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# CHARACTERISTICS AND THERAPEUTIC POTENTIAL OF MENSTRUAL BLOOD-DERIVED STEM CELLS

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## Abstract

*The tremendous regenerative capacity of the human endometrium is based on the activity of adult stem cells. Endometrial stem cells are mainly located in the basal layer, but could also be successfully isolated from the functional layer, which is shed during menstruation. Menstrual blood-derived stem cells (MenSCs) can be obtained by noninvasive procedures. They are characterized by high proliferative potential, long-term culturing properties, mesenchymal stem cell-like marker expression and multilineage differentiation potential. MenSCs have been successfully employed as therapeutics in animal models of myocardial infarction, stroke, Duchenne muscular dystrophy and critical limb ischemia. Their allogenic application is not associated with immunological side effects, and does not promote tumor formation in vivo. Pilot studies have confirmed their safety upon applications in humans, and phase 1/2 clinical studies on their safety and therapeutic efficacy are ongoing. A systematic banking of immunoprofiled MenSCs will expand the therapeutic repertoire beyond autologous stem cell transplantations.*

***Key Word :*** *the human endometrium, Menstrual blood-derived stem cells , applications in humans,*

## INTRODUCTION

The human endometrium forms the inner lining of the uterus. Originating from the Müllerian ducts, it is comprised of two major zones in the adult organism (**Figure 1**): The functional layer (functionalis) largely contains glands extending from the luminal surface columnar epithelium as well as a supportive stroma. The basal layer (basalis) is composed of the basal region of the glands, a comparatively dense stroma, lymphoid aggregates, and blood vessels, including the characteristic spiral arteries in the luteal phase of the menstrual cycle. Furthermore, the endometrial stroma of both layers is populated by different classes of leukocytes, including the tissue-specific uterine natural killer cells, mast cells, macrophages, T and B cells, and neutrophils (Gargett 2007).

During the reproductive years of women, the endometrium is a highly regenerative tissue, and a remarkable example of controlled tissue remodelling governed by cyclic endocrine changes (Sherman and Korenman 1975). During reproductive life, the human endometrium can undergo about 480 cycles of growth, breakdown and regeneration. During one menstrual cycle, the endometrium grows from 0.5–1 mm to 5–7 mm in thickness (McLennan and Rydell 1965), demonstrating an enormous regenerative capacity.

The functional layer of the endometrium is shed about every 28 days during menstruation, which lasts between 3-5 days and yields on average about 35 ml menstrual blood containing cells and

tissue fragments derived from the functionalis (Salamonsen et al. 1999, Toyoda et al. 2007). Menstruation is followed by scar-free regeneration of the functional layer in the proliferative phase of the menstrual cycle, which is characterized by steadily increasing levels of the gonadotropins follicle-stimulating hormone and luteinizing hormone,resulting in increased ovarian production and release of the steroid hormone estrogen (Sherman and Korenman 1975). Peak levels of these hormones trigger ovulation and formation of the corpus luteum, which produces progesterone.

This steroid hormone dominates the endometrial changes during the second half of the menstrual cycle, the luteal phase. This phase, also called the secretory phase of the endometrium, is characterized by transformation of endometrial glands into a secretory state, glandular glycogen storage and secretion, increased angiogenesis, vascular proliferation and formation of spiral arteries (Gargett and Rogers 2001). While these changes serve to prepare the decidualized endometrium for implantation of the embryo in the case of fertilization, degeneration of the corpus luteum and the associated drop in progesterone and estrogen levels trigger menstruation if no fertilization occurs.

This transition period between the late secretory phase and menstruation is accompanied by vasoconstriction and necrosis of the functionalis layer of the endometrium and by increases in prostaglandin and matrix metalloproteinase expression and activity, ultimately promoting controlled shedding of the functionalis layer (Salamonsen et al. 1999, Salamonsen 1998). Intense research efforts in recent years have provided evidence for the concept that the highly regenerative nature of the endometrium is based on the activity of adult stem cells, as will be outlined in the following sections.

Current four evidence for stem cell activity in the endometrium : a). Stem cells - a brief introduction. The human body is composed of approximately 210 different cell types (Alberts et al. 2007). This considerable diversity is initiated during embryonic development, and is a consequence of complex differentiation processes. While differentiated cells, such as cardiomyocytes or pancreatic islet beta cells fulfil defined physiological functions within the organism, the only function of stem cells is the generation of precursors for these differentiated cell types. Thus, stem cells are undifferentiated cells that have the ability to self-renew and to generate more differentiated daughter cells through the process of asymmetric cell division **(Figure 2**).

Stem cells can be categorized based on their origin or on their differentiation potential. Embryonic Stem Cells (ES cells) are derived from the blastocyst of the 3- to 5-day-old embryo, and are plutipotent, being capable of differentiating into cells of all three germ layers (i.e. endoderm, ectoderm, mesoderm). In contrast, the zygote and its early cleavage stages up to the 16 cell stage can additionally form extraembryonic tissues and are thus called totipotent. Among the different types of stem cells, ES cells have the highest degree of developmental plasticity.

They are clonally derived, maintain a normal karyotype in culture, are immortal and can be propagated indefinitely in the embryonic state (Pera et al. 2000). Regarding therapeutic applications, the property of ES cells to form teratomas, and the ethically controversial issue of using cells derived from human embryonic tissue have limited their clinical use (Findikli et al. 2006).

A cell type not associated with such strong ethical concerns, albeit with similar developmental plasticity is the induced pluripotent stem cell (iPS cell). In a landmark paper, Yamanaka and coworkers were able to convert human skin fibroblasts, i.e., a differentiated cell type, into a pluripotent state via transduction with the four transcription factors Oct3/4, Sox2, Klf4, and c-Myc (Takahashi et al. 2007). Subsequent work showed that, depending on the cell type used as a source for iPS cell generation, less than four factors may be required to successfuly generate these pluripotent cells (Zaehres et al. 2010).

Current research activities are focussing on methods of generating iPS cells without the necessity of retroviral transfections, which would be a major breakthrough regarding therapeutic applications in humans. Nevertheless, the property of tumor formation by pluripotent cells *in vivo* still represents a drawback of this technology (Mosca et al. 2010). In contrast to these plutipotent stem cells, adult stem cells show a more restricted developmental potential. They can either be multipotent, being capable of differentiating into multiple cell types of a given lineage, or unipotent, being only capable of generating one type of differentiated cell.

