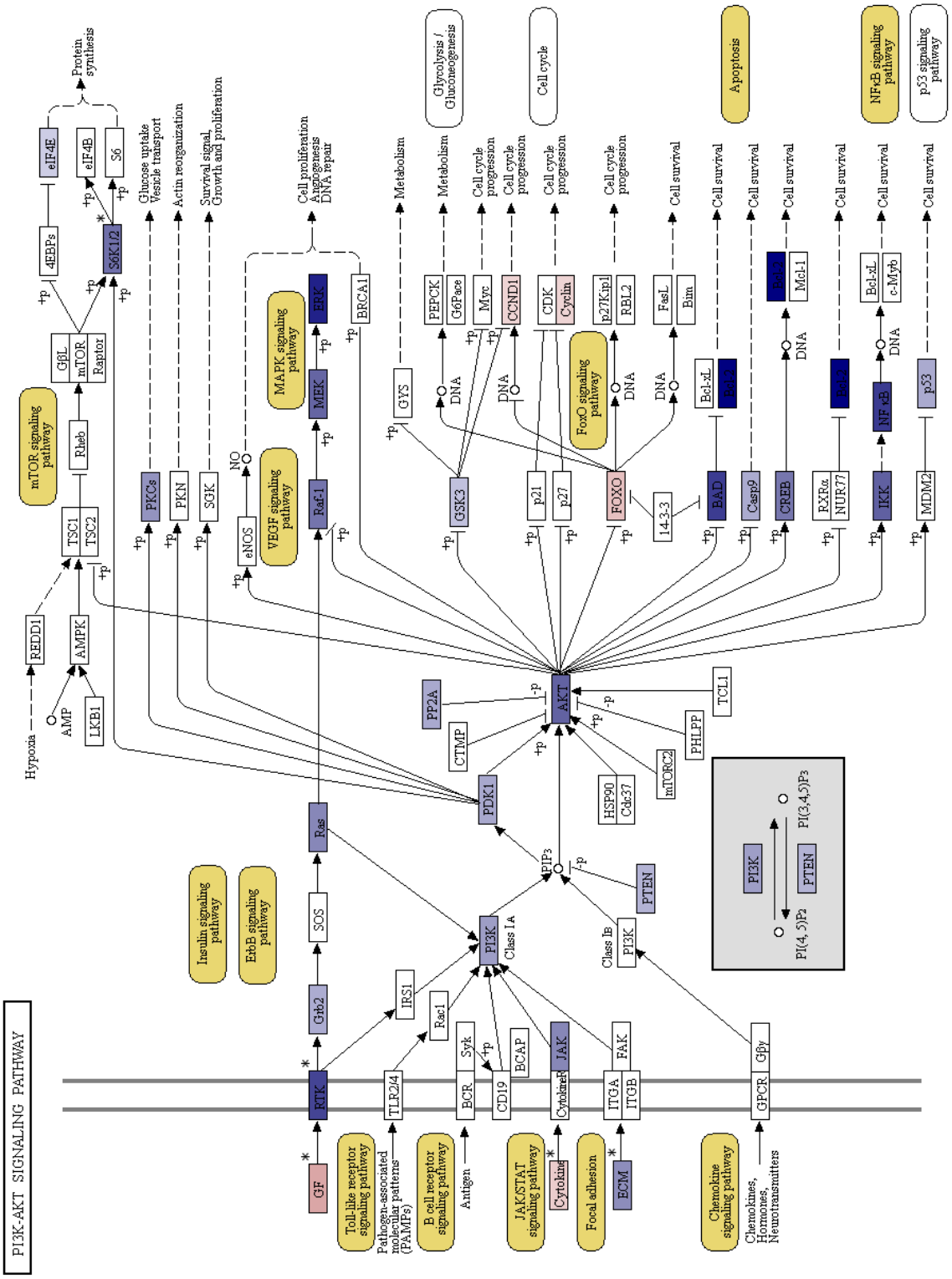

Figure 45. Diagram of the most represented signaling pathway: PI3K-Akt. *The most represented signaling pathway PI3K-Akt (35.3%) is shown in detail. The 66 genes measured by RT-qPCR Arrays that are part of it are colored in red (upregulated) or in blue (downregulated), with color intensity corresponding to the fold regulation. Each node can correspond to several genes. Pathways highlighted in yellow are those related to PI3K-Akt that were mentioned in Figure 43 and harbor differentially expressed genes. The asterisk marks the related genes from the bibliography that are indicated as potential factors.*

It is important to note that previous to the arrays, a bibliographic search of growth factors involved in regeneration and tissue repair was done. The genes from our search that overlap with those from the arrays are summarized in Table 14. Moreover, those that are in the PI3K-Akt signaling pathway and were considered potential growth factors in this study are labelled with an asterisk in Figure 44. With the results depicted in this map, we can see that FOXO is activating cell cycle progression and that apoptosis function is downregulated 3 months after treatment with $CD133^{+}$ BMDSCs.

