VNIVERSITAT DE VALÈNCIA

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EUROPEAN DOCTORAL THESIS

"Identification and characterization of the somatic stem cell niche in the human endometrium"

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CERTIFICA:

Que el trabajo de investigación titulado: "**Identification and characterization of the somatic stem cell niche in the human endometrium**" ha sido realizado íntegramente por Dña. Claudia Gil Sanchis 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 21 de Junio de 2013.

Fdo. Prof. Carlos Antonio Simón Valles

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Dra. Irene Cervelló Alcaraz, Doctora en Biología e Investigadora de la Fundación IVI.

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Fdo. Dra. Irene Cervelló Alcaraz

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"Una tesis no es meramente un trámite administrativo para lograr una promoción profesional, ni es tampoco un evento exclusivamente científico de pura investigación. Escribir una tesis tiene sobre todo una finalidad de aprendizaje. El primer fruto de una tesis es el crecimiento de su autor, su aprendizaje al escribirla, al mantener un discurso coherente y bien argumentado a lo largo de estas páginas. Quien es capaz de escribir una tesis doctoral acredita con ello su habilidad investigadora y su capacidad de comunicar a otros lo descubierto, siguiendo las pautas de quienes le han precedido y de quienes trabajan en ese mismo campo de investigación.

Una tesis debe aportar algo original y novedoso, pero su primer mérito ha de radicar en la tarea rigurosa de acopio de lo que otros han dicho sobre la cuestión afrontada, en el examen detenido de sus aciertos y limitaciones. Sólo después de hecha esa tarea imprescindible, puede y debe el autor de la tesis aportar algo nuevo.

Es precisamente durante el camino a recorrer por el doctorando, donde encontrará personas que le ayudarán gratamente a resolver dudas y dificultades científicas, pero por encima de todo ello, está el conocer a aquellas personas que le harán crecer, haciendo que su vida cobre un significado especial. A todos y cada uno de ellos, mil gracias".

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ABBREVIATIONS

ALL: Acute Lymphoblastic Leukemia

AMbL: Acute Myeloblastic Leukemia

ART: Assisted Reproductive Techniques

AT: Adenine-Thymine

BM: Bone Marrow

BMP: Bone-Morphogenetic Protein

BMSCs: Bone Marrow derived Stem Cells

BMT: Bone Marrow Transplant

bp: Base Pairs

BSA: Bovine Serum Albumin

CBCs: Columnar Base Cells of the Crypt

cDNA: Copy Deoxyribonucleic Acid

CEP: Chromosome Enumeration Probes

CFUs: Colony Forming Units

CML: Chronic Myeloid Leukemia

DAPI: Diamidino-2-phenylindole Dihydrochloride

DEPC: Diethylpyrocarbonate

DIG: Dioxygenin

DMEM: Dulbecco´s Modified Eagle Medium

DNA: Deoxyribonucleic Acid

dNTPS: Deoxynucleotide Triphosphates

DTT: 1,4-Dithiothreitol

EBMT: European Group for Blood and Marrow Transplantation

EDTA: Ethylenediaminetetraacetic acid

eMSCs: endometrial Mesenchymal Stem-like Cells

E_2: Estradiol

ES: Early Secretory

ESCs: Embryonic Stem Cells

EV: Estradiol Valerate

FACS: Fluorescence Activated Cell Sorting

FBS: Fetal Bovine Serum

FC: Flow Cytometry

FISH: Fluorescence *in situ* Hybridization

FS: Forward Scatter

g: × Gravity

GC: Guanine-Cytosine

G-CSF: Granulocyte-Colony Stimulating Factor

GNRH-a: Gonadotropin-Releasing Hormone Agonist

HBSS: Hank's Balanced Salt Solution

HCl: Hydrochloric Acid

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HLA: Human Leukocyte Antigen

HSCs: Hematopoietic Stem Cells

HRP: Horseradish Peroxidase

HRT: Hormone Replacement Therapy

iPSCs: Induced Pluripotent Stem Cells

ISH: *In situ* Hybridization

Lgr5: Leucine-rich repeat-containing G-protein coupled receptor 5

LH: Luteinizing Hormone

LS: Late Secretory

MAPCs: Multipotent Adult Progenitor Cells

MCS: Multiple Cloning Site

MHC: Major Histocompatibility Complex

MION: Molday ION- Superparamagnetic iron oxide nanoparticle (also called SPIOs when is labelled with fluorescence)

MMLV: Moloney-Murine Leukaemia Virus

MS: Mid Secretory

MSCs: Mesenchymal Stem Cells

mRNA: Messenger Ribonucleic Acid

NBT/BCIP: Nitro Blue Tetrazolium Chloride/ 5-bromo-4-chloro-3-indolyl-phosphate

NGS: Normal Goat Serum

NOD-SCID: Non-obese Diabetic/Severe Combined Immunodeficiency

NSP: Non Side Population

NP40: Nonyl Phenoxypolyethoxylethanol

ON: Over Night

P: Proliferative

P_4: Progesterone

PBS: Phosphate Buffered Saline

PBSCs: Peripheral Blood Stem Cells

PCR: Polymerase Chain Reaction

PFA: Paraformaldehyde

PGCs: Primordial Germ Cells

PI: Propidium Iodide

PVDF: Polyvinylidene Fluoride

Q-FISH: Quantitative Fluorescence *in situ* Hybridization (Telomapping)

RNA: Ribonucleic Acid

RPM: Revolutions Per Minute

RT: Room Temperature

SCs: Stem Cells

SDS: Dodecylsulfate Buffer

SDS-PAGE: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SS: Side Scatter

SSCs: Somatic Stem Cells

SSC-buffer: Saline Sodium Citrate Buffer

ssDNA: Single Strand Deoxyribonucleic Acid

SP: Side Population

SPF: Specified Pathogen-Free

TA: Transit Amplifying

TBI: Total Body Irradiation

TGF-B: Transforming Growth Factor-β

Tween 20: Polysorbate 20

UD-UCB: Unrelated Donor Umbilical Cord Blood

UD-BMT: Unrelated Donor Bone Marrow Transplant

Vm: Vimentin

Vp: Verapamil

v/v: Volume/Volume

w/v: Weight/Volume

CONTENT INDEX

RESUMEN

Introducción

El endometrio es la capa de mucosa que recubre el interior del útero y consiste en un epitelio simple cilíndrico con o sin cilios, glándulas y un estroma rico en tejido conjuntivo y altamente vascularizado. Durante cada ciclo menstrual, el endometrio sufre una serie de cambios en respuesta a fluctuaciones en los niveles de las hormonas esteroideas procedentes del ovario. El endometrio se divide histológicamente en dos capas, la capa basal, la cual ni responde a las hormonas esteroideas ni sufre descamación, pudiendo regenerar una nueva capa funcional en el siguiente ciclo menstrual. Mientras que la capa funcional si responde tanto a la progesterona como el estradiol, descamándose durante la menstruación.

La capacidad excepcional de renovación y regeneración del endometrio de manera periódica y que además puede hacerlo durante toda la vida de la mujer con un apropiado tratamiento hormonal (se considera que esto ocurre aproximadamente 400 veces durante la vida reproductiva de la mujer), lo hace un tejido candidato para la existencia de una población de células madre somáticas (CMS) que sean las responsables de esta actividad.

Las CMS, son definidas generalmente como células indiferenciadas pero que se encuentran entre las células diferenciadas de un tejido u órgano, y que pueden renovarse tanto a sí mismas como diferenciarse, dando lugar a los diferentes tipos celulares especializados que forman los tejidos. Así pues la función de éstas permite la adecuada homeostasis con el fin de mantener y reparar el tejido en el que se encuentran.

En el caso del endometrio, dada su excepcional fisiología se hipotetiza sobre que estas células madre somáticas sean las responsables de esta actividad cíclica de renovación y en determinados casos sean también las responsables de determinadas patologías endometriales como endometriosis y cáncer endometrial. En los últimos años, diferentes estudios apoyan esta hipótesis, donde se postula que las CMS endometriales pudieran estar localizadas próximas a los vasos sanguíneos en la capa basal.

Debido a la ausencia de marcadores específicos de CMS endometriales, el fenotipo Side Population (SP) se ha establecido como la técnica más usada para aislar CMS en los diferentes tejidos. La SP se basa en la capacidad de las células para excluir el colorante vital Hoechst 33342 a través de los transportadores de membrana ATP, y fue originalmente descrita como marcador de las CMS de la médula ósea. En el endometrio humano, varios grupos han demostrado la existencia de células SP con características de CMS, donde entre otros aspectos, se ha descrito su papel clave en la regeneración del endometrio *in vivo*.

Posible aporte exógeno de CMS

Por otro lado, las células madre de la médula ósea (MO) tienen la habilidad de diferenciarse en múltiples células no hematopoyéticas. Diferentes estudios describen que en pacientes que recibieron un trasplante de médula ósea, las células derivadas del donante fueron capaces de diferenciarse a hepatocitos, células de músculo esquelético, neuronas o piel, dependiendo del ensayo clínico realizado.

Además en estudios donde mujeres eran trasplantadas por médula ósea de varón, las células provenientes del donante eran capaces de diferenciarse a células epiteliales y estromales en el endometrio de las receptoras. Ello sugiere una posible fuente exógena para la regeneración del endometrio que se puede producir después de la movilización de células de la médula ósea, ya sea como un proceso fisiológico normal o en condiciones patológicas como la quimioterapia previa al trasplante.

Parte de nuestro estudio pretende abordar la cuestión de si las células de la MO derivadas del donante contribuyen a la población endógena de CMS representada por la SP endometrial y por lo tanto conocer el posible origen de esta población celular, o saber si únicamente actúa como aporte exógeno de CMS.

Búsqueda de marcadores específicos de CMS

Uno de los retos de la biología celular y la biomedicina es la búsqueda de marcadores específicos de CMS. Dado que ello beneficiaría el aislamiento de estas células y su potencial uso terapéutico en medicina regenerativa. En los últimos

años, se han descrito multitud de marcadores atribuidos a caracterizar las CMS en endometrio, pero ninguno hasta la fecha relevante.

Recientemente, Clevers y colaboradores, han conseguido identificar CMS en el intestino delgado de ratón a través del marcador Lgr5. Muy brevemente, Lgr5 es un miembro de la familia de receptores acoplados a proteínas G, que contiene dominios ricos en Leucinas, y que se encuentra implicado en la ruta se señalización Wnt (ampliamente descrita en la biología de las células madre). La expresión de Lgr5 es específica de las células epiteliales columnares que forman la cripta intestinal; además se ha visto que estas células Lgr5 positivas son capaces de dar lugar a las distintas células que forman la cripta intestinal, mostrando multipotencialidad, característica propia de CMS.

La expresión específica de este marcador Lgr5 propio de células madre intestinales (tejido derivado de endodermo) también ha sido descrita en tejidos como el folículo piloso (tejido derivado de ectodermo) o recientemente en riñón (tejido derivado de mesodermo), lo cual hace pensar que podría representar un marcador universal de CMS.

Objetivos

Los objetivos principales que se han establecido en la presente tesis son:

1. Demostrar la contribución de la médula ósea como una fuente exógena de Células Madre Somáticas para el nicho endometrial.

2. Investigar la expresión del marcador Lgr5 para identificar el nicho de Células Madre Somáticas endometriales.

Los objetivos secundarios que se derivan son:

1.1 Identificar las células portadoras del cromosoma Y en el endometrio de las receptoras trasplantadas de médula ósea y caracterizarlas mediante inmunohistoquímica.

1.2 Analizar la longitud telomérica en los endometrios de las receptoras trasplantadas de médula ósea.

1.3 Investigar la contribución de la médula ósea a la población Side Population en el endometrio de las receptoras trasplantadas de médula ósea.

2.1 Investigar la presencia del marcador Lgr5 en el endometrio humano a lo largo de ciclo menstrual.

2.2 Correlacionar las células Lgr5 positivas a nivel de ARN mensajero con su longitud telomérica como posible característica de célula madre.

2.3 Aislar las células Lgr5 positivas e inyectarlas en la cápsula renal de un ratón NOD-SCID para evaluar la formación de tejido endometrial humano.

Metodología

Para ello, se ha establecido el siguiente diseño experimental:

1) En relación a la contribución exógena de Células Madre Somáticas endometriales:

Para el análisis de la contribución de la médula ósea a la regeneración endometrial, se ha creado un modelo de trasplante de médula ósea entre donante y receptor de distinto sexo, siendo posible distinguir la contribución de la médula ósea por la presencia de células portadoras del cromosoma Y (donante) en el endometrio de la receptora a través de hibridación *in situ* fluorescente (FISH). El análisis fenotípico de estas células se realizará con marcadores específicos como son CD45 (origen hematopoyético), CD9 (epitelio) y Vimentina (Vm, estroma).

El análisis de longitud telomérica se llevara a cabo con la técnica del telomapping, y la evaluación de células Side Population por citometría de flujo como la población de CMS endógenas en el endometrio de la receptora nos permitirá determinar la contribución de la médula ósea así como la posible correlación de las diferentes fuentes de células madre.

2) En relación a la contribución endógena del nicho de Células Madre Somáticas endometriales:

Para ver la implicación de Lgr5 como posible marcador del nicho endógeno de Células Madre Somáticas endometriales, se emplearon secciones de endometrio a

lo largo del ciclo menstrual para la identificación y localización de Lgr5, utilizando diferentes técnicas como son la hibridación *in situ* (a nivel de RNA mensajero), inmunohistoquímica y western blot (a nivel de proteína). El análisis de la longitud telomérica de las células Lgr5 determinadas por hibridación *in situ* permitirá establecer una primera aproximación como característica de célula madre. Finalmente, el aislamiento de esta población de células por citometría de flujo permitirá realizar experimentos *in vivo* para evaluar la formación de tejido endometrial humano en un modelo animal, como prueba de concepto.

Conclusiones

Las conclusiones extraídas de la presente tesis son:

1. Las células XY procedentes de la médula ósea del donante son capaces de migrar al endometrio de la receptora, y transdiferenciarse a células estromales (expresan Vm), y células epiteliales (expresan CD9) contribuyendo a la regeneración del tejido endometrial.

2. Las células XY derivadas del donante no son incorporadas en la población endometrial Side Population (SP) considerada al menos en parte como la población de Células Madre Somáticas.

3. Las células XY derivadas del donante no están asociadas con regiones de telómeros largos.

4. Estas evidencias demuestran que las células XY derivadas del donante pueden ser consideradas como una fuente exógena temporal de células endometriales en lugar una fuente cíclica de Células Madre provenientes de la médula ósea.

5. La expresión de Lgr5, determinada tanto a nivel de ARN mensajero como de proteína, está presente en el endometrio a lo largo del ciclo menstrual. Las células Lgr5 positivas se encuentran localizadas en la zona inferior de la capa funcional, cerca de la basal, estando en mayor frecuencia en el compartimento estromal.

6. Algunas de las células Lgr5 positivas poseen telómeros largos, sugiriendo ciertas características de célula madre.

7. La prueba de concepto de que Lgr5 es un marcador de Células Madre Somáticas endometriales se demuestra por la inyección de células Lgr5 positivas en la cápsula renal de un ratón NOD-SCID dando lugar a la formación de tejido endometrial humano.

Como conclusión general, se demuestra que las Células Madre provenientes de la médula ósea contribuyen en la regeneración endometrial como una fuente exógena de células capaces de diferenciarse; mientras que Lgr5 puede ser considerado como un marcador del nicho endógeno de Células Madre endometriales.

I. Introduction

I. INTRODUCTION

1. Stem Cells

1.1 Definition

"Stem cells by definition are not terminally differentiated and have the ability to divide throughout the lifetime of the organism, yielding some progeny that differentiate and others that remain stem cells" (Alberts, 1989; Figure 1).

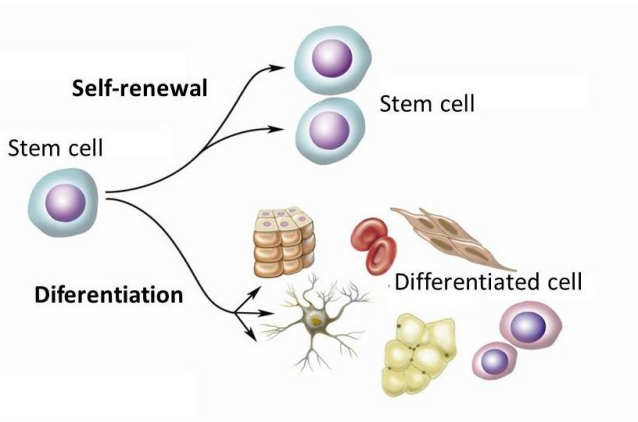

***Figure 1. Stem cells**. Stem cells can regenerate themselves and produce specialized cell types (Image adapted from "Understanding stem cells: an overview of the science and issues" from the National Academies).*

1.2 Types and Classification

Stem cells (SCs) are found from the early stages of human development to the end of the lifetime. There are different classifications for SCs based on their ability to differentiate (potency) or origin. Regarding their developmental potential, they can be distinguished as totipotent (capable of differentiating into a new organism), pluripotent (able to give rise to any cell type of the organism), or multipotent (giving rise to differentiated cells of the same embryonic lineage). Depending on their origin, SCs can be divided into five groups: embryonic stem cells (ESCs), primordial germ cells (PGCs), induced pluripotent stem cells (iPSCs), teratocarcinoma stem cells and somatic stem cells (SSCs), as shown in Figure 2.

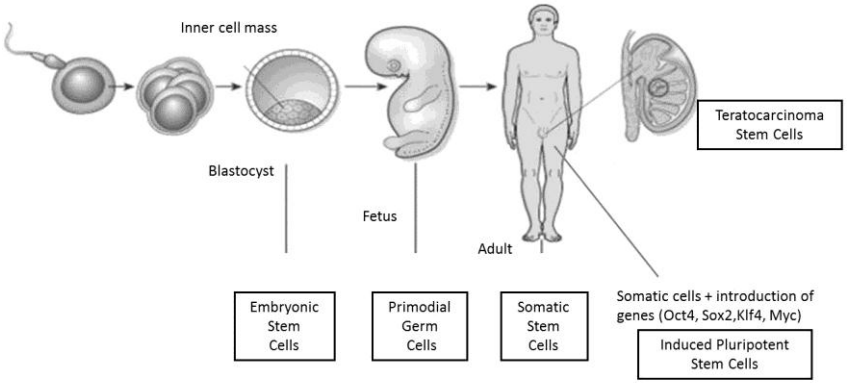

Figure 2. Stem cell types depending on their origin. *(Image adapted from Donovan and Gearhart "The end of the beginning for pluripotent stem cells").*

1.2.1 Embryonic Stem Cells

Embryonic stem cells (ESCs) were first isolated in 1998 by Thomson and Marshall from the inner cell mass of a human blastocyst. They possess the capacity to divide indefinitely whilst retaining their ability to make all cell types within the organism. However, their use for research presents legal and ethical problems which arise from their origin.

According to Marshall et al. (2001) and Smith (2001) the defining characteristics of the ESCs are:

- They can be obtained from the inner cell mass of a blastocyst, morula or even single blastomeres.
- They have an unlimited capacity for asymmetric cell division, and remain undifferentiated *in vitro* in appropriate culture conditions.
- They have a normal karyotype, which remains stable during the isolation and differentiation process.
- They are able to differentiate into cell types of all three primary germ layers: ectoderm, mesoderm, and endoderm, as well as germ cells (gametes). This is the reason why they are termed pluripotent stem cells, which makes them a unique cellular type.

- In the murine model, when they are injected into a blastocyst they will form chimeras.
- Injection of embryonic stem cells into an immunocompromised mouse gives rise to the formation of a benign tumour called a teratoma, which contains cellular components from all the three germ layers.
- They express stem cell specific markers, including the transcription factor Oct-4, the Sex Determining Region Y-box2 (SOX2), and Nanog, which regulate a large number of genes, maintaining embryonic stem cells in a proliferating but undifferentiated state.

1.2.2 Primordial Germ Cells

Primordial germ cells (PGCs) are stem cells that give rise to gametes in vertebrates. During the early stages of embryogenesis, they originate outside the embryo and migrate by a well-defined route into the genital ridge. They are obtained from human embryonic gonadal ridge at 5-9 weeks of gestation and are the precursors of gametes (spermatozoa and oocytes).

PGCs have biochemical, morphological, immunological, and developmental properties common to ESCs. They are pluripotent, and contribute to the formation of the germline when they are injected into blastocysts, giving rise to chimeric animals (Matsui et al., 1992; Stewart et al., 1994). PGCs can also differentiate into embryoid bodies and form teratomas *in vivo*. However, there are differences in the methylation pattern of these cell types that may reflect changes in the developmental program of the PGCs *versus* ESCs. However, the existence of this gene methylation pattern does not appear to affect the ability of these cells to contribute to germline development in chimeras (Sato et al., 2003).

In primordial germ cell lines there is a great variability in the expression of genes that are subject to imprinting or gene regulatory mechanisms which modify their expression without changing their DNA sequences. As a consequence, in the offspring of chimeric mice resulting from injection of these cells, PGC genes are transmitted in a normal manner, while in other cases the animals show abnormalities in growth and bone structure.

1.2.3 Teratocarcinoma Stem Cells

Teratocarcinoma is a type of malignant teratoma commonly found in the gonads but also occasionally in extra-gonadal sites, although the rate of spontaneous formation is very low (Fawcett, 1950). Teratomas are formed from malignant SCs called embryonal carcinoma cells which are regarded as equivalent to germ cells or early ESCs (Damjanov, 1993). They are pluripotent and therefore give rise to the multiple cell types found within them (Kleinsmith and Pierce, 1964; Stevens and Little, 1954), hence these tumours are composed of a haphazard mixture of somatic tissues and misshapen organs.

The strain 129 mice showed an incidence of spontaneous testicular teratoma formation of 1%. The malignant nature of these tumours is displayed by their ability to quickly grow and invade when they are transplanted subcutaneously or intraperitoneally (Stevens and Little, 1954).

Teratocarcinoma SCs retains their capacity to differentiate, producing derivatives of all three germ layers: ectoderm, mesoderm, and endoderm (Nicolas et al., 1976). Their properties include:

- They possess the ability to participate in embryonic development resulting in chimeras.
- The emergence of chromosomal aberrations.
- The loss of differentiation capacity in culture.
- *In vitro* differentiation only occurs under special conditions or using chemical inducers, and hence is much more limited.
- The need for co-culture with feeder cells to maintain the undifferentiated state.

1.2.4 Induced Pluripotent Stem Cells

Induced pluripotent stem cells (iPSCs) are created by genetically reprogramming cells to become pluripotent stem cells. Yamanaka and colleagues first discovered the dedifferentiation of mouse somatic fibroblast cells to iPSCs in 2006, by the transfection of 4 genes. The key step in the discovery of the factors that enable cell dedifferentiation was the assumption that the transcription factors that maintained

the state of pluripotency in embryonic stem cells were the same as those that could induce dedifferentiation of somatic cells. The researchers tested 24 combinations of transcription factors on somatic cells, and found that only four of them, Oct3/4, Klf4, Sox2, and c-Myc, were required to reverse cell differentiation (Takahashi K and Yamanaka S, 2006).

The most important characteristics of iPSCs are:

- iPSCs acquire a morphology similar to ESCs, such as the formation of colonies with a compact morphology, expression of pluripotency markers like Oct4, the ability to differentiate into cells of the endoderm, mesoderm, and ectoderm, and the ability to remain undifferentiated for long periods in culture.
- In ESCs the CpG island promoters (regions devoid of methylation) required to express pluripotency genes are not methylated and thus the gene expression machinery has full access to these regions. In iPSCs cells these epigenetic marks are deleted, therefore allowing expression of previously silenced factors.
- In addition, as embryos are not used, the ethical obstacles with ESCs are avoided.
- The possibility of obtaining *in vitro* iPSCs opens a great number of applications in basic research as well as in the diagnosis and therapeutic implications for certain diseases.

1.2.5 Somatic Stem Cells

The primary function of somatic stem cells (SSCs; also named adult or progenitor stem cells) is to maintain tissue homeostasis by replenishing senescent or damaged cells after injury. SSCs are undifferentiated cells found among differentiated cells in any tissue or organ, which have the ability to self-renew and differentiate to result in tissue specific-cell types (Fuchs and Segre, 2000).

The identification and characterization of these stem cells is one of the greatest scientific challenges that exists today. Their uniqueness, scarcity, and lack of distinctive morphological characteristics, such as defining cell surface markers, makes their identification and location a very complex task in most tissues.

Unlike ESCs, SSCs generally have a limited potency and are thought to give rise only to cells within the tissue of residence, or those from the same embryonic lineage. However, in recent years, evidence has accumulated that contradicts this dogma, suggesting that under certain circumstances these cells may `transdifferentiate´ and contribute to a much wider spectrum of differentiated progeny (Jiang et al., 2002; Poulsom et al., 2001; Mezey et al., 2003). Transdifferentiation describes the conversion of a cell from one tissue lineage into a cell of a distinct lineage, with concomitant loss of the tissue-specific markers and function of the original cell type, and acquisition of markers and function for the transdifferentiated cell type.

The suggestion that SSCs may transdifferentiate has given rise to the concept of stem cell plasticity, which holds that the lineage determination of a differentiating stem cell may not be rigidly defined, but is instead flexible, allowing these cells to respond to a variety of microenvironmental signals (Reyes, 2001; Wagers AJ and Weissman IL 2004). One of the most universal examples is that of hematopoietic stem cells (responsible for the formation of blood components), where both stem cells and their progeny, are capable of self-renewing and differentiating depending on the cell cycle phase and the microenvironment surrounding them (Quesenberry et al., 2005).

Definitions and concepts

SC self-renewal is the ability of one cell to produce identical daughter cells, and is essential for maintaining the SC reservoir in adult tissues.

Asymmetric cell division is the mechanism implemented by these SSCs to produce a daughter cell identical to itself and a differentiated daughter cell (Figure 3). At the same time, stem cells also undergo symmetric divisions from progenitor cells, producing two daughter cells or transit amplifying cells (TA; Gargett, 2006). Finally, the differentiation process is driven by a change in cell phenotype due to expression of certain genes which would normally be associated with the cell function to which they are destined.

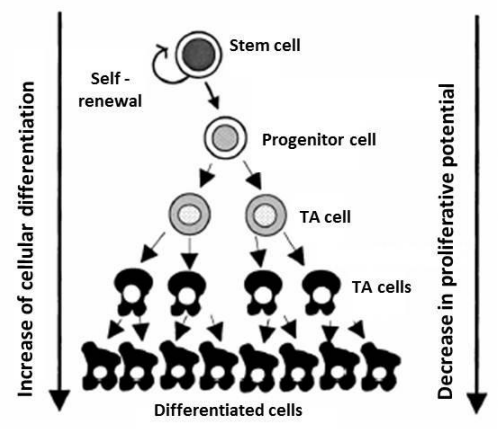

Figure 3. Hierarchical distribution of somatic stem cells. *Stem cells are capable of self-renewing and differentiating into committed progenitor cells. They proliferate and give rise to transient amplifying cells, which finally give rise to differentiated and functional cells without any further proliferation capacity (Image adapted from Chan et al., 2004).*

Role in tissue repair and regeneration

SSCs are located in a physiological microenvironment called a niche, where they remain in a quiescent state mediated by signalling pathways that inhibit cell division and cell differentiation: among them, the transforming growth factor-β (TGF-β) and members of the family of bone morphogenetic proteins (BMP) (Li and Xie, 2005) are well known. SSCs play a key role in cellular homeostasis, promoting cell turnover in highly regenerative tissues, as well as cell loss by apoptosis (Snyder and Loring, 2005).

It is undisputed that stem cells not only reside and function in tissues with exceptional regenerative capacity with a constant production rate of differentiated cells (e.g. bone marrow, intestine or epidermis), but that they also exist in low cell-turnover tissues such as neural, retina, or kidney. In these tissues, the function of SSCs is likely the maintenance of cellular homeostasis through the re-organization of tissue when cells are lost by damage or apoptosis. After tissue damage or injury, the quiescent SSCs, re-enter in the cell cycle, resulting in the formation of TA cells, which undergo rapid proliferation and expansion cycles to allow tissue repair and replacement with fully differentiated and functional cells. Thus, SSCs maintain the

proper balance between cell turnover and the provision of a sufficient number of mature differentiated cells to maintain the proper function of tissues and organs. This balance between self-renewal and differentiation is regulated by the stem cell niche.

1.3 The Stem Cell Niche Concept

A cell niche is a physically located, structurally organized, and interactive unit that facilitates cell fate decisions in response to homeostasis in an appropriate spatio-temporal manner. An intricate network of molecule and signalling pathways interactions all come together at the right place at the right time (Figure 4).

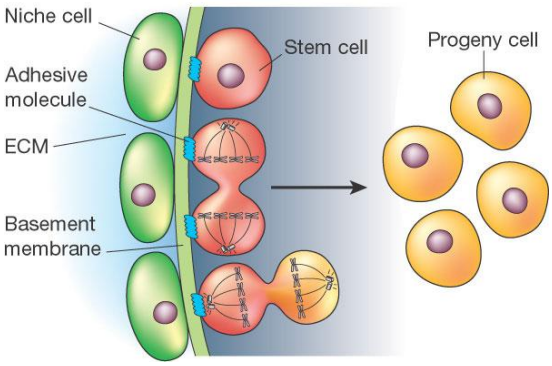

Figure 4. Schematic representation of a stem cell niche. *Niche cells direct the fate of somatic stem cells through cell signalling to initiate and/or block cell differentiation and cell cycle regulation (Image adapted from Spradling et al. "Stem cells find their niche").*

These niches keep the SSCs in a quiescent state via molecular signalling pathways which inhibit cell division and differentiation. In the same way, they also facilitate the expansion and differentiation of stem cells when a necessity for tissue repair or regeneration arises. It is possible that these niches are constituted by endogenous SSCs that are resident inside the tissue.