Adult stem cells typically reside in an anatomical microenvironment called the stem cell niche (Schraufstatter et al. 2011). Cells and extracellular matrix within the microenvironment provide signals to the stem cell which keep it in the undiferentiated state, and which are able to trigger self-renewal and differentiation if neccessary, e.g. when tissue regeneration is required during wound healing. This becomes apparent upon considering that some established cell surface markers of adult stem cells are actually matrix receptors, such as the hyaluronan receptor CD44 (Götte and Yip 2006) or the 1-integrin CD29 (**Table I**). Signals are mediated though different pathways, involving e.g. -integrins, TGF- family members, the wnt-signaling pathway, or the notch-pathway (Götte et al. 2008, Haegebarth and Clevers 2009, Schraufstatter et al. 2011).

Upon asymmetric division of the stem cell, the daughter cells progressively undergo differentiation. These daughter cells are known as progenitor cells, or commited stem cells. Transient (transit) amplifying cells are progenitor cells which - in contrast to the original stem cell - show a high proliferation rate. During repetitive cycles of cell division, these cells progressively acquire differentiation markers, ultimately resulting in acquisition of a terminally-differentiated phenotype (Diaz-Flores et al. 2006) which has a defined physiological function within the human body (**Figure 2**). Although adult stem cells have a more limited developmental plasticity compared to embryonic stem cells, there are no major ethical concerns regarding the use of these cells for therapeutic purposes. The transplantation of bone marrow (as a source of hematopoietic stem cells) is a well-established example for a therapeutic application of adult stem cells that has become a routine procedure in clinical practice. b). Endometrial stem cells - early concepts and indirect evidence.

As pointed out earlier (1.1), the endometrium of reproductive-age women is a highly regenerative tissue, suggesting the presence of stem cell activity as an underlying principle. Early clinical observations of regeneration of functional endometrial tissue after complete endometrial ablation in monkey and later in humans, as well as metabolic radiolabelling studies in monkeys demonstrating the presence a germinal compartment localized to the lower basalis provided indirect evidence for the endometrial stem cell concept (Hartman 1944, Padykula et al. 1984, Tresserra et al. 1999). In addition, kinetic studies of endometrial cell proliferation have demonstrated zonal differences predicting an orderly replacement of differentiated endometrial glandular and stromal cells from slowly dividing putative stem cells residing in the basalis (reviewed in Gargett 2007).

These findings were supported by analysis of changes in the methylation pattern of endometrial glands (Kim et al. 2005), the demonstration of clonality in endometrial glands based on PCR analysis of non-random X-chromosome inactivation of the androgen receptor gene (Tanaka et al. 2003), as well as rare PTEN null mutations in individual endometrial glands (Mutter et al. 2000). At the functional level, the group of Caroline Gargett has performed pioneering work performing clonality assays on endometrial epithelial and stroma cells in vitro. In several key publications, her group could demonstrate that about 0.22 % of endometrial epithelial cells and about 1.25 of stromal cells are capable of forming individual colonies of >50 cells/colony within 15 days when purified single-cell suspensions of hysterectomy-derived endometrial tissue were seeded at clonal density (Chan et al. 2004), define the growth factor requirements for culturing these cells under serum-free conditions (Schwab et al. 2005), to characterize their surface marker expression profile (1.2.3) and demonstrate the in vitro differentiation potential of these cells (Gargett et al. 2009). Overall, these studies provide indirect, yet convincing evidence for a stem cell activity in the human endometrium. c). Marker expression profiles of endometrial stem cells.

Stem cells have frequently been phenotypically characterized based on the expression of a specific combination of marker genes which can be detected by low cytometric analysis or PCR-based methods (Greve et al. 2012). While specific cell surface receptor expression may reflect interaction of the stem cell with its niche, or the need for activation of particular signal transduction pathways, specific intracellularly located gene products associated with stemness are frequently transcription factors capable of steering complex cellular programs. In addition, surrogate markers of stem cell activity have been detected in endometrial stem cells.

For example, the activity and expression of telomerase, associated with a suppression of replicative senescence, is higher in the endometrium compared to most other tissues in the human body (Yokoyama et al. 1998, Götte et al. 2008). In addition, cells with the side population phenotype have been detected in the endometrium (Kato et al. 2007, Tsuji et al. 2008). Side population cells can be detected by flow cytometry based on the property of actively excluding fluorescent dyes such as Hoechst 33343 via the action of multidrug resistance proteins (Greve et al. 2012).

The side population is thought to be enriched in stem cells. About 2% of the endometrium displays the side population phenotype (Masuda et al. 2010). Human endometrial side population cells and cell lines have been reported to display intermediate telomerase activity and express the markers Oct-4, GDF3, DNMT3B, Nanog, and GABR3 (undifferentiated cells) (Cervello et al. 2011), CD105, CD146, CD90, WT1, Cardiac Actin, Enolase, Globin, REN(mesenchymal) (Tsuji et al. 2008, Masuda et al. 2010, Cervello et al. 2011), CD31, CD34 and KDR (endothelial) (Tsuji et al. 2008, Masuda et al. 2010) and EMA (epithelial) (Tsuji et al. 2008).

The results of the study by Masuda et al (2010) suggest that different subpopulations such as cells predominantly expressing mesenchymal or endothelial progenitor cell markers exist in the endometrial side population. In addition, stromal clonal endometrial cells have been shown to express the markers CD146, CD140b (PDGFRβ), ITGB1 (CD29), CD44, NT5E (CD73), THY1 (CD90), ENG (CD105), W5C5, Msi1, Notch1 and SOX2, and to be negative for endothelial or hemopoietic markers such as CD31, CD34, and CD45 (Gargett et al. 2009, Schüring et al. 2011, Masuda et al. 2012). Finally, in human endometrial tissues, expression of the pluripotency markers Oct4, Sox2, nanog and KLF4 (Matthai et al. 2006, Götte et al. 2011), and of components of the notch (Götte et al. 2008) (Figure 1B)and wnt signaling pathways (Nguyen et al. 2012) has been reported. d).

In vitro and in vivo evidence for endometrial stem cell activity. The considerable differentiation potential and developmental plasticity of endometrial stem cells has been demonstrated in several independent in vitro studies. Clonal endometrial stroma cells were shown to be multipotent and to exhibit mesenchymal stem cell-like features, as they have been differentiated into smooth muscle cells, adipocytes, chondrocytes, and osteoblasts (Schwab and Gargett 2007, Gargett et al. 2009, Schüring et al. 2011). Furthermore, cells and cell lines derived from the endometrial side population could be differentiated into adipocytes and osteocytes (Cervello et al. 2010, Cervello et al. 2011).

Endometrial side population cells could also be differentiated in vitro to produce gland (CD9+)- and stroma (CD13+)-like cells (Kato et al. 2007). Further examples of a differentiation of endometrial stem cells into insulin-producing cells, cardiomyocytes and dopaminergic neuron-like cell types will be presented in section 3.3.1. The differentiation potential of endometrial stem cells is further underscored by their potential to generate endometrial tissue in vivo. For example, Cervello et al.