Paracrine Factor	Reference
AGPT1	(Gnecchi *et al.*, 2008)
BDNF	(Kawamura *et al.*, 2012; Yu *et al.*, 2012; Procházka *et al.*, 2016)
BMP2	(Gnecchi *et al.*, 2008)
BMP6	(Gnecchi *et al.*, 2008)
CSF-1	(Kawamura *et al.*, 2012)
EGF	(Deans and Moseley, 2000; Kim *et al.*, 2006; Kawamura *et al.*, 2012; Yu *et al.*, 2012; Moreno *et al.*, 2015; Thongkittidilok *et al.*, 2015)
FGFα / FGF1	(Procházka *et al.*, 2016)
FGFβ / FGF2	(Gnecchi *et al.*, 2008; Moreno *et al.*, 2015; Simental-Mendía, Vílchez-Cavazos and Martínez-Rodrígueza, 2015; Kanazawa *et al.*, 2017)
FGF7	(Gnecchi *et al.*, 2008)
GDNF	(Kawamura *et al.*, 2012)
HGF	(Gnecchi *et al.*, 2008; Bagno *et al.*, 2016)
IGF-I	(Kim *et al.*, 2006; Gnecchi *et al.*, 2008; Kawamura *et al.*, 2012; Yu *et al.*, 2012; Moreno *et al.*, 2015; Simental-Mendía, Vílchez-Cavazos and Martínez-Rodrígueza, 2015; Bagno *et al.*, 2016; Procházka *et al.*, 2016)
IL-1	(Gnecchi *et al.*, 2008; Procházka *et al.*, 2016)
IL-4	(Procházka *et al.*, 2016)
IL-6	(Gnecchi *et al.*, 2008; Procházka *et al.*, 2016)
IL-8	(Procházka *et al.*, 2016)
IL-10	(Procházka *et al.*, 2016)
IL-11	(Gnecchi *et al.*, 2008)
IL-12	(Procházka *et al.*, 2016)
KITLG / SCF	(Gnecchi *et al.*, 2008)

LIF	(Gnecchi *et al.*, 2008)
CCL2 / MCP-1	(Gnecchi *et al.*, 2008)
RPS6K / p70	(Procházka *et al.*, 2016)
PDGF	(Deans and Moseley, 2000; Gnecchi *et al.*, 2008; Moreno *et al.*, 2015; Simental-Mendía, Vílchez-Cavazos and Martínez-Rodrígueza, 2015; Procházka *et al.*, 2016)
PGF	(Gnecchi *et al.*, 2008)
PTN	(Gnecchi *et al.*, 2008)
TGFβ	(Deans and Moseley, 2000; Gnecchi *et al.*, 2008; Moreno *et al.*, 2015; Simental-Mendía, Vílchez-Cavazos and Martínez-Rodrígueza, 2015; Li *et al.*, 2016; Procházka *et al.*, 2016; Park *et al.*, 2017)
THBS1	(Gnecchi *et al.*, 2008)
TIMP-1	(Gnecchi *et al.*, 2008)
TIMP-2	(Gnecchi *et al.*, 2008)
TNFα	(Gnecchi *et al.*, 2008; Procházka *et al.*, 2016)
VEGF	(Gnecchi *et al.*, 2008; Biswas *et al.*, 2011; Moreno *et al.*, 2015; Bagno *et al.*, 2016; Procházka *et al.*, 2016)

Table 14. Table summarizing paracrine factors involved in tissue regeneration: bibliographic search*. Thirty-two paracrine factors in common in RT-qPCR arrays and the previous bibliographic search based on factors involved in tissue damage and regeneration are shown. Abbreviations explained in abbreviations section.*

2.4. Validation by immunofluorescence of the upregulated gene Midkine

One of the most upregulated genes, MK, was chosen for further validation because of its involvement of multiple functions: cell growth, differentiation (Muramatsu, 1993), migration, angiogenesis, regeneration (Ochiai *et al.*, 2004; Ikutomo *et al.*, 2014; Liedert *et al.*, 2014), cell survival (S.-L. Zhao *et al.*, 2014)… Additionally, it has been suggested as a therapeutic target for several disorders (Muramatsu and Kadomatsu, 2014). Thus, MK was tested by IF to compare the expression in the endometrium before and after treatment with $CD133^+$ BMDSCs in patients with endometrial pathologies.

After IF analysis, we could observe the differential expression of MK (with a perinuclear signal) in samples A-D in both conditions (Figure 46). None of the PRE-TT samples expressed MK, except the last one that showed a little signal (expanded in Figure 46)

with a scattered pattern. In every POST-TT sample, MK signal was enhanced, located in stromal cells around glands and luminal epithelium (Figure 46).