The concept of a stem cell niche was first proposed by Schofield in 1978 who hypothesized that the basic concepts that define a cell niche should be:

1) In a location which is anatomically defined.

2) A location where the stem cells can be maintained and reproduced.

3) A place where the differentiation process is inhibited.

4) A limited space which also limits the number of stem cells.

5) A place where reversion to a stem cell phenotype is induced at much slower rate than for a fully differentiated cell.

This niche concept remained as hypothesis until 1998, when Xie and Spradling performed pioneering work in *Drosophila melanogaster,* showing that Schofield´s premises were true. This study demonstrated that germ stem cells in the fruit fly ovary reside adjacent to other cell types in a specialized location. This location is required for the preservation of the stem cell phenotype and produces specific signals, including the bone morphogenetic protein (BMP), capable of inhibiting differentiation. When contact with niche cells is lost, stem cells initiate their differentiation process.

Niches have different characteristics in the different adult tissues depending on their cellular composition, structure, and location of the stem cells. Actually, numerous niches have been identified and characterized, such as the epidermal stem cell niche in the buldge follicle region (Fuchs et al., 2004), or the glandular crypts in small intestine (Booth and Potten, 2000). In the case of the small intestine niche, the crypt forms a pocket composed of epithelial cells at the base of the villi. Intestinal stem cells and TA cells continuously regenerate the villus crypt due to cellular loss in the normal digestive process over 3 to 5 days. There are 4 or 6 intestinal crypt stem cells, which are located above the differentiated cells in the crypt base ring. The canonical Wnt signalling pathway regulates intestinal stem cells. This pathway triggers a specific program of gene expression through the stabilization and nuclear re-localization of β-catenin, while the signalling pathway triggered by BMP acts as a negative regulator of intestinal stem cell proliferation, exerting the opposite effect of Wnt. Many components of the signalling pathway are expressed inside the stem cell niche, such BMP-4, which is expressed in mesenchymal cells adjacent to intestinal stem cells; Noggin (a BMP inhibitor) which is expressed by intestinal stem cells and adjacent mesenchymal cells, meaning that transient Noggin expression inside the niche favours periodical activation of intestinal stem cells (Radtke and Clevers 2005).

2. Somatic Stem Cells in the Human Endometrium

2.1 The Human Endometrium

The human endometrium is the mucosal lining of the uterine cavity, whose basic function is to create a suitable environment for embryo implantation. It is hormonally regulated and undergoes periodical changes that underlie the menstrual cycle in humans and higher primates. These changes serve to prepare for the acquisition of the receptive state which is essential for embryo implantation and pregnancy.

Histologically the endometrium is divided into two layers, a luminal level overlying the endometrial cavity and a functional layer formed by invagination of glands from the surface. All this forms the area called the `functionalis´ whereas the basal area `basalis´ is formed by the deeper glandular folds that reach the underlying smooth muscle wall, the myometrium (Figure 5).

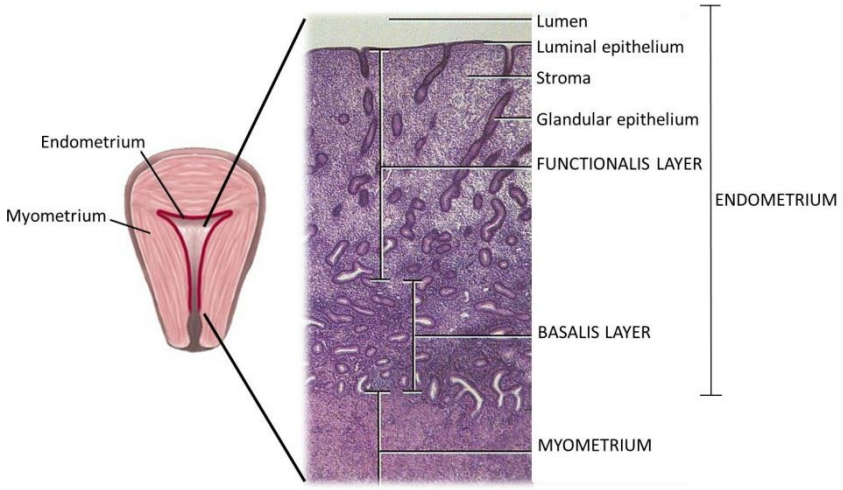

Figure 5. Anatomical and histological diagram representing the uterus. *The uterus is formed by the myometrium (outer layer) and endometrium (inner layer). Cellular content in layers of endometrium, basalis, and functionalis are represented (Image adapted from the online reproductive database).*

It is known that the functional layer responds to progesterone and estradiol, exerting its action, through local mediators, over neighbouring cells in a paracrine

fashion, or over endometrial cells themselves in an autocrine pathway (Giudice, 1994; Giudice et al., 2002), leading to shedding during menstruation. In contrast, the basal layer does not respond to steroid hormones and therefore does not shed, and is able to fully regenerate endometrium.

The main cellular components of the endometrium are the epithelium, and the stromal and vascular components, in addition to the existence of a resident population of immune cells.

_ **Epithelium:** A single-layer of prismatic epithelium that has three different types of cells: secretory, ciliated, and basal cells. Simple tubular uterine glands reach from the endometrial surface through to the base underlying the stroma-myometrial junction. This monolayer regulates embryo implantation and controls the impact of the embryo on the endometrial stroma and blood vessels. With the exception of the basal fraction, this epithelium is shed during each menstruation, and is regenerated again in the next menstrual cycle.

_ **Stroma:** A connective tissue composed of fibroblastic shaped cells and extracellular matrix. The main cell type present is the fibroblast, which is involved in extracellular matrix remodelling throughout the menstrual cycle and the process of decidualization. A portion of the stroma is also shed during menstruation and is regenerated in the same manner in the next cycle to re-form suitable conditions for embryo invasion.

_ **Endothelium and vascular smooth muscle:** Intrauterine vascular architecture comprises an intricate network beginning in the myometrium. Uterine arteries extend into the spiral arteries, which are responsible for maintaining basal layer irrigation, and further branch into in the functional layer.

_ **Resident immune cells:** The leukocyte population found in normal endometrium represents between 10-15% of the stromal cellularity. This population mainly consists of natural killer (NK) cells, macrophages, and T lymphocytes. The immune system cells are relevant for endometrial physiology, especially in the regulation of the local immune response to protect the genital tract from infections and to prevent immune rejection during embryo implantation.

The menstrual cycle

During the menstrual cycle, the endometrium undergoes several changes in response to fluctuations in the levels of steroid hormones from the ovary. As a consequence of menstrual changes two phases can be distinguished: the proliferative and the secretory phase which are both separated by the ovulation process, (Figure 6).

The proliferative or follicular phase, extends from the end of menstruation (day 28/0) until ovulation (day 14). This is when the follicle containing the oocyte is developed, and a result an increased estrogen secretion is responsible for the proliferation of the endometrium (intensive mitosis in the glandular epithelium and stroma), the glands grow longer and the spiral arteries wind themselves lightly into the stroma when it reaches its greatest size. The endometrial thickness increases from 0.5-1 mm to about 7.5 mm (McLennan and Rydell, 1965). At the end of the proliferative phase an estradiol peak (released by the growing ovarian follicles) triggers a positive feedback mechanism at the level of the pituitary gland resulting in the commencement of ovulation 35 to 40 hours after the initial LH peak.

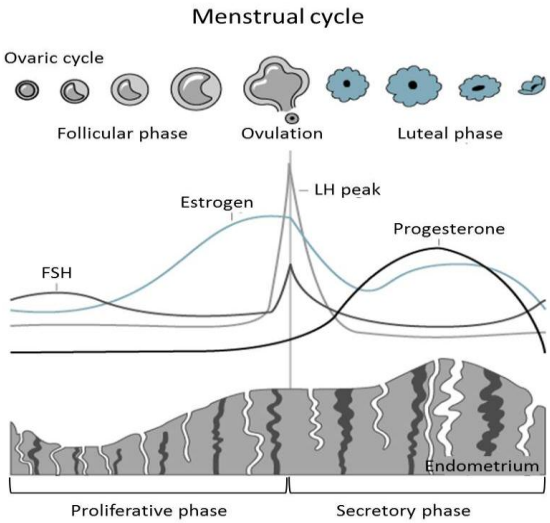

Figure 6. Endometrial menstrual cycle. *Changes occurring in the human endometrium during the different phases from both menstrual-ovarian cycle and hormone expression are shown (Image adapted from the online reproductive database).*

The secretory or luteal phase, extends from the moment of ovulation (day 14) to menstruation (day 28/0). The progesterone concentration increases because the corpus luteum secretes and maintains high estrogen levels. Progesterone limits the proliferative effect of estrogens on the endometrium and promotes differentiation, glandular secretion of glycogen, and glycoproteins, and stromal decidualization. Furthermore, growth of endometrial blood vessels continues, acquiring a tortuous appearance. All these changes prepare the endometrium for its receptive stage. In the absence of embryonic implantation, the corpus luteum degenerates and therefore the levels of ovarian hormones (estrogen and progesterone) drop dramatically causing involution and shedding of the endometrium.

There are several pathologies associated with the endometrium. Endometriosis is an estrogen-dependent disease defined by the growth of endometrium outside its physiological niche, whereas in endometrial cancer malignantly transformed cells are only linked to hormonal changes in some cases. In the case of Asherman´s syndrome there is a complete destruction of the uterine cavity because of the formation of adherences resulting in infertility and amenorrhea.

2.2 Existence of Somatic Stem Cells in the Human Endometrium

Preliminary evidence

The human endometrium is a tissue with an exceptional capacity for regeneration, since it is able to undergo more than 400 cycles of regeneration, tissue differentiation, and shedding over the female reproductive life. On a monthly basis, and under the influence of circulating estrogen, the endometrium undergoes growth ranging from 0.5-1 mm to 5-7.5 mm.

This evidence led to Prianishnikov and Padykula (Prianishnikov, 1978; Padykula et al., 1984, 1989) to speculate the existence of SSCs in the endometrium. They described that the endometrium undergoes regression in menstruating primates as a result of the loss of the functional layer. Classical morphological studies divide the endometrium into four zones: Zone I, which consists of luminal epithelium and underlying stroma; Zone II which includes previous cellular components as well as the microvasculature (Zones I and II are associated with the functional layer); Zones III and IV consist of basal segment glands and stroma associated with the basal

layer. Thus, after the menstrual cycle, Zones III and IV remain intact and occasionally some fraction of Zone II may persist. In this context, in the early 80s, the existence of a SSC population residing in Zones III and IV was suggested, with TA cells in Zones II and III and differentiated cells in Zones II and I, as shown in Figure 7.

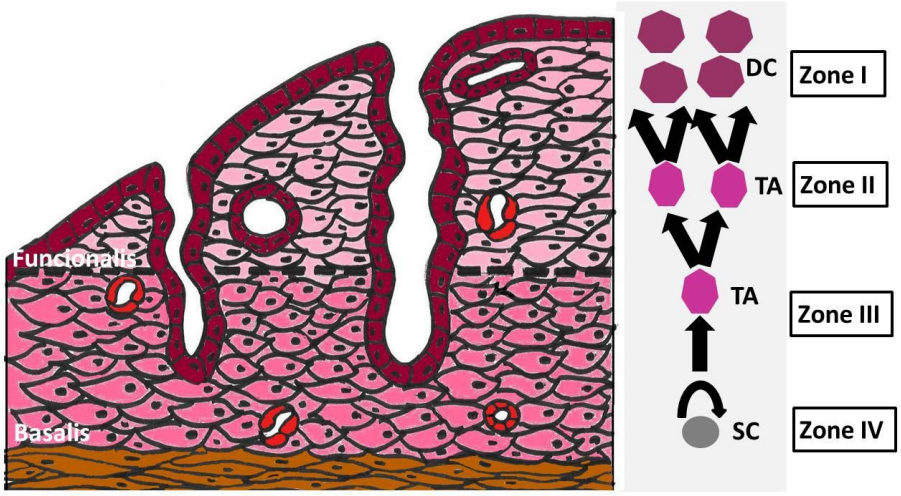

Figure 7. Human endometrium and somatic stem cells. *Left panel: distribution of the endometrium divided into four zones as described by classical studies. Zone I (luminal epithelium and subjacent stroma) and zone II (luminal epithelium, stroma, and microvasculature) are associated with the functionalis layer. Zones III and IV consist of basal segments of glands and stroma associated with the basalis layer. Right panel: hierarchical organization of stem cells in adult tissues showing the putative location of the stem cell niche (zones III and IV), transit amplifying cells (zones II and III), and differentiated cells (zones II and I) in the human endometrium. Abbreviations: SC (stem cell), TA (transit amplifying cell), DC (differentiated cell).*

In 2004 a descriptive study by Cho and collaborators analyzed the presence of two stem cell markers (c-Kit/CD117 and CD34) in human endometrium from the fetal to the postmenopausal period. CD117, a cell surface marker for hematopoietic stem cells and germ cells, and plays an important role in cell differentiation and proliferation. CD34 is the most common marker for hematopoietic stem cells

(defined as multipotent stem cells that give rise to all blood cell types). The expression of both markers in the human endometrium by immunohistochemistry suggests the presence of cells with stem cell features in the endometrium at the different periods analyzed. In addition, these cells appear mainly in the stromal compartment of the basalis layer and thus avoid cyclic shedding due to menstruation (Cho et al., 2004). Another innovative publication explored the involvement of bone marrow stem cells (BMSCs) in endometrium. Molecular analyses of endometrium in four bone marrow transplant (BMT) recipients demonstrated the presence of donor-derived bone marrow cells in both the epithelial and stromal compartments of the newly formed endometrium, implying the possibility of BMSCs as a new ectopic source of endometrial SSCs (Taylor et al., 2004).

Clonogenic cells

The first evidence for SSC activity in human endometrium was produced by demonstrating that single cells proliferate and produce individual colonies in culture. Gargett´s group (Chan et al., 2004; Schwab et al., 2005) identified small populations of these colony forming units (CFUs) in freshly isolated cells from the epithelium and stroma which comprised 0.22% and 1.25% of the total cell population and were EpCAM positive or negative respectively.

Both cell fractions gave rise to colonies of different sizes. In the case of epithelium, the large CFUs differentiated *in vitro* into gland-like structures (which were cytokeratin^{+}), while the large CFUs from stroma exhibited multipotent features. Stromal CFUs with an appropriate differentiation treatment were able to differentiate *in vitro* into muscle cells, adipocytes, osteocytes, and chondrocytes hence showing a mesenchymal stem cell (MSC) phenotype. By contrast, the small colonies had a limited proliferative capability. Characteristics of self-renewal, such as a high proliferative potential, and a high differentiation capacity in culture conditions supported that these cells exhibit key SSC features.

Endometrial stem cell phenotypes

Several markers for human endometrial SSCs have been described, although no agreement on them has been reached. Gargett and collaborators described the co-

expression of two specific markers, CD146 and PDGF-Rβ in CFU stromal cells (Schwab and Gargett 2007). This $CD146^+$ $PDGF\text{-}R\beta^+$ cell population was multipotent, showing differentiation toward typical mesodermal lineages; it also showed typical expression of MSC markers and was located near blood vessels in both the basal and functional layer, and expressed typical pericyte markers and genes related to angiogenesis.

Another endometrial stem cell population described is characterized by the marker W5C5 (Masuda et al., 2012), which purifies a stromal population similar to the $CD146^+$ $PDGF\text{-}R\beta^+$ population. To date there have been no reported markers for endometrial epithelial stem cells.

Menstrual blood progenitor cells

Menstrual blood is being studied for endometrial stem cell research. It is hypothesized that endometrial SSCs, at least in part, may be discarded during menstruation (Gargett and Masuda 2010). After collection of menstrual blood, endometrial cells are isolated and cultured where they acquire an appearance similar to fibroblasts and have a high proliferative potential (Hida et al., 2008; Meng et al., 2007; Patel et al., 2008). Additionally these cells display telomerase activity and typical phenotypic MSC markers (Cui et al., 2007; Hida et al., 2008; Meng et al., 2007; Patel et al., 2008). Studies show that these cells exhibit an ability to differentiate *in vitro* into typical mesodermal lineages, and also into cardiomyocytes and skeletal muscle (Cui et al., 2007; Hida et al., 2008), in addition to neurons of ectodermal lineage (Patel et al., 2008). The presence of stromal cells but not epithelial cells in menstrual blood, suggests that epithelial progenitors must reside in the basal endometrium and are not released during menstruation (Musina et al., 2008). In fact, menstrual blood cells have been reprogrammed into a pluripotent status and differentiated into various cell types from all 3 germ layers both *in vitro* and *in vivo* (Li et al., 2013).

2.3 Side Population Isolation as a Method to Identify Somatic Stem Cells in Endometrium

A widely used approach to identify SSCs is the search for the side population (SP) cells in a particular tissue or organ; the first work done using this methodology was

described by Goodell for bone marrow. It is based on the use of a fluorescent vital dye called Hoechst 33342, which specifically binds to adenine-thymidine rich regions in the double DNA chain. Undifferentiated cells are enriched in ABC multidrug resistance membrane transporter proteins which are responsible for the expulsion of Hoechst (Goodell et al., 1996). Accordingly, undifferentiated cells are able to efflux Hoechst in the short term. These SP cells separate as a discrete population when viewed on a lateral two-colour diagram on a flow cytometer.

Several groups have identified and characterized SP cells in the human endometrium. In 2007, Kato described the first work on the SP phenotype of endometrial cells, which mainly had a $CD13^-$ and $CD9^-$ phenotype, distinguishing immature endometrial cells and progenitor cells. In culture these SP cells were capable of producing gland-like structures ($CD9^+$) and stromal cells ($CD13^+$; Kato et al., 2007). Moreover in 2008, Tsuji et al. analysed the endometrial SP population as a heterogeneous mixture of cells expressing typical stromal cell markers such as CD13, CD31 and CD34 (endothelial), the epithelial cell marker (EMA) and CD146 and CD105 (mesenchymal), as well as also being negative for CD45 (hematopoietic lineages). The SP phenotype was correlated at the molecular level with a high expression of ABC protein type BCRP1/ABCG2, which was located in the basal area of the endometrium. In addition, these SP cells had a high colony formation *in vitro* (Tsuji et al., 2008).

In 2010, two independent groups finally performed a xenograft of SP cells in NOD-SCID mouse model as a functional proof of the concept of endometrial SSCs (Cervelló et al., 2010; Masuda et al., 2010). Our group (Cervelló et al., 2010) isolated SP cells from both cell fractions, epithelium and stroma, with SP representing 1.6% and 0.3% of the original cell samples respectively. The cellular purity of each fraction was assessed by phenotypic analysis, CD9 in the case of epithelium and Vimentin (Vm) in the case of stroma. Immunophenotypic analysis of SP cells revealed that they were around 70-80% positive for CD90, only 1-2% cells were positive for CD34, and 5-7% were positive for CD45. These results suggest that, as expected epithelial and stromal SP cells have a mesenchymal and not a hematopoietic phenotype. In addition at the molecular level, expression of certain SC markers was analysed: interestingly SP cells were enriched for c-KIT and OCT-4 as well as markers of ABC membrane transporters, MDR1, and BCRP-1.

SP cells showed an intermediate telomerase activity pattern which was between that of human ESCs and non-side population (NSP) cells as differentiated cells. Clonal efficiency was tested in hypoxic culture conditions, where SP cells showed a much higher capacity to form colonies than NSP cells, although the differences were statistically significant only in the case of the stromal fraction. A genetic signature for endometrial SP cells common in epithelium and stroma was also generated using microarrays, identifying a total of 44 upregulated and 14 downregulated genes.

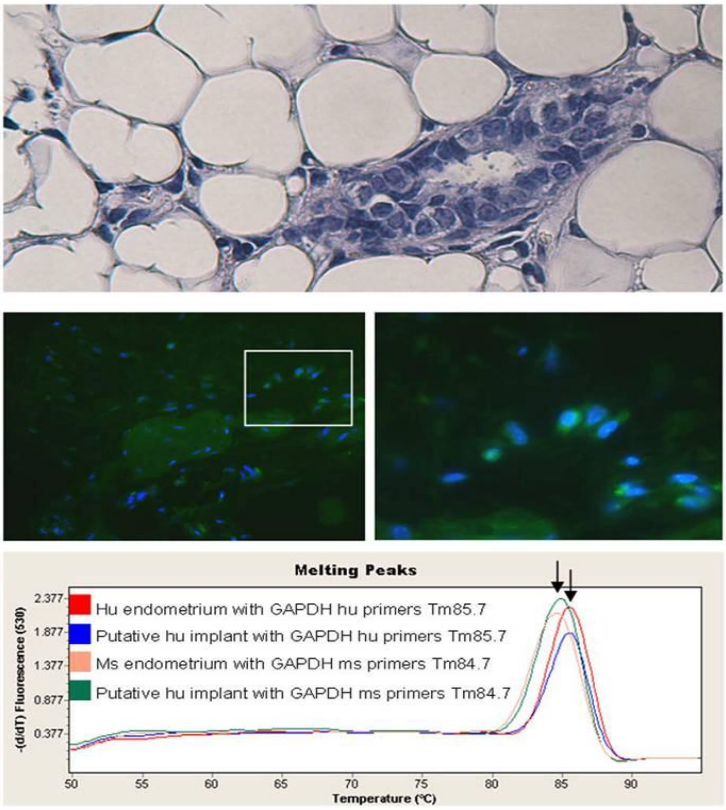

Figure 8. Formation of human endometrium-like tissue after subcutaneous injection of SP cells in NOD-SCID mice. *Immunohistochemical analysis for human Progesterone Receptor together with the existence of two amplicons for GAPDH corresponding to different species (human and mouse) allowed the detection of the newly formed endometrial-like tissue (Image from Cervelló et al., 2010).*

To determine whether SP cells were clear stem cells candidates, a differentiation assay was performed *in vitro* and *in vivo* as a proof of concept of multipotentiality; they were differentiated into adipocytes and osteocytes, and formed endometrium-like structures after injecting them subcutaneously in NOD-SCID mice, respectively. As a result it was shown that endometrial SP cells exhibit genotypic and phenotypic features as well as the functional capacity to develop human endometrium after injection into NOD-SCID mice.

Independently, Masuda and collaborators obtained a similar percentage of SP cells from the endometrium which exhibited predominantly endothelial markers. SP cells proliferate *in vitro* and differentiate into various types of endometrial cells, including glandular epithelial, stromal and endothelial cells. Furthermore, SP cells were able to reconstitute human endometrial tissue with well-delineated glandular structures when they were transplanted under the kidney capsule of immunodeficient mice. In these endometrium-like structures, mature blood vessels were formed *de novo,* showing the potentiality of SP cells for angiogenesis and endometrial cell regeneration *in vivo* (Masuda et al.,2010).

Our group generated seven human endometrial SP cell lines (ICE 1-7; Cervelló et al., 2011): four from the epithelial and three from the stromal fraction, respectively. Briefly, SP cell lines were generated following selection of individual large colonies that grew in culture under hypoxic conditions for 12-15 passages. The lines showed a normal 46XX karyotype, an intermediate telomerase activity pattern, and were enriched in stem cell markers such as Oct-4, GDF3, DNMT3B, Nanog, and GABR3 as well as mesodermal markers like WT1, Cardiac Actin, Enolase, Globin, and REN. The stromal or epithelial phenotype was confirmed by the expression of specific markers, CD9 in the case of epithelium, and Vm in the case of stroma. Surprisingly, the cell lines did not express estrogen (ERα) or progesterone (PR) receptors suggesting that they were undifferentiated of some degree. Furthermore these lines were able to differentiate *in vitro* into adipocytes and osteocytes, which confirmed their mesodermal origin. Finally, they were able to generate endometrial- like tissue when they were transplanted into the kidney capsule of NOD-SCID mice. These findings confirm that SP cells exhibit key properties of endometrial SSCs and open new possibilities for understanding gynaecological disorders such as endometriosis or Asherman's syndrome (Cervelló et al., 2011).

3. Putative Markers and the Origin of Human Endometrial Somatic Stem Cells

3.1 Searching for Endometrial Stem Cell Markers

Searching for specific endometrial SSC markers is one of the major objectives in the field of reproductive and regenerative medicine, and a lot of work has attempted to elucidate novel endometrial stem cell markers.

Musashi-1 is an RNA binding protein that is associated with the maintenance and asymmetric cell division of epithelial and neural progenitor cells (Okano et al., 2005). In a broad study, mRNA expression levels of Musashi-1 were determined in both epithelial and stromal cells, and were higher in the proliferative versus secretory endometrium with even more pronounced expression in the basal region. Furthermore, a greater percentage of cells positive for Musashi-1 were found in both endometrial carcinoma and endometriotic tissue compared with normal endometrium, suggesting a possible origin based on the activity of stem cells in endometriosis and endometrial carcinoma (Gotte et al., 2008).

Other work, reported a sub-population of endometrial cells in the stromal compartment which co-expressed the markers CD146 and PDGF-Rβ, and was characterized as having MSC properties, CFU activity, and also the ability to differentiate *in vitro* into fat, muscle, cartilage, and bone tissues (Schwab et al., 2007). The same group used a panel of antibodies to identify perivascular markers in stromal cells. Among them, W5C5 was particularly effective in selecting endometrial mesenchymal stem-like cells (eMSCs). These $W5C5^+$ cells comprised about 4.2% of the endometrial stromal cells and resided predominantly in a perivascular location in both the basal and functional layers of the endometrium. $W5C5^+$ cells are related to the existing eMSC markers PDGF-Rβ and CD146: most $W5C5^+$ cells expressed PDGF-Rβ, while all the $W5C5^+CD146^+$ cells were positive for PDGF-Rβ. Despite this, $W5C5^+$ cells showed even greater clonogenicity and were able to differentiate *in vitro* into adipocytes, osteocytes, chondrocytes, myocytes and endothelial cells. After injection of these cells into the mouse kidney capsule, $W5C5^+$ cells produced endometrial stromal-like tissue *in vivo,* thus identifying W5C5 as a putative marker of endometrial mesenchymal stem-like cells (Masuda et al., 2012).

It has also been reported that Wnt family members play an important role in the female reproductive tract. During embryonic development, there is a gradual expression of different Wnt molecules such as Wnt4, Wnt9b, Wnt7a and Wnt5a in the Müllerian ducts, which are responsible for setting the proper formation of the uterus, cervix and vagina. Therefore a key role for this signalling pathway in the endometrium has been hypothesized (Tulac et al., 2003).

Endometrial gene expression studies conducted throughout the menstrual cycle phases revealed that some of the Wnt molecules were upregulated by estrogen during the proliferative phase, while in the secretory phase, progesterone acted as an activator of the Wnt pathway inhibitors (Wang et al., 2010). Thus, ovarian steroids regulate the Wnt pathway in the endometrium, maintaining a balance between estrogen-induced proliferation and progesterone-guided differentiation (van der Horst et al., 2012). Furthermore, differential Wnt expression pathway between pre- and post-menopausal endometrial epithelial cells suggests that an epithelial stem cell population resides in the basalis layer (Nguyen et al., 2012). All these studies indicate that somatic stem cells play an important role in the regeneration of the endometrium and that the Wnt signalling pathway is involved in their maintenance.

Accumulated evidence based on lineage tracing studies indicates that a surface protein named Leucine-rich repeat-containing G-protein coupled receptor 5 (*Lgr5*) is a marker that can identify SSCs in several tissues such as small intestine mucosa (endodermal origin; Barker et al., 2007), hair follicles (ectodermal origin; Jaks et al., 2008), and mature kidney nephrons (mesodermal origin; Barker et al., 2012). This protein plays a crucial role in the Wnt/β-catenin signalling system by acting on the self-renewal and maintenance of the SSC population. All these data suggest the possibility that Lgr5 could be considered a universal SSC marker (Schuijers and Clevers 2012).

Lgr5 molecule expression in human endometrium was first described in 2007 by Krusche and colleagues, a publication which coincided with Clevers' identification of this protein as a SSC marker in the small intestine and colon. Krusche et al. demonstrated constitutive Lgr5 expression at the mRNA level throughout the menstrual cycle and interestingly, this expression was higher in endometrial

epithelial cells compared to stromal cells and was not hormonally regulated (Krusche et al., 2007).

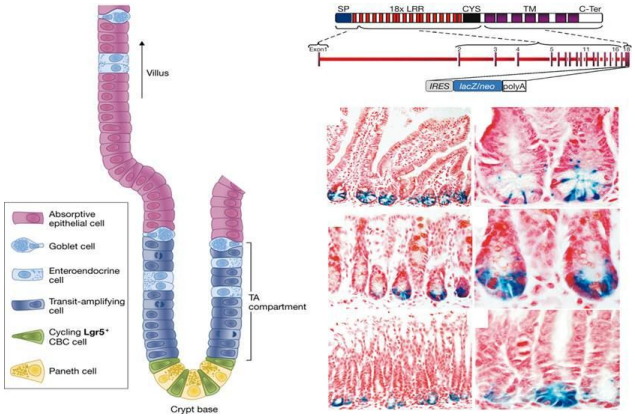

Figure 9. Proposed model for Lgr5 expression by small intestine cells. *Expression of LacZ-Lgr5 cells (shown in blue) in adult mouse tissues: small intestine and colon. In both cases expression is confined to a few cells located at the crypt base (Adapted from literature published by Clevers et al.).*

Sun and collaborators demonstrated that Lgr5 expression in the mouse uterus was dynamic and dependent on the developmental stage of the uterus. Indeed, the influence of ovarian hormones in the oscillation of Lgr5 expression revealed that in the presence of estrogen and progesterone, its expression decreased. Furthermore, Lgr5 expression was undetectable in the absence of β-catenin in lacZ reporter mice (Sun et al., 2009). Despite these findings, the function and regulation of the Lgr5 gene in the uterus remains largely unknown.