(2011) were able to generate endometrial-like tissue upon injection of 2x105-1x106 human endometrial side population cell lines into the kidney kapsule of immunodeficient mice. While the data reported in the previous sections indicate that endometrial stem cells appear to have properties similar to typical tissue-resident mesenchymal (Schwab and Gargett 2007, Gargett et al. 2009, Schüring et al. 2011), or possibly also endothelial (Tsuji et al. 2008, Masuda et al. 2010) progenitor cells, there is also functional evidence for a role of bone-marrow-derived progenitor cells in endometrial tissue regeneration: In a pioneering study, Taylor (2004) reported about bone marrow transplantations between 4 human donors and recipients with an HLA-mismatch, and showed that donor-derived endometrial cells could be detected in endometrial biopsy samples from all bone marrow recipients, accounting for 0.2% to 48% of epithelial cells and 0.3% to 52% of stromal cells.

Similar results were reported later for bone marrow transplants between male donors and female recipients, using the Y-chromosome as a marker (Ikoma et al. 2009). Although Cervello et al (2012) could recently confirm the presence of XY donor-derived cells in recipient endometrium in a different collective of bone-marrow transplanted patients, their data suggested that bone-marrow derived cells did not contribute to the endometrial side population of the recipient , leading the authors to the conclusion that XY donor-derived bone marrow cells may be considered a limited exogenous source of transdifferentiated endometrial cells rather than a cyclic source of donor-derived stem cells.

## 1. Menstrual blood derived stem cells (MenSCs)

As the basal layer of the endometrium is not shed during menstruation, it has been postulated that it may be the preferential location of stem cells responsible for endometrial regeneration (Prianishnikov, 1978; Padykula et al., 1984). While this concept is supported by the finding of zonal differences in endometrial cell proliferation (reviewed in Gargett 2007), and a differential glandular epithelial expression of components of the wnt pathway in the basal vs functional layer of the endometrium (Nguyen et al. 2012), cells with adult stem cell-like properties have also been detected in and isolated from the superficial layers of the endometrium. For example, the adult stem cell marker Musashi-1 is expressed both in the basalis and functionalis layer of the endometrium, albeit with anenrichment of stem cells in the basalis (Götte et al. 2008). Moreover, clonal endometrial stroma cells with mesenchymal-stem cell-like marker expression profiles and multilineage differential potential have been isolated from the superficial layers of endometrial tissue obtained by routine biopsy techniques (Schüring et al. 2011). Similarly, endometrial side-populations cells have been obtained by Pipelle biopsy (Cervello et al. 2011).

Moreover, the contribution of bone marrow-derived cells to endometrial tissue regeneration (Taylor HS 2004, Du and Taylor 2007) additionally suggests that endometrial stem cell activity may not be exclusively localized to the basal layer. Finally, there are indications for a mentrual-cycle-dependent modulation of the functional state and release of adult stem cells: For example, an upregulation of circulating endothelial progenitor cells has been observed in a mouse model of endometriosis (Becker et al. 2011), a disease linked to altered endometrial stem cell function (Du and Taylor 2007, Götte et al. 2008, Götte et al. 2011, Chan et al. 2011). Interestingly, the quantity and differentiation potential of these cells varies during the menstrual cycle in reproductive age women: The number of CD133+/CD34−, CD133+/CD34+ progenitor cells and CD133+/CD34+/VEGF-R2+ endothelial progenitor cells per ml of blood was shown to fluctuate throughout the cycle in synchronization with circulating 17β-estradiol levels, and maturation of CD133+/VEGF-R2+ and CD133+/CD34−/VEGF-R2+ EPCs towards respective CD144+ advanced endothelial progenitor cell subpopulations was reduced at mid-luteal phase (Lemieux et al. 2009), demonstrating a relation between the release and functional properties of bone-marrow-derived progenitor cells and the menstrual cycle.

Of note, the number of endothelial progenitor cell colony-forming units shows a negative correlation to the levels of stromalcell-derived factor-1 (SDF-1), a key cytokine for release and homing of bone marrow-derived cells (Elsheikh et al. 2011). In this study, SDF-1 was also shown to beupregulated in the proliferative vs the secretory phase of the menstrual cycle.The concept of a mentrual-cycle-dependent release of endothelial progenitor cells is further supported by the finding that oral glucose-induced increase in circulating numbers of CD133+ and CD133+CD34+ cells and the endothelial differentiation potential of peripheral blood-derived endothelial progenitor cells is attenuated in insulin-resistant amenorrhoeic subjects (Bairagi et al. 2012).

Similarly, the number of Lin-/7AAD-/CD34+/CD133+/KDR+ circulating endothelial progenitor cells was found to be significantly higher in women with regular menstrual cycles compared to menopausal women and men (Rousseau et al. 2010). Finally, a pilot study suggested a possible decrease in G-CSF-mobilized CD34+ hematopoietic stem cells during menstruation (Dincer 2004). In light of these data, it is not surprising that endometrium-derived stem cells have been isolated from menstrual blood. The following sections will provide an overview over the isolation of MenSCs, their marker expression profiles and differentiation potantial, and their therapeutic potential.

### *1.2 Isolation and culture of MenSCs*

Different protocols have been applied to collect and culture MenSCs. Although we use the general term MenSCs in this review, it has to be considered that these techniques may yield at least partially different stem cell populations, as the final enriched cell population may be derived either from

different cell types of the endometrium (stroma vs glands vs endothelium vs immune cells) or from the blood.

Meng et al. (2007) collected 5 ml of menstrual blood in an antibiotic-containing solution using a urine cup-tubing method. They separated mononuclear cells by standard Ficoll centrifugation methodology and cultured these cells in complete Dulbecco's modified Eagle's medium (DMEM) supplementedwith 20% FCS. While these cells were only marginally adherent after overnight culture, an outgrowth of adherent cells with a fibroblast-like morphology wasobserved after two weeks, with media changes performed twice weekly. For further studies, these so-called endometrial regenerative cells were clonally selected in 96 well-plates. The basic protocol was later slightly modified to obtain MenSCs for a therapeutic feasibility study on human multiple sklerosis patients (Zhong et al. 2009).

Hida et al. (2008) collected mesenchymal cells from 10 ml of menstrual blood of 6 women (aged 20-30) on the first day of menstruation, and cultured the samples in DMEM high glucose supplemented with 10% FBS in two 10-cm dishes. A starting number of $1x10^7$ cells were obtained, and the cells were subsequently subjected to adenoviral transfection for differentiation studies (see 3.2.1).