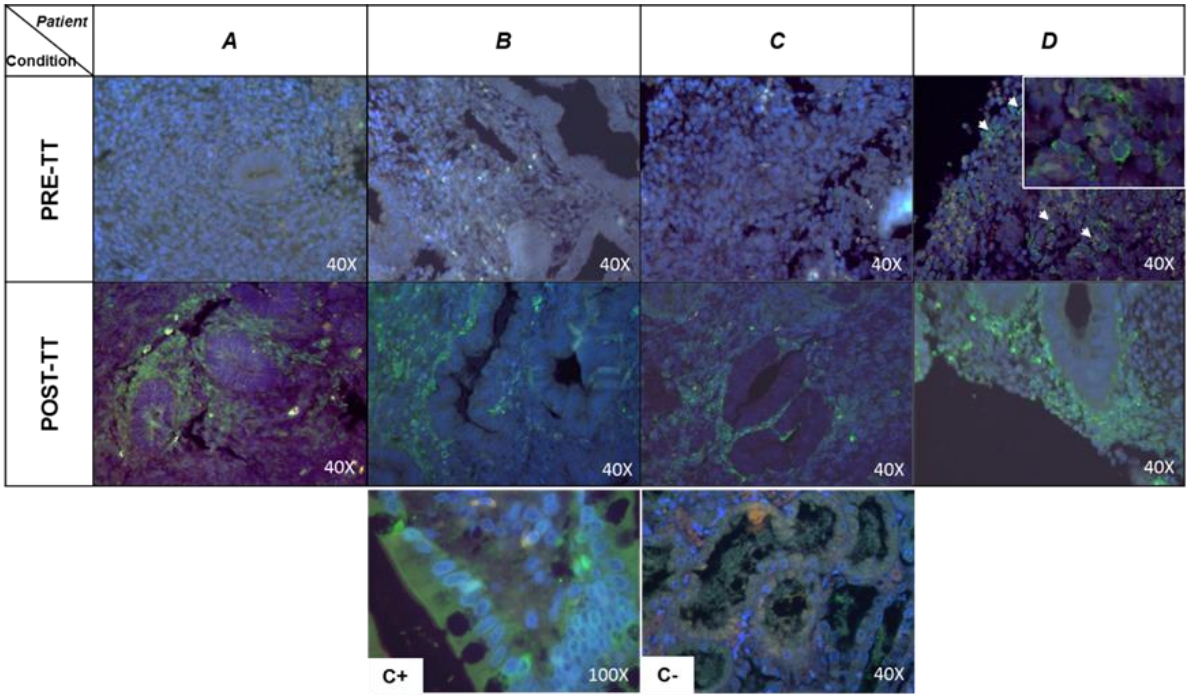

Figure 46. Immunofluorescence of Midkine (MK) in endometrium from diseased women with Asherman's syndrome and/or endometrial atrophy, before (PRE-TT) and after (POST-TT) treatment with CD133^{+} BMDSCs. *A large increase of MK signal is noticeable in POST-TT samples, with a periglandular pattern in stroma. Only the PRE-TT sample from patient D showed a little signal before treatment, which is expanded in the picture, with few cells stained in a scattered pattern. Patients named as A, B, C and D correspond to patients 5, 7, 12 and 13 from the study of Santamaria et al., 2016, respectively. The positive control (C+) is human ileum and the negative one (C-) is human kidney.*

VII. DISCUSSION

"La ciencia no nos ha enseñado aún si la locura es o no lo más sublime de la inteligencia"

Edgar Allan Poe

VII. DISCUSSION

All women go through a cyclic process of endometrial regeneration and degeneration regulated by hormonal changes during their reproductive life. This cyclic process, called the menstrual cycle, occurs each month starting from menarche until menopause and is composed of proliferative and secretory phases, and menstruation. The endometrium consists of the functional layer, which sheds and regenerates each menstrual cycle, and the basal layer, which remains almost intact. The exceptional regenerative capability of this tissue has been postulated for years and the presence of SSCs was already suggested in the 70's (Prianishnikov, 1978; Padykula *et al.*, 1984). This hypothesis has been confirmed in humans and mice investigating the high proliferative potential of endometrial cells, the differentiation into other cell types, the expression of markers of pluripotency and the clonogenic activity, as well as *in vivo* tissue reconstruction in animal models.

It is postulated that a pool of SSCs resides inside the endometrium (endogenous source), contributing to the regeneration of the tissue each month (Cervelló *et al.*, 2010). SSCs reside in specific microenvironments called niches, which maintain a balance between self-renewal and differentiation, determining the division pattern in order to perpetuate the stem cell phenotype or to give rise to committed progeny (Schofield, 1978; Fuchs, Tumbar and Guasch, 2004). The mechanisms to regulate this equilibrium would be determined by the communication with surrounding cells (niche cells) by the release of soluble factors. However, the identity and location of these SSCs and their niches remain unknown in many adult tissues. The identification of specific endometrial SSC markers would allow their isolation (and the identification of the endometrial niche), which would open the door to use them in gynecological diseases. Several molecules have been studied and postulated as specific endometrial SSC markers, such as CD146 - PDGF-Rβ (Schwab and Gargett, 2007), W5C5 (Masuda *et al.*, 2012), LGR5 (Gil-Sanchis *et al.*, 2013; Cervelló *et al.*, 2017; Vallvé-Juanico *et al.*, 2017), EPCAM (Gargett *et al.*, 2009), SSEA1 (Valentijn *et al.*, 2013), Musashi-1 (Götte *et al.*, 2008; Lu *et al.*, 2011) or Sox-2 (Götte *et al.*, 2011), as well as the SP as a heterogeneous population with proved endometrial reconstitution potential (Cervelló *et al.*, 2010; Masuda *et al.*, 2010; Cervelló, Mas, *et al.*, 2011).

As well as the endogenous source of SSCs, the contribution originating from the bone marrow after tissue damage has been reported in several organs and tissues (exogenous source), migrating to and reaching spatially distant tissues via blood. In this case, they could remain undifferentiated or differentiate themselves into the cell type of

the target tissue or organ. This regenerative role has been demonstrated in smooth muscle (Ferrari *et al.*, 1998), central nervous system (Brazelton *et al.*, 2000; Mezey *et al.*, 2000), heart (Jackson *et al.*, 2001; Orlic *et al.*, 2001), liver (Petersen *et al.*, 1999; Theise *et al.*, 2000) and endometrium (Cervelló *et al.*, 2012; Gil-Sanchis *et al.*, 2015). Specifically, the contribution of these cells after a bone marrow transplantation to the endometrium has been proved both in humans (Taylor, 2004; Mints *et al.*, 2008; Ikoma *et al.*, 2009; Cervelló *et al.*, 2012) and mice (Du and Taylor, 2007). Regarding to endometrial pathologies, the potential of BMDSCs have been studied in AS and EA (Cervelló *et al.*, 2012; Alawadhi *et al.*, 2014; Gil-Sanchis *et al.*, 2015; Santamaria *et al.*, 2016), both caused by a lack of growth of endometrial cells.