3.2 Implication of the Bone Marrow as an Exogenous Contributor to the SSC pool

Bone marrow derived stem cells (BMSCs) include many populations of progenitor cells: hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs) or stromal cells, side population (SP) cells, and multipotent adult progenitor cells (MAPCs) (Dimmeler et al., 2005).

BMSCs administered after minimal manipulation, represent an important cell source for cell-based therapies. It is known that BMSCs have the ability to differentiate *in vitro* into multiple non-hematopoietic cell types (Pittenger et al., 1999; Mezey, 2011). Furthermore, it has been shown that in human patients undergoing bone marrow transplant (BMT), cells from the donor were able to differentiate into multiple cell types, as a result producing chimeric organs. Cases of transdifferentiation into hepatocytes (Theise et al., 2000), neurons (Mezey et al., 2003), cardiomyocytes (Quaini et al., 2002), and skin (Korbling et al., 2002, Nemeth et al., 2012) have been reported, where bone marrow donor cells migrate to the recipient organ and repair damaged tissues. The percentages of chimerism were about 2% to 8% in the case of keratinocytes, depending on the clinical trial. Nonetheless, there is no doubt that circulating cells from bone marrow can reach the skin and participate in its regeneration under non-pathological conditions, for example, the toxicity of chemotherapy results in hair loss which requires stem cell mediated regeneration (Nemeth et al., 2012).

It has been postulated that this repair can involve one or more of the following mechanisms (Mezey, 2011; Figure 10):

1) BMSCs (like HSCs), would be induced to enter into circulation through the bloodstream, or after being injected locally at the site of damage or injury. These cells become tissue-specific stem cells, proliferating and regenerating the tissue.

2) BMSCs may enter into organs, secreting paracrine factors that induce proliferation and/or differentiation of tissue-resident somatic stem cells.

3) BMSCs would secrete anti-apoptotic factors from the injured tissues to allow survival of damaged cells.

4) BMSCs could regulate the function of the immune system by inducing or inhibiting specific pathways for cytokine production.

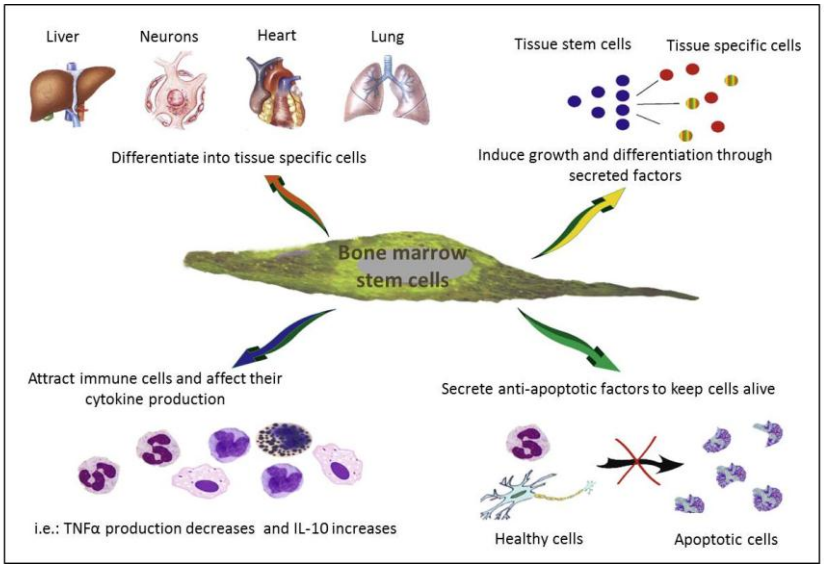

Figure 10. Possible mechanisms implemented by bone marrow stem cells to differentiate into other cell types. *Tissue repair might involve one or more of the mechanisms summarized above. Adapted from Mezey "The therapeutic potential of bone marrow-derived stromal cells".*

HSCs are capable of giving rise to all the different types of blood cells (myeloid, erythroid, lymphoid, mast cells, and platelets); whereas MSCs have the potential to differentiate only towards lineages of mesenchymal origin, including bone, cartilage, fat, connective tissue, smooth muscle, and hematopoietic supportive stroma (Pittenger et al., 1999). MSCs usually express CD73, CD90, and CD105 antigens and lack expression of the major histocompability complex (MHC) class II surface molecules, and endothelial (CD31) and hematopoietic (CD34, CD45, CD14) specific antigens (Dominici et al., 2006).

In 2002, Verfaillie´s group described the derivation of rare cells from rat and mouse bone marrow (BM) with characteristics different from most adult stem cells. These cells, termed MAPCs, could proliferate without becoming senescent and could differentiate *in vitro* at the single cell level into cells of the three germ layers (Ulloa-Montoya et al., 2007). This remarkable differentiation potential makes this particular subset of cells a candidate for tissue transplantation.

Studies in murine endometrium

The origin of endometrial SSCs remains unclear, although tissue specific somatic stem cells from endometrium have been described (literature cited above), an alternative source could be the bone marrow itself, either continuously or in specific circumstances.

To address this issue, the murine endometrium has been studied. In 2007, Taylor designed a male to female BM transplant model to demonstrate that BM derived stem cells can engraft the endometria, as Y chromosome endometrial cells were found in the uterus of transplanted mice. This fact suggests two important points: first, BM-derived cells can generate endometrium, thus proving their mesenchymal origin, and second, that they have a role in tissue repair after injury (Du and Taylor, 2007). However, their contribution to cyclic endometrial functions is reduced, which might be due to the irradiation used prior to BM transplantation which compromises fertility.

BMSCs are largely comprised of: HSC and MSC populations, although which BMSC subpopulation promotes endometrial reconstruction remains unresolved. In an attempt to address this issue, Bratincsak used transgenic mice to determine whether CD45 progeny contribute to repopulation of uterine epithelium in healthy conditions. Females were examined throughout adult life and stromal and epithelial cells from CD45 progenitor cells were observed; in the case of epithelium the percentage increased in relation to number of oestrous cycles (Bratincsak et al., 2007). Conclusions from these studies led to the hypothesis that circulating HSCs might enter into the stroma, and when they are needed enter into the epithelium to regenerate it.

The involvement of the HSC pool *per se* has also been described by Du in 2012, where HSCs were mobilized using the granulocyte colony stimulating factor (G-CSF), as a potent cytokine that regulates granulopoiesis. After induced ischemia followed by BMT, mice received G-CSF; at 8 months follow-up it was understood that this cytokine did not increase BMSC migration to the uterine endometrium, and in fact this treatment resulted in significantly lower stem cell recruitment to the uterus (Du et al., 2012). This report also addresses the important question of whether BMSC physiologically contribute to the uterus or just participate in repair

tasks after injury. In fact, the induced ischemia resulted in recruitment of approximately 2-fold more stem cells to the endometrial stroma, but not to the epithelium, indicating that the stroma is the main target of injury. Additionally, injury may be more important in mobilization of BMSCs to endometrium than hormonal signals because even when ovarian function is restored after injury migration of more BMSCs to endometrium is not induced (Du et al., 2012).

Studies in human endometrium

In the first study published describing the human endometrium following BMT, the Taylor laboratory demonstrated the existence of circulating stem cells of extra-uterine origin that could differentiate into endometrial tissue. BM-transplanted women were recruited in a clinical trial where donors and recipients were female with different human leukocyte antigen (HLA) phenotypes. The mismatched HLA type for each donor-recipient pair allowed the origin of the cells to be determined. A greater number of donor-derived stromal cells compared to glandular epithelial cells were found, suggesting a differential rate of transdifferentiation (in all cases derived donor cells were CD45 negative). Furthermore, the percentage of donor cells depended on the length of time from treatment to biopsy, as well as multiple factors including chemotherapy regimen, radiation dosage, graft versus host disease and other endometrial conditions unrelated to the transplantation (Taylor, 2004).

In 2007, flow cytometry analysis revealed a phenotypic population of HSCs in the endometrium of healthy women. This population was $CD45^{+}$ and $CD34^{+}$, but different from the HSC population in BM due to low c-kit expression and expression of committed lymphoid progenitor markers (Lynch et al., 2007).

A few years later, Mints and Cols extended this knowledge, focusing their study on endothelial progenitors, indicating that these progenitor cells from bone marrow could also contribute to the formation of new endometrial blood vessels, expressing both CD34 and VEGFR2 markers, in a process known as angiogenesis (Mints et al., 2008).

Another original approach, using a sex-mismatched donor-receptor system (Ikoma et al; 2009), showed the formation of endometrium from male donor cells,

resulting in both epithelial and stromal cells, as assessed by the expression of the typical markers cytokeratin and CD10, and negative expression for CD45. However, the percentage of cells coming from the donor were less than those reported by Taylor (2004), suggesting a lower efficiency or ability of male cells to form female reproductive organs. Surprisingly, the percentage of donor-derived cells was greater in stroma than epithelium, and in contrast to previous studies, there was no significant correlation between the time from transplantation to biopsy and the percentage of donor-derived cells present. A specific hematopoietic stem cell contribution to the endometrium has also been described, although patients receiving hematopoietic progenitors did not show donor chimerism between stromal and bone marrow cells, indicating that these cells do not contribute to the endometrial stem cell pool (Wolff et al*.,* 2012).

Together these reports recognise the contribution of bone marrow to endometrial regeneration, but do not define the specific subpopulation of cells making the contribution, since unfractioned bone marrow cells were used.

The existence of a circulating source for endometrial SSCs might represent an alternative theory to explain the etiology of endometriosis, especially in the case of endometriotic foci which present outside the peritoneal cavity, such as in lung or brain (Du and Taylor, 2007).

II. Hypothesis

II. HYPOTHESIS

The hypothesis of this project is that human endometrial stem cells are produced from endogenous and exogenous sources and the main questions to be answered are:

1. Can bone marrow derived cells contribute to the endometrial somatic stem cell reservoir or do they transdifferentiate into endometrial cells?

2. Is Lgr5 a universal marker for somatic stem cells and can it identify the endogenous endometrial stem cell niche?

III. Objectives

III. OBJECTIVES

The primary objectives of this thesis are:

1. To demonstrate the contribution of BM as an exogenous source of cells for the endometrial stem cell niche.

2. To investigate the Lgr5 marker to identify the endometrial endogenous stem cell niche.

The secondary objectives of this thesis are:

1.1 To identify the Y-chromosome bearing cells in the human endometrium and phenotype them.

1.2 To analyse the telomerase length of endometrial cells in BM transplanted recipients.

1.3 To investigate the contribution of BM to the side population cell cohort in BM transplanted patients.

2.1 To investigate the presence of the Lgr5 marker in endometrium throughout the menstrual cycle.

2.2 To correlate the Lgr5 mRNA-expressing cells with their telomere length as criteria to assess their `stemness´.

2.3 To isolate the endometrial Lgr5 positive cells and inject them into the kidney capsule of NOD-SCID mice in order to assess endometrial-like tissue reconstruction.

IV. Experimental Design

IV. EXPERIMENTAL DESIGN

We established the following study design:

1) *Exogenous contribution to the endometrial stem cell reservoir*

To analyse the contribution of BM to endometrial regeneration we created a BM transplant model in which the donor-recipient pair were sex mismatched, making it possible to distinguish contribution of bone marrow cells by the presence of Y chromosome-bearing cells using fluorescent *in situ* hybridization. The phenotype of these cells will be assessed with specific markers such as CD45, CD9, and Vimentin. The analysis of telomere length will be performed using telomapping and evaluation of the side population cells as an endogenous pool of stem cells in the recipient endometrium will allow us to determine the contribution of BM, and may enable correlation with the different sources of stem cells.

2) *Endogenous contribution to the endometrial stem cell niche*

To assess the implication of Lgr5 as a marker for the endogenous endometrial stem cell niche, endometrial sections will be used to identify and locate Lgr5 using different techniques such as *in situ* hybridization, immunohistochemistry, or western blotting.
The analysis of telomere length associated with Lgr5 expression by *in situ* hybridization will serve as an approximation of the stemness features of these cells. Finally, the isolation of this population by flow cytometry will allow us to perform *in vivo* proof of concept experiments to assess whether these cells will form human endometrium as a xenograft in an animal model.

V. Materials & Methods

V. MATERIALS AND METHODS

5.1. Exogenous Contribution of the BM to the Human Endometrial Stem Cell Niche

The use of human biological samples in this project was approved by the institutional review board of the *Hospital Universitario La Fe* registered under law number 2009/0364. Samples were obtained after the project was explained and with written informed consent from all cases (see Annex I).

5.1.1 Biological Samples

Five female patients were enrolled in this study; they received allogeneic BM stem cell transplantation from male donors for the treatment of haematological malignancies at the Haematology Department, *Hospital Universitario La Fe*, Valencia, Spain, from May to October 2010. The epidemiological and oncological characteristics of the transplanted patients are presented in Table I.

Patient	Age	Duration of HRT (months)	Pregnancy after BMT	Time from transplantation to biopsy (months)	Indications for transplant	Hematopoietic Progenitors (age)
1	38	4	Yes	132	CML	CBSC (0)
2	29	15	No	30	AMbL	CBSC (0)
3	41	72	Yes	210	CML	BM (46)
4	34	36	No	116	ALL	PBSC (21)
5*	28	18	No	45	AMbL	CBSC (0)

Table I. *Epidemiological and oncological characteristics of the BM-transplanted recipients. *Endometrium from patient 5 was evaluated as atrophic.*

- The recipients' ages ranged between 28 and 41 years.

- Patients presented a variety of haematological malignancies: chronic myeloid leukaemia (CML) in two patients (patients 1 and 3); acute myeloblastic leukaemia (AMbL) in two patients (patients 2 and 5); and acute lymphoblastic leukaemia (ALL) in one patient (patient 4).

- All the patients received a busulfan based myeloablative conditioning regimen without total body irradiation (TBI). Patients 1, 2 and 5 received transplantation

from unrelated donor umbilical cord blood (UD-UCB). Patient 3 received an HLA-(human leukocyte antigen)-matched unrelated bone marrow transplant (UD-BMT). Whereas patient 4 was transplanted using mobilized peripheral blood stem cells (PBSC) from an HLA-matched sibling donor.

- Patients with secondary amenorrhea after transplantation received hormone replacement therapy (HRT) using a gonadotropin-releasing hormone agonist (GnRH-a; Decapeptyl 3.75, IPSEN, France) injected on day 21 of the previous cycle. Hormones received were estradiol valerate (EV; Progynova; Schering Spain, Madrid, Spain), at 6 mg daily, initiated on day 2 of the cycle for at least 10 days, while progesterone (Utrogestan, Corne, Mexico), 800 mg per day, was added as previously described (Reis et al., 2012; Soares et al., 2005). Endometrium from patient 5 was evaluated as atrophic.

Collection and processing of biological samples

Endometrial tissue specimens were obtained using a Pipelle catheter (Gynetics Medical Products N.V, #4164 probet, Belgium) under sterile conditions from the uterine *fundus* at a minimum of 4 months after starting HRT. The tissue was collected immediately after the extraction and was kept in phosphate buffered saline (PBS) until its processing.

Endometrial samples were then washed with PBS to remove mucus and blood traces. Biopsies from all five bone marrow transplanted (BMT) recipients were processed as formalin-fixed paraffin-embedded tissues and were also digested enzymatically in order to obtain viable single cells. The main part of the sample (two-thirds) was subjected to an epithelial and stromal cell separation protocol, and the remainder was fixed in 4% paraformaldehyde (PFA) and paraffin-embedded.

5.1.2 Isolation of Side Population Cells

5.1.2.1 Epithelial and Stromal Cell Separation from Human Endometrial Biopsies

All cellular procedures were carried out in sterile conditions to avoid contamination by external agents. Thus, sample collection and processing was done with sterilized material in a laminar flow hood in an aseptic area of the culture laboratory.

Epithelial and stromal cell fractions were isolated using an established protocol (Simón et al., 1993) with minor modifications. Samples were carefully dissected and minced into 1-2 mm^3 fragments. After mechanical disaggregation, enzymatic digestion was carried out with collagenase type IA at 10 mg/mL (Sigma-Aldrich, Spain) diluted in DMEM (Dulbecco´s Modified Eagle Medium; Sigma-Aldrich, Spain) at a final concentration of 10 mg/mL. Following this, sample was left at 4° C overnight (ON). Collagenase is an enzyme that hydrolyses collagen in its triple-helical conformation, releasing small tissue fragments and isolated cells.

After this, both fractions: stromal and epithelial cells were separated on a size-basis protocol using gravity sedimentation and membrane filtration. Disaggregated cells were stored vertically for 10 min after vigorous agitation, as a result a pellet with epithelial cells was formed, making it possible to take the supernatant (stromal cells) and pass it into a new tube; this procedure was repeated twice. The large-size cells tend to sediment faster to the lower part of the tube and correspond with gland and epithelial cell fragments, while the smaller stromal cells remain in the upper part.

Cellular suspensions corresponding to stroma were filtered through 30 µm filters (Partec, Celltrics) and centrifuged at 580 g for 7 min. The supernatant was discarded and DNase (Sigma-Aldrich, Madrid, Spain) was added to the pellet, in order to eliminate the free DNA resulting from cellular lysis that favours the union of cells like a network. Due to high content of blood in the stroma, cell suspension was treated for 5 min at room temperature (RT) with erythrocyte lysis solution, eliminating blood cells by hypotonic shock (1.5 M NH_4Cl, 100 mM $NaHCO_3$, and Ethylenediaminetetraacetic [EDTA] pH7.4) , which favours the isolation of a single-cell suspension, and prevents the formation of clumps of glandular tissue. Next, the epithelial cell fraction was treated with DNase (Sigma-Aldrich, Madrid, Spain) in the same manner as the stromal cell fraction. Finally, both cellular fractions were centrifuged at 580 g for 7 min and the pellets were resuspended in DMEM, ready for cell counting.

5.1.2.2 Flow Cytometry

Flow cytometry (FC) is a powerful and useful technique for the analysis of multiple parameters of individual cells in suspension within heterogeneous populations. The

flow cytometer performs this analysis by passing thousands of lined up particles (usually cells) per second in front of a focused laser beam capturing the light that is scattered by each cell as it passes, and producing an individual cell signal. This technology enables rapid measurement of certain physical and chemical characteristics from a suspension of cells. These signals are collected by the various detectors and are subsequently converted into digital signals to allow simultaneous measurement of several parameters (size, shape, complexity, phenotype etc.) from the same cell. It is essential to use a suspension of single cells or particles, which may be live or fixed.

Fluorescence activated cell sorting (FACS) of live cells separates a population of cells into sub-populations based on fluorescent labelling. Sorting involves more complex mechanisms in the flow cytometer than a non-sorting analysis. Cells stained using fluorophore-conjugated antibodies can be separated from one another depending on which fluorophore they have been stained with.

This technique was used to determine cell viability (see section 5.1.2.3), to isolate side population cells (see section 5.1.2.5), and also for the isolation of Lgr5 positive cells (see section 5.2.6.4).

Phases for flow cytometry assay

- Pre-cytometry step: involves the protocol design, preparation of reagents, cell suspensions and their staining with the fluorescent reagents. There are different fluorescence markers depending on its type binding to the cell:
  _ Fluorescent markers with covalent binding: fluorochromes which react with lipids and proteins. The most common are the phycoerythrin (PE), fluorescein (FITC), and rhodamine (ROD).
  _ Non-covalent binding markers: fluorochromes which, due to their molecular composition, bind to certain cellular components. Among them are RNA and DNA markers (Hoechst 33342, DAPI [diamidino-2-phenylindole Dihydrochloride], chromomycin A3, olivomycin, mithramycin, and propidium iodide [PI]), membrane potential markers (cyanines and rhodamine 123 [ROD]) and markers for membranes and lipids.
- Flow cytometry step: involves processing the labelled cells and collecting data for each of the measurements (parameters). The cell suspension is

appropriately processed and stained, injected into the flow cytometer chamber, which is designed to pass the cells individually, interacting physically with a beam of monochromatic light. The impact of light on the cells produces light dispersion in all directions, and at the same time fluorescent light is emitted as a result of the excitation laser which the sample is subjected to. Two essential parameters describing cellular size and components are obtained from flow cytometry analysis: the forward scattered light (called *forward scatter*, FS to 0 degrees) is related to cell size, whereas the light scattered at 90 degrees to the light beam (*side scatter*, SS 90 degrees) is related to the internal structure and cytoplasmic complexity of the cell.

- Analysis step: Involves the analysis of the data collected. Photomultiplier tubes detect both stray light and fluorescent emissions, and the information is then processed and digitized by a computer, generating histogram 'dot-plots' which are correlated with the desired parameters.

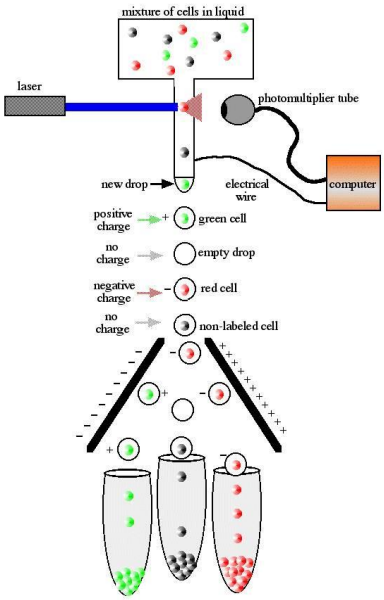

Figure 11. Schematic diagram showing a fluorescence-activated cell sorter (FACS). *Cells are stained with the corresponding fluorochromes and as result are collected as independent samples (Image adapted from the Cell Biology website).*

5.1.2.3 Cellular Viability

An important aspect related with our research and work with biological samples is cellular viability: after subjecting cells to disaggregation and enzymatic digestion protocols it is essential to avoid a high rate of cell death.

To determine the viability of cell suspensions for the epithelium and stroma, we used a method based on using propidium iodide (PI, Sigma-Aldrich, Madrid, Spain). PI is a fluorochrome which intercalates into double-stranded nucleic acids. The membrane of living cells does not allow the passage of PI into the cytoplasm, meaning that only cells with damaged or compromised membranes (dead, damaged, or apoptotic cells) stain with it. Isolated nuclei can also be stained with PI.

Cell viability was assessed by adding PI to samples at a concentration of 5 µg/mL, leaving them in the dark at RT for 15-20 min, and then analysing them with cytometric techniques. PI emits a red-orange fluorescence when it is excited with a 488 nm laser. As a result dead cells can be excluded due to the presence of PI.

5.1.2.4 Hoechst Staining Protocol

Viable epithelial and stromal cells from endometrial biopsies, were counted in a Neubauer chamber and resuspended in medium (DMEM, 2% FBS [Fetal Bovine Serum], and 10 mM HEPES; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) at final concentration of 10^6 cells per mL. After that, cell staining with the vital dye Hoechst 33342 (bisbenzimide H 33342; Sigma-Aldrich, Madrid, Spain) was done. Hoechst was added to cell suspensions at a final concentration of 10 µg/mL with an incubation period of 90-120 min in darkness in a water bath at 37° C. In addition, an aliquot of cells were simultaneously incubated with both Hoechst 33342 and an ABC transporter blocker called Verapamil (Vp; Sigma-Aldrich, Madrid, Spain), at a concentration of 100 µM. Vp is used as a negative control as it blocks the membrane transporters responsible for the exclusion of Hoechst.

After the incubation period, the cell suspensions were centrifuged at 580 g for 7 min at 4° C. The resulting pellet was resuspended in HBSS buffer (Hank's Balanced Salt Solution [HBSS], 2% fetal bovine serum [FBS], and 10 mM HEPES; Gibco

Invitrogen) reaching a concentration of 10^7 cells per mL, and the suspension was kept cold until its analysis on the flow cytometer. Finally, PI was added at a concentration of 5 µg/mL so dead cells could be excluded from the analysis. The samples were filtered through a 30 µm and 50 µm membrane sieve for cell suspensions of stroma and epithelium respectively, to avoid cellular aggregates that could hamper the analysis.

5.1.2.5 Cell Sorting of Side Population Cells

Hoechst 33342 is a vital dye that has the ability to penetrate into cells through the plasma membrane, intercalating with the DNA, particularly in adenine-thymine (AT) regions; due to its low cytotoxicity, it is frequently employed as an intercalating agent used to visualise the phases of the cell cycle. In 1996 Goodell described the enrichment of ABC-type membrane transporters, which are able to efflux Hoechst, in undifferentiated hematopoietic cells. When the cells are analysed by flow cytometry this efflux results in a specific fluorescence pattern in which cells enriched in these transporters are delimited in a low-fluorescence lateral region, adjacent to the population of cells in G_0/G_1 cell cycle phase, termed side population or SP cells (Goodell et al., 1996).

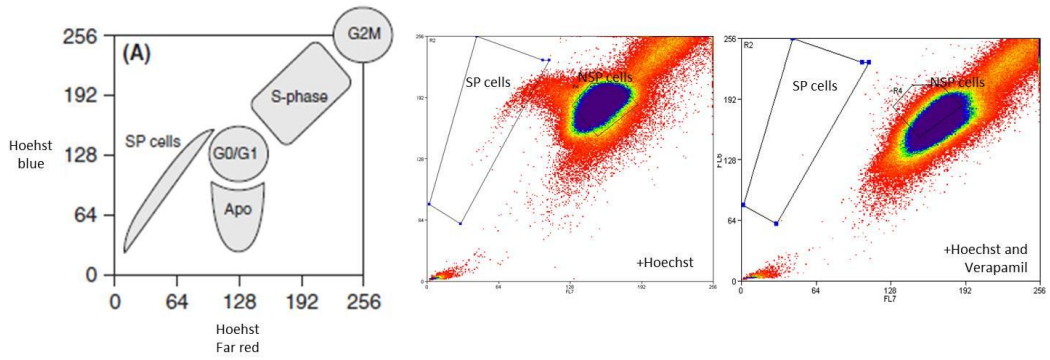

Figure 12. Side population cells visualised by FACS. *Left panel shows the correlation of Hoechst fluorescence with the cell cycle phase. Right panels are images obtained from FACS, where the side population cells are obtained by Hoechst efflux (low fluorescence, middle panel) and disappear in presence of the Verapamil blocker (right panel). Adapted from "Adult stem cells in human endometrium" in Stem cells in Human Reproduction book.*

To detect the SP, endometrial cells incubated with Hoechst were analysed and isolated by FACS (MoFlo® High-Performance Cell Sorter, Dako, Denmark). The excitation was performed with *a Coherent Enterprise* II laser (California, USA) with two emission peaks (488 nm and 351 nm) and two excitation peaks, working at 30 mW. The red and blue fluorescence from Hoechst 33342 were detected through a band pass filter of 405/30 nm and 670/20 nm respectively, measuring signals in a linear scale. PI fluorescence was detected on a filter with a band pass of 631/20 nm on a logarithmic scale. As a result two histograms were produced, one with two-parameters, displaying blue and red Hoechst fluorescence, and another representing the red fluorescence of PI versus FS to exclude dead cells from the analysis.

Finally, PI negative live cells with low Hoechst fluorescence were isolated (as putative SP cells), as well as PI negative high Hoechst fluorescence live cells, which were isolated as non-SP (NSP) cells, correlating with the cell population found in S phase and the G_0/G_1 phase transition.

5.1.2.5 Fluorescence *in situ* Hybridization (FISH) for the Y chromosome

Fluorescence *in situ* hybridization (FISH) is widely used to localise genes and specific genomic regions on target chromosomes, both in metaphase and interphase cells.

Briefly, an interphase or metaphase chromosome preparation is produced, placed onto a slide, and denatured. Fluorescent probes specific to one region of a chromosome and complementary to the known sequence are then hybridized with the chromosomes and detected by fluorescence microscopy. There are four types of probes that are typically used for *in situ* hybridization: oligonucleotide probes, single stranded DNA probes, double stranded DNA probes, and RNA probes (cRNA probes or riboprobes). The probes hybridize to areas on the chromosome which have a high degree of sequence similarity.

In this study, the FISH technique was used to detect the presence of X or Y chromosomes. First, the DNA sample (from interphase cells in this case) was denatured, separating the complementary strands of the DNA double-helix structure. The fluorophore-labelled probe of interest was added to the denatured

sample, allowing its association with the DNA in the target site, in a process called hybridization, in which a double helix is again formed. As a result, the signal emitted by the probe could be observed using a fluorescence microscope.

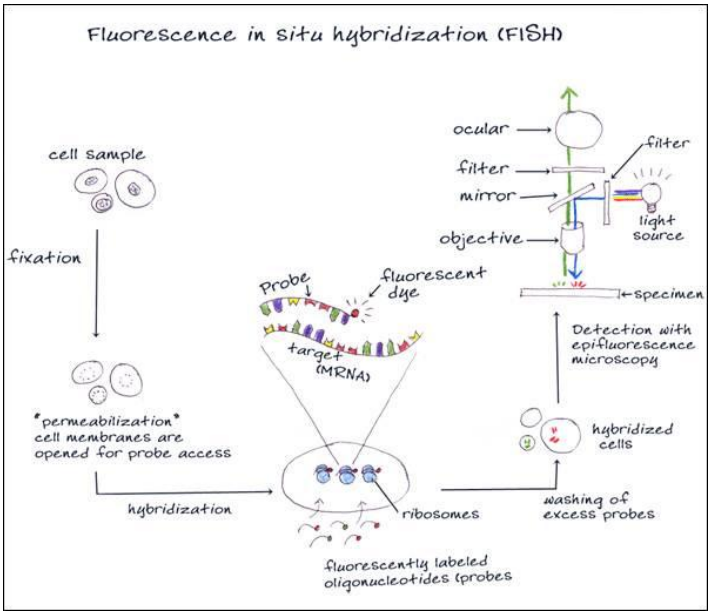

Figure 13. Fluorescence in situ Hybridization (FISH). *The technique involves fluorescent detection after binding with a small nucleic acid sequence 'probe' with a fluorescent molecule attached to its corresponding sequence on the target chromosome site (Image adapted from online Molecular Methods tools).*

Cell suspensions from SP and NSP fractions cultured for 3 weeks under hypoxic conditions (1-2% O_2), were trypsinized, treated with 0.075 M potassium chloride, and fixed with methanol and acetic acid in 3:1 proportion before proceeding with FISH analysis.