Phuc et al. (2011) collected menstrual blood for 2-3h using a menstrual cup (**Figure 1C**). The blood was transferred into a Falcon tube containing PBS and 5-fold antibiotic-antimycotic mix (Sigma) and kept on ice during transport. Samples negative for bacterial or mycotic contaminations were subjected to erythrocyte lysis and Ficoll-Paque purification of the mononuclear cell fraction, which was subjected to FACS-based cell sorting and *in vitro* differentiation into dendritic cells (see 3.1.2).

A very detailed protocol for isolation of menstrual blood-derived stem cells has recently been published by Allickson et al. (2011). Menstrual blood was collected during the time of heaviest flow during the cycle using a menstrual cup, which was kept in place for less than 4h. The blood was transferred to cooled sterile buffered media containing heparin and antibiotics and cells were further processed in the presence of antibiotics (Vancomycin, Cefotaxime sodium, Amikacin, Gentamycin and Amphotericin B) within 24h. For this purpose, cells were washed, concentrated and ultimately cryopreserved by addingprecooled 10% Dimethylsulfoxide (DMSO), 30% PBS, 10% Human Serum Albumin to the cell suspension, aliquoted, and cryopreserved in a controlled rate freezer, prior to transferring them to a liquid nitrogen vapor storage freezer.For further applications, cells were thawed, cultured in Chang's Complete Media (Chang et al. 1982), and enriched for a subpopulation expressing the c-kit receptor CD117 using antibody-coupled magnetic beads (MACS sorting). In a study on 150 menstrual blood samples, the authors achieved an average yield of approximately $8x10^6$ cells per preparation (Allickson et al. 2011). MenSCs displayed a fibroblast-like morphology.

Nikoo et al. (2012) collected 3–5 mL of menstrual blood on day 2 of the menstrual phase from 5 women (22-45 years) using a menstrual cup. The samples were transferred to PBS containing amphotericin B, penicillin, streptomycin and 2 mMEDTA, and mononuclearcells were separated using Ficoll-Paque. MenSCs were cultured in T75 flasks at 37°C, 5% $CO_2$in DMEM-F12medium containing the antibiotics mentioned above and 20% FBS. 1–1.5 $x10^6$ mononuclear cells were recovered per milliliter of menstrual blood. The MenSCs had a stable fibroblast-like spindle-shaped morphology at later passages.

In summary, these studies demonstrate that MenSCs can be obtained using easy non-invasive procedures and that they can be cultured using routine cell biological techniques.

### *2.3 Evidence for MenSC stemness*

Adult stem cells are routinely characterized based on characteristic marker expression profiles, on their multilineage differentiation potential *in vitro*, and on additional properties such as long-term culturing properties and clonality. The defining criteria for multipotent mesenchymal stroma cells, for example, state that these cells must be plastic-adherent when maintained in standard culture conditions, must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules and that they must differentiate to

osteoblasts, adipocytes and chondroblasts *in vitro* (Dominici et al. 2006). As outlined in the following sections, MenSCs fulfil the criteria of a specific marker expression, and of multilineage differentiation potential, in addition to showing long-term culturing properties in the absence of chromosomal aberrations.

### *2.4 Marker expression profiles*

An overview of stem cell marker expression profiles of MenSCs is provided in Table I. Meng et al (2007) demonstrated the expression of CD9, CD29, CD41a, CD44, CD59, CD73, CD90 and CD105, and absence of monocyte and hematopoietic stem cell markers on mononuclear cell-derived 'endometrial regenerative cells' isolated from menstrual blood. The cells did not express the embryonic stem cell markers SSEA-4 and nanog.In contrast, the CD117-positive MenSCs isolated by Allickson et al. (2011) were characterized by expression of the mesenchymal stem cell markers CD13, CD29, CD44, CD49f, CD73, CD90, CD105, CD166, MHC Class I and the pluripotent embryonic stem cell markers SSEA-4, Nanog and Oct-4. Furthermore, telomerase activity could be demonstrated comprising about 50% of the activity of embryonic stem cells, similar to previous findings by Patel et al. (2008), who also demonstrated expression of pluripotency markers such as expressed markers of pluripotency, such as Oct-4, SSEA-4 and c-kit. The cells were negative for CD133, the endothelial progenitor marker CD34 and the leukocyte common antigen CD45.Zemel'ko et al (2011) reported isolation of MenSCs which were positive for expression of CD73, CD90, CD105, CD13, CD29 and CD44. 50% of the cells were positive for the ES cell marker SSEA-4. MenSCs isolated by Nikoo et al.

(2012) were positive for the mesenchymal stem cell markers CD9, CD29, CD73, CD105, and CD44 and the ES cell marker Oct-4A, while CD34, CD133, CD45,CD38, and STRO-1 expression was not detectable. Menstrual blood-derived cells used for *in vivo* therapy of a mouse model of Duchenne muscle dystrophy displayed expression of CD13, CD29, CD44, CD54,CD55, CD59, CD73, CD90, and CD105, and were negative for CD133, CD34, CD45 and monocyte-macrophage antigens such as CD14, a marker for macrophage and dendritic cells (Cui et al. 2007). Furthermore, the cells did not express CD31 (PECAM-1), CD50 or c-kit. The cell population was positive for HLA-ABC, but not for HLA-DR.Most recently, Khanmohammadi et al. (2012) demonstrated that MenSCs show a marker expression profile which is very similar to bone-marrow-derived mesenchymal stem cells, including expression of CD44, CD73, CD90, and CD105. A noteable exception was the high expression of the pluripotency factor Oct-4 in MenSC, which was not detected in the bone-marrow derived cells.

While it has to be taken into account that differences in marker expression are at least partially due to physiological donor variability and the fact that the MenSC isolation procedures were not uniform, these studies demonstrate that MenSCs show a marker profile which is very similar to mesenchymal stem cells, and that they frequently express the pluripotency factor Oct-4.

### *2.2.2 Functional properties*

Several independent studies have clearly demonstrated that MenSCs possess adult stem cell properties. The 'endometrial regenerative cells' isolated by Meng et al. (2007) could be maintained in tissue culture for >68 doublings without showing karyotypic abnormalities. Compared to umbilical cord derived mesenchymal stem cells, these cells were characterized by a higher proliferation rate and increased production of MMP3, MMP10, GM-CSF,angiopoietin-2 and PDGF-BB. The authors explored the differentiation potential of these cells by performing in vitro differentiation experiments. Although the authors reported successful differentiation into 9 lineages (cardiomyocytic, respiratory epithelial, neurocytic, myocytic, endothelial, pancreatic, hepatic, adipocytic, and osteogenic), a potential caveat is associated with this study, as proof for successful differentiation was only based on a single marker immunostaining in many cases. Therefore, a more thorough investigation would have been worthwhile. Patel et al. (2008) demonstrated that stromal cells derived from menstrual blood showed clonogenic properties and had the ability to differentiate into mesoderm- and ectoderm-derivedtissues. The MenSCs isolated by Allickson et al. (2011) could be subcultured up to 47 times before complete senescence and death. Karyotypic analysis demonstrated the maintenance of diploid