The main objective of this thesis was to study endogenous and exogenous sources of endometrial SSCs and investigate their potential to regenerate and repair human endometrial tissue. Several specific objectives were established to accomplish this goal throughout the presented work. After the selection and isolation of cells according to postulated endometrial SSC markers, an animal xenotransplantation model was aimed to validate the stemness nature of these cells. Under pathological conditions we assessed the nature of the previously postulated SSCs in endometriosis patients, we as well identified paracrine factors released by the exogenous source of SSCs probably implicated in endometrial regeneration under AS and EA.

A. TISSUE-SPECIFIC SOMATIC STEM CELLS RESIDENT IN HUMAN ENDOMETRIUM

Identification of specific somatic stem cell markers using a xenotransplantation model – The importance of niche-like cells for endometrial reconstitution

An important objective of this thesis was to identify bonafide markers for human endometrial SSCs by using an established functional assay for human endometrial reconstruction in animal models. In this respect, we have demonstrated the heterogeneous formation of human endometrial-like tissue under the kidney capsule of immunodeficient mice following injection of different human endometrial cell subsets. We have deduced that injecting cells isolated by using well-described SSC markers, W5C5 and ICAM1, requires supplementing/support with total endometrial cells (TF) as niche-like cells due to the low endometrial reconstitution capability of $W5C5^+$ or $ICAM1^+$ cells alone.

W5C5 was previously described as a perivascular endometrial SSC marker (Masuda *et al.*, 2012). This finding could enable the isolation of endometrial SSCs using a single

marker; indeed, Masuda found that W5C5$^+$ cells could regenerate stromal tissue *in vivo.* However, the human endometrial cells used for xenotransplantation assays in that study were “single-cell suspensions of cultured W5C5$^+$ cells” (Masuda *et al.*, 2012). In contrast, in our experimental design we used fresh (non-cultured) cells, and surprisingly, neither combination led to significant results. Other factors, such as the sorting procedure, could influence the regeneration ability of these cells and might diminish the cell’s viability as described by Masuda in 2012 (Masuda *et al.*, 2012). Nevertheless, in the present thesis full endometrial reconstitution was observed with supplemented and non-cultured cells. We propose that (1) the previous *in vitro* step in the study of Dr. Masuda could have affected the final phenotype of isolated cells, promoting *in vivo* cell proliferation, and (2) the endometrial surrounding cells play an important role, acting as niche-like cells for tissue reconstitution in *in vivo* models.

We also analyzed endometrial epithelial and stromal cell fractions of cells expressing ICAM1, obtaining a percentage of positive cells consistent with the expected percentage of endometrial SSCs in the entire tissue (Cervelló and Simón, 2009; Gargett *et al.*, 2013). High expression of ICAM1 has been reported in endothelial cells of veins, arterioles and capillaries and stromal cells within human endometrium (Tawia, Beaton and Rogers, 1993; Lockwood *et al.*, 2005; Schatz *et al.*, 2006). This marker plays a significant role in immunological synapse formation, T-cell activation, leukocyte trafficking and numerous cellular immune responses (Ramos, Bullard and Barnum, 2014). Importantly, ICAM1 also exhibited high expression in MSCs (Brooke *et al.*, 2008; De Francesco *et al.*, 2009; Fong *et al.*, 2012; Sununliganon and Singhatanadgit, 2012). Together, this evidence suggested the potential of this molecule to be considered as an endometrial SSC marker. However, consistent with our W5C5 assays, ICAM1$^+$ cells alone gave rise to a low endometrial reconstitution efficiency and ICAM1$^+$ cells supplemented with total endometrium promoted efficient reconstruction.

Presumably, both negative fractions (for W5C5 and ICAM1) resulted in greater endometrial regeneration in comparison to their corresponding pure positive fractions. All together, these results reinforce the important role of whole endometrial cells (TF) as niche-like cells that act as a supportive population maintaining, guiding and stimulating the endometrial SSC population to reconstruct the human endometrium in an animal model (Mondal *et al.*, 2014).

Finally, we tested established SP cell lines derived from endometrial stem cells (Cervelló, Mas, *et al.*, 2011). The SP approach is based on the capability of SP cells to extrude the Hoechst dye through ABCG2 channels called BCRP1, which is a property of stem cells

(Zhou *et al.*, 2001). It has been proposed that this heterogeneous population includes different stem/progenitor cells in different organs and tissues, including the endometrium (Kato *et al.*, 2007; Cervelló *et al.*, 2008, 2010, 2012; Tsuji *et al.*, 2008; Maruyama *et al.*, 2010; Masuda *et al.*, 2010; Cervelló, Mas, *et al.*, 2011). In this study, in comparison to the xenotransplantation assays mentioned previously, SP cells contributed to greater endometrial-like tissue reconstruction. Thus, we postulate that the SP from human endometrium could be enriched, at least in part, with several cells with stemness capabilities, corroborating findings previously mentioned. It is important to underline that the SP cells used here were derived from stable cell lines (Cervelló *et al.*, 2010) (established karyotype, stem cell characteristics and reconstitution potential) while the W5C5 and ICAM1populations were isolated from human biopsies.

In the experimental design, to elucidate the role of the niche or surrounding cells, we supplemented the other cell types with total endometrial cells (entire endometrial fraction). The importance of the niche was described in detail by Dr. Lane and collaborators, highlighting resident niche cells and direct cell contacts (Lane, Williams and Watt, 2014). Theoretically, stem cell niches contain both tissue-specific and generic cell populations, each having specialized roles. The total endometrial fraction (TF) harbors all the cell types present in human endometrium, being able to function as a niche what results in an increased reconstruction efficiency, as described previously (Miyazaki *et al.*, 2012) and in our present work.