The slides with fixed cells were dehydrated in an ethanol gradient (70%, 85%, and 100%) for one minute in each bath, and air dried. The mixture of centromeric probes for the X chromosome (orange spectrum), Y chromosome (green spectrum), and chromosome 18 (aqua spectrum; Poseidon probes, Kreatech, Spain) were added to the preparations. Coverslips were carefully added and the slides were incubated in a humidified chamber at 73° C for 5 min to denature, and at 37° C ON

to hybridize. After that, the slides were washed, firstly with pH 7.5 0.4× SSC-buffer (saline sodium citrate buffer)/0.3% NP40 (nonyl phenoxypolyethoxylethanol) in a 73° C bath for 2 min, and secondly in pH 7.2 2× SSC/0.1% NP40 at RT. Finally, nuclei were stained with DAPI Gold (Invitrogen Carlsbad, CA, USA).

5.1.3 Paraffin-Embedded Endometrial Tissue Analysis

5.1.3.1 Endometrial Processing

Endometrial tissue samples were taken from five patients enrolled in the BMT study, and from a patient not treated with allogeneic transplant, with fetal testicular and human spleen tissues used as controls.

A small endometrial biopsy fragment was used for histological studies. After cleaning the sample of mucus and blood traces, it was placed in 4% PFA (v/v) for 24 h and then embedded in paraffin according to established protocols. After this tissues were dehydrated using ethanol gradient baths of increasing concentration (80%, 90%, and 100% [v/v]), and then embedded in xylol. Paraffin-embedded samples were sectioned at 3-5µm with a microtome and mounted on glass slides treated with poly-Lys (poly-lysine) and incubated overnight at 37° C to facilitate the deparaffinization and the adhesion of the tissues to the slide. The final deparaffinization was carried out by heating to 60° C for 1 h, followed by 3 passages with xylol for 10 min, 3 bath solutions of 100% ethanol for 5 min, and a subsequent rehydration with alcohols of decreasing concentration (95%, 80%, and 70%, [v/v] for 5 min each) ending in a water bath, ready to begin the fluorescence *in situ* hybridization (FISH) or immunohistochemistry (see Sections 5.1.3.2 and 5.1.3.4 respectively).

5.1.3.2 Fluorescence *in situ* Hybridization (FISH) for the Y chromosome

FISH with specific probes for chromosomes X and Y (chromosome enumeration probes [CEP], Abbott) was performed (Table II). To test the specific signal for X and/or Y chromosomes, endometrial sections from non-transplanted women were used for XX signal, whereas fetal testicular tissue was used for XY signal.

Probe	Location	Fluorophores
CEP X	Xp11.1-q11.1Alpha Satellite DNA	Orange/Red Spectrum
CEP Y	Yq12 Satellite III	Green Spectrum

Table II. *Probes used for fluorescence in situ hybridization (FISH) for chromosomes X and Y.*

Figure 14. Detailed location of CEP X and CEP Y probes in sex chromosomes. *The CEP X/Y probe is a mixture of a Spectrum Orange-labelled CEP X-DNA-probe and a Spectrum Green-labelled CEP Y-DNA-probe specific for the alpha satellite centromeric region of chromosome X and the satellite III (Yq12) region of chromosome Y (Information provided from Roche website).*

After tissue fixation, inter- and intra-molecular cross-links are produced with certain structural proteins which mask the tissue antigens. In order to 'unmask' these antigenic sites antigen retrieval was performed. Thus, a heat-induced antigen retrieval protocol with citrate buffer (10 mM Citric Acid, 0.05% Tween 20 [Polysorbate 20], at pH 6.0) was performed on deparaffinized endometrial sections (section 5.1.3.1) in a bath in the microwave using alternating settings (maximum/minimum power) to facilitate permeabilization. To increase the accessibility to the nucleic acids, an enzymatic digestion was performed in a 0.2 M hydrochloric acid (HCl) bath for 10 min, followed by washing for 5 min with distilled water and addition of proteinase K (Qiagen, Hilden, Germany) at 10 µg/mL, diluted in PBS (Invitrogen Carlsbad, CA, USA) in a bath at 37° C for 10 min. Thereafter, the sections were washed with saline sodium citrate buffer (2× SSC) for 5 min and fixed with 1% buffered formaldehyde for 10 min, then washed again with SSC-buffer and allowed to air dry.

Following the process described above, dehydration with increasing concentration ethanol baths (70%, 80%, and 100%) was performed before applying the solution of

centromeric X and Y probes to the slides, and sealing with a coverslip. After this they were placed in a humid chamber, and held in in the following temperature program: denaturation ramp at 80° C for 5 min and hybridization at 37° C for 72 h. In the post-hybridization process, coverslips were carefully removed and the sections were washed, firstly with a pH 7.5 0.4× SSC/0.3% NP40 solution in a bath at 80° C for 2 min, and a secondly in a pH 7.2 2× SSC/0.1% NP40 solution at RT for 30 sec. Finally, nuclei were stained with DAPI Gold (Invitrogen Carlsbad, CA, USA) and the sections were stored at -20° C until they were visualized with a fluorescence microscope.

5.1.3.3 X/Y Cell Quantification

The sections were observed on a fluorescence microscope (Leica DM 6000 B/M) with a 63× oil immersion objective. The female cells were represented by two signals in the orange-red spectrum, corresponding to the X chromosome, while the male cells, were visualised with two colours, orange-red and green, represented by an X and a Y chromosome respectively. An average of 1000 to 1500 isolated cells was counted to analyse the percentage of XY cells present in the endometrium of BMT patients. The total number XX and XY cells were quantified using Image-Pro Plus Software, Version 6.3 (MediaCybernetics).

5.1.3.4 Immunofluorescence and Confocal Analysis with CD45, CD9, and Vimentin Markers

After the FISH assay for the X and Y chromosomes was performed, the XY cells observed in the endometrium were phenotyped. CD45 antibody was used to distinguish hematopoietic cells, and CD9 and Vimentin antibodies were used for endometrial epithelial cells and stromal cells respectively. After chromosome hybridization, the sections were washed with PBS and were subjected to a blocking process with PBS supplemented with 3% bovine serum albumin (BSA), 5% normal goat serum (NGS) and 0.05% Tween 20 for one hour at RT to avoid any possible non-specific binding. Following this, a permeabilization step with PBS supplemented with 0.05% Tween 20 was performed, and the primary antibody was added in solution with PBS.

The antibodies and dilutions used were: mouse anti-human CD45 diluted at 1:50 (BD Pharmingen, 555480), mouse anti-human CD9 diluted at 1:20 (Abcam, 49325), and mouse anti-human Vimentin diluted at 1:10 (Vm; Abcam, 8069), all incubated ON at 4° C.

After the initial incubation, excess unbound antibody was removed by washing, and the samples were incubated with the Alexa 633 F(ab')2 fragment goat anti-mouse (Invitrogen, A-21053) fluorescent secondary antibody. This fluorophore was chosen because the orange and green fluorophores for CEP centromeric probes for chromosomes X and Y respectively had already been used. Alexa 633 has a far red emission, which does not overlap with the spectra of the CEP probes. The secondary antibody was used at a 1:500 dilution and was incubated for 45 min at RT. Finally, to remove any unbound antibody that could result in background noise, serial of washes were performed, twice with PBS for 5 min, and twice with distilled water for 5 min. Lastly, DAPI Gold (Invitrogen Carlsbad, CA, USA) was added to the preparations to stain the cell nuclei and the sections were stored at -20° C until they were visualized by confocal fluorescence microscopy.

To test the signal specificity for each antibody we used human spleen sections as a CD45 positive control, and human endometrial tissue (due to its epithelial and stromal cell content) as a CD9 and Vm positive control.

Images were taken with a 63× objective in oil immersion with a Leica TCS SP2 AOBS (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) confocal microscope. The wavelengths used for excitation of the fluorophores were as follows: 488 nm for FITC (green), 561 nm for TRITC (orange/red), 633 nm for Alexa633 (far red), and 405 nm for DAPI (blue). The two-dimensional images were acquired with a size of 1024×1024 pixels. Image processing was done with Leica Lite Confocal Software, version 2.61.

5.1.3.5 Telomere Length Analysis by Fluorescence *in situ* Hybridization

Telomeres are located at the end of chromosomes and play a key role in protecting their integrity. They are composed of tandem repeats of a DNA sequence (TTAGGG in vertebrates) and their associated proteins (also called telomere binding proteins) which have all been conserved throughout evolution. Telomerase functions to

protect the ends of the chromosomes from normal cellular DNA-repair and degradation activity, thereby ensuring the correct functionality and viability of the cells (Flores et al., 2005).

The average chromosomal length and percentage of short telomeres in paraffin-embedded endometrial sections was measured by hybridization with specific probes and was observed by fluorescence microscopy. The telomere length is measured by telomeric quantitative-FISH (Q-FISH) on interphase nuclei, both on tissue sections or cell suspensions, using a technique known as Telomapping (Flores et al., 2008). Telomeric Q-FISH is a type of quantitative fluorescence *in situ* hybridization where telomeres are hybridized with a telomeric probe labelled with fluorescence (Cy3 PNA-tel). Each telomeric probe recognizes a fixed number of telomeric repeats (base pairs). For this reason, the intensity of the fluorescent signal from the telomeric probes that hybridize to a given telomere is directly proportional to the telomere length. Finally, the fluorescence values are transformed into telomere length values for each individual telomere spot within a cell, and so the mean telomere length, as well as the percentage of short telomeres, in a cell population can be measured.

Thus, endometrial tissue sections were hybridized with a PNA-tel Cy3 probe and signals for Cy3 and DAPI (cell nuclei staining) were acquired simultaneously in separate channels using an ultra-spectral Leica TCS-SP2-A-OBS-UV microscope. This double labelling creates combined images of each nucleus with the corresponding telomeric information. Cy3 fluorescence intensity (telomere fluorescence) was measured as the average gray value units (total gray/nuclei area; arbitrary units of fluorescence). The average value of fluorescence intensity represents the average Cy3 intensity of the total nuclear area and not the average value of individual telomere intensities and therefore it is possible discard differences in nuclear size which may influence the telomere length measurements. This analysis was reported by Life Length, S.L. Madrid, Spain.

5.2 Implication of the Lgr5 as a Marker for an Endogenous Endometrial Stem Cell Niche

The use of human origin samples for the development of this study was approved by the ethics committee of the *Instituto Universitario Instituto Valenciano de la Infertilidad* (IUIVI). These samples were obtained after explanation of the project and signed informed consent by all the patient participants (see Annex II).

5.2.1 Biological samples and inclusion criteria

Endometrial biopsies from donors were collected at the *Instituto Universitario Instituto Valenciano de la Infertilidad* (IUIVI). Endometrial samples were taken on the day that oocytes were retrieved (day 15 of the menstrual cycle).

Additional endometrial samples were collected throughout the menstrual cycle to investigate Lgr5 expression by western blot (see section 5.2.4). These samples were divided into four groups according to the phase of the menstrual cycle: proliferative (P; days 1-14), early secretory (ES; days 15-18), mid secretory (MS; days 19-22) and late secretory (LS; days 23-28) according to Noyes criteria (Noyes et al., 1975).

Inclusion criteria for patients were as follows:

- Age ranged between 18-39 years.

- Body Mass Index (BMI) of between 19-25 Kg/m^2.

- Absence of endometrial pathologies.

5.2.2 Detection of the Lgr5 molecule by *in situ* Hybridization with RNA Probes

In situ hybridization (ISH) is based on the ability of the nitrogenous bases of nucleic acids to establish hydrogen bonds with each other in a specific way. This allows specific RNA messengers (mRNAs) to be located at cellular level, by use of appropriately labelled RNA sequences (probes). This technique is complementary to immunohistochemical studies, allowing molecules to be elucidated at the mRNA level.

5.2.2.1 Probe Preparation

RNA extraction

Total RNA was extracted from endometrial biopsies using the Trizol method (Invitrogen Carlsbad, CA, USA). Samples were placed in Trizol and left for 10 min at RT, and then 0.2 volumes of chloroform per Trizol-volume used was added.

The sample was agitated with a vortex and allowed to stand for 5 min at RT, and was then centrifuged at 12,000 g for 15 min at 4° C before transferring the aqueous phase to another tube. 0.5 volumes of isopropanol were added to this aqueous phase per original volume of Trizol used, and it was then incubated for 10 min at RT to precipitate the RNA. This was followed by centrifugation at 12,000 g for 10 min at 4° C. The supernatant was removed and the precipitate was washed twice with 70% ethanol (v/v) in water treated with diethylpyrocarbonate (DEPC), and was centrifuged a final time for 5 min at 7,600 g at 4° C. The supernatant was discarded and the precipitate was dried at RT for 10 min. Finally, it was resuspended in a volume of 20 µL of DEPC water.

After the RNA extraction, its purity was quantified as the absorbance reading at 260 nm using a NanoDrop (ND-1000 Thermo Fisher, USA) spectrophotometer. The criteria established to accept the sample as valid were:

- Absorbance ratio A_{260}/A_{280} with a value close to 2.

- Absorbance ratio A_{260}/A_{230} with a value between 1.8 and 2.2.

Variations in these values result from the presence of protein traces, phenol, or other contaminants in the RNA that could interfere with the purity of the sample.

cDNA Synthesis

Reverse transcription of RNA into complementary DNA (cDNA) was done using the reverse transcription system. For this purpose, 1 µg of RNA was used with the Advantage Reverse Transcription for PCR kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. 1 µL of oligo dT and DEPC water was added to each sample to a final volume of 13.5 µL, and was heated at 70° C for 2 min to denature the RNA strands and prevent the formation of secondary

structures. Thereafter, 6.5 µL of the reaction mixture containing: 4 µL 5× reaction buffer, 1 µL dNTPs (10 mM each), 0.5 µL recombinant RNase inhibitor, and 1 µL of the MMLV reverse transcriptase (Moloney-Murine Leukaemia Virus) enzyme was added to each tube. Samples containing a final volume of 20 µL were incubated in a thermocycler (T3000 Thermocycler, Biometra) in a program for 1 h at 42° C followed by 5 min at 94° C to stop the reaction and destroy the DNase activity. The final product was quantified on a NanoDrop spectrophotometer (ND-1000 Thermo Fisher, USA) to check the purity, as described in the previous section, was diluted to 200 ng/µL, and stored at 4° C until use.

Polymerase chain reaction (PCR)

PCR was used to obtain a large number of copies of a particular DNA fragment, because DNA polymerases can replicate DNA strands when cycled through alternate high and low temperatures, separating newly formed strands of DNA after each phase of replication, and allowing new DNA strands to be synthesised using the previously formed ones as a template.

The probe to detect Lgr5 mRNA by FISH was generated by PCR using the primers shown in Table III. These sequences were designed using specialised software (Gene Fisher) and the amplified 470 base pair (bp) fragment insert, which was the basis for the synthesis of the riboprobe (primer sequences are shown in purple in the 5´-3´ direction), was as follows:

5´GGAGAGTCTGACTTTAACTGGAGCACAGATCTCATCTCTTCCTCAAACCGTCTGCAATCAGTTACC
TAATCTCCAAGTGCTAGATCTGTCTTACAACCTATTAGAAGATTTACCCAGTTTTTCAGTCTGCCAAA
AGCTTCAGAAAATTGACCTAAGACATAATGAAATCTACGAAATTAAAGTTGACACTTTCCAGCAGTT
GCTTAGCCTCCGATCGCTGAATTTGGCTTGGAACAAAATTGCTATTATTCACCCCAATGCATTTTCCA
CTTTGCCATCCCTAATAAAGCTGGACCTATCGTCCAACCTCCTGTCGTCTTTTCCTATAACTGGGTTAC
ATGGTTTAACTCACTTAAAATTAACAGGAAATCATGCCTTACAGAGCTTGATATCATCTGAAAACTTT
CCAGAACTCAAGGTTATAGAAATGCCTTATGCTTACCAGTGCTGTGCATTTGGAGTGTGTGAGA3´

Transcript		Primer sequence 5´-3´	Size (bp)
Lgr5	Forward (Fw)	GGAGAGTCTGACTTTAACTGGA	470bp
	Reverse (Rv)	TGCATTTGGAGTGTGTGAGA	

Table III. *Primers used for the Lgr5 riboprobe synthesis by PCR in direction 5´-3´.*

Primers are sequences of oligonucleotides used to hybridize with the desired DNA region to be amplified, leading to the initiation of an elongation reaction by DNA polymerase. Primers are normally short sequences, ranging between 18 and 22 nucleotides. The PCR process classically involves a series of 20 to 35 cycles; in each cycle there is a change of temperatures which generally consists of the following phases:

Denaturation

First, the DNA is denatured (the two strands which form it are separated). This step can be done in different ways, heating (94-95° C) the sample the most common method. The temperature chosen for denaturation depends, among other things, on the ratio of GC (guanine-cytosine) that the strand has, as well as its length. Other methods, rarely used in the PCR technique, include the addition of salts or chemicals.

Primer hybridization

Next the primers hybridize i.e., the primer binds to its complementary sequence in the DNA template. This requires a temperature drop (generally to 55° C, although it may vary, depending on the case, to between 45° C and 65° C). These primers serve as the boundaries of the selected region of the DNA molecule to be amplified.

Extension of the strain

Finally, the DNA polymerase uses the DNA template to synthesize the complementary strand, starting from the primer as the initial support necessary to synthesise this new DNA. The temperature is increased to 72° C, the temperature at which Taq polymerase is maximally active, geometrically increasing the amount of amplified DNA fragments in the reaction with each repetition of the main cycle steps.

The semi-quantitative PCR technique is used to determine the presence or absence of specific sequences. Here, PCR was done with 500 ng of cDNA, to which the following reaction mixture (final concentration) was added: 1× reaction buffer (Bioline, London, England), 3 mM $MgCl_2$ (Bioline), 240 µM dNTPs (Bioline), forward primers (Fw, direction 5´-3´ to 10µM) and reverse primers (Rv, direction 3´-5´ to

10µM), 1U Netzyme DNA polymerase (Bioline). The PCR reaction was performed in a T3000 thermocycler (Biometra, Gottingen, Germany). As negative control, the cDNA was replaced by bi-distilled water to check for the absence of cellular material.

Each PCR reaction cycle was standardized: initial DNA denaturation was at 95° C for 5 min, followed by 38 cycles of a) denaturation at 95° C for 1 minute, b) hybridization at 54° C for 1 minute, and c) extension at 72° C for 30 sec, followed by a final 8 min extension step at 72° C. Samples were subsequently stored at 4° C until use**.**

The amplified cDNA fragments were separated by electrophoresis for 1 h at 90 V in an agarose gel at 1.5% (w/v) and stained with GelRed (Biotium Hayward, CA, USA) and finally observed under ultraviolet light. The visualized bands were cut from the gel and purified with the Pharmacia elution band kit (Amersham, Barcelona, Spain). To verify the correlation of the amplicon with the studied gene, sequencing was performed by the Genomic Unit at Valencia University, confirming amplification of the correct amplicon.

Riboprobe synthesis

The PCR product for the Lgr5 gene was purified with the StrataPrep PCR purification kit to remove the primers, unincorporated nucleotides, PCR buffer components, and enzyme from the PCR product. The purified product (470 bp) was polished with Pfu DNA polymerase to generate blunt-ended DNA. The action of DNA ligase allowed this blunt ended DNA to be cloned into the MCS (multiple cloning site) of the pPCR-Script Amp SK (+) vector (See Figure 15), according to manufacturer's instructions (Agilent Santa Clara, CA, USA).

The transformation was performed by adding plasmidic DNA to XL10-Gold competent cells and incubating for 1 h on ice, and then performing a heat shock for 45 sec at 42° C. Cells were plated on LB-Ampicillin solid medium, and bacteria that had incorporated the plasmid were selected based on their antibiotic resistance.

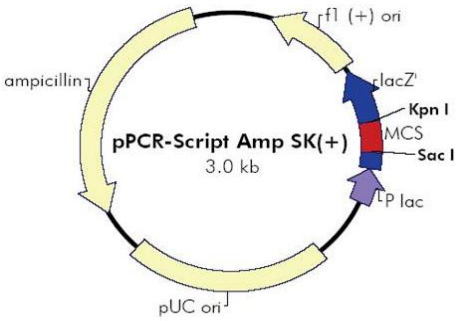

Figure 15. pPCR-Script Amp SK (+) Vector Map. *The pPCR-Script Amp SK (+) cloning vector is derived from the pBluescript II SK (+) phagemid. This cloning vector includes an ampicillin-resistance gene, a promoter for expression of the lac gene, T3 and T7 RNA polymerase promoters for in vitro production of RNA, an f1 intergenic region for single stranded DNA (ssDNA) rescue, the SK multiple cloning site (MCS), which is modified to include the Srf I restriction-endonuclease target sequence, and five conveniently located sequencing primer sites (Technical datasheet from Agilent manufacturer).*

Colonies with the incorporated insert were visualized as white colonies due to disruption of β-galactosidase activity by incorporation of the Lgr5 insert into the plasmid vector. White colonies were selected and grown in LB medium at 37° C with shaking (150 rpm ON). Bacteria were collected by centrifugation, and plasmid DNA was extracted using the EndoFree® Plasmid Purification Kit (Qiagen Hilden, Germany). The plasmidic DNA (1 µg/µL) obtained was linearized with restriction enzymes: EcoR1 for the sense probe and Sac1 for the antisense probe (New England Biolabs, Ipswich, Massachusetts, USA in both cases), in a reaction at 37° C ON. Correct DNA sequence cloning and determination of correct orientation was confirmed by sequencing using the T3 and T7 primers, and finally assessed with the BLAST tool from NCBI.

The RNA probes were synthesized *in vitro* by transcription of the coding sequence inserted into the plasmid with a DNA-dependent bacteriophage-derived RNA polymerase (normally SP6, T7, or T3). The plasmid has two different promoter sequences which can initiate synthesis, and these are recognized by different RNA polymerases which flank the cloned cDNA. Thus, altering the RNA polymerase can result in RNA synthesis complementary to the mRNA expressed *in vivo*, or in RNA identical to the endogenous mRNA. Complimentary RNA is used as a probe; due to

its hybridization with the mRNA expressed by the studied tissue, and is called an 'antisense' (opposite direction) probe. The other probe, called a 'sense' (same direction) probe serves as a negative control because any signal which is detectable when using this probe is the result of non-specific binding and not hybridization.

Sense and antisense probes were generated by *in vitro* transcription from 1 µg of linearized plasmid using the T3 RNA polymerase to create the sense and the T7 RNA polymerase for the antisense riboprobe. Labelling of the probes was performed using nucleotides labelled with dioxygenin (DIG; Roche, Penzberg, Germany) in a final volume of 20 µL in a thermo-block at 37° C for 3 h. The labelled probes were precipitated using 4 M lithium chloride (LiCl), 250 mM pH 8 EDTA, and 70% ethanol at -20° C ON. Quantification was performed using a dot-blot assay with a pattern of DIG-labelled RNA of known concentration.

5.2.2.2 De-waxing and Slide Pre-treatment

Endometrial tissue embedded in paraffin blocks was cut on a microtome to a thickness of 5 µm and stretched in a water bath at 37° C. All experiments were carried out using ribonuclease-free slides and gloves, and DEPC-treated water in order to prevent ribonuclease activity.

The paraffin-embedded endometrial sections were incubated for 2 h at 60° C and were subsequently de-waxed with two xylene baths and rehydrated in a series of ethanol solutions of decreasing concentration. Subsequently, they were digested with 0.2 M HCl in DEPC water with proteinase K (10 mg/ml) at 37° C for 30 min, and washed with 0.1 M triethanolamine and acetic anhydride (0.25% v/v).

5.2.2.3 Hybridization

Tissues were pre-hybridized for 3 h at 42° C in a pre-hybridization buffer containing 60% deionized formamide, 25 mM pH 7.4 Tris-HCl, 1 mM EDTA at pH 8, 0.4 M NaCl, dextran sulphate (12% w/v), and Denhardt's solution (1×) with 100 µg/mL of tRNA and 200 µg/mL of salmon sperm DNA.

- Formamide and DTT (dithiothreitol) are organic solvents which reduce the thermal stability of the bonds allowing hybridization to be carried out at a lower temperature.

- Dextran sulphate is added because it becomes strongly hydrated and thus reduces the amount of hydrating water for dissolving the nucleotides and therefore effectively increases the probe concentration in solution resulting in higher hybridization rates.

- NaCl is a monovalent cation that interacts with the phosphate groups of the nucleic acids, decreasing the electrostatic interactions between the two strands.

- EDTA removes free divalent cations from the hybridization solution, because they strongly stabilize duplex DNA.

- Salmon sperm DNA, tRNA, and Denhardt's solution are added to avoid non-specific binding of the DIG-labelled probe to the tissue samples.

Hybridization was performed ON at 42° C with hybridization buffer containing: 0.1% (v/v) 100 mM DTT (1,4-Dithiothreitol) stock solution, 1% (v/v) sodium thiosulfate 10% stock solution and 1% (v/v) dodecylsulfate buffer [SDS] 10% stock solution, and the sense and antisense probes at a 200 ng/mL concentration. All components used are from Roche, Penzberg, Germany.

5.2.2.4 Post-Hybridization Treatment

The following day, tissues were washed with 2× SSC-buffer at RT and afterwards at 42° C (same temperature as used for hybridization step) with 2× SSC, 1× SSC, and 0.1× SSC-buffers. After these washes RNase (20 µg/mL) digestion was performed at 37° C for 1 h with shaking to eliminate any unbound RNA probe. The sections were then incubated in a 1× blocking solution (Roche Penzberg, Germany) made with Buffer 1 (containing 100 mM maleic acid and 150 mM NaCl at pH7.5) to decrease the background noise in non-radioactive hybridization and to detect hybridized nucleic acids.

5.2.2.5 Immunological Detection

The sections were incubated for two hours at RT with an anti-DIG alkaline phosphatase conjugate (dilution 1/500) in a blocking solution with Buffer 1. Immunological detection was done by adding a solution formed by Buffer 3 (100 mM NaCl, 50 mM $MgCl_2$, and 100 mM Tris-HCl at pH 9) containing 1% NBT/BCIP

(nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate; v/v) and 0.1% (v/v) of 1 mM levamisole. The developing process took place at RT for 8 h. Counterstaining was performed with 0.1% methyl green. The sections were mounted using Kaiser's glycerol gelatine (Sigma, USA) and images were taken at 63× magnification with a Nikon Eclipse 80i microscope.

5.2.2.6 Controls

Human heart muscle was used as a negative Lgr5 ISH control. Lgr5 expression present in the crypts of Lieberkühn in the small intestine was used as positive ISH control. Sense probes were generated with genetic material identical to the original mRNA, meaning it was unable to hybridize, serving as a negative control because any signal from this probe was the result of non-specific binding and not hybridization.

5.2.3 Lgr5 Detection at the Protein Level by Immunohistochemistry

Immunohistochemistry is based on antigen-antibody binding reactions, which allows the position of the target molecule to be precisely located in a specific tissue at the protein level.

Endometrial biopsies from different days of the menstrual cycle were collected in PBS and fixed in 4% PFA for 24 h to paraffin-embedded, as described in detail in section 5.1.3.1.

Once the endometrial sections were de-waxed and rehydrated antigen retrieval was performed by placing the sections in a bath citrate buffer-0.05% Tween 20 pH6 for 20 min at 95° C to break down the formalin networks and to facilitate antibody access. Afterwards the membranes were permeabilized with PBS containing 0.05% Tween 20. To avoid unspecific interactions, the slides were passed through two blocking solutions: firstly, endogenous peroxidase activity was blocked in the tissue itself by using H_2O_2 for 15 min at RT in darkness. Then slides were then washed and unspecific antibody binding was blocked with PBS containing 5% BSA, 5% NGS, and 0.1% Tween 20 for 1 h at RT.

The primary antibodies used were rabbit anti-human Lgr5 that recognizes the extracellular N-terminal region of the protein, specifically loop 2 (Abgent

AP27452d), or the polyclonal rabbit anti-human Lgr5 (Novus 28904). The antibody dilution was 1:50 in 3% blocking solution, and samples were incubated ON at 4° C to allow binding of the antibody. After several washes with PBS, the secondary antibody, biotinylated goat anti-rabbit IgG was applied at a 1:500 dilution in 3% blocking solution, and samples were incubated for 30 min at RT. Finally, after several washes to remove excess and unbound secondary antibody, staining was revealed with DAB (3,3'-diaminobenzidine) according to the manufacturer's instructions (Vector DAB Peroxidase Substrate Kit SK-4100). Sections were incubated with the ABC complex for 30 min at RT in darkness, washed, and DAB developer (comprising 2.5 mL distilled water, 1 drop stock buffer, 2 drops DAB, and 1 drop of hydrogen peroxide solution) was quickly applied. The reaction was stopped at the same time that the positive control took colour.

After washing with PBS, the slides were counterstained with Harris hematoxylin diluted 1/5. Subsequently slides were dehydrated and finally mounted with Entellan (Sigma). The sections were visualized using a Nikon Eclipse 80i microscope with the 20×, 40×, and 63× objectives.

The controls used in this case were:

- Heart muscle as a negative control.

- Intestine as a positive control for Lgr5 expression in the crypts of Lieberkühn.