cells without chromosomal abnormalities.Regarding the in vitro differentiation potential, the authors could clearly demonstrate the potential for multilineage differentiation with efficiencies ranging between 40-70%: Adipogenic differentiation was confirmed by Oil Red O staining of lipid vacuoles, whereas osteogenic differentiation was demonstrated by Alzarin Red Staining and qPCR for alkaline phosphatase expression. Chondrogenic differentiation was assessed by alcian blue staining for sulfated glycosaminoglycans, and neurogenic differentiation was demonstrated by neurofilament-3 and nestin PCR as well as positive immunocytochemistry for tubulin-III, glial fibrillar acidic protein, MAP-2 and nestin. In addition, cardiomyogenic differentiation was shown by immunocytochemistry of in vitro differentiated cells for actin, desmin, troponin and connexin 43. In accordance with these findings, a recent study indicated than MenSC have a chondrogenic differentiation potential which is very similar to bone-marrow derived mesenchymal stem cells, albeit the mRNA expression patterns of the chondrogenic markers collagen 2A1, collagen 9A1 and SOX9 differed between the differentiated cells, depending on the tissue source of the stem cells (Khanmohammadi et al. 2012).

## 3. Therapeutic applications of MenSCs and endometrial stem cells

### *3.1 In vitro differentiation potential - MenSCs as a therapeutic cell source*

While the studies presented in section 2.2.2 have demonstrated the multilineage differentiation potential of MenSC, these differentiation experiments mainly served to provide proof of principle for the developmental plasticity of these cells. In contrast, more advanced studies have aimed at generating specific cell types for therapeutic purposes. In the following sections, we will present two of these approaches, the generation of cardiomyocyte-like cells, and the generation of dendritic cells from MenSCs *in vitro*. Selected therapeutic applications of MenSCs and endometrial stem cells are listed in **Table II**.

#### *3.1.1 Generation of cardiomyocyte-like cells from MenSCs*

Autologous cells with cardiomyocyte-like properties can be of considerable value for the treatment of myocardial infarction, one of the major causes of death in Western societies. A successful application of MenSC-derived cells in an animal model of myocardial infarction will be described in section 3.2.1. By modifying the original protocol of Hida et al. (2008), Ikegami et al. (2010) were able to perform a cardiomyogenic differentiation of MenSCs under serum-free conditions.Murine fetal cardiomyocytes were initially cultured as feeders on laminin coated plates in a medium consisting of M199 medium containing 5 mM creatine, 2 mM L-carnitine, 5 mM taurine, 0.1 nM thyroxin (T3), 0.1 mM insulin, 2.5 mM pyruvate, and 2 mg/mL heat-treated bovine serum albumin (Fraction-V). MenSCs were subsequently cultured at a density of $3x10^3$cells /cm² for cardiomyogenic induction. The previous study by Hida et al (2008) had indicated that no 5-aza-cytosine was necessary to promote differentiation. Cells started beating after 3 days, and were immunopositive for cardiac troponin-I, -actinin, and connexin 43 at evaluation after one week of induction. Successful cardiomyogenic differentiation was demonstrated at the functional level by analysis of fractional shortening of the cell and of action potentials, which were shown to be more physiological using serum-free differentiation compared to serum-containing media.Apart from the increased functionality of the differentiated cells, the new protocol presents a major advance for potential therapeutic applications, as it eliminates safety issues linked to the use of animal-based sera.

#### *3.1.2 Generation of dendritic cells from menstrual blood cells*

Based on their immunomodulatory properties, dendritic cells are currentlybeing used in the immunotherapy of cancer and for the purpose of cancer vaccination (Steinman and Dhodapkar 2001, Palucka et al. 2011). The therapeutic approach involves the in vitro generation of dendritic cells from a patients monocytes or hematopoietic stem cells, and a reintroduction of these autologous cells into the patient after stimulation with tumor-specific antigens. While previous studies have relied on the use of peripheral blood- or umbilical cord blood-derived monocytes or hematopoietic stem cells from bone

marrow and blood sources, recent work by Phuc et al. (2011) has shown that these cells can also be generated from menstrual blood-derived cells.

The authors used the mononuclear cell fraction of menstrual blood as a source for a FACS-based sorting of CD4+ T-cells and monocytes.Employing a two step in vitro differentiation protocol involving culturing of menstrual blood-derived monocytes in the presence of IL-4 + GM-CSF to generate imDCs, and a subsequent 24h exposure to TNF-□, the authors could generate dendritic cells characterized by their characteristic morphology, expression of HLA-DR, HLA-ABC,CD80 and CD86, IL-12 release, uptake of dextran-FITC, and by their capability to stimulate allogenic T cell proliferation. Although the authors emphasized the advantage of using a quantitatively attractive noninvasive source for dendritic cell generation, the yield after differentiation was still comparably low (68%). Therefore, it remains to be shown if menstrual blood-derived dendritic cells will be a viable alternative for more traditional established cell sources.

### *3.2 Therapeutic efficacy of MenSCs in animal models of disease*

In the following sections, examples for a successful therapeutic application of human MenSCs in established animal models of human diseases will be presented. Two major conceptual differences become apparent in these studies: One frequently used approach involves a pre-differentiation of MenSCs into a defined cell type which is used to replace the respective damaged cell type in the disease model. In light of envisaged therapeutic approaches in humans, this approach may be the more reliable and safer one, provided that the transplanted cells retain phenotypic stability in the new microenvironment. The second approach involves the direct application of undifferentiated MenSCs in a particular disease model. While this therapeutic route may lead to less predictable and more indirect effects, it has nevertheless proven to be equally efficient at least in some disease models.

#### *3.2.1 Myocardial infarction*

Heart diesease is the leading cause of death in the USA, and constitutes a major epidemiological challenge (Minino et al. 2011). Therefore, the application of novel therapeutic approaches involving stem cells has gained considerable attention in recent years. A pioneering study on the therapeutic use of MenSCs in an animal model of myocardial infarction was published in 2008 by Hida et al. The researchers isolated MenSCs (2.1), transfected them with an enhanced green fluorescent protein(EGFP) adenoviral construct (for detection purposes only) and achieved cardiocytic differentiation using a coculture system with embryonic cardiomyocytes (see 3.1.1).