Moreover, in the proliferation assay, Ki67 staining indicated the presence of human proliferating cells in the kidney capsule near blood vessels, where engrafted human cells were located. This indicates that human cells may drive the proliferation of surrounding cells and the differentiation to endometrial cell types such as epithelium or stroma. This is consistent with the most plausible location of the SSC niche described (Scadden, 2006; Oh and Nör, 2015), which is around blood vessels, corroborating the dialogue between this tissue and the bone marrow in endometrial restoration (Taylor, 2004; Ikoma *et al.*, 2009; Cervelló *et al.*, 2012, 2015, 2017; Santamaria *et al.*, 2016).

In summary, we observed in a xenotransplantation model that pure $W5C5^+$ and $ICAM1^+$ cells from human endometrium do not have the ability to efficiently reconstruct the endometrium. Indeed, we demonstrated that isolated $W5C5^+$ and $ICAM1^+$ cells require supplementation with total endometrial cells acting as specific niche-like cells. The use of whole endometrial tissue enabled a close association between putative endometrial stem cells with their niche cells (Higa *et al.*, 2013). In contrast, the SP appears to harbor a stem cell population, indicated by its high reconstitution potential. As we postulated in

prior work, the SP is a heterogeneous cell population containing a mixture of endometrial progenitors (epithelium, stroma and endothelium).

The stemness features of cell populations was assessed by measuring MS1-positive cells in the formed endometrial-like tissue in the animal model and the results support previously mentioned results. MS1 has been postulated as a novel SSC marker both in mouse and in humans for several tissues because of its role in maintaining the balance between self-renewal and differentiation (Okano *et al.*, 2005). MS1 expression has been demonstrated in endometrium, colocalizing with the stem cell markers Notch-1 and telomerase (Götte *et al.*, 2008). Additionally, the proportion of MS1-expressing cells is higher in endometrial carcinoma, endometriosis and endometrial hyperplasia compared to the healthy endometrium (Götte *et al.*, 2008; Lu *et al.*, 2011). In this study, the highest MS1 expression was found in SP cell lines, followed by positive fractions supplemented with TF, and by the control mice injected with TF. The difference lies in the percentage of negative and pure positive cells for W5C5 and ICAM1, lower than the previously mentioned groups. This result could indicate that positive cells for the studied markers possibly need to be supplemented with TF as niche-like cells to remain undifferentiated and therefore retain the stemness characteristics (indicated by the expression of MS1). A possibility could be that positive pure fractions were enriched in SSCs when injected, but in absence of niche-like cells (TF) they could not remain undifferentiated. Then, during the experiment, many of them differentiated, losing their MS1 expression and creating less endometrial-like tissue.

In conclusion, the role of total endometrial cells as niche-like cells seems to be essential in the retention of stemness features of SSCs (identified by W5C5 and ICAM1 markers and the SP) and in the reconstitution potential of these cells.

Despite the results obtained, we are aware about the limitations of this study. Due to the low percentage of SP cells present in human endometrium, we used cultured cell lines, while the other endometrial cell populations analyzed in this study (W5C5 and ICAM1) came from sorted primary samples. The last step before injecting the cells (flow cytometry) could also have had a harmful effect on the cellular viability compromising their reconstitution efficiency in animal models. Regardless, the reconstitution ability of the endometrial TF has been demonstrated previously (Masuda *et al.*, 2007) and confirmed along this work. We assessed here the capability of putative endometrial SSCs with and without TF to study their necessity as support or niche-like cells. This was done because it is widely known that the niche microenvironment is essential for the maintenance of stem cell features (Lander *et al.*, 2012). This endometrial TF would

harbor niche-like/neighbor cells belonging to the SSC niche that could enhance the regenerative potential of these postulated SSCs as previously described (Miyazaki *et al.*, 2012). The identification of specific endometrial stem cell markers is essential for the study of the endometrial biology, stem cell isolation and the understanding of endometrial pathologies.

Presence of previously postulated endometrial somatic stem cell populations in endometriosis samples

The stem cell features of W5C5, ICAM1 and SP markers have also been studied in relation to endometriosis development. The mechanism leading the development of this disease has not been completely understood as of yet, and it may be due to a combination of several factors. The most accepted and main theory is that of retrograde menstruation (Sampson, 1927; Vercellini *et al.*, 2014), but not all the women that suffer from this phenomenon develop endometriosis. This statement taken together with the fact that the endogenous stem cells may be remnant germ stem cells from the embryonic development (Mehedintu *et al.*, 2014; Vercellini *et al.*, 2014; Makiyan, 2017) makes it possible to postulate a possible explanation of this pathology. The existence of a different proportion of endogenous stem cells in the eutopic endometrium in women suffering from endometriosis may be an explanation for the differential development of this disease. We started from the hypothesis that those women with a higher percentage of endometrial stem cells (identified by W5C5, ICAM1 and SP in this case) would be more likely to develop endometriosis. Theoretically, this would be more visible in the proliferative phase of the menstrual cycle (with more cells expressing these markers) because during this phase the endometrium regenerates from the basal layer.

As results in our study, a higher population positive for postulated stem cell markers was obtained in proliferative phase in comparison to secretory phase. This finding might indicate the stemness features of both of these markers because the endometrium is supposed to be enriched in SSCs during the proliferative phase (for the regeneration of the endometrial functional layer). Moreover, more expression was also observed in rASRM stage I endometriosis than in healthy patients. These results with eutopic endometrium from healthy and endometriosis women, also taking into account secretory and proliferative phases, support the idea that these markers could allow the identification of endometrial SSCs, and probably constituting one of the factors responsible for the development of endometriosis (among others).