5.2.4 Lgr5 Protein Expression over the Menstrual Cycle assessed by Western Blot

5.2.4.1 Protein Extraction

Endometrial biopsies from different days of the menstrual cycle were collected and kept at -80° C. To carry out the protein extraction, the samples were washed with cold PBS, mechanically disaggregated, and immediately put into lysis buffer for 20 min on ice to lysate the cells (volumes added were between 20 and 100 µL according to the biopsy size). The lysis buffer was composed of 50 mM pH 8.0 Tris-HCl, 150 mM NaCl, 1% Igepal CA360, 0.5% Na-DOC, 0.1% SDS, and 0.5M EDTA. After this the samples were centrifuged at 12,000 rpm for 15 min at 4° C, and the supernatant containing the cell protein extract was collected.

5.2.4.2 Quantification

Proteins were quantified using the Bradford method, based on binding of Coomassie blue G-250 dye (Bio-Rad, UK) to proteins. In acidic solution the colorant exists in two isoforms, one blue and one orange. The proteins in the sample join with the blue dye to form a protein-dye complex with an extinction coefficient greater than that of the free dye. The absorbance was measured at 280 nm using a spectrophotometer, which corresponds to the absorption coefficient wavelength of proteins. To determine the total protein concentration present in the sample a calibration curve was prepared using a standard protein (BSA).

5.2.4.3 Western Blotting

Approximately 40 µg of each protein extract was mixed with Laemmli buffer (Bio-Rad, UK) with 8% β-mercaptoethanol, and denatured at 95° C for 5 min. The technique used was SDS-PAGE (Sodium dodecyl sulphate [SDS] polyacrylamide gel electrophoresis [PAGE]), in which proteins are treated with reducing agents, causing them to lose their secondary and tertiary structures (for example by reducing disulphide bonds [SS] to thiol groups [SH+SH]) and are maintained in this denatured state. Thus, the three-dimensional protein structure has no influence on electrophoresis, and so the polypeptides are separated based only on size. Protein extracts were separated by discontinuous electrophoresis (a 4% polyacrylamide stacking gel and a 10% polyacrylamide resolving gel) for 1 h at 180 V.

Next, proteins were transferred from the polyacrylamide gel to a polyvinylidene fluoride membrane (PVDF; Amersham Biosciences, NJ, USA) making the proteins accessible for antibody detection. This transfer was accomplished by applying an electric field (wet electroblotting), using transfer buffer Tris/Glycine (Bio-Rad, UK). The transfer conditions were 160 V for 4 h at 4° C with agitation.

After the transfer step, the membrane was blocked with 5% skimmed milk powder in a PBS detergent with 0.1% Tween 20 in order to saturate all of the possible membrane protein-binding sites to prevent nonspecific antibody binding. Subsequently, the membranes were incubated at 4° C ON with the primary antibodies at a suitable concentration: 1/2000 for mouse monoclonal anti-GAPDH (36KDa; Abcam, Cambridge, UK) and 1/500 for monoclonal rabbit anti-Lgr5

(100kDa; Novus Biologicals, USA) diluted in 3% milk powder in PBS. The membranes were then incubated for 1 h at RT with anti-mouse and anti-rabbit secondary antibodies respectively, which were conjugated with horseradish peroxidase (HRP; Santa Cruz, CA, USA) at a dilution of 1:2000 in 3% milk powder in PBS. The antigen-antibody complexes were revealed with the *ECL Plus reagent* (Amersham Biosciences, USA) analysis system and photographed using the Fujifilm LAS-3000 apparatus. The analysis of bands was performed by densitometry with the Multi Gauge Fujifilm program, version 3.0.

5.2.5 Telomere Length Analysis by Fluorescence *in situ* Hybridization

The average length and percentage of short telomeres in endometrial tissue sections was analysed as explained in section 5.1.3.5.

In this case, consecutive sections were used to co-localize the presence of labelled cells by *in situ* hybridization for Lgr5 with hot spot regions obtained by telomapping.

5.2.6 Isolation of Lgr5 Positive Cells

5.2.6.1 Collection and Processing of Biological Samples

As explained in section 5.1.2.1 endometrial samples were enzymatically digested and subjected to a cell separation process based on a sedimentation gradient to obtain epithelial and stromal cells.

5.2.6.2 Cellular Viability

Cell viability was assessed by adding PI to the samples at a final concentration of 5 µg/mL (Sigma-Aldrich, Madrid, Spain) incubating in darkness at RT for 15-20 min, and then analysing them by flow cytometry. Propidium iodide fluorescence emission is red-orange when is excited with a 488 nm laser meaning that precise percentages of live or dead cells could be distinguished based on the presence of PI in damaged or dead cells by flow cytometry.

5.2.6.3 Endometrial Cell Labelling using Molday ION Rhodamine

A non-transfection-based method, using fluorescent iron-oxide nanoparticles was chosen to label endometrial cells for cell tracking experiments in the *in vivo* model. Molday ION (MION) is a superparamagnetic iron oxide nanoparticle developed as a device to label cells by cellular uptake. The label appears to incorporate inside endosomes but is not found in the endoplasmic reticulum, Golgi apparatus, nucleus, or any other cellular organelles. Molday ION Rhodamine B (CL-50Q02-6A-50, [2mg Fe/ml], BioPAL) is a homogeneous, fluorescent iron oxide-based superparamagnetic (SPIO) contrast reagent designed to label cells efficiently and simply, because it can be visualized by both magnetic resonance imaging (MRI) and fluorescence. Rhodamine B is a fluorescent dye with an excitation and emission wavelength of 555 and 565-620 nm, respectively. This dual property allowed labelled cells to be sorted by flow cytometry and detected by Prussian blue (a common histopathology stain) staining due to the iron deposits.

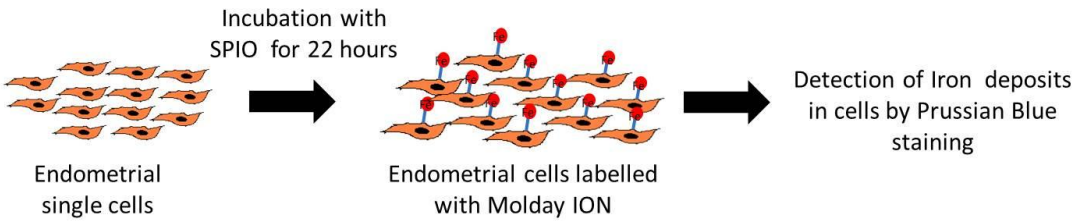

Figure 16. Molday ION Labelling. *After incubation, cells incorporate the Molday ION which can be detected by Prussian blue staining.*

Epithelial and stromal cells were plated in appropriate conditions with DMEM-F12 medium containing 10% SBF, 0.1% antibiotics and antimycotics (GIBCO-Invitrogen) and incubated at 37° C and 5% CO_2. After allowing the cells to adhere ON, Molday ION Rhodamine B was added in different concentrations (10 µg/mL, 25 µg/mL, and 35 µg/mL) to the supplemented medium and incubated for 22 h. The medium was aspirated after incubation and cells were washed with PBS (containing calcium and magnesium) after which they were visualized using a fluorescent Nikon Eclipse 80i microscope.

Two further tests were performed at this point:

(1) A Trypan blue cell viability exclusion test to detect signs of toxicity;
(2) Quantification of Rhodamine B fluorescence staining by flow cytometry to detect any possible cytotoxicity of the compound, and to detect the percentage of cellular staining in order to establish an appropriate final concentration for future assays.

Thus, one aliquot of the cells was subjected to the Trypan blue test, allowing the dead cells to be distinguished from the total cells with a normal light microscope, and the other aliquot was centrifuged and resuspended in PBS for flow cytometric analysis using a Cytomics FC500 (Beckman-Coulter, CA, USA) flow cytometer. The cytometer is equipped with an Argon laser (488 nm, 15 mW) as explained in section 5.1.2.2. Fluorescence was used at 575 nm in order to detect the Rhodamine B signal.

5.2.6.4 Isolation of Lgr5 Positive Cells

Viable epithelial and stromal cell populations were placed in a blocking buffer consisting of PBS with 5% BSA for 30 min at RT. After this cells were centrifuged at 580 g for 7 min at 4° C. A small aliquot of the total cells was taken aside as a control and incubated with the secondary but not the primary antibody (referred to as isotypes). Incubation with the primary polyclonal rabbit anti-human antibody (Novus 28904) against the Lgr5 antigen was performed in PBS with 3% BSA for 1 h at 4° C on ice. The antibody was used at a ratio of 1 µL per million cells. After incubation the cells were centrifuged at 580 g for 7 min at 4° C and were washed with PBS; this washing process was repeated twice to remove any unbound antibody. The secondary antibody used was a goat anti-rabbit Alexa 488 at a dilution of 1:500 in with 3% BSA for 30 min at 4° C. After this incubation the cells were centrifuged at 580 g for 7 min at 4° C and washed with PBS. The cells were maintained at 4° C throughout all of the protocols in order to preserve cell viability. Finally, the cells were centrifuged at 580 g for 7 min at 4° C and resuspended in HBSS buffer. The labelled cells were kept at 4° C ON until processing.

Before analysing the epithelial and stromal cell suspensions by FACS the cells were filtered through 50 µm and 30 µm pore-size filters respectively (Partec, Celltrics) to

eliminate possible cell aggregates formed by sedimentation. Finally PI was added at a concentration of 5 µg/mL to allow dead cells to be discriminated.

Cell separation was performed with a high speed FACS Legacy MoFlo (Beckman-Coulter, USA) equipped with 3 lasers and eight fluorescence detectors. The assay protocol also included PI-gating to discard dead cells and thus allow the identification and separation of living Lgr5 positive or negative cells. To detect Lgr5-Alexa 488 and PI, a 488 nm laser was used: fluorescence emission was captured in the 525 nm channel for Lgr5-Alexa 488 and in the 620 nm channel for PI. The Lgr5 positive cell population was separated from the Lgr5 negative population based on the analysis of an isotype sample.

5.2.6.5 Isolation of Lgr5-Rhodamine Positive Cells

Viable epithelial and stromal cell suspensions labelled with Molday ION Rhodamine B were placed in a blocking buffer (PBS with 5% BSA) for 30 min at RT. After this the cells were centrifuged at 580 g for 7 min at 4° C, and the protocol described in section 5.2.6.4 was followed, with the exception that the FACS settings were changed.

A small aliquot from the entire cell population was separated and used for controls: a) unstained cells (before Molday ION labelling), b) Molday ION labelling only, c) incubation with only the secondary antibody (isotype) and no Molday ION staining. Samples were incubated with the polyclonal rabbit anti-human antibody against the Lgr5 antigen (Novus 28904) in PBS containing 3% BSA for 1 h at 4° C on ice. The antibody was used at a ratio of 1µL per million of cells. After incubation the cells were centrifuged at 580 g for 7 min at 4° C and then washed with PBS twice to remove any unbound antibody. The secondary antibody used was a goat anti-rabbit Alexa 488 at a dilution of 1:500 in PBS for 30 min at 4° C. After this the cells were centrifuged at 580 g for 7 min at 4° C and washed with PBS. The cells were maintained at 4° C throughout all of the protocols in order to preserve cell viability. Finally, the cells were centrifuged at 580 g for 7 min at 4° C and resuspended in HBSS buffer. The labelled cells were kept at 4° C ON until processing.

Before analysing the epithelial and stromal cell suspensions by FACS the cells were filtered through 50 µm and 30 µm pore-size filters respectively (Partec, Celltrics) to

eliminate possible cell aggregates formed by sedimentation. Finally, because PI interferes with Rhodamine B fluorescence, DAPI (Invitrogen) was added at a concentration of 5 µg/mL to allow any dead cells or debris to be discriminated.

To detect and sort Lgr5-FITC and Rhodamine simultaneously a Moflo Legacy High Speed Cell Sorter (Beckman-Coulter, USA) equipped with three lasers (351, 488, and 635 nm), two detectors for FS and SS, and eight fluorescence detectors was used. The signals for Lgr5-FITC and Rhodamine B were acquired using the 525 nm and 620 nm channels respectively, after excitation with the 488 nm laser. The DAPI probe was used to discard the dead cells, which was detected in the 450 nm channel, after excitation with the 351 nm laser. Live cells (DAPI negative cells) were gated in a FS/DAPI dot plot, and this gate was applied to the Lgr5-FITC/Rhodamine B dot plot, where the double positive cells were selected for cell sorting.

5.2.7 Xenotransplantation Assays with Lgr5-Rhodamine Positive Cells

Demonstration of the existence of endometrial SSCs which can produce endometrial-like tissue in an animal model was essential to provide functional evidence of the regenerative capacity of these candidate cell populations *in vivo*. One of the most commonly used animal models in research and regenerative medicine is the NOD-SCID (non-obese diabetic/severe combined-immunodeficiency) mouse. This strain exhibits severe defects in the production and function of T cells, is defective in B lymphocytes, and in some cases is also defective in NK cell production. This mouse model is immunologically incompetent to treat infection or reject transplanted cells or tissues. Therefore it is widely used in the scientific community for proof-of-concept experiments to assess the degree to which a cell population is undifferentiated (Cervelló et al*.,* 2010; Masuda et al*.,* 2010; Cervelló et al*.,* 2011; Masuda et al*.,* 2012).

All procedures involving animals in this study were approved by the *Centro de Investigación Príncipe Felipe* (CIPF) and *Instituto Valenciano de Infertilidad* (IVI) review board ethics committees.

5.2.7.1 Experimental Animal Model

Female NOD-SCID mice (strain code 394; NOD. CB17- Prkdc^{scid}/NCrCrl from Charles River Laboratories, Spain) were ovariectomised at 4-5 weeks and then used for xenotransplantation experiments. Subsequently, mice were treated with a cocktail of analgesic and anti-inflammatory drugs and were anesthetized by sevofluorane inhalation, followed by kidney externalization through a dorsal-horizontal incision, to enable cell injection.

In this experimental approach, freshly FACS-sorted Lgr5^{+}Rhodamine^{+} single cell suspensions (2,000-20,000 cells) were resuspended in 30 µl of medium (DMEMF-12, Sigma-Aldrich, Spain) and injected under the kidney capsule (n=3) on the left side. The same number of Lgr5^{-}Rhodamine^{-} cells, in single cell suspension, were injected in the left kidney capsule of control mice (n=3).

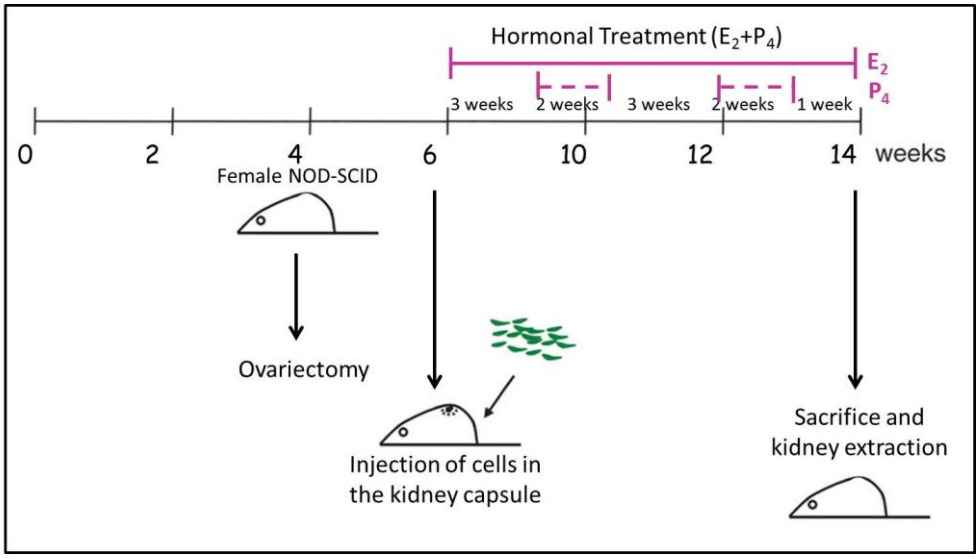

Figure 17. Experimental Design of the Animal Model Experiments. *Four weeks after birth, mice were ovariectomised and two weeks later, after recovery, the experiment was started. Mice were treated hormonally by administration of estradiol (E_2, 0.36 mg/60 days for 11 weeks) and progesterone (P_4, 1 mg/day, for two two-week intervals). After 60 days mice were sacrificed and were nephrectomised in order to analyse cell engraftment and development in the injected kidneys.*

During transplantation, estradiol pellets (E_2; SE121, 17β-estradiol 0.36 mg/60 days; Innovative Research of America) were also implanted subcutaneously in the neck. Moreover, mice treated with estradiol pellets were injected subcutaneously every day with of progesterone (P_4, Dr. Carreras, Hospital 14, Barcelona, Spain) for two weeks. After a three-week interval, mice were subjected to a second cycle of daily P_4 injections for 2 weeks until they were sacrificed. Throughout this assay, xenotransplanted mice were maintained in specified pathogen-free (SPF) facility and fed *ad libitum* for 60 days after the initial cell injection. After that, mice were nephrectomised according to the experimental protocol.

60 days after cellular injection, mice were sacrificed by inhalation of carbon dioxide (CO_2) resulting in immediate death by causing haemoglobin in erythrocytes to combine with CO_2 thus producing anoxia. Following this, the injected kidneys were removed for further analysis, and finally stored in 4% PFA until they were paraffin embedded (as described in section 5.1.3.1).

5.2.7.2 Immunohistochemical Characterization of Candidate Endometrial Tissue

In order to determine if the $Lgr5^+Rhodamine^+$ cell population was able to form endometrial-like tissue *in vivo*, two experimental approaches were taken: Prussian blue staining and immunohistochemistry for specific endometrial markers.

5.2.7.2.1 Detection of Iron Deposits by Prussian Blue Staining

Prussian blue staining is a histological technique used to visualize the presence of iron deposits found inside cells after treatment with Molday ION. It is based on a reaction in which ionic iron reacts with acidic ferrocyanide, producing a blue colour.

The reagent kit used is called ACCUSTAIN® IRON STAIN (Procedure No. HT20, Sigma-Aldrich) which provides two solutions 1) the working iron stain solution, prepared by mixing equal volumes of potassium ferrocyanide solution (Catalogue No. HT20-1) and hydrochloric acid solution (catalogue No. HT20-2) and 2) the working pararosaniline solution, prepared by adding 1 mL of pararosaniline solution (catalogue No. HT20-3) to 50 mL of water. Both solutions must be freshly prepared before use.

Paraffin sections from the injected kidneys were deparaffinized for one hour at 60° C and rehydrated (5 min in each ethanol bath as described in section 5.1.3.1), and placed in distilled water. After this the slides were added to the working iron stain solution for 10 min and then rinsed with distilled water. They were subsequently stained with pararosaniline solution for 5 min and rinsed with distilled water. Finally, the sections were rapidly dehydrated through alcohol and xylene stages and mounted with Entellan (Sigma). The sections were visualized with a Nikon Eclipse 80i microscope with 20×, 40×, and 63× objectives.

The controls used to assess the results obtained in this section were:

- Non-injected kidney, used as negative control.

- Spleen, used as positive control for iron deposits.

5.2.7.2.2 Immunohistochemistry for Endometrial Tissue Markers

With the purpose of characterizing any newly formed tissue present in the kidney capsule, immunohistochemistry for Vm and CD9, to search for endometrial-like tissue, was performed.

Sections from the injected kidneys were deparaffinized by heating them at 60° C for 1 h, followed by 3 passages with xylol for 10 min, 1 bath in 100% ethanol for 5 min, and subsequent rehydration with alcohols of decreasing concentration (95%, 80%, and 70%, [v/v] for 5 min in each) ending in a water bath. Antigen retrieval was performed in a citrate buffer bath at 95° C in order to break down formalin networks and facilitate antibody binding. After this, membrane permeabilization with PBS containing 0.05% Tween 20 was performed for Vm staining.

The slides were then washed and blocked with PBS containing 5% BSA and 0.05% Tween 20 for 1 h at RT to reduce unspecific antibody binding. Following this, a solution of PBS with 1% BSA and the primary antibody was added. The antibodies used were mouse monoclonal anti-human CD9 at a dilution of 1:20 (Abcam, 49325) and mouse monoclonal anti-human anti-Vm clone V9, at a dilution of 1:100 (Dako M0725) for endometrial epithelial and stromal cells respectively. Cells were incubated at 37° C for 1 h in both cases. After buffer washing, the second blocking was performed with hydrogen peroxidase by incubation with 3% H_2O_2 for 5 min in

order to block endogenous peroxidase activity in the tissue. Slides were then washed and incubated with biotinylated universal link reagent for 20 min at RT by adding enough drops of the link solution to cover the specimen (DAKO Kit, Agilent). To stop the reaction, washing-buffer was added.

Afterwards, slides were placed in solution with streptavidin peroxidase for 20 min at RT; to stop the reaction, washing-buffer was added. Finally, slides were incubated with the substrate-chromogen solution (DAB Chromogen): one drop of DAB chromogen per 1 mL of buffered substrate was incubated on the slides until a dark colour developed in the positive control.

After washing with PBS, the slides were counterstained with Harris hematoxylin diluted to 1/5 and subsequently dehydrated (in an increasing concentration ethanol gradient as described in section 5.2.3) and finally mounted with Entellan (Sigma). The sections were visualized with a Nikon Eclipse 80i microscope with 20×, 40×, 63×, and 100× objectives.

The controls used to assess the results obtained in this section were:

- Non-injected kidney, used as negative control for CD9 and Vm expression.

- Endometrium, used as positive control for CD9 and Vm expression.

VI. Results

VI. RESULTS

6.1 The Contribution of the Bone Marrow as an Exogenous Source for the Endometrial Stem Cell Niche

6.1.1 Presence of XY Cells of Donor Origin in the Endometrium of BMT Recipients

The presence of donor-derived cells (Y-chromosome positive cells) in BMT recipients´ endometria was examined using the FISH approach. The results revealed the presence of XY donor-derived cells in all the endometrial sections from BMT-recipients analysed (Figure 18).

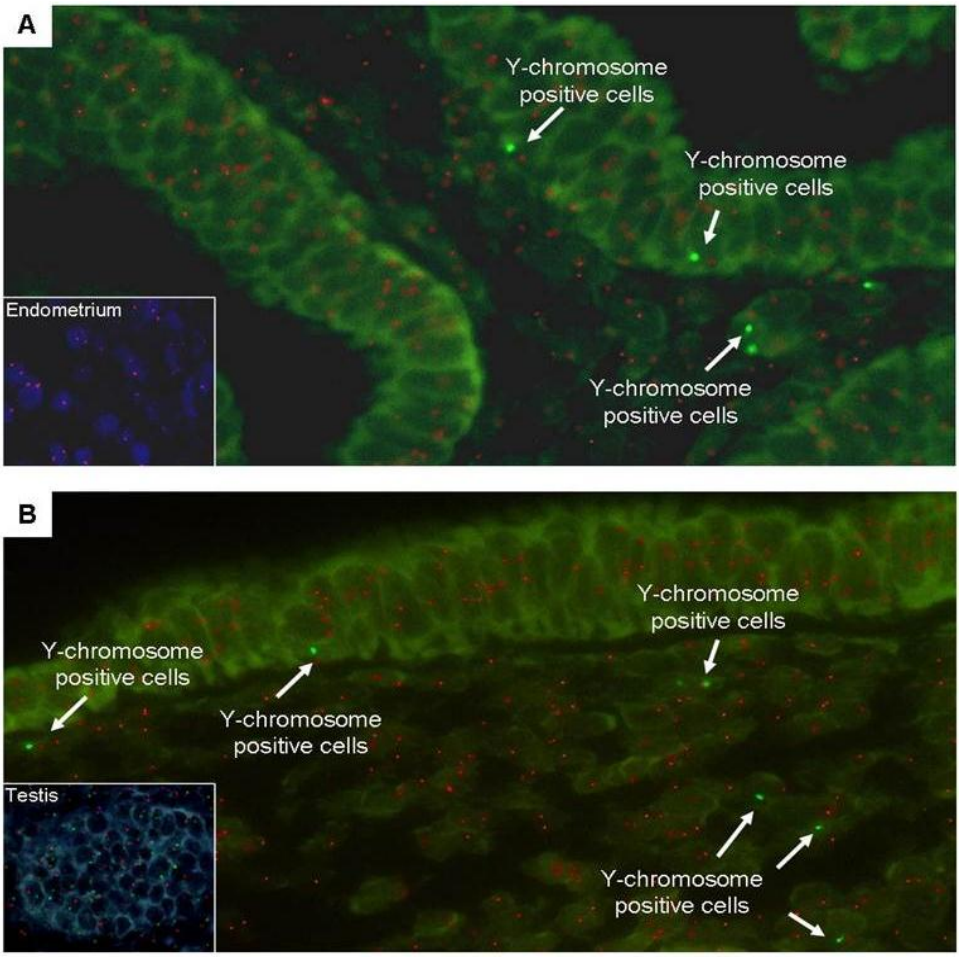

Figure 18. Detection of XY cells in the endometrium of BMT recipients. (*A & B) Human endometrial sections from 2 different patients, showing a positive FISH signal corresponding to the Y chromosome (green signal) and X chromosome (orange signal) in both epithelial and stromal compartments. The presence of donor-derived Y-chromosome cells in the endometrium is indicated by white arrows in the figure above. Lower panels containing control slides: (A) endometrium as a positive control for the XX chromosome signal and (B) fetal testes as a positive control for the XY chromosome signal. Images were acquired using a 63× /1.6× oil immersion objective.*

The presence of donor-derived cells in the endometrium is indicated by white arrows in the figure above. Although only images from two patients are shown, these results were the same for all the BMT-patients included in the study.

Control FISH-analyses included an endometrial sample from a healthy non-BMT patient; to avoid the possibility of contamination with Y-chromosome positive cells via materno-fetal chimerism and male tissues from fetal testes, a non-BMT patient who had a previous pregnancy with a boy was selected. As expected, all the cells in our controls exhibited orange (X-chromosome positive cells) signals (lower panel A), whereas the ratio of the Y-chromosome to X-chromosome positive cells was confirmed in male testes (lower panel B). Moreover, the possibility of cell fusion was ruled out because of the absence of aneuploidies.

Patient	Age	Time from transplantation to biopsy (months)	% XY	%XY Stromal Fraction	%XY Epithelial Fraction
1	38	132	1.7	1	1.07
2	29	30	2.17	1.67	0.5
3	41	210	2.46	1.42	0.81
4	34	116	2.62	1.77	0.85
5	28	45	2.29	1.83	0.45

Table IV*. Table summarizing the recipient characteristics and the percentages of XY-chromosome positive cells obtained in the whole endometrium plus the epithelial and stromal fractions from BMT-patients.*

Endometrial tissue sections were analysed by two observers with a total of five visual fields per sample scored (around 1000-1500 cells). Donor-derived cells

corresponding to Y-chromosome positive cells accounted for 1.7-2.62% of the total cell count, with fewer in the epithelial compartment (ranging from 0.45% to 0.85%), while the percentage of Y-chromosome positive cells in the stromal compartment ranged from 1.0% to 1.83% respectively. Interestingly, no correlation was found between the time from transplantation to biopsy (from 30 to 210 months) and the percentage of Y-chromosome positive cells found in the endometrium (Table IV).

6.1.2 Immunophenotype of the XY Donor-Derived Cells in the Endometrium of BMT-Recipients

To assess the phenotype of the XY donor-derived cells in the recipient endometrium, we used CD45 to identify cells of a hematopoietic origin, and CD9 and Vm to locate endometrial epithelial and stromal cells respectively. These markers were co-localized in combination with Y-chromosome detection by FISH (See Figure 19).

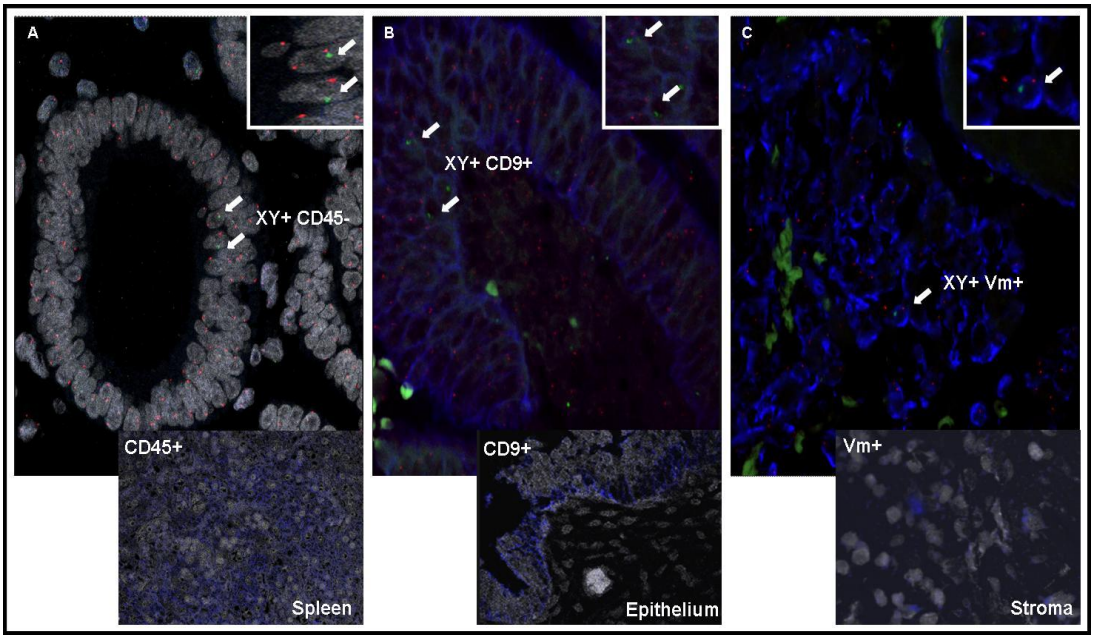

Figure 19. Immunophenotype of the XY donor-derived cells in the endometrium of BMT-recipients. *(A) XY donor-derived cells (marked by arrows: orange and green signals) did not co-localize with the hematopoietic marker CD45 (blue signal). Lower panel: spleen as a*

positive control for CD45. (B) Co-localization of XY donor-derived cells with epithelial marker CD9 (blue). Lower panel: endometrial epithelium as a positive control for CD9. (C) Co-localization of XY donor-derived cells with stromal marker Vm (blue). Lower panel: stromal endometrium as a positive control for Vm. Images were acquired using a 63× /1.6× oil immersion objective.