Transdifferentiation was not due to fusion-dependent cardiomyogenesis, as demonstrated by chromosome analysis. In vitro, about 50% of the differentiated MenSCs started beating strongly, spontaneously and synchronously. By immunostaining, expression of the marker protein troponin-I, a striated staining pattern for □-actinin, and a diffuse dot-like staining pattern of connexin 43 around the margin of each cardiomyocyte were demonstrated, suggesting tight electrical coupling. Analysis of action potentials revealed cardiomyocyte-specific sustained plateaus and slowly depolarizing resting membrane potentials, i.e. cardiomyocyte-specific pacemaker potentials.

The authors subsequently employed the transdifferentiated MenSCs in a nude rat model of myocardial infarction based on ligation of the left anterior coronary artery. In the therapeutic group, 1-2x106 EGFP-positive MenSCs were injected into the center and margin of the infarcted myocardium. The area of the myocardial infarction was significantly lower in the MenSC-treated group compared to controls injected with bone marrow-derived mesenchymal stem cells. Of note, the EGFP-positive MenSCs in the myocardial infarction area displayed a clear striation staining pattern of cardiac troponin-I and sarcomeric □-actinin, suggesting a high degree of in situ cardiomyogenic transdifferentiation as an underlying cause of improved cardiac function.While nuclear fusion between the cocultivated MenSCs and murine cardiomyocytes without separation of the athelocollagenmembrane was observed in only 0.16%, the potential contribution to cell fusion in the in vivo model is not fully clear at this point.

Overall, the data of this study open up exciting therapeutic perspectives, as the authors suggested that MenSCs could be obtained in a noninvasive manner from young female volunteers and stored to obtain a MenSC bank covering all HLA types for cardiac stem cell-based therapy. For applications in humans, the development of serum-free differentiation protocols represents an important advancement (3.1.1), however, carefully controlled protocols will be required as a co-culture with animal-derived cells is still part of the protocol (Ikegami et al. 2010). Nevertheless, this carefully executed study provides an exciting starting point for additional investigations in preclinical models.

***3.2.2Stroke***

Stroke is the fourth leading cause of death in the USA (Minino et al. 2011). Within the infarcted brain region, tissue in the ischemic core is mostly irreversibly damaged, while the tissue in the surrounding penumbra area may be rescued provided that proper therapeutic action is taken in a timely manner (Rodrigues et al. 2011). As the major current pharmacological therapy for stroke involves treatment with tissue plasminogen activator within a very narrow time window of 3 hours, the alternative therapeutic application of stem cells for stroke is an area of intense research. Stem cells may exert a beneficial effect during the later inflammatory phases of the disease when tissue repair commences, and may promote angiogenesis and survival of neurons via secretion of neuroprotective factors (Rodrigues et al. 2012).

In fact, apart from the use of ES cells, beneficial effects of adult stem cell therapy in experimental models of stroke have been reported for bone marrow (Wu et al. 2008), umbilical cord blood (Chen et al. 2001), adipose tissue (Leu et al. 2010), andstem cells isolated from amniotic fluid (Rehni et al. 2007). In 2010, Borlongan et al. could demonstrate the therapeutic efficacy of MenSCs in an in vivo model of stroke. Using a modification of the procedure initially described by Patel et al. (2008) (2.1), the authors collected on average 8-10ml of menstrual blood at the time of heaviest flow (day 1-3) prior to cryopreservation.

Cells were later expanded and immunoenriched for CD117 expression to preselect for a highly prolifertive and vital subpopulation of MenSCs. Cultures of MenSCs expressed the EScell markers Oct-4, SSEA-4 and nanog, as detected by immunocytochemistry, and the chemokine receptor CXCR4, which was also expressed on ES cells. Using a differentiation protocol involving culture in neural induction medium (DMEM/F12 containing N2 and FGF-2) and a subsequent stimulation with retinoic acids, the authors could induce expression of the neural markers MAP2 and nestin in the MenSCs. A large proportion of cells acquired an astrocytic phenotyp (GFAP-positivity) upon FGF-2 withdrawal. Coculture of primary rat neurons with MenSCs or conditioned media derived from these cells significantly protected the neural cells from oxygen glucose deprivation (OGD), an in vitro model of stroke.

ELISA analysis of conditioned media of OGD-exposed cultured MenSCs revealed an upregulation of several trophic factors, including vascular endothelial growthfactor, brain-derived neurotrophic factor, and neurotrophin-3. As these factors have previously shown beneficial effects in stroke (Wang et al. 1997, Sun et al. 2003), their upregulation may be mechanistically linked to the neuroprotective effect of the MenSCs in the in vitro model.Noteably, a protective effect was also observed in an established rat model of stroke based on induction of transient unilateral focal ischemia as achieved by middle cerebral artery occlusion (Yasuhara et al. 2006).

MenSCs were applied either intercerebrally or intravenously 2 hours after the ischemic insult, exerting a neuroprotective effect, and leading to a significant improvement in motor asymmetry, motor coordination and neurologic tests. Immunohistochemistry employing human-specific antibodies revealed survival of the transplanted cells in the penumbra 14d after transplantation. However, as over 90% of these MenSCs still expressed stem cell markers such as Oct-4, secretion of neuroprotective factors rater than replacement of damaged tissue appeared to be the mechanistic principle behind the beneficial effect of MenSC application in this experimental stroke model. Thus, although these results

are very encouraging, more experimental data are needed to fully understand the pathophysiological role of MenSC in experimental stroke therapy.

### *3.2.3Duchenne muscular dystrophy*

Duchenne muscular dystrophy (DMD) is an X-linked inherited form of muscular dystrophy caused by mutations or exon deletions in the dystrophin gene, which encodes a protein conferring structural stability to the dystrophin complex. This multiprotein complex links the intracellular cytoskeleton of muscle cells to the extracellular matrix, thus providing physical strength and mechanical support for the contractile forces generated by the muscle fiber (Allamand and Campbell 2000). Affecting approximately one in 3500 newborn males, the disease is characterized by progressive muscle weakness, ultimately resulting in paralysis and an average life expecancy of only 25 years (Bogdanovich et al. 2004).

As the underlying molecular cause of the disease is known, intense research efforts are undertaken to cure the disease, and have yielded promising results in experimental and preclinical models. Apart from pharmacological approaches, classical gene replacement therapy and antisense-mediated exon skipping, stem cells have been applied for therapeutic purposes (Bogdanovich et al. 2004). In a pioneering paper published in 2007, Cui et al. demonstrated a therapeutic effect of menstrual blood-derived cells in a well-established mouse model of DMD. mdx mice lack dystrophin in skeletal muscle fibers, however, the phenotype of the mice is more mild compared to human DMD (Allamand and Campbell 2000).