B. EXOGENOUS STEM CELL CONTRIBUTION IN HUMAN ENDOMETRIAL REGENERATION

Identification of paracrine factors secreted by bone marrow-derived stem cells responsible for endometrial regeneration under pathological conditions

In this study, our purpose was to study the specific role of the exogenous source of SSCs in endometrium. As it has been explained at the beginning of this section, the bone marrow contribution in endometrial regeneration has been previously demonstrated in humans and in mice, as well as under pathological conditions (Taylor, 2004; Du and Taylor, 2007; Mints *et al.*, 2008; Ikoma *et al.*, 2009; Cervelló *et al.*, 2012; Alawadhi *et al.*, 2014; Gil-Sanchis *et al.*, 2015; Santamaria *et al.*, 2016). Specifically, we aimed to identify molecules secreted by BMDSCs that could improve the endometrial quality after tissue damage (AS and/or EA), in an autocrine or paracrine manner (Cervelló *et al.*, 2015; Santamaria *et al.*, 2016).

After a gene expression analysis for general and angiogenic human growth factors, we saw that 3 months after cell therapy the upregulated genes codified for factors related to cell growth, proliferation, migration, differentiation, angiogenesis and tissue repair. On the contrary, most downregulated genes codified for cytokines and chemokines involved in inflammation and immune processes, as well as apoptosis and cell death.

As well as the immunomodulatory scenario created in case of tissue transplantation and repair, the immunosuppressive nature of MSCs *in vitro* and *in vivo* has been described (Shi *et al.*, 2010; English, Mahon and Wood, 2014). The best studied clinical application of MSCs therapy is hematopoietic stem cell transplantation. These cells have proved trophic effect through immunomodulation, anti-apoptotic and cytoprotective effects and the promotion of neoangiogenesis (English, Mahon and Wood, 2014). It is also well known that during repair, excessive activation of the immune cells interferes with the process or even worsens the tissue injury. Similarly, MSCs have low immunogenicity and immunosuppressive capabilities, having an inhibitory function of immune system. Cell-cell contacts as well as the release of paracrine factors seem to be a key in the immunosuppression process. During tissue repair, MSCs are believed to migrate into damaged tissues where they release growth factors such as EGF, FGF, PDGF, TGF, VEGF, IGF-1, AGPT1 and SDF-1, influencing that way the growth of endothelial cells and fibroblasts (Shi *et al.*, 2010). In our gene analysis, FGF and PDGFD were the fifth and sixth most upregulated genes respectively.

Midkine (MK) was one of the most upregulated genes after the treatment with $CD133^+$ cells in women with AS or EA and chosen for further validation due to the biological functions it is involved with. MK is a member of a small family of secreted growth factors, involved in the regeneration in liver (Ochiai *et al.*, 2004), lacrimal glands (Zhang *et al.*, 2014) and skeletal muscle (Ikutomo *et al.*, 2014). It has also been described in relation to cell growth, proliferation, differentiation (Muramatsu, 1993), migration, angiogenesis, regeneration (Ochiai *et al.*, 2004; Ikutomo *et al.*, 2014; Liedert *et al.*, 2014), and cell survival (S.-L. Zhao *et al.*, 2014). Furthermore, MK has a chemotactic activity to promote the migration of macrophages and neutrophils (Ochiai *et al.*, 2004; Ikutomo *et al.*, 2014; Zhang *et al.*, 2014). After cell therapy, the main improvement observed in selected patients at histological level was an increase in neoangiogenesis, better endometrial histology and the formation of new glands (Santamaria *et al.*, 2016). MK expression after cell treatment was located around the glands, and this made us consider that it could be stimulating the formation of new glands. The release of MK may be promoted by BMDSCs and surrounded cells after the infiltration of these SSCs in patients with endometrial pathologies. An hypothesis is that BMDSCs are engrafted around blood vessels (from where they reach the endometrium) at the beginning, proved by the study of Dr. Cervelló in 2015 in animal models (Cervelló *et al.*, 2015). Then they would migrate to endometrial glands and lumen, releasing MK for endometrial reconstitution and stocking up this molecule in this location aiding in the regenerative process.

In turn, MK is related to the signaling pathway most represented with studied genes: PI3K-Akt, involved in the maintenance of multipotency in MSCs. In this pathway, PI3K phosphorylates Akt, leading to its activation and triggering downstream targets for the regulation of several cellular functions, like cell migration, survival, migration, differentiation, angiogenesis, proliferation and apoptosis (Chen *et al.*, 2013). The upregulated gene MK is described to activate mitogen-activated protein kinase (MAPK) and PI3K, finally inducing cell proliferation (*GeneCards: The Human Gene Database*, 1996). A dysregulation in this pathway could lead to several disorders (Chen *et al.*, 2013). In this case, from our point of view, stem cells-secreted factors could exogenously promote the regulation of the PI3K-Akt pathway, possibly regulating the endometrial regeneration through the inhibition of cell death and the increase of cell proliferation.

The most downregulated gene, CXCL8 (also Interleukin-8), is a member of the chemokine family whose receptors are CXCR1 and CXCR2, present in a wide variety of leukocytes. This molecule contributes to the elimination of pathogens, acts in disease-associated processes such as tissue injury, neutrophil-dependent tissue damage, fibrosis or angiogenesis. CXCL8 is a chemoattractant cytokine, which plays an important

role in inflammation response, activating cell recruitment, activation and migration of neutrophils and T cells. Many cell types can release CXCL8, including monocytes, endothelial cells, epithelial cells or T lymphocytes (Russo *et al.*, 2014). This dramatic decrease in the level of CXCL8 after treatment could help lowering the inflammatory response after cell therapy, favoring the regenerative process. Inflammatory conditions in injured tissues obstruct local cells in the reconstitution process, so it is usual to find chemokines downregulated in this phase of tissue regeneration (Murphy, Moncivais and Caplan, 2013).