None of the XY donor-derived cells were $CD45^+$ which rules out the possibility that these cells were hematopoietic progenitors or of contamination by resident leukocytes originating from the BMT (Figure 19A). All the XY donor-derived cells present in the glands were $CD9^+$ (Figure 19B) and all the XY donor-derived cells in the stroma co-localized with the Vm marker (Figure 19C), suggesting a final tissue-specific differentiation of XY donor-derived cells. Use of specific tissues for positive controls enabled the specific immunoreactivity of the antibodies used to be tested. The DAPI signals are shown in grey in all images.

6.1.3 Analysis of Telomere-Length Regions (Telomapping) in the Endometria of BMT-Recipients

To search for the location of possible endometrial SSC niches within the endometrium and whether XY donor-derived cells were part of them, telomere length in the endometrial sections from both BMT-recipients and non-transplanted patients were examined by telomapping. In all the endometrial sections, 'hot' regions, associated with long telomeres were located specifically in the stromal compartment. Nevertheless differences in terms of telomere-length regions, with or without BMT, were not found. Also, some 'hot' regions were identified in the luminal epithelium of a healthy non-BMT woman.

Evaluation of telomere length in endometrial sections from all BMT-recipients (Patients 1-5) was performed and is represented in Figure 20, together with a healthy non-BMT patient control. A colour code in the left part of the image symbolizes a colour scale corresponding to different telomeric lengths where the longest and shortest telomeres are represented in red and blue respectively. This colour code aims to transform the fluorescence intensity values into a map which visually shows telomere-length regions within the sample.

The 'hot' regions associated with long telomeres were mainly located in the stromal compartment, principally in the patients undergoing BMT: patient 1,

patient 2, patient 3, and patient 4. However, in patient 3 some red points were close to the luminal epithelial compartment, and in patient 4 these red signals were located in the epithelium. In case of the patient 5, only a small sample of endometrium was analysed due to her atrophic endometrium. In BMT recipients, most XY donor-derived cells were randomly distributed and could not be associated with long telomere regions.

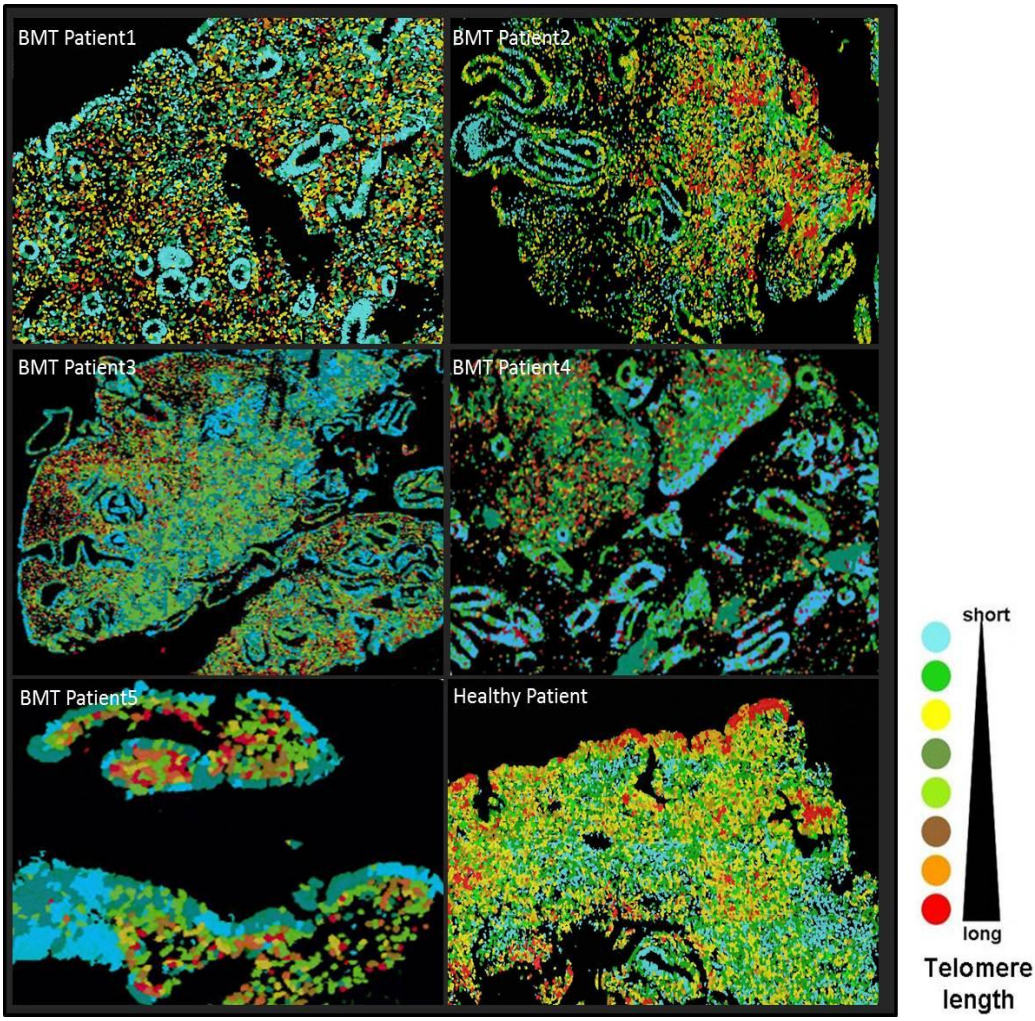

Figure 20. Analysis of telomere length in endometrial sections. *Assessment of telomere length in the endometrial sections from BMT-recipients and a healthy patient. A colour code*

on the right-hand side of the image symbolizes telomeric length, where the longest and shortest telomeres are represented in red and blue respectively.

6.1.4 Isolation of Endometrial SP Cells and FISH Analyses from BMT-Recipients

In order to investigate to what extent the donor-derived cells from male donors contributed to the endometrial SP (which has been demonstrated to constitute the SSC population), this subset of cells from the epithelial and stromal fractions in all five BMT recipients was isolated. The table in Figure 21A shows the percentages of endometrial SP cells obtained in all 5 patients by cell sorter analysis**.**

A

Percentages of Side Population cells		
Patient	**Epithelium**	**Stroma**
Patient 1	0.04%	0.01%
Patient 2	0%	0.02%
Patient 3	*ND	*ND
Patient 4	0%	0%
Patient 5	0%	0.1%

*ND: Not determined

B

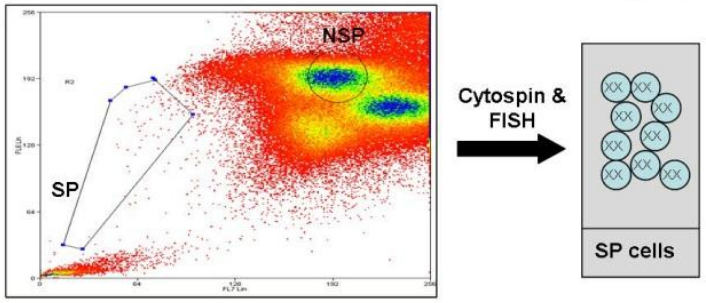

Figure 21. Presence of side population cells in BMT recipients. *(A) Table indicating the percentages of endometrial SP cells obtained in all 5 patients by cell sorter analysis. Percentages are provided for both the epithelial and stromal fractions. (B) Diagram showing a typical SP graph and the results of the FISH analyses of epithelial and stromal SP cells, revealing the absence of XY donor-derived cells.*

One interesting finding was that there were no epithelial SP cells detected in four patients, but that in patient 1 this population was determined to represent 0.04% of the total cell population. In patients 1, 2, and 5, the stromal SP percentages

ranged from 0.01% to 0.1%, however SP cells were not present in patients 3 and 4 (Figure 21A). Unexpectedly, the viability of all the samples analysed was very low compared that of cells similarly obtained from non-BMT patients (Cervelló et al*.,* 2010). Figure 21B shows a typical SP graph; FISH analyses were later performed on these isolated SP cells. Results for epithelial and stromal SPs from BMT-patients revealed the absence of XY donor-derived cells. It is noteworthy that one XY cell was observed in a total of 100 FISH-analysed cells in the control NSP stromal fraction sample from patient 4.

6.2 Investigation of the Lgr5 Marker to Identify the Endogenous Endometrial Stem Cell Niche

Stem cell niches are usually identified based on the expression of specific protein markers on the SSCs that can be tracked and localized. The Lgr5 gene is a multi-pass-membrane protein, with 3 domains: a large extracellular domain with multiple leucine-rich repeats that mediate ligand interaction, a transmembrane domain, and an intracellular domain for signal transduction.

6.2.1 Detection of Lgr5 RNA by *in situ* Hybridization

The *in situ* hybridization technique allows specific gene expression to be localized within the cellular environment. To generate the probes the vector pPCR-Script Amp SK (+) was used to clone the Lgr5 sequence into the MCS. After labelling with DIG the riboprobes were precipitated. Two specific riboprobes were generated for Lgr5 mRNA: T7 as the antisense and T3 as the sense riboprobe.

The controls used in the assay allowed the binding specificity to be determined during the hybridization. Therefore, human small intestine was chosen as positive control because of reports of Lgr5 expression in the literature (Baker et al*.,* 2007), whereas human heart tissue was selected as negative control, because Lgr5 expression is not reported in this tissue in the Atlas Protein Database.

Therefore the antisense (T7) and sense (T3) riboprobes were used with these tissues to allow the hybridization-produced signal to be distinguished from the background (See Figure 22).

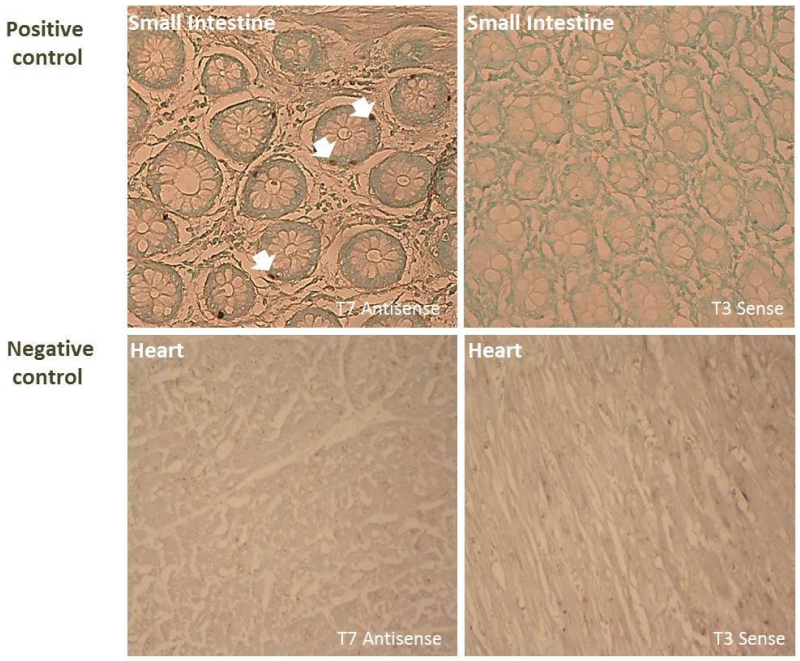

Figure 22. Controls used for the Lgr5 in situ hybridization*. Small intestine was used as a positive control for Lgr5 expression; signals are indicated with white arrows, whereas heart tissue was the negative control. Two different riboprobes were generated: T7 antisense with a sequence complementary to Lgr5 allowing binding, and T3 sense with the same sequence which was unable to bind (negative control of the technique itself). Images were taken at 40× magnification.*

In situ hybridization results revealed the existence of Lgr5 positive cells in human endometrium at the mRNA level; the antisense probe hybridized with the endometrial tissue but fewer cells were stained positive in the epithelial and stromal area. Interestingly these cells were located mainly in the perivascular regions as shown in Figure 23. The NBT/BCIP colorimetric reaction causes a brown signal to precipitate, indicating specific binding of the mRNA Lgr5-DIG probe, whereas nuclei showed up in green only.

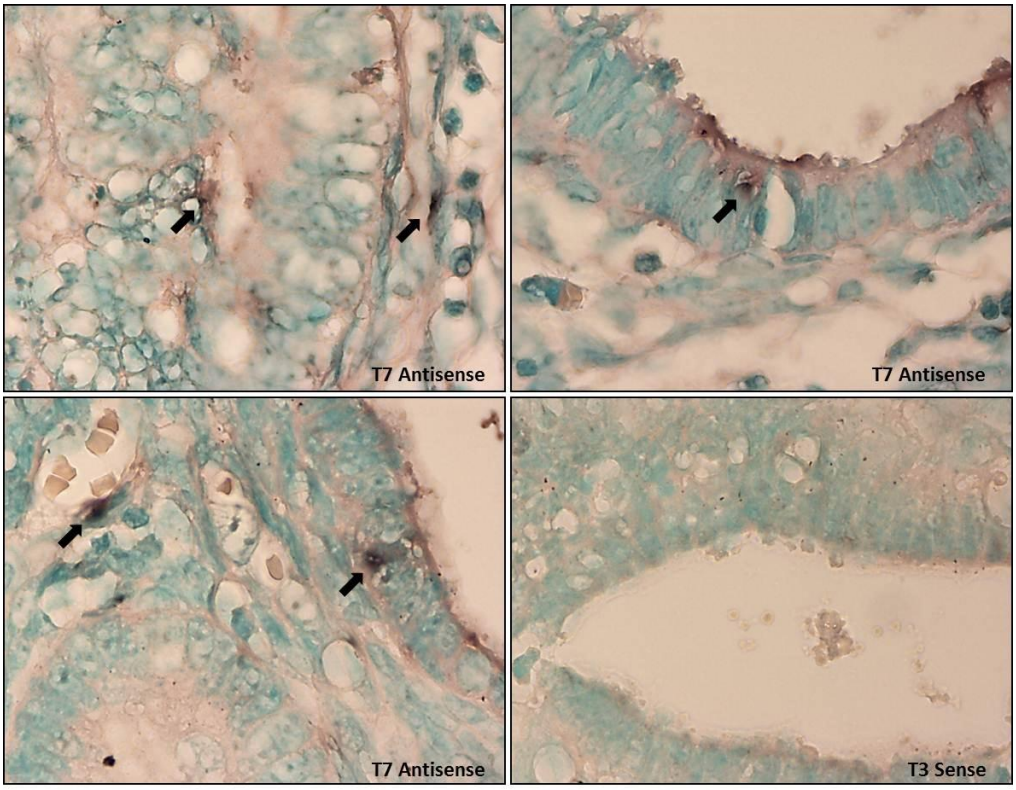

Figure 23. Detection of Lgr5 mRNA expression in endometrium by in situ hybridization. *Positive signals from the T7 antisense riboprobe are shown with black arrows, whereas no signal was detected with the T3 sense riboprobe. Images were taken at 63× magnification.*

6.2.2 Expression of Lgr5 in the Human Endometrium

Lgr5 expression was evaluated in the human endometrium throughout the menstrual cycle, in both the proliferative and secretory phase. The immunoreactivity was shown by DAB as a characteristic brown signal, whereas nuclei were stained with hematoxylin. After determining that the immunohistochemical controls (human small intestine as a positive control and human heart as a negative control), were functioning correctly, several endometrial samples were studied.

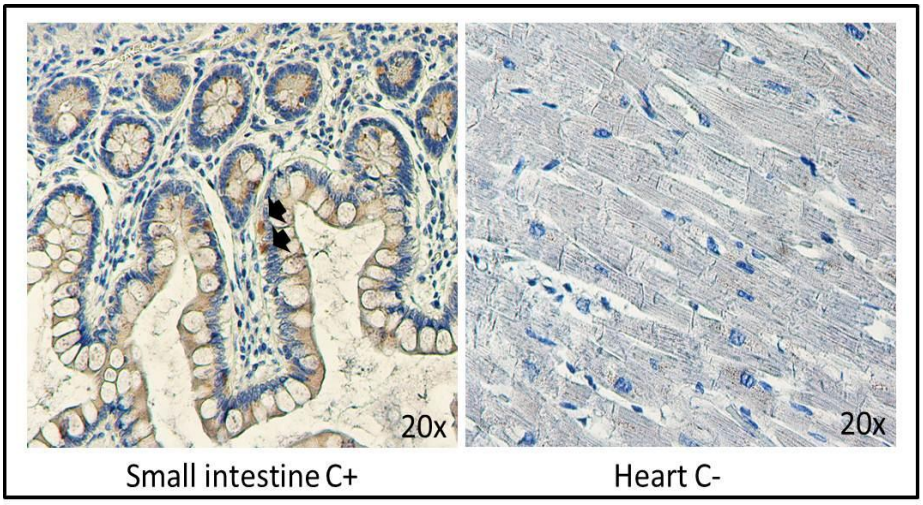

Figure 24. Controls used in immunohistochemistry for Lgr5. *Small intestine was used as a positive control for Lgr5 expression (signals are shown with black arrows), whereas heart tissue was the negative control. Images were taken with a microscope at 40× magnification.*

Cells marked as positive for the Lgr5 antibody (indicated with black arrows in the pictures) were localized in the epithelial glands and perivascular regions of the stromal compartment, with no apparent differences in the frequency or intensity of the signal throughout the menstrual cycle (Figure 25). Therefore, immunohistochemical analysis revealed specific signals (cytoplasmic and membrane positions, according of the antibody used) located in the lower functionalis, near the basalis layer, which were most frequently found in stromal cells.

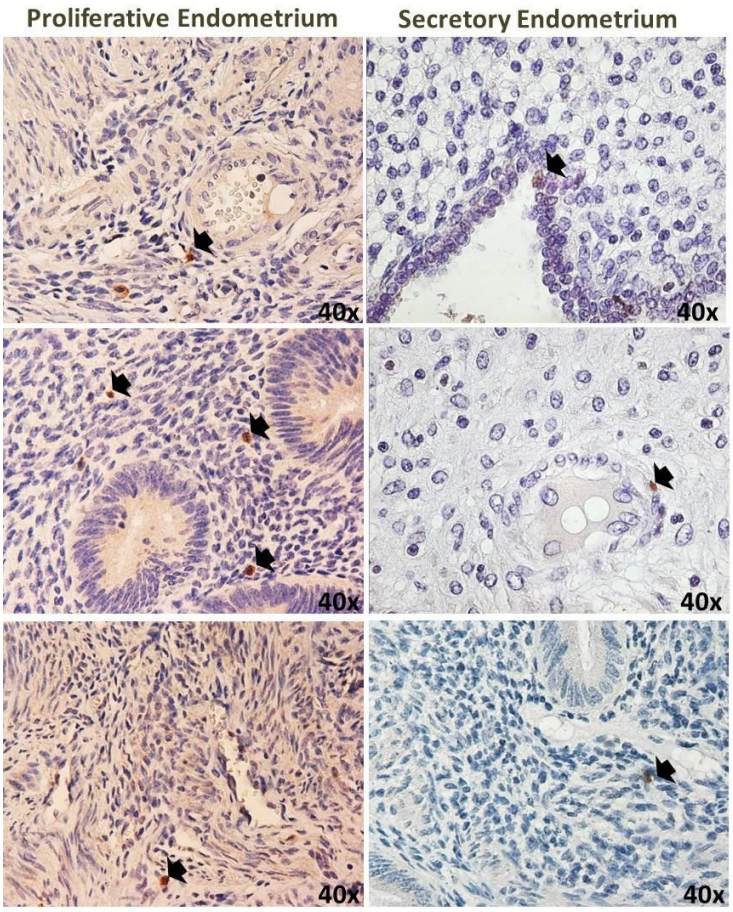

Figure 25. Lgr5 signal localization in proliferative and secretory endometrium by immunohistochemistry. *Positive signals are indicated with black arrows and are found in the stromal area, epithelial glands, and surrounding blood vessels. Images were taken at 40× magnification.*

Furthermore, the expression of the Lgr5 protein was also confirmed by western blot analysis. Samples collected in all stages of the menstrual cycle: proliferative (P), early secretory (ES), mid-secretory (MS), and late secretory (LS) were included, as shown in Figure 26, where GAPDH expression was also included in order to normalize the expression of the Lgr5 molecule. Increased expression was discernible in the P phase versus the other phases, although the results were not statistically significant (n=2).

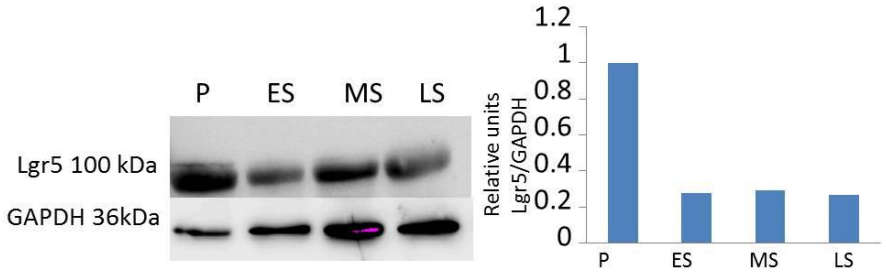

Figure 26. Expression and quantification of the Lgr5 protein in the endometrium throughout the menstrual cycle by western blot. *Left image shows the expression of Lgr5 and GAPDH throughout the menstrual cycle (P: proliferative; ES: early secretory; MS: mid-secretory; LS: late secretory); the right image shows quantification of the Lgr5 protein throughout the menstrual cycle, normalized to GAPDH protein expression*.

6.2.3 Correlation of Lgr5 Positive Cells with Telomere Length

The telomerase enzyme, which prevents telomere shortening at each cell division, maintains telomere length, and it is expressed in stem cells including germ cells, embryonic stem cells, and in the niche of several adult tissues. The technique known as telomapping as mentioned above, is a type of quantitative confocal fluorescence *in situ* hybridization which displays the gradient of telomere lengths that exists in a given adult tissue; the longest-telomere cells are mapped in red and the shortest are shown in blue (Flores et al*.,* 2008).

SSCs from skin, small intestine, testis, cornea, and brain have all been investigated in the mouse model in this way (Flores et al*.,* 2008). Specific 'hot' regions (red), which corresponds to cells with the longest telomeres, were identified in the human endometrium using this technique. Interestingly, a few of the Lgr5 mRNA signals obtained by *in situ* hybridization (Figure 27A) co-localized with some of the cells with the longest telomeres (Figure 27B), suggesting that Lgr5 cells are associated, at least in part, with a long telomere phenotype. Consecutive endometrial sections were used for *in situ* hybridization and telomapping analyses, thus enabling any possible correlation to be identified.

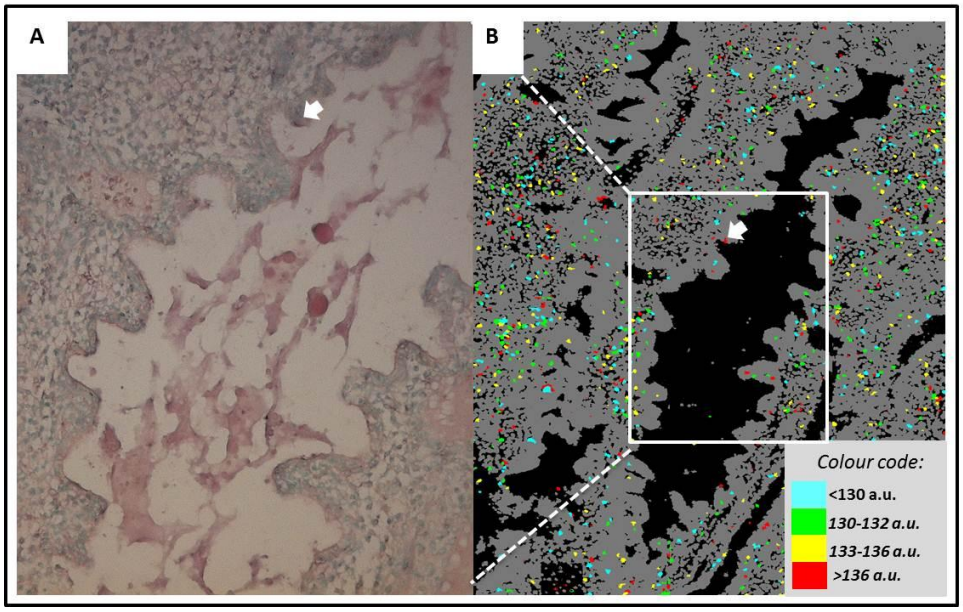

Figure 17. Consecutive endometrial sections co-localizing Lgr5 mRNA signals and telomapping. *An Lgr5 positive cell is indicated with a white arrow (left panel) and the same cell is indicated in the right panel which contains telomere-length measurement data; note the amplified area. The box in the right panel indicates the colour scale which corresponds to telomere length.*

6.2.4 Endometrial Cell Labelling using Molday ION Rhodamine

Molday ION Rhodamine B, a superparamagnetic iron oxide (SPIO) nanoparticle with a fluorophore incorporated into its structure (to allow for staining and cell tracking) was used to label cells by cellular uptake.

Both types of endometrial cell (stroma and epithelium) take Molday ION Rhodamine B up, as shown by the fluorescence of these cells after the incubation period, as illustrated in Figures 28A and 28A. It was essential to check cellular viability with a Trypan blue exclusion test for the three different Molday ION Rhodamine B concentrations used (10µg/mL, 25µg/mL, and 35µg/mL) to test for cytotoxicity. Neither stromal nor epithelial SPIO-labelled cells exhibited signs of cytotoxicity; in the case of stromal cells, even at the highest concentration of

35µg/mL, the viability was around 91% (Figure 28B) while in epithelial cells it was around 88% in the same conditions (Figure 29B).

Rhodamine B fluorescence staining was quantified by flow cytometry; cell suspensions were analysed using a Cytomics FC500 cytometer (Beckman-Coulter, CA, USA), to detect the Rhodamine fluorescence signal at 575 nm. Stromal cell staining was high (between 82-93%), independent of the SPIO concentration used (Figure 28C). Taken together with the viability assay data, Molday ION Rhodamine B was used at 10µg/mL with stromal cells all the following experiments.

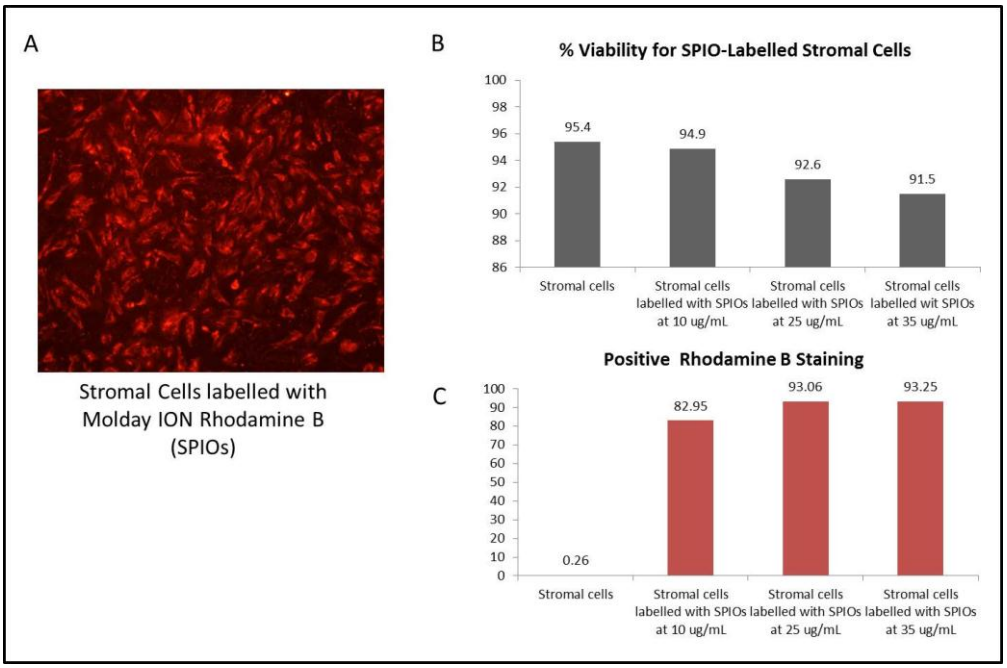

Figure 28. Staining of stromal cells with SPIOs*. A) Rhodamine B fluorescence in stromal cells due to incubation with SPIO. B) Percentages of cell viability are shown for the different SPIO concentrations used. C) Corresponding percentages of Rhodamine B fluorescence are shown for the different SPIO concentrations used.*

However, the results for Rhodamine B staining in epithelial cells were not acceptable: even at the top concentration used (35 µg/mL) the percentage of staining achieved was around 40% (Figure 29C). For this reason the concentration

of Molday ION Rhodamine B used to stain epithelial cells was increased to 50µg/mL (data not shown).