In cell culture, menstrual blood-derived cells selected for therapeutic purposes showed a stromal cell-like morphology and expressed a mesenchymal cell surface marker spectrum similar to MenSC preparations reported by other laboratories (Cui et al. 2007) (2.2.1). Successful 5-azacytidine-induced myogenic differentiation could be achieved in vitro, based on the expression of the markers MyoD, desmin, myogenin, and dystrophin. When untreated menstrual blood-derived cells were implanted directly into the thigh muscles of mdx-scid mice, myotubesexpressing human dystrophin as a cluster could be detected after 3 weeks. Immunohistochemistry with an antibody against human nuclei and DAPI revealed that dystrophin-positive myocytes had nuclei derived fromboth human and murine cells, suggesting that dystrophin expression could be attributed to fusion between murine host myocytes and human donor cells, rather than myogenic differentiation. This view was supported by further in vitro studies demonstrating fusion between human endometrial cells and cocultured murine C2C12 myoblasts.

### *3.2.4Critical limb ischemia*

Critical limb ischemia, an advanced form of peripheral artery disease, comprises medical conditions such as chronic ischemic rest pain, ulcers, or gangrene caused by proven occlusive disease (Norgren et al. 2007, Setacci et al. 2011). As up to 45% of the affected patients require amputation, there is considerable interest in the development of therapies efficiently reestablishing blood flow in the affected limbs. Besides surgical intervention and application of proangiogenic cytokines, therapeutic application of bone-marrow-derived mesenchymal stem cells and endothelial progenitor cells have yielded promising resultsin vivo (Attanasio and Snell 2009, Setacci et al. 2011).

Application of the stem cells resulted in increases in angiogenic factor secretion and increased angiogenesis both in animal models and in several small-sized clinical studies (Sprengers et al. 2008, Murphy et al. 2011), demonstrating its benefit as a supportive therapy especially for patients without therapeutic options. In 2008, Murphy et al. expanded this therapeutic repertoire by applying MenSC in a mouse model of critical limb ischemia: Using 'endometrial regenerative cells' isolated according to the protocol developed by Meng et al. (2007) (2.1), the authors first demonstrated a substantial proliferative effect of these MenSC conditioned media on human umbilical vein endothelial cells, which was attributed to the secretion of proangiogenic factors such as PDGF-BB and angiopoietin.

In a pilot study on 16 mice, 1x106 MenSC were injected intramuscularly after ligation of the femoral artery and its branches for induction of limb ischemia. To additionally reproduce a

neurotrophic ulcer-like injury, the N. peroneus was excised in the experimental animals. Injections of MenSC in the treatment group were repeated on day 0, day 2 and day 4. As a result of the experimentally-induced ischemia, necrosis was observed in the legs of the control mice after 14 days, whereas all MenSC-treated mice treated with MenSC had intact limbs, albeit two mice displaying signs of impeded walking. The results of this pilot study suggest that MenSC may be a viable alternative to bone marrow stem cells in critical limb ischemia. As the authors have registered a clinical trial on a small patient collective (see 3.2), data on the therapeutic efficacy of these cells should be available in the not too distant future.

### *3.3 Alterative endometrial stem cell sources*

The previous chapters have demonstrated that stem cells derived from menstrual blood are a readily available source of adult stem cells which can be obtained by non-invasive techniques. However, alternative sources of endometrial stem cells have been utilized to gain additional knowledge on the function and therapeutic potential of these cells. Initial studies relied on hysterectomy-derived endometrium (Gargett 2007), which had the advantage of containing a high amount of endometrial stem cells, but also the disadvantage of limiting the number of patients available for clinical correlation studies on endometrial stem cells. Subsequent studies demonstrated that endometrial stem cells could also be obtained by minimally invasive superficial endometrial biopsies, which may constitute an alternative to MenSCs, as this technique allows for obtaining endometrial stem cells independent of the menstrual cycle (Schüring et al. 2011, Cervello et al. 2011).

Another highly interesting feature of endometrial stem cells is the finding that they are more amenable to reprogramming into induced pluripotent stem (iPS) cells compared to skin fibroblasts (Park et al. 2011), which can be attributed to the high basal expression levels of pluripotency markers (Schüring et al. 2011, Götte et al. 2011). Of note, Nishino et al. (2011) were able to successfully generate iPS cells from MenSCs. As the issue of teratoma formation of iPS cells still constitutes a major obstacle for therapeutic applications (Gonzales and Pedrazzini 2009), we willfocus on the therapeutic application of multipotent non-menstrual-blood derived endometrial stem cells in the following section.

#### *3.3.1 Therapeutic applications of tissue-derived endometrial stem cells*

Apart from MenSCs (3.2), tissue-derived endometrial stem cells obtained by low invasive biopsy or hysterectomy have been employed for therapeutic purposes in several animal models of disease. For example, the study by Hida et al. (2007) not only demonstrated therapeutic efficacy of MenSCs in a mouse model of myocardial infarction, but also showed a similar therapeutic effect upon application of *in vitro* differentiated endometrial cells derived from hysterectomy tissue (3.2.1). In contrast to MenSCs, the tissue-derived cells required retroviral transfection with HPV16E6, E7, and hTERT, which constitutesa problem regarding therapeutic applications in humans.

Wolff et al. (2011)described the successful therapeutic application of endometrial stem cells in an animal model of Parkinson's disease. This common neurodegenerative disease is caused by a progressive loss of dopamine-producing neurons in the substantianigra projecting into the striatum, which ultimately results in tremor,hypokinesia and rigidity in affected patients (Infante-Duarte et al. 2008, Soo et al. 2008). Wolff et al. (2011) initially isolated endometrial stroma cells obtained by by curettage from nine reproductive-aged women. After repeated passaging, the cells were strongly positive for CD146, CD90 and PDGFRß1, and negative for CD31 and CD45. Using an *in vitro* differentiation protocol involving sequential culturing in the presence of FGF and EGF and subsequently butylated hydroxyanisole, dibutyryl cyclic AMP, 3-isobutyl-1-methyl-xanthine, and all-trans-retinoic acid (Blondheim et al. 2006), the authors were able to obtain dopaminergic neurons. The cells exhibited axon projections, pyramidal cell bodies, and dendritic projections, expressed the rate-limiting enzyme in dopamine synthesis, tyrosine hydroxylase andthe neural marker nestin, and displayed electrophysiologic properties specific to dopamine-producing neurons. These cells were

transplanted into the striatum of both immunocompetent and immunodeficient mouse models of Parkinson's disease based on the injection of 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine. Successful transplantation was confirmed by detection of human specific DNA, mitochondrial markers and human Nestin. The endometrium-derived cells were able to migrate into the brain lesion, populating both the striatum and the substantia nigra. Noteably, the researchers could show that the endometrium-derived cells had transformed into dopamine-producing neurons, resulting in partially restored dopamine levels *in vivo*. While the results of this study demonstrate the plasticity and therapeutic potential of endometrial stem cells, future studies still need to address if the increased dopamine levels suffice to improve the clinical symptoms, in particular the coordination of balance and movement in this mouse model.