In summary, $CD133^+$ BMDSCs could contribute to endometrial restoration by the release of soluble factors, which may influence the endometrial stem cell niche or activate SSCs, supporting endometrial regeneration under pathological conditions. The mechanism responsible for this tissue improvement may be affected by the regulation of the PI3K-Akt signaling pathway, providing an immunosupressed scenario for tissue repair and enhancing cell proliferation, migration, differentiation and angiogenesis after cell therapy.

C. FUTURE PERSPECTIVES: APPLICATIONS IN REGENERATIVE MEDICINE

Stem cell therapies in regenerative medicine have been confined thus far to bone marrow transplantations as a treatment for hematological diseases, and epidermis transplantations over the past decades (Cossu *et al.*, 2018), but this field is beginning to broaden its spectrum, employing other therapies. For this and considering the information complied in the presented thesis, the study of endometrial SSCs would allow the understanding and identification of the SSC niche, and the characterization and isolation of SSCs in this tissue, which has not been achieved for now. For this purpose, the identification of specific endometrial SSC markers as we attempted is necessary, which would allow to understand gynecological diseases originating from an excessive proliferation, and at the same time understanding the physiology of the endometrial SSC niche.

In recent times, the newly developed therapies are focused in cell and gene therapy, new generation drugs and tissue engineering (Cossu *et al.*, 2018). The identification of specific SSC markers and their isolation might permit the repopulation of decellularized tissues (in this case endometrium) with autologous cells by tissue engineering (a specialized form of bioengineering combining cell therapy with biomimetic scaffolding). The reconstruction of the uterus using bioengineering has attracted more and more attention within the field of reproductive and regenerative medicine (Campo, Cervelló and Simón, 2017). Protocols have been established for the decellularization of uteri in mouse (Hiraoka *et al.*, 2016), rat (Hellström *et al.*, 2014) and pig by the Dr. Cervelló's

group (Campo *et al.*, 2017), creating a natural scaffold for the establishment of exogenous/autologous SSCs and regenerating uterine tissues for women with gynecological problems. In addition, the treatment with endometrial SSCs would be useful to treat diseases characterized by a lack of endometrial growth such as endometrial atrophy or Asherman's syndrome. The isolation of specific endometrial SSCs is a vital part for all these possible therapies, this is why we have to further investigate the markers characterized along this study (ICAM1 and W5C5) and to attempt to discover others. Cells isolated according to identified endometrial SSC markers could be good candidates to repopulate scaffolds as a stem cell-based therapy, used in combination other cells needed to efficiently reconstruct the whole uterus.

Another hot topic in regenerative medicine is the use of paracrine factors released by stem cells. MK and other upregulated genes identified along this thesis should be validated *in vitro*, by wound healing assays (to test cell migration) of human primary cell culture from endometrial biopsies to reliably confirm their regenerative potential. Their injection in an animal model established by Dr. Cervelló (Cervelló *et al.*, 2015), as the one used in the previously mentioned project, would be the following step, with the goal of testing them in humans in the future. The last aim is to promote endometrial regeneration in women with endometrial pathologies. Therefore, the identification of potential paracrine factors could suppose a non-invasive way to treat tissue injury in regenerative medicine, possible being used in a commercially available (or artificially synthetized) cocktail without needing to extract autologous cells. Moreover, the activation of PI3K-Akt pathway (obtained in this study) has been applied in cellular therapy and tissue engineering, but the overexpression of key components of this pathway has not been applied yet (Chen *et al.*, 2013). As PI3K-Akt plays an essential role in MSCs, the activation and overexpression of some important parts of the pathway would be another possible therapy to accelerate the endometrial regeneration potential of BMDSCs. In this respect, a combination of both (1) the injection of paracrine factors involved in regeneration and (2) the activation of PI3K-AKT would be a good approach to be tested.

In conclusion, the great advances in the stem cells field are permitting the development of new stem cells-derived therapies and clinical trials in regenerative medicine, which could improve the prognosis of people suffering from many tissue diseases in the near future.

VIII. CONCLUSIONS

"Guarda tus miedos para ti, pero comparte tu coraje con otros"

Robert Louis Stevenson

VIII. CONCLUSIONS

The conclusions extracted from this thesis are the following:

1. The xenotransplantation model demonstrated that pure “putative stem cell populations” like W5C5^{+} and ICAM1^{+} from the human endometrium do not have the ability to efficiently reconstruct endometrium.

2. Supplementing with total endometrial cell fraction must include niche-like/neighbor cells belonging to the stem cell niche that would enhance the regenerative potential of pure “putative stem cell populations”.

3. The Side Population is a heterogeneous population harboring stem cells in the human endometrium, because of its high regeneration capability demonstrated in the animal model.

4. The identification of specific endometrial stem cell markers, as endogenous source, is essential for the study of endometrial biology, stem cell isolation, and the understanding of endometrial pathologies.

5. Exogenous stem cells secrete multiple factors that may exert a therapeutic effect via paracrine actions, as we observed after treating patients suffering from endometrial pathologies with bone marrow stem cells.

6. Stem cells secreted factors could exogenously promote the regulation of the PI3K-Akt pathway, which is associated with the maintenance of multipotency in mesenchymal stem cells.

7. The genes downregulated after cell therapy may imply an immunomodulatory scenario favoring this tissue remodeling. In contrast, the upregulated ones (including MK) are implicated in cell growth, angiogenesis, migration, differentiation and tissue repair.