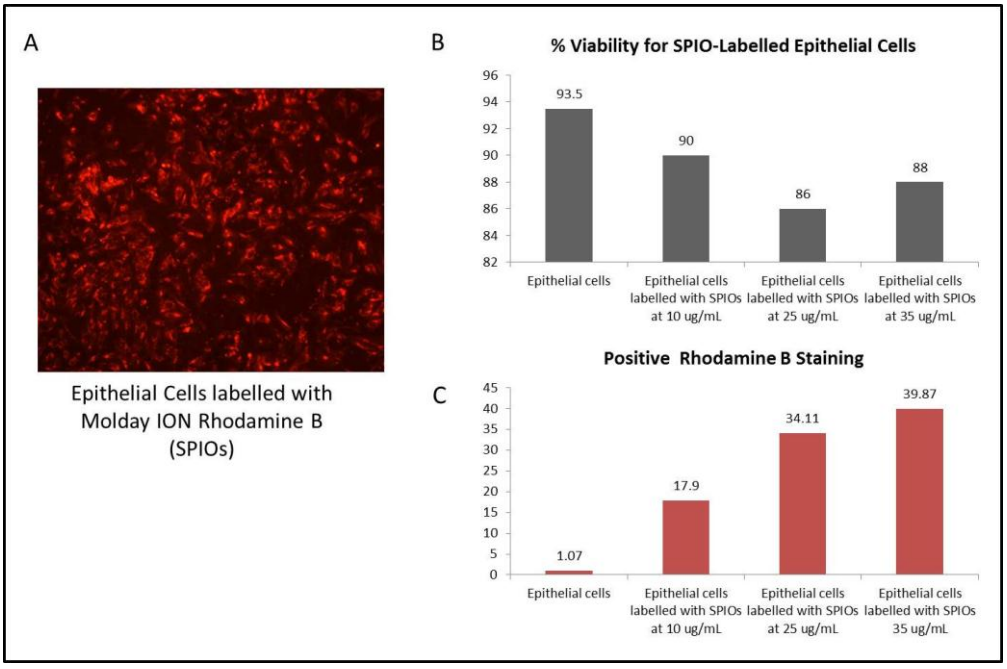

Figure 29. Staining of epithelial cells with SPIOs*. A) Rhodamine B fluorescence in stromal cells due to incubation with SPIOs. B) Percentages of cell viability are shown for the different SPIO concentrations used. C) Corresponding percentages of Rhodamine B fluorescence are shown for the different SPIO concentrations used.*

6.2.5 Isolation of Lgr5-Rhodamine Positive Cells in Endometrium

Isolation of Lgr5 positive cells in the endometrium

After mechanical disaggregation and enzymatic digestion of the endometrial tissue, a suspension of viable single cells was obtained. Epithelial and stromal cells were labelled with an unconjugated rabbit anti-human Lgr5 antibody (Novus 28904), followed with a goat anti-rabbit Alexa 488 secondary antibody. The whole process was carried out on ice to preserve cell viability.

To minimize the background signal some samples were also incubated with isotype controls in order to establish the appropriate baseline settings on the Legacy MoFlo

high speed fluorescence activated cell sorter (Beckman-Coulter, USA) used for the experiments.

Following this, a total of 16 samples (n=16) each of epithelial and stromal cells were examined. A mean of 1.68% Lgr5 positive staining was observed for epithelial cells, with an average viability value of 56.38%. In the case of stromal cells, a mean of 0.83% Lgr5 positive staining was observed, with an average viability value of 60.20%. Data from a single exemplary analysis is shown in Figure 30.

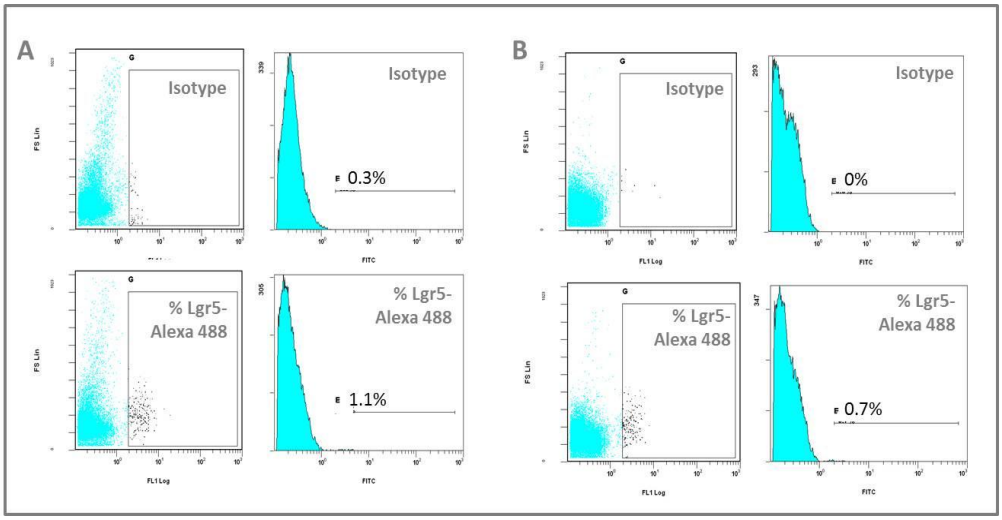

Figure 30. Flow cytometry diagram showing Lgr5 staining for epithelial and stromal cells. *Panel A shows the staining for epithelial cells whereas Panel B shows stromal cell staining.*

Isolation of Lgr5-Rhodamine B Positive Cells in the Endometrium

Using the conditions determined in previous experiments for Molday ION Rhodamine B and Lgr5 staining in epithelial and stromal endometrial cells, double-fluorescence positive Rhodamine B (Molday ION Rhodamine B) and FITC (Lgr5-Alexa 488) cells were sorted. Unstained cells, as well as cells incubated with only the secondary Alexa 488 antibody, were used to create sorting gates in order to optimize the detection and isolation of the desired cells using a MoFlo Legacy high speed cell sorter (Beckman-Coulter, USA).

Figure 31 shows FITC-Alexa 488 fluorescence in the FL1 channel (x-axis) and the Rhodamine B signal in the FL3 channel (y-axis), with DAPI staining to exclude dead cells and debris in the FL6 channel (not shown). Note that that R6 region represents the desired sorting population of double-fluorescence positive Lgr5-Alexa 488 and Rhodamine B cells.

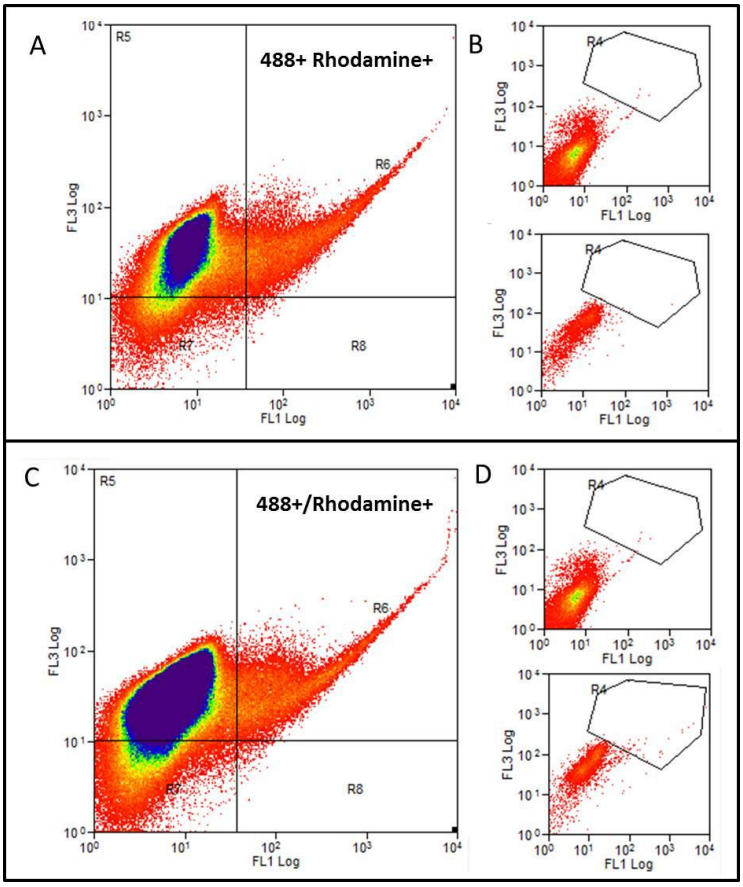

Figure 31. Flow cytometry diagram showing the double staining for Lgr5 and Rhodamine B in both epithelial and stromal cells. *Panel A shows the double staining for Lgr5-Alexa 488 and Rhodamine B in epithelial cells; Panel B shows controls: unlabelled cells (upper panel) and cells incubated only with Alexa 488 (lower panel). Panel C shows Lgr5-Alexa 488 and Rhodamine B double staining in stromal cells; Panel D shows controls: unlabelled cells (upper panel) and cells incubated only with Alexa 488 (lower panel).*

6.2.6 Xenotransplantation Assays with Lgr5-Rhodamine Positive Cells

Once the $Lgr5^{+}Rhodamine^{+}$ cells were sorted the xenotransplantation assays were performed. Freshly isolated $Lgr5^{+}Rhodamine^{+}$ and $Lgr5^{-}Rhodamine^{+}$ single cell suspensions (2,000 to 20,000 cells in each case) were injected under the kidney capsule on the left side of NOD-SCID mice (n=3 in each case), as summarized in Table V.

$Lgr5^{+}Rhodamine^{+}$ cells (n=3)	$Lgr5^{-}Rhodamine^{+}$ cells (n=3)
2,000 $Lgr5^{+}Rhodamine^{+}$ cells (C)	2,000 $Lgr5^{-}Rhodamine^{+}$ cells (B)
4,700 $Lgr5^{+}Rhodamine^{+}$ cells (F)	4,700 $Lgr5^{-}Rhodamine^{+}$ cells (E)
20,000 $Lgr5^{+}Rhodamine^{+}$ cells (D)	20,000 $Lgr5^{-}Rhodamine^{+}$ cells (A)

***Table V**. Table summarizing the injections with one of the two types of sorted cells, (Lgr5⁺Rhodamine⁺ and Lgr5⁻Rhodamine⁺) cells that each mouse received.*

Unfortunately, mice A, E, F died during the three month experimental period. Only results from the remaining mice are shown (see Figure 32).

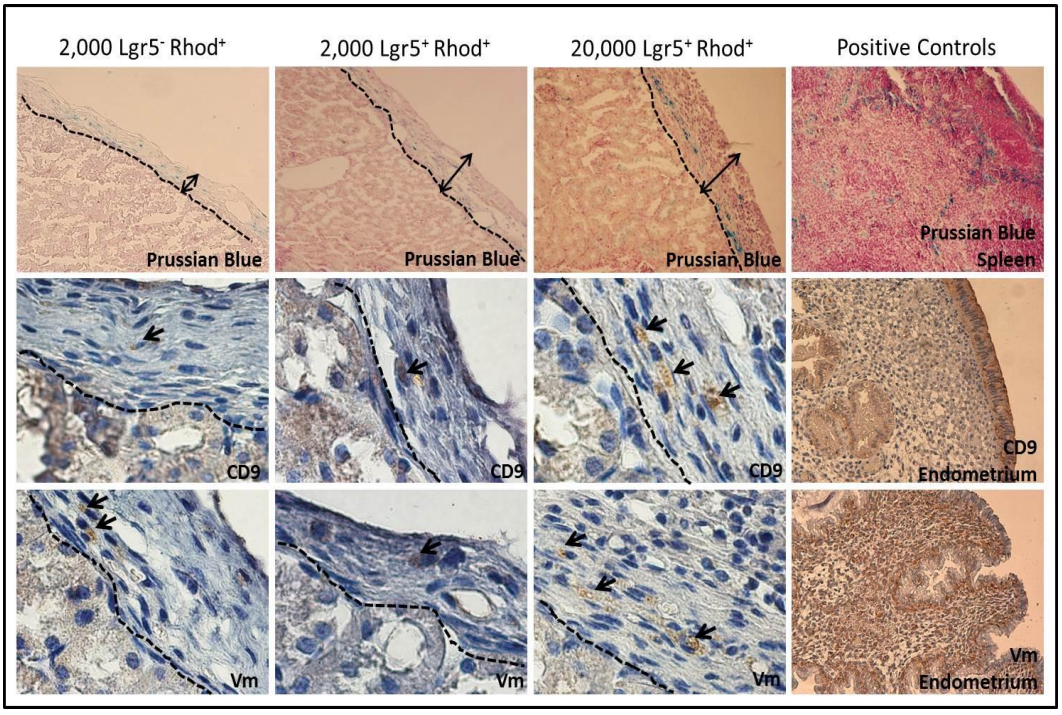

Figure 32. Staining for Prussian blue (Rhodamine B$^+$), and endometrial cell markers CD9 and Vm in the cell-injected kidneys. *Sections from the left kidneys of all three mice are displayed, and show Prussian blue, CD9, and Vm positive signals. Positive controls are shown in the last column: spleen and endometrium for Prussian blue and CD9/Vm staining respectively. Images were taken at 40× and 100× magnification.*

All three kidneys showed *de novo* tissue formation within the kidney capsule. In Figure 32 a dashed line delineates the kidney tissue from the newly formed tissue above the line and an arrow shows the new tissue growth which was greater when a larger number of cells were injected. Only this area shows positive staining for Prussian blue, which shows up as blue spots, indicating that the injected cells were Rhodamine B positive independent of Lgr5 positivity. Spleen was used as a positive control. This data suggests that all newly-formed tissue originated from the injected cells.

Immunohistochemistry for CD9 and Vm was performed in order to test if the *de novo* formed tissue present in the experimental mouse kidney capsules was endometrial-like: i.e. if it contained specific human epithelial and/or stromal cells. Endometrial tissue was used as a control for CD9 epithelial and Vm stromal cell staining. The experimental tissue staining pattern revealed some positive signals for CD9 and Vm, which corresponded to the Prussian blue stained regions, highlighted with black arrows in all cases in Figure 32. Thus, these results suggest that at least some endometrial-like tissue was formed *de novo* from the cells injected in these experiments.

VII. Discussion

VII. DISCUSSION

SSCs are the main players in the tissue homeostasis process, supporting ongoing tissue regeneration by replacing the cells lost during natural cell cycling or injury. They reside in a special microenvironment termed the 'niche' which varies in its nature and location depending on the tissue the cells reside in. It is hypothesized that abnormal functioning of endometrial SSCs and/or their neighbouring niche cells may contribute in the onset of gynaecological pathologies associated with the endometrium such as endometriosis, adenomyosis, endometrial cancer, or Asherman´s syndrome. Ultimately, better understanding the function of these putative SSCs will help to treat these endometrial disorders. However, the first key step in this process is the identification, location, and characterization of this subset of cells.

Many groups in the endometrial SSC field have focused their efforts on discovering markers and techniques to isolate this tissue-specific stem cell population. Thus in the last 5 years accumulated evidence has confirmed the existence of SP cells in human endometrium, and indeed has also demonstrated that the SP technique could represent a useful tool to isolate human endometrial SSCs (Kato et al*.,* 2007; Tsuji et al*.,* 2008; Cervelló et al*.,* 2010; Masuda et al*.,* 2010; Cervelló et al*.,* 2011; Cervelló et al*.,* 2012). Human endometrial SP cells have high clonogenic efficiency, express typical and well-described undifferentiated genes, and present a MSC phenotype (CD90, CD105, and CD73 positive, and CD34 and CD45 negative). They also have the ability to differentiate *in vitro* into mesenchymal lineages such as adipocytes, osteocytes, and chondrocytes. The final proof of the existence of human endometrial SSCs would therefore be demonstrated by the reconstruction of human endometrium in an animal model (Cervelló et al*.,* 2010; Masuda et al*.,* 2010; Cervelló et al*.,* 2011).

Future studies are likely to identify endometrial SSC-specific markers, allowing their identification and isolation from this tissue. Candidate tissue-specific stem cell surface markers such as PDGF-Rβ, CD146, and W5C5 (Gargett et al., 2009; Spitzer et al., 2012; Masuda et al*.,* 2012) were reported in the human stromal cell compartment, but definitive endometrial SSC markers have so far remained elusive.

Our study: The Contribution of the Bone Marrow as an Exogenous Source for the Endometrial Stem Cell Niche

The data presented in this study can be used to extend previous reports on the contribution of bone marrow to endometrial regeneration. We herein confirm that, in a pathological model of females who received BMT from HLA-identical male donors to treat hematologic cancers, some XY cells originating from the donor BM migrate to the recipient endometrium and transdifferentiate, contributing to 1.7% to 2.62% of the stromal and the total epithelial cell compartment. 0.45% to 0.85% of the XY donor-derived cells in the epithelial compartment displayed specific epithelial markers like CD9, and 1.0% to 1.83% of the XY donor-derived cells in the stromal compartment expressed Vm. This scenario supports the capacity of BM donor-derived cells to give rise to human endometrial tissue. Moreover functional endometrium in these patients was demonstrated by its ability to support ongoing pregnancy, such as in patients 1 and 3.

Different studies have demonstrated that multipotent human BM-donor derived cells differentiate into cell types of the functionally competent endometrium. In 2004, Taylor identified donor cells in the endometrium of recipients after BMT for the first time, and confirmed their typical epithelial and stromal features, and their corresponding hematopoietic CD45 negative phenotype (Taylor et al*.,* 2004). In line with this, Ikoma assessed firstly, the phenotype of these unexpectedly plastic BM cells, showing a typical expression pattern of the epithelial (Cytokeratin, Estrogen Receptor-α) and stromal (CD10) compartments in the absence of CD45 marker, and secondly by demonstrating successful pregnancies in two of the three patients analysed (Ikoma et al*.,* 2009). The presence of donor-derived endothelial cells in the endometrium has also been described to contribute to the formation of new blood vessels in this tissue during ongoing pregnancy, although only one case has been reported (Mints et al*.,* 2008). Moreover patients from previous reports with donor-derived cells in their endometrium had undergone a whole unfractioned bone marrow transplant, which contains a heterogeneous population of cells, including a population of primitive HSCs and MSCs which could have also contributed to the endometrium. Conversely, a very recent report in which patients received peripheral blood stem transplantation consisting in an enriched population of HSCs obtained by G-CSF mobilization confirmed that recipients do

not exhibit cell engraftment in the endometrium, suggesting that HSCs do not contribute to the endometrial SC pool (Wolff et al*.,* 2013). There is very little known about the cellular subpopulations from the BM which can contribute to uterine function.

Similarly, endometrial engraftment has been demonstrated in two murine models; Du and Taylor demonstrated BM stem cell-derived *de novo* development of the endometrium after injury, suggesting a link between endometriosis and immune disorders (Du and Taylor, 2007). Following BMT, Bratincsak and Cols detected murine donor-derived cells in the epithelial and stromal endometria of recipient animals, where $CD45^+$ cells played an important role in the stromal compartment, and were found to be a putative source of cells for epithelial regeneration (Bratincsak et al*.,* 2007). The role of G-CSF in uterine repair was also investigated in murine endometria, where reduced BM-cell migration was observed (Du et al*.,* 2012); here G-CSF seems to be less effective than it is in ischemia reperfusion and cerebral ischemia therapies (Lu and Xiao 2007; Ieishi et al*.,* 2007). Taken together, these results suggest that perhaps MSCs derived from BM are responsible for uterine repair, possibly due to their similarities with stromal endometrial cells (Du et al*.,* 2012).

This study contributes new evidence, demonstrating that XY donor-derived cells can be considered as a limited exogenous source of transdifferentiated endometrial cells rather than a cycling source of BM-derived stem cells. Firstly, these cells did not incorporate into the endometrial SP, which is broadly considered to be the endometrial SSC population because two independent groups have demonstrated ability of these SP cells to regenerate the human endometrium after their injection into the subcutaneous tissue (Cervelló et al*.,* 2010) or the renal capsule (Masuda et al*.,* 2010; Cervelló et al*.,* 2011; Masuda et al*.,* 2012) of NOD-SCID mice. Nonetheless, it is possible that BM-derived cells induce or favour the endometrial SP population compartment as an exogenous niche in response to a physiological demand. Secondly, XY donor-derived cells were not associated with the putative SC niches, as assessed by telomapping long-telomere regions (Flores et al*.,* 2005; Flores et al*.,* 2008). This approach enables cells with different telomeric lengths to be analysed, and was recently used to identify SSCs in adult tissues and organs like skin (Flores et al*.,* 2005) and human colon (Jung et al*.,* 2007).

Finally, engraftment percentages were very low and no correlation was found between the time from transplant and the engraftment efficiency, suggesting that the cells terminally differentiated randomly, perhaps due to hormonal regulation, as demonstrated by the absence of CD45 expression. In physiological terms, the transient infiltration of BM derived cells occurs in each menstrual cycle (Meng et al*.,* 2007; Taylor et al*.,* 2004). Likewise our results indicate that a final differentiation from the BM cell population to endometrial tissue might occur; this event is not related with the capabilities of endogenous endometrial stem cells because we demonstrated that no endometrial SP cells originated from the BM. Therefore, we suggest that BM cells do not contribute to the endometrial SSC pool *in situ*, but might contribute to form differentiated endometrial cells, although to a limited extent, in pathological conditions.

Similarly, XY donor-derived cells were not detected in the recipients' endometrial SP fraction. It is unlikely that the Y-chromosome is lost during engraftment as reported in lung tissue of a murine transplantation model (Herzog et al*.,* 2007), because endometrial cells do not form heterokaryons, and cell fusion was ruled out by chromosomal analyses. Hypothetically, dormant BM stem cells could become activated after stress or injury and therefore result in their mobilization from BM (Zapata 2009). Therefore our data raise questions about the mechanisms that control the differentiation of BM cells into different endometrial cell types, and why BM contribution is more important in the stromal compartment than the epithelial cell compartment. One hypothesis is that BM stem cells could provide factors that favour the reactivation of endogenous SSCs in the damaged endometrial niche.

It is surprising that in our study we discovered a reduced SP size compared to non-transplanted patients, which may be due to the use of chemotherapeutic agents. Nevertheless, the largest retrospective study, which consists of 229 centres in the European group for blood and marrow transplantation (EBMT), and includes patients who conceived naturally or by assisted reproductive techniques (ART) after BMT, demonstrates that the recipients' endometria were functional and concludes that the outcome of a pregnancy after BMT is likely to be successful (Salooja et al*.,* 2001). The latest publication on this topic assessed endometrial renewal in an Asherman's syndrome patient throughout the intrauterine

administration of BMSCs. The clinical evidence derived from this study confirms that the stimulation of dormant endometrial SSCs into a cell proliferation status to help regenerate damaged endometrium by BMSCs is a feasible hypothesis (Gargett and Healy, 2011). Therefore, BMSCs might reactivate a dormant or injured endometrial SSC niche, serving in the re-activation of a natural endogenous process.

Our study: Investigation of the Lgr5 Marker to Identify the Endogenous Endometrial Stem Cell Niche

In 2007, Clevers' laboratory performed breakthrough research focused on the identification of the genes responsible for self-renewal in small intestinal mucosa. From 80 genes identified as regulated by Wnt signalling, expression was restricted to the intestinal crypt bottoms in only 11 of them (Van der Flier et al*.,* 2007). Notably one of these genes was expressed in the crypt in a distinctive manner, this gene, termed leucine-rich repeat-containing heterotrimeric guanine nucleotide-binding protein coupled receptor 5 (Lgr5), is a cell surface receptor that specifically associates with the Frizzled and LPR protein receptors, which are activated by extracellular Wnt molecules to trigger canonical Wnt signalling, thus increasing gene expression.

Lgr5 knockout mice are non-viable, their death being caused by gastrointestinal tract dilation and malformations affecting the tongue and lower jaw that result in serious respiratory problems (Morita et al*.,* 2004). Clevers' group generated heterozygous Lgr5-lacZ mice to serve as an *in vivo* model to identify the exclusive expression of Lgr5 (which specifically marks the cycling base columnar cells of the crypt [CBCs]) by lineage tracing experiments. The cycling nature of the Lgr5 stem cell population is contradictory to the quiescent cell population located at position +4 of the crypt, which until then had been considered the only stem cell population in the small intestine (Barker et al*.,* 2007). The coexistence of both populations is regulated by a specific microenvironment in which opposing signalling pathways are either in *on* or *off* status, controlled respectively by Wnt and BMP (Li and Clevers, 2010). *In situ* hybridization studies in mice revealed a limited number of Lgr5 cells located at the crypt bottom with an actively cycling nature. This exclusive expression pattern was clearly different from genes marking Paneth or TA cells

(Barker et al., 2007). Lgr5 cells from mouse intestinal crypts also showed significant telomerase activity: a key characteristic of stem cells, as previously explained (Schepers et al., 2011).

Small intestine mucosa Lgr5-positive cells are actively cycling, retain BrdU labelling, and express Ki67 and phospho-histone H3 (Barker et al., 2007). In fact, there are significantly increased numbers of Lgr5 cells found in colon and rectum cancers, and this expression is even higher in metastatic tumours compared with primary tumours, suggesting that Lgr5 overexpression is associated with abnormal cellular proliferation and metastasis (Becker et al., 2008; Uchida et al., 2010; Takahashi et al., 2011). Characteristic features, related to endoderm origin, suggest that Lgr5 could also be considered a mucosal stem cell marker in organs such the small intestine, colon, and stomach. Lgr5 expression in all these tissues was confined to CBCs in the intestinal crypt, the colon crypt base, and the base of pyloric glands in the stomach respectively (Barker et al., 2007; Barker et al., 2010).

Lgr5 also acts as a hair follicle (i.e. ectodermal origin) stem cell niche marker. Gene expression profile studies documented that Lgr5 expression was enriched in bulge cells, and lineage tracing experiments distinguished it in the hair germ during late telogen/early anagen phases, as well as in the hair matrix during the late anagen phase (Morris et al., 2004). The Lgr5 positive population isolated from the bulge constituted around 0.6% of the parent cell population, and *in vitro* these cells formed large colonies with a high clonogenic efficiency when compared to Lgr5 negative cells taken from the same population. To test follicle reconstitution, Lgr5 positive cells sorted by FACS were transplanted into nude mice and were able to completely regenerate hair follicles at the transplantation sites (Jaks et al., 2008). Lgr5 cells have also been traced in the kidney in Lgr5-enhanced green fluorescent protein (EGFP)-ires-CreERT2 experimental model reporter mice, where they remain restricted to cell subsets within developing nephrons. These cells give rise to the ascending limb of Henle's loop and the distal convoluted tubule in mature nephrons, although this expression was permanently silenced at postnatal day 7. These data suggest that Lgr5 cells are likely stem or progenitor cells involved in kidney formation, but their absence in the mature organ might lead to slow cell turnover (Barker et al., 2012).

These findings suggest that Lgr5 is a SCC marker in tissues derived from endoderm, ectoderm, and also probably mesoderm, making it a potentially universal SSC marker.

The Wnt signalling pathway plays an important role in the maintenance and activation of the stem cell reservoir (Ivanova et al*.,* 2002; Logan et al*.,* 2004; Haegebarth and Clevers, 2009). Wnt family members are required during embryological development of the female reproductive tract, with Wnt4 and Wnt-7a identified as key regulators of female Müllerian duct development (Stark et al*.,* 1994; Vainio et al*.,* 1999). Wnt9b, which is important in the outgrowth of Müllerian ducts, is transiently expressed (Carroll et al*.,* 2005) and later, Wnt5a and Wnt7a are involved in the proper differentiation of the ducts into uterus, cervix, vagina, and oviduct (Miller and Sasson, 1998; Parr and McMahon, 1998). Indeed, mice deficient for the Wnt-7a gene completely lack uterine glands (Miller and Sasoon, 1998) and oviducts in most animals (Parr and McMahon, 1998), resulting in infertility.

In human endometrium, published data reveals that Wnt7a is specifically located in the luminal epithelium, whereas Wnt5a is restricted to the stromal compartment. No significant regulation of Wnt2, -4, -5a, or -7a has been demonstrated in the different phases of the menstrual cycle, with the exception of Wnt3 upregulation in proliferative endometrium (Tulac et al*.,* 2003). Ovarian hormones regulate Wnt signalling in endometrium to maintain the balance between oestrogen-induced proliferation and progesterone-driven differentiation (Van der Horst et al*.,* 2012; Sonderegger et al*.,* 2010). Wnt molecules (-4,-5a, -6, and -7a) are up regulated by oestrogen during the proliferative phase, while in the secretory phase progesterone acts as an activator of Wnt signalling inhibitors (Wang et al*.,* 2010). Moreover, differential expression of Wnt signalling molecules between pre- and postmenopausal endometrial epithelial cells suggests an epithelial stem cell population resides in the basalis layer (Nguyen et al*.,* 2012). All the evidence gathered suggests that the cyclic remodelling of the endometrium requires epithelial-mesenchymal communication, which is probably guided by Wnt family members (Tulac et al*.,* 2003), and that the Wnt signalling pathway participates in stem cell maintenance and differentiation.

Since the relation between Wnt pathway signalling and Lgr5 is clearly established, we hypothesized that a mechanism, similar to the gastrointestinal tract where epithelial cells in the crypts proliferate in response to activated Wnt signalling (Clevers, 2006), could be present in the human endometrium, and that Lgr5 might be a potential SSC marker for these cells.

Our identification of Lgr5 in endometrium (mesodermal origin tissue) is consistent with the idea that Lgr5 may be a universal SCC marker. Stem cell niches are usually identified based on the expression of specific protein markers on SSCs that can be tracked and localized. Different approaches were used by our group in order to characterise the expression of Lgr5 in human endometrium. We used *in situ* hybridization, to specifically localize Lgr5 gene expression in cells within the human endometrium cellular environment throughout the menstrual cycle, using specific riboprobes generated in our laboratory. Results revealed the existence of Lgr5 positive cells in a restricted epithelial and stromal area, mainly located in the perivascular regions and in the lower functionalis, near the basalis layer. Similarly results from Lgr5 protein expression in the human endometrium throughout the menstrual cycle were obtained. As expected, Lgr5 cells were localized in the epithelial glands and perivascular regions of the stromal compartment, most frequently in stromal cells, with no apparent differences in the frequency or intensity of the signal across the cycle. To support this data, the expression of Lgr5 protein was also confirmed by western blot analysis, where increased expression in the proliferative versus the secretory endometrium was observed, although these differences were not statistically significant due to small sample size analysed (n=2). However, this data is consistent with an increase in stem cell activity during the renewal of the endometrium in the proliferative growth phase.