8. These factors secreted by transplanted cells may influence the microenvironment or activate endometrial somatic stem cells for tissue regeneration.

IX. REFERENCES

"Veo al final de mi rudo camino, que yo fui el arquitecto de mi propio destino"
Amado Nervo

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Zhao, S.-L. *et al.* (2014) 'Mesenchymal stem cells with overexpression of midkine enhance cell survival and attenuate cardiac dysfunction in a rat model of myocardial infarction', *Stem Cell Research & Therapy*. Stem Cell Research & Therapy, 5(2), p. 37.

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X. ANNEXES

"Para las personas creyentes, Dios está al principio. Para los científicos está el final de todas sus reflexiones"

Max Planck

ANNEX I. Animal ethical committee 2015/VSC/PEA/00073

GENERALITAT VALENCIANA
CONSELLERIA DE PRESIDENCIA Y AGRICULTURA, PESCA, ALIMENTACIÓN Y AGUA

Unión Europea

DIRECCIÓN GENERAL DE PRODUCCIÓN AGRARIA Y GANADERÍA

Ciutat Administrativa 9 d'Octubre
Castán Tobeñas, 77. Edif. B3 P2 46018 VALENCIA

AUTORIZACION PROCEDIMIENTO 2015/VSC/PEA/00073

Vista la solicitud realizada en fecha **12/03/15** con nº reg. entrada **4485** por D/Dª. **Pilar Campins Falcó**, Vicerrectora de Investigación y Política Científica, centro usuario **ES462500001003**, para realizar el procedimiento:

"Modelo en vivo para la búsqueda de marcadores endometriales de célula madre adulta "

Teniendo en cuenta la documentación aportada, según se indica en el artículo 33, punto 5 y 6, y puesto que dicho procedimiento se halla sujeto a autorización en virtud de lo dispuesto en el artículo 31 del Real Decreto 53/2013, de 1 de febrero,

Vista la propuesta del jefe del servicio de Sanidad y Bienestar Animal.

AUTORIZO:

la realización de dicho procedimiento al que se le asigna el código: **2015/VSC/PEA/00073** tipo **2,** de acuerdo con las características descritas en la propia documentación para el número de animales, especie y periodo de tiempo solicitado. Todo ello sin menoscabo de las autorizaciones pertinentes, por otras Administraciones y entidades, y llevándose a cabo en las siguientes condiciones:

Usuario: **Universidad de Valencia**

Responsable del proyecto: **Irene Cervello Alcaraz**

Establecimiento: **Animalario de la Unidad Central de Investigación de la Facultad de Medicina y Odontología**

Necesidad de evaluación restrospectiva:

Condiciones específicas:

Observaciones:

Valencia a, 1 de abril de 2015

El director general de Producción Agraria y Ganadería

José Miguel Ferrer Arranz

ANNEX II. Publications in which the PhD student has contributed

1. International scientific publications

A. Publication directly related to the doctoral thesis

- **López-Pérez N**, Gil-Sanchis C, Ferrero H, Faus A, Díaz A, Pellicer A, Cervelló I, Simón C. Human endometrial reconstitution from somatic stem cells: the importance of niche-like cells. Reprod Sci. 2018 Jan 1:1933719118766251. doi: 10.1177/1933719118766251

B. Publications not directly related to the doctoral thesis

- Campo H, Baptista PM, **López-Pérez N,** Faus A, Cervelló I, Simón C. De- and recellularization of the pig uterus: a bioengineering pilot study. Biol Reprod. 2017 Jan 1;96(1):34-45. doi: 10.1095/biolreprod.116.143396

- Pisaturo V, Gatti M, **López-Pérez N**, Bottazzi C, Barbieri F, Cervelló I, Florio T, Costa M. Endometrial stem cells and human reproductive failure. Mini-review. Current Trends in Clinical Embryology. 2016;3(3):90-97. doi: 10.11138/cce/2016.3.3.090

2. Oral presentations and posters

- **Oral presentation:** SRI's 65th Annual Scientific Meeting. 2018, San Diego (California). "Stem cell-secreted paracrine factors in human endometrial regeneration". Nuria López-Pérez, Xavier Santamaria, Patricia Díaz-Gimeno, Patricia Sebastian-Leon, Amparo Faus, Jose Remohí, Antonio Pellicer, Carlos Simón and Irene Cervelló.

- **Poster:** SRI's 65th Annual Scientific Meeting. 2017, Orlando (Florida). "Human endometrial reconstitution using W5C5+, ICAM1+ cells and Side Population cell lines in a xenograft model". Nuria López-Pérez, Claudia Gil-Sanchis, Amparo Faus, Ana Díaz, Hannes Campo, Nicolás Garrido, Jose Remohí, Antonio Pellicer, Irene Cervelló and Carlos Simón.

- **Poster:** CONBIOPREVAL (II Congreso en Biomedicina de Predocs en Valencia). 2016, Valencia. "Proof of Concept of Endometrial Somatic Stem Cell Markers". Nuria López-Pérez, Claudia Gil-Sanchis, Amparo Faus, Ana Díaz, Hannes Campo, Nicolás Garrido, Jose Remohí, Antonio Pellicer, Irene Cervelló and Carlos Simón.

- **Poster:** SRI's 63rd Annual Scientific Meeting. 2016, Montreal (Canada). "De- and Recellularization of the pig uterus: a pilot study in uterus bioengineering". Hannes Campo, Pedro Baptista, Nuria López-Pérez, Amparo Faus, Irene Cervelló and Carlos Simón.