The telomerase enzyme, which prevents telomere shortening at each cell division, maintains telomere length and is expressed in stem cells, including germ cells, and in the niche of several adult tissues. Using telomapping we identified specific 'hot' regions in the human endometrium, corresponding to the cells with the longest telomeres. Interestingly, we co-localized Lgr5 mRNA signals to some of the cells with the longest telomeres, suggesting that Lgr5 cells are associated, at least in part, with a long-telomere phenotype. The presence of Lgr5 in human endometrium, using both mRNA and protein approaches, is a novel finding and

combined with our preliminary data suggests that it co-localizes in cells with the longest telomeres. Therefore we speculate that Lgr5 may both mark and be implicated in the endometrial SSC niche.

Finally Lrg5 positive cells were isolated by flow cytometry, with percentages of 1.68% of the total cell population for epithelial cells, and 0.83% for stromal cells. The isolation of Lgr5 positive cells from endometrial samples represents a feasible method for working with this putative SSC population, enabling different assays to be performed. Thus, freshly isolated and labelled cells were used for xenotransplantation assays under renal capsule in immunocompromised mice. The identification of new putative endometrial tissue formed above the kidney capsule, which positively stained for Prussian blue was observed in both the Lgr5 positive fraction and Lgr5 negative fraction (in both epithelial and stromal cells). Additionally, this *de novo* formed tissue showed a CD9 and Vm positive phenotype (markers of differentiated endometrial cells) in some cells. Previous studies have revealed *de novo* formation of endometrial-like tissue in the kidney capsule with different kinds of putative endometrial SSCs. Thus two different groups injected around 100,000 or 500,000 SP cells either in the kidney capsule or subcutaneously respectively (Masuda et al*.,* 2010; Cervelló et al*.,* 2010; Cervelló et al*.,* 2011), and both showed endometrial-like tissue formation and expression of typical endometrial markers.

However, it is known that the SP is a heterogeneous population of stem cells, and that a marker for a purified endometrial SSC population is required. Masuda discovered a novel marker W5C5, for stromal endometrial SSC, after isolating and culturing them, 500,000 $W5C5^{+}$ cells were injected underneath the kidney capsule, reconstituting stromal tissue *in vivo* (Masuda et al*.,* 2012).

Clearly, our studies represent the first time that a very low number of cells (2,000 and 20,000) isolated by a distinctive marker like Lgr5 has been used in an *in vivo* tissue reconstitution assay. Taking all the results obtained in this thesis together, we can assume that Lgr5 marker defines the purest endometrial SSC population yet described. Nevertheless the regeneration of a well-structured endometrium *in vivo* was inefficient, likely because the niche provided by other endometrial cells was missing, and because of the low number of cells used in this new approach. Even

with the low number of Lgr5 cells injected some organization into a new structure in the kidney seemed to occur, and perhaps this population of cells would behave with more SSC-like potency if they were surrounded with an appropriate microenvironmental niche. Moreover we detected minimal human tissue-specific reconstruction (due to Prussian blue staining) when Lgr5 negative cells were injected, further suggesting the essential and vital role of the surrounding microenvironmental niche, TA cells, and differentiated cells, in this complex tissue-renewal process.

Future Perspectives: Applications in Regenerative Medicine

Stem cell based therapies, including collection, purification, manipulation, characterization, and delivery of cells for therapeutic purposes have existed since the first BMTs in 1968 (Gatti et al*.,* 1968). BMSCs include many populations of progenitor cells: HSCs, MSCs, SP cells, and MAPCs. From these, MSCs are commonly used in the lab, because they are easy to separate from other cells types by their adherence in culture, and they constitute an unlimited pool of transplantable cells. They have been used in a large number of clinical assays for the treatment of different diseases.

Considering the information complied in this work, we know that the BM contributes to the regeneration of endometria after injury by chemotherapy which results in damaged to the reproductive system. Indeed published data indicate that HSC mobilization does not improve endometrial function *per se*, hence perhaps implying that MSCs represent the pool of cells which respond to this type of endometrial damage. MSCs are similar to endometrial stromal cells by nature, therefore perhaps making it easier for this cell cohort to enhance endometrial tissue renewal. Further studies supporting this hypothesis may result in better knowledge of which kinds of cells can best restore endometrial function.

A recent case report has examined BM derived cells as a possible cell source for regenerating endometrium in a patient with Asherman´s syndrome (Nagori et al*.,* 2011; Gargett and Healy 2011). This pathology is characterized by adhesions and/or endometrial fibrosis and atrophic endometrium, most often associated with dilation and curettage of the intrauterine cavity, resulting in future obstetric complications. The case report described an innovative treatment using autologous

BM-derived stem cell populations administered directly into the uterine cavity, which regenerated the endometrium sufficiently to enable implantation of a donor oocyte IVF embryo and support the following pregnancy. The BM cells used were selected with three different markers, CD9, CD90, and CD133 in attempt to isolate angiogenesis promoting cells. While CD133 specifies a very rare subset of hematopoietic stem cells with endothelial progenitor cell activity, CD90, and CD9 are both MSC and fibroblast markers (Gargett and Healy 2011).

In studies to date, the main mechanism of action attributed to transplanted BM-derived mononuclear cells involves cell-cell contact and secretion of bioactive molecules that promote angiogenesis and tissue repair, inhibit scarring, modulate inflammatory and immune reactions, and activate tissue-specific progenitor cells, rather than by engraftment (Prockop 2009; Caplan 2009). Further clinical assays are required to further dissect this mechanism as well as to better understand the biology of these different kinds of BM cells.

Another perspective in this amazing field is tissue engineering, a rapidly expanding area of applied biology and biomedical engineering that aims to create artificial organs for transplantation, basic research, or drug development. In this context reconstruction of the uterus is one of the most ambitious targets in reproductive medicine. The design of a tissue engineering scaffold construct into which cells with a broad differentiation potential could be incorporated as an autologous cell-based therapy could provide many potential new avenues in regenerative medicine applications. The process usually starts with a three-dimensional structure called a scaffold that is used to support cells as they grow and develop. Skin, blood vessels, bladders, trachea, oesophagus, muscle and other types of tissue have all been successfully engineered in this way, and some of these tissues have already been used to treat human disease (Atala Lab, Wake Forest School of Medicine).

In literature one recent study reports an assay in which endometrial MSCs are incorporated into an elastic scaffold as a treatment to regenerate the damaged endopelvic fascia of the vagina wall for women suffering pelvic organ prolapse (Gargett laboratory note, not press). In accord with this strategy, we aim to eventually construct a uterus *de novo*, for patients with serial gynaecological problems. If our findings truly indicate an endometrial SSC population, autologous

Lgr5 positive cells would be an important candidate population for use in endometrial restoration, among the many other cells needed to histologically reconstruct the uterus. Because SSCs are able to self-renew and to differentiate into one or more type of specialized cell, Lgr5 cells with their undifferentiated phenotype might represent an important source of cells for this type of tissue engineering.

VIII. Conclusions

VIII. CONCLUSIONS

The conclusions extracted from this thesis are:

1. XY cells from bone marrow donors migrate to the recipient's endometrium, and transdifferentiate to the stromal (Vm expressing cells), and epithelial (CD9 expressing cells) cell compartments contributing to the regeneration of endometrial tissue.

2. XY donor-derived cells were not incorporated into the endometrial side population (SP), considered at least in part, to be the somatic stem cell (SSC) population.

3. XY donor-derived cells were not associated with long telomeres.

4. This evidence demonstrates that XY donor-derived cells can be considered a temporal exogenous-source of transdifferentiated endometrial cells rather than a cyclic source of bone marrow derived stem cells.

5. Lgr5 expression is present in the endometrium throughout the menstrual cycle, both at the mRNA and protein levels. Lgr5 positive cells are located in the lower functionalis, near the basalis layer, and are most frequently found in stromal compartment.

6. Some Lgr5 bearing cells have some of the longest telomeres suggesting they have this 'stemness' feature.

7. The proof-of-concept that Lgr5 is an endometrial SSC marker was demonstrated by the injection of Lgr5 positive cells in the kidney capsule of NOD-SCID mice, leading to the formation of human endometrial-like tissue.

8. In summary we demonstrate that the bone marrow derived stem cells contribute to endometrial regeneration as an exogenous source of cells capable of differentiating; whereas Lgr5 can be considered as a marker of the endogenous endometrial stem cell niche.

IX. References

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X. Annexes

ANNEX I. Consentimiento informado para la obtención análisis de muestras de tejido endometrial.

Estudio: *Estudio del origen de las células madre somáticas endometriales en mujeres con enfermedades hematológicas que reciben transplante alogenico de progenitores hematopoyéticos de donante masculino.*

Procedimiento médico: BIOPSIA ENDOMETRIAL

Usted fue sometida a un trasplante alogénico de progenitores hematopoyéticos como tratamiento de su enfermedad hematológica. Con el siguiente consentimiento se le pide su autorización para participar en un estudio piloto que, básicamente, requiere de la realización de una biopsia de endometrio. Con ello pretendemos buscar células madre en su endometrio que puedan derivar del donante usado para su trasplante, tal y como sucede con su médula ósea las cuales, como usted ya sabe, proceden de dicho donante.

Dado el interés generado sobre el estudio de células madre adultas, este proyecto en el que se le propone participar busca si dichas células están presentes en el endometrio humano. Los investigadores que participan en este trabajo ya han descrito la existencia de estas células madre adultas endometriales en un modelo animal.

El endometrio tiene una gran capacidad regenerativa lo que lo convierte en un tejido idóneo para llevar a cabo este tipo de estudio. La localización, en una primera parte del desarrollo del proyecto y más adelante, el aislamiento, la cuantificación y generación de líneas de células madre endometrial procedentes de tejido endometrial humano suponen el objetivo central de dicho proyecto.

Con el fin de que pueda decidir si desea participar en este estudio, usted debe comprender las ventajas e inconvenientes del mismo para que sea capaz de tomar una decisión informada al respecto. Este proceso es lo que se conoce como consentimiento informado.

Este consentimiento informado le da información detallada sobre el estudio y además, su médico comentará esta información con usted. Cuando haya comprendido el estudio se le solicitará que firme este consentimiento informado si desea participar en él. Se le dará una copia de este documento para que pueda guardarlo. Su participación en este estudio es voluntaria.

Descripción del proceso y posibles riesgos:

La toma de una biopsia se trata de una intervención consistente en la extirpación de un fragmento de tejido, con fines diagnósticos y/o terapéuticos. En su caso concreto la biopsia se hará de endometrio. Toda intervención quirúrgica, tanto por la propia técnica como por el estado de salud de cada paciente (diabetes, cardiopatías, hipertensión, anemia, obesidad, edad avanzada...etc.) lleva implícita una serie de posibles complicaciones comunes y otras potencialmente más importantes que podrían requerir tratamientos complementarios, tanto médicos como quirúrgicos, así como, excepcionalmente, un porcentaje mínimo de mortalidad.

Las complicaciones específicas de la biopsia más frecuentes son: hemorragia (inmediata o tardía); infección; quemaduras accidentales en caso de utilizar electrocirugía (tanto en el electrodo activo como quemaduras distales); excepcionalmente, reacción vagal con lipotimia (desmayo); reacción alérgica al anestésico local. Si en el momento del acto quirúrgico surgiera algún imprevisto, el equipo médico podrá modificar la técnica quirúrgica habitual o programada.

Alternativas:

No se contemplan alternativas para la obtención de tejido endometrial en el contexto de este estudio distintas a la biopsia endometrial clásica.

Riesgos personalizados:

Derivados de la situación particular de cada paciente (cumplimentar si procede):

.....

Aspectos legales:

1.- Las muestras serán almacenadas utilizando códigos alfanuméricos sin que en el registro conste el nombre u otros datos personales del enfermo, a excepción de su número de historia clínica. El responsable de dicho registro será el Dr. D. Miguel Ángel Sanz Alonso. A estos datos sólo tendrán acceso los componentes de los diferentes grupos investigadores y centros encargados del paciente, manteniendo la confidencialidad de los datos. Estos datos están protegidos por la Ley 15/99 de Protección de Datos de Carácter Personal. De acuerdo con dicha ley usted puede ejercer el derecho de acceso, rectificación, cancelación y oposición en cualquier momento solicitándolo por escrito a la siguiente dirección:

- Servicio de Hematología y Hemoterapia

- Hospital Universitario La Fe. Edificio Central. Planta Baja.
- Avda. Campanar 21, Valencia 46009
- Teléfono de contacto: 963862745

De la misma manera usted podrá solicitar la destrucción de sus muestras almacenadas solicitándolo por escrito a la misma dirección.

2.- Los estudios de investigación que se vayan planteando serán los más indicados en cada momento al conocimiento científico vigente. Usted puede no dar su consentimiento sin que ello suponga ningún perjuicio para usted.

3. Los resultados de las investigaciones serán públicos.

4. El material biológico recogido y almacenado pasará a ser propiedad de las autoridades competentes y su destino y plazo para dicha propiedad será decidido por las autoridades competentes, sin perjuicio de sus derechos individuales.

5.- Bajo ningún concepto y en ningún momento las muestras serán motivo de lucro directo, bien sea por la venta del material o de los derechos para realizar estudios sobre los mismos.

6.- En algunos casos será necesario enviar la muestra a otro centro para su almacenamiento y/o para llevar a cabo los estudios en su totalidad o parcialmente.

7.- Es posible que de las investigaciones no se derive ningún beneficio directo para el paciente.

8.- Si con posterioridad a haber dado el consentimiento para la realización de la prueba usted quisiera revocarlo, podría hacerlo libremente en cualquier momento.

9.- En ningún momento ni bajo ningún concepto dicha información o dichas muestras biológicas serán empleadas con otros fines diferentes a los aquí expuestos. En caso de que así fuera, sería informado y se solicitaría un nuevo consentimiento para los mismos.

CONSENTIMIENTO DE OBTENCIÓN Y ALMACENAMIENTO DE MUESTRAS

Apellidos:.....

Nombre:.....

DECLARACIONES Y FIRMAS**:**

Declaración del enfermo:

- He sido informado por el médico abajo mencionado de:
  - las ventajas e inconvenientes del procedimiento arriba indicado
  - las posibles alternativas al mismo
- He podido formular todas las preguntas que he creído oportunas.
- He comprendido la información recibida y puedo retirar mi consentimiento:
  - Cuando quiera
  - Sin tener que dar explicaciones
  - Sin que esto repercuta en mis cuidados médicos

Dicho lo cual, OTORGO LIBREMENTE MI CONSENTIMIENTO para el procedimiento

Nombre..... Firma: .....

No otorgo el consentimiento para el almacenamiento y use descrito de muestras

Nombre:..... Firma: .....

Declaración del médico, de que ha informado debidamente al paciente.

Nombre: ..... Firma: .....

Declaración del familiar, persona allegada o representante legal, en su caso, de que han recibido la información por incompetencia del paciente.

Nombre ..... Firma .....

REVOCACIÓN

Nombre D/Dª.....

ANNEX II. Consentimiento informado para la obtención de muestras de tejido endometrial.

TÍTULO DEL ESTUDIO: *Demostración de que Leucine-rich repeat-containing G protein coupled receptor 5(Lgr5) es un marcador de células madre endometriales. Desde los modelos animales hasta su contribución en terapia celular e ingeniería tisular.*

CÓDIGO DEL PROMOTOR: 1203-C-098-IC-F

PROMOTOR: Fundación IVI e Instituto Universitario IVI-Universidad de Valencia-INCLIVA

INVESTIGADOR PRINCIPAL/DPTO. /EMAIL: Dr. Carlos Simón / Departamento de Ginecología y Obstetricia-Fundación IVI/ Carlos.Simon@ivi.es

CENTRO: Fundación IVI

1.INTRODUCCIÓN

Nos dirigimos a Vd. para informarle sobre un estudio de investigación, aprobado por el Comité de Ética, en el que se le invita a participar.

Nuestra intención es tan sólo que Vd. reciba la información correcta y suficiente para que pueda evaluar y juzgar, si quiere o no participar en este estudio.

Para ello le ruego lea esta hoja informativa con atención, pudiendo consultar con las personas que considere oportuno, y nosotros le aclararemos las dudas que le puedan surgir.

2.PARTICIPACIÓN VOLUNTARIA

Debe saber que su participación en este estudio es voluntaria, y que puede decidir no participar, o cambiar su decisión y retirar su consentimiento en cualquier momento, sin que por ello se altere la relación con su médico ni produzca perjuicio alguno en su tratamiento.

3. DESCRIPCIÓN GENERAL DEL ESTUDIO

Nuestro equipo investigador trabaja desde hace años en la búsqueda de células madre adultas en el útero y más concretamente en el endometrio, que es la capa de tejido mucoso que recubre la cavidad uterina. El gran problema que existe hoy en día es que debido a la novedad de este tipo celular, tenemos grandes incógnitas en nuestras investigaciones. Una de ellas y que es el principal objetivo de nuestro proyecto científico es buscar un marcador

celular, una señal concreta que tengan estas células y que nos permitan identificarla dentro del conjunto de células que componen el organismo, o en nuestro caso el endometrio. Estudiaremos las células que obtendremos de las biopsias endometriales.

El objetivo de la toma de muestra es conocer la implicación y existencia del marcador Lgr5 en las pacientes/donantes mediante una toma de muestra endometrial. La toma de muestra o biopsia endometrial se realizará en el momento de la punción ovocitaria; este protocolo no altera ni interrumpe para nada el éxito de la técnica de extracción de ovocitos. La toma de biopsia endometrial no supone riesgo alguno para la paciente y tampoco dolor ya que se realiza en el momento de la punción ovocitaria, por lo tanto cuando Vd. está en quirófano y sedada, bajo anestesia general. Vd. sólo será sometida a una toma de biopsia el día de la punción y no es necesario que vuelva para una nueva toma. Todo esto se lleva a cabo dentro del propio ciclo de donación, con la medicación propia de este proceso.

Las muestras serán: anónimas (no es posible su vinculación a una persona identificable por nombre, dirección, número de historia clínica...). Tras la extracción de la muestra se le asignará un código que será su único método de identificación. La muestra se conservará en un lugar seguro y de acceso restringido. Se conservará por un período de tiempo limitado y proporcional al tiempo necesario para llevar a cabo los objetivos establecidos. La muestra se consumirá completamente para el estudio indicado en este proyecto.

Las células madre han adquirido gran importancia en el área de la Biología en los últimos años. Estas células madre han generado grandes expectativas en investigadores y pacientes, y en la sociedad en general. Existen diferentes tipos de células madre, entre los más destacados están las células madre embrionarias (derivan de la masa celular interna del embrión) y las células madre somáticas o adultas (CMS), siendo estas últimas las células de interés de nuestro estudio.

Las CMS son células adultas pero indiferenciadas/inmaduras y por lo tanto parecidas a las embrionarias pero que se encuentran en los tejidos/órganos de los seres adultos. Son las responsables de la regeneración de nuestros órganos y tejidos de forma natural, además de saberse hoy en día que su degeneración puede dar lugar a diferentes tipos de cáncer.

Por lo tanto debido a la falta de marcadores específicos de CMS endometriales o del endometrio, nuestro laboratorio ha trabajado en la búsqueda de células madre de endometrio en ratones utilizando diferentes técnicas (Cervelló et al., 2007), y también en el modelo humano mediante una técnica basada en características celulares (Cervelló et al., 2010, Cervelló et al., 2011, Cervelló et al., 2012). Pero no hemos descubierto aún un

marcador universal para la detección de estas células madre adultas o somáticas en el endometrio.

Aún así existen evidencias científicas en intestino y piel que han proporcionado datos sólidos que muestran que una proteína de superficie celular llamada Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) es un marcador específico de las CMS en estos órganos y tejidos.

Nosotros hemos trabajado con esta misma proteína con el fin de conocer su papel en el endometrio y nuestros resultados preliminares han proporcionado datos muy positivos para centrarnos en un estudio mucho más concreto de este posible marcador celular.

4. BENEFICIOS Y RIESGOS DERIVADOS DE SU PARTICIPACIÓN EN EL ESTUDIO.

No existe ningún riesgo para la salud que derive de la toma de muestras de biopsia endometrial en las pacientes/donantes.

Es muy posible que los resultados obtenidos en esta investigación tengan poco valor diagnóstico o predictivo para usted, pero podrá ayudar a conocer la presencia de células madre somáticas en el útero y el tratamiento de futuros pacientes con diferentes tipos de patología.

Los beneficios esperados derivados de este proyecto son inmensamente útiles para la sociedad y para la Medicina en general.

5. CONFIDENCIALIDAD Y TRATAMIENTO DE DATOS

El tratamiento, la comunicación y la cesión de los datos de carácter personal de todos los sujetos participantes se ajustará a lo dispuesto en la Ley Orgánica 15/99 de 13 de diciembre de protección de datos de carácter personal. De acuerdo a lo que establece la legislación mencionada, usted puede ejercer los derechos de acceso, modificación, oposición y cancelación de datos, para lo cual se deberá dirigir a su médico del estudio.

Los datos recogidos para el estudio estarán identificados mediante un código y sólo su médico del estudio/colaboradores podrán relacionar dichos datos con Usted y con su historia clínica. Por lo tanto, su identidad no será revelada a persona alguna salvo excepciones en caso de urgencia médica o requerimiento legal. El acceso a su información personal quedará restringido al médico del estudio/colaboradores, autoridades sanitarias, al Comité Ético de Investigación Clínica y personal autorizado por el promotor, cuando lo precisen para comprobar los datos y procedimientos del estudio, pero siempre

manteniendo la confidencialidad de los mismos de acuerdo a la legislación vigente en nuestro país.

6. SEGURO

El Promotor del Estudio, esto es, la Instituto Universitario IVI-Valencia, dispone de una Póliza de Seguros de Responsabilidad Civil en vigor que se ajusta a la legislación vigente.

7. COMPENSACIÓN ECONÓMICA

El Investigador del estudio es el responsable de gestionar la financiación del mismo. La paciente no recibirá remuneración alguna. Su participación en el estudio no le supondrá ningún gasto adicional al tratamiento que se le practica en la clínica.

Aun así queremos informar al participante la gratuidad de la transmisión de la propiedad de órganos, tejidos y células siempre que estén destinados meramente a la investigación científica.

OTRA INFORMACIÓN RELEVANTE

Cualquier nueva información relevante referente al estudio y que pueda afectar a su disposición para participar en el estudio, que se descubra durante su participación, le será comunicada por su médico lo antes posible. Si usted decide retirar el consentimiento para participar en este estudio, ningún dato nuevo será añadido a la base de datos y, puede exigir la destrucción de todas las muestras identificables previamente retenidas para evitar la realización de nuevos análisis.

Al firmar la hoja de consentimiento adjunta, se compromete a cumplir con los procedimientos del estudio que se le han expuesto.

CONSENTIMIENTO INFORMADO PARA INVESTIGACIÓN CON MUESTRAS BIOLÓGICAS HUMANAS ANONIMIZADAS

TITULO DEL PROYECTO: Demostración de que Leucine-rich repeat-containing G-protein coupled receptor 5(Lgr5) es un marcador de células madre endometriales. Desde los modelos animales hasta su contribución en terapia celular e ingeniería tisular.

Código: 1203-C-098-IC-F

Fecha: _____ Nº de historia: _____

Dª. *(nombre y dos apellidos)* _____

CON DNI Nº _____ Y DOMICILIO EN_____

DECLARO que he leído la Hoja de Información del Proyecto de Investigación que se me ha entregado, que he podido hacer preguntas y que he recibido suficiente información por parte del investigador D./Dª _____, quien me ha explicado todos los pormenores del mismo, en particular de:

1.-La finalidad de la investigación o de la línea de investigación para la que consiento.

2.- De los beneficios esperados con la misma.

3.- De los posibles inconvenientes vinculados con la donación y obtención de la muestra, incluida la posibilidad de ser contactado con posterioridad con el fin de recabar nuevos datos u obtener otras muestras.

4.- De la identidad del responsable de esta investigación, el Dr. _____

Declaro además que comprendo que mi participación es voluntaria, por lo que puedo retirarme de la investigación:

1º Cuando lo desee.

2º Sin tener que dar explicaciones.

3º Sin que esto repercuta en mis cuidados médicos.

Presto libremente mi conformidad para participar en esta investigación y doy mi consentimiento para el acceso y utilización de mis datos en las condiciones detalladas en la Hoja de Información que se me ha entregado.

Firma de la donante:

Firma del médico/investigador:

Nombre:

Nombre:

Fecha:

Fecha:

ANNEX III. Solution composition used in the in situ hybridization process.

SOLUTION	COMPOSITION
1× Proteinase K Buffer	**1M Tris pH8 + 250mM EDTA pH 8 + dH2O**
10× PK Stop Solution	**Glycine +10× PBS-DEPC**
20× SSC-buffer	**NaCl + Tri sodium citrate pH 7**
Buffer 1 pH 7.5	**Maleic acid + NaCl + H2O miliQ**
10× Bloking Solution	**Bloking reagent + Buffer 1 (10×) Use 1x Solution**
5× RNAse Buffer	**NaCl + 1M Tris pH 7.5 + 250mM EDTA pH 8**
Ribonucleic Acid Solution	**ssDNA 10mg/mL + tRNA 25mg/Ml + total RNA25mg/mL**
TTAA	**Triethanolamine + H_2O DEPC + Acetic Anhydride**
Buffer 3	**Tris pH 9.5** **MgCl** **NaCl** **dH_2O DEPC**
Hybridization Buffer	**Deionized Formamide** **1M Tris pH 7.4** **250mM EDTA pH8** **5M NaCl** **Dextran sulphate** **50x Denhardt's Solution** **H_2O DEPC**

ANNEX IV. Publications resulting from this research work.

- International Scientific Publications:

Cervelló I, **Gil-Sanchis C**, Mas A, Simón C. "Current understanding of Endometrial Stem cells". Expert Review of Obstetrics & Gynecology 2009;Vol. 4(3). ISSN 1747-4108

Cervelló I, **Gil-Sanchis C**, Mas A, Delgado-Rosas F, Martínez-Conejero JA, Galán A, Martínez-Romero A, Martínez S, Navarro I, Ferro J, Horcajadas JA, Esteban FJ, O'Connor JE, Pellicer A, Simón C. "Human endometrial Side Population exhibit genotypic, phenotypic and functional features of somatic stem cells". PLoS ONE 2010 24;5(6). doi:10.1371/journal.pone.0010964

Cervelló I, Mas A, **Gil-Sanchis C**, Peris L, Faus A, Saunders PT, Critchley HO, Simón C. Reconstruction of endometrium from human endometrial side population cell lines. PLoS ONE. Jun ; 2011;6(6):e21221 doi:10.1371/journal.pone.0021221

Cervelló I, **Gil-Sanchis C,** Mas A, Faus A, Sanz J, Moscardó F, Higueras G, Sanz MA, Pellicer A and Simón C. "Bone marrow-derived cells from male donors do not contribute to the endometrial side population of the recipient". PLoS ONE. *7(1): e30260, 2012*

Mas A., Cervelló I., **Gil-Sanchis C.,** Faus A., Ferro J., Pellicer A., Simón C. "Identification and characterization of the human leiomyoma side population as putative tumor-initiating cells". Fertil Steril. 2012 May 23. PubMed PMID: 22633281.

Cervelló I, Mas A, **Gil-Sanchis C,** Simón C. "Somatic stem cells in the human Endometrium". Semin Reprod Med. 2013 Jan;31(1):69-76. doi: 10.1055/s-0032-1331800. Epub 2013 Jan 17. PubMed PMID: 23329639.

Gil-Sanchis C, Cervelló I, Mas A, Faus A, Pellicer A, Simón C. Leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5) as a putative human endometrial stem cell marker. Mol Hum Reprod. 2013 Mar 20. PubMed PMID: 23475985.

- Oral Presentations and Posters

7º ISSCR (International Society for Stem Cells Research). Barcelona (España), 2009. International Society for Stem Cells Research. Poster– Adipogenic Differentiaton of Side Population cells from human Endometrium.

Cervelló I, Mas A, **Gil-Sanchis C**, Martínez-Romero A, Martínez-Escribano S, Cañete P, Domingo S, Bellver J, Ferro J, Pellicer A, O´Connor JE, Simón C.

14th World Congress of Gynecological Endocrinology. Firenze, (Italia), Marzo 2010. International Society Gynecological Endocrinology (ISGE) Oral Presentation – What we do know about endometrial stem cells? **Gil-Sanchis C.,** Simón C.

57th Annual Meeting of the Society for Gynecologic Investigation. Orlando, Florida (USA), 2010. Society for Gynaecology Investigation (SGI) Poster – Somatic Stem cell line from human endometrium. Mas A, Cervelló I, **Gil-Sanchis C**, Martínez-Romero A, Galán A, Ruiz V, Ferro J, Pellicer A, O´Connor JE, Pellicer, Simón C.

ESHRE 27th Annual Meeting. Stockholm (Suecia), Julio 2011. European Society of Human Reproduction and Embryology (ESHRE). Poster– Identification and characterization of putative leiomyoma stem cells. **Preselected for the Basic Science Award.** Mas A, Cervelló I, **Gil-Sanchis C**, Peris-Pardo L, Faus A, Ferro J, Pellicer A , Simón C.

ESHRE 27th Annual Meeting. Stockholm (Suecia), Julio 2011. European Society of Human Reproduction and Embryology (ESHRE). Oral Presentation – Endometrial versus bone marrow source of somatic stem cells in human endometrium? Cervelló I, **Gil-Sanchis C**, Mas A, Santamaría X, Moscardó F, Sanz J, Higueras G, Sanz MA, Pellicer A, Simón C.

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