Two independent studies have recently explored the therapeutic potential of endometrial stem cells in experimental models of type I diabetes, a disease characterized by insulin deficiency caused by a loss of pancreatic ß-cell function (Scherbaum and Seissler 1995). Li et al. (2011) used an immunodepletion protocol to isolate mesenchymal-like stem cells from hysterectomy tissue of 11 patients. The cells were positive for Oct-4, nanog, nestin, CD44, CD29, CD105, and CD81, and showed multilineage differentiation potential *in vitro*. After a 7 day culture period in pancreatic differentiation medium, the authors observed the formation of cell clusters and aggregates, which exhibited a pancreatic cell like gene signature. Quantitative real-time PCR analysis confirmed transcriptional upregulation of typical pancreatic cell markers, including insulin, Glut2, Pax4, Nkx2.2, NeuroD, Isl-1, somatostatin, and glucagon, compared to undifferentiated cells and endometrial fibroblasts. Noteably, insulin and c-peptide secretion in response to a glucose stimulus were significantly increased in the differentiated cells. The therapeutic potential of the endometrium-derived cells was further evaluated in streptozotocin-induced hyperglycemic mice, an established model of diabetes mellitus. Following injection of $2x10^7$ differentiated endometrial stem cells into the subcapsule of the left kidney, the formation of diffuse aggregated islet-like clusters which were immunopositive for insulin and glucagon was observed. Human insulin levels were readily detectable and stable during an 8 week observation period, whereas blood glucose levels were significantly decreased in the transplanted mice compared to controls. Of note, no expression of c-Myc or Oct-4 was detected in the islet-like tissues, and no teratoma formation was observed after 12 weeks of observation. A second study by Santamaria et al. (2011)employed a similar approach. The authors differentiated human endometrial stromal stem cells *invitro*, and succeeded in generating cells which expressed the markers PAX4, PDX1, GLUT2, and insulin. Insulin was secreted in a glucose-dependent manner. In a diabetic mouse model, transplantation of the differentiated endometrial cells resulted in an increase in human insulin levels, which was accompanied by a stabilisation of blood glucose levels within 5 weeks. Noteably, mice transplanted with undifferentiated cells developed progressive hyperglycemia, whereas control mice lost weight and developed cataracts as a result of insulin deficiency and hyperglycemia. In summary, the promising results of these animal studies suggest that the therapeutic application of *in vitro*-differentiated endometrial stem cells may be a future alternative to pancreatic islet transplantation, which is currently limited by availability of cadaveric donor tissue (Ryan et al. 2002).

### *3.4 Clinical perspective*

As the previous sections have demonstrated, the application of MenSCs and endometrial stem cells in in vitro and animal models of human diseases has yielded highly promising results. In contrast to the application of ES or iPS cells, the risk of inadvertedly causing a malignant disease as a side-effect of the stem cell therapy appears to be low (Meng et al. 2007). Moreover, for some female patients, autologous stem cell transplantations are feasible, avoiding possible immunogical incompatibility and rejection issues. However, in order to make therapeutic options available for postmenopausal women and men, a systematic immunophenotyping and banking of MenSCs would be required. In addition, the symptoms of most diseases potentially amenable to stem cell therapy will become apparent only at a postmenopausal age, establishing a requirement for MenSC banking even in the case of autologous

therapies. In urgent cases, endometrial stem cells obtained by superficial endometrial biopsy (Schüring et al. 2011) could serve as an alternative source for autologous cell transplantations in women.

Furthermore, *in vitro* expansion of the MenSCs will very likely be required in order to obtain a sufficient amount of cells for transplantation. In spite of the proliferative character of MenSCs, sufficient cell numbers may not be easily achieved using menstrual blood derived from only one donor. The development of serum-free culture conditions for MenSCs is therefore an important step towards their use in clinical applications (Ikegami et al. 2010). The field of MenSC therapeutics can benefit from decades of experience regarding GMP procedures, surface antigen characterization, cell banking and standardized procedures developed in the fields of transfusion medicine and transplantation immunology. Borlongan et al. (2010) pointed out that MenSCs express a similar marker spectrum as mesenchymal stem cells known for their immunosuppressive effects. They furthermore reported that MenSCs showed a very weak stimulatory response in a mixed lymphocyte reaction. In line with these findings, a systematic study by Nikoo et al.

(2012) demonstrated that MenSCs affected the proliferative response of peripheral blood mononuclear cells in allogeneic mixed lymphocyte reaction in a dose-dependentmanner. The results of their study prompted the authors to suggest that MenSCs may even be employed as a therapeutic option to prevent or modulate graft versus host disease in allogeneic transplantation.Nevertheless, the specific requirements for clinical applications within this new field of regenerative medicine need to be carefully defined and need to result in unified procedures at least at the national level. Furthermore, access to MenSC banks in order to locate an appropriate donor will have to be standardized similar to existing systems in the field of transplantation immunology. Apart from the issue of immunocompatibility, other safety precautions need to be taken. Similar to the experimental studies discussed in section 3.1, it has to be ensured that the MenSCs maintain their phenotypic stability prior to therapeutic use in humans. For example, cells containing chromosomal aberrations should obviously not be used for therapeutic purposes, requiring defined regular screening procedures.

Moreover, the risk of viral and especially microbial contaminations can be expected to be higher in MenSCs compared to blood- or bone-marrow-derived adult stem cell sources, requiring an equally important strict and efficient control system. Allickson et al. (2011) have provided a good example for a thoughtful MenSC isolation and storage protocol which addresses several of the issues mentioned above.In addition, detailed descriptions of endometrial stem cell and MenSC characterization and isolation protocols have beenprovided in several patent applications, including US-Patent applications #20090053182 (MenSC / endometrial stem cell isolation and characterization), #20080241113 and #20100040588 (isolation and cryopreservation of MenSCs), as well as #20110268710 (treatment of stroke with MenSCs. Finally, Zhong et al. (2009) defined the release criteria of MenSCs to be used in allogeneic transplantations in humans: (i) absence o bacterial and mycoplasma contamination; (ii) endotoxin levels < 1.65 EU/ml; (iii) morphology consistent with adherent, fibroblastic-like shape; (iv) CD90 and CD105 positive (> 90%) and CD45 and CD34 negative (< 5%) by flow cytometry;(v) cell viability > 95% by trypan blue staining and flow cytometer; (vi) absence of karyotypic abnormalities.

Keeping these caveats in mind, a number of pilot studies and clinical trials on MenSC safety and therapeutic efficacy in humans have already been initiated. A pionerring pilot study explored the feasibility of MenSC transplantation in 4 patients suffering from multiple sclerosis (Zhong et al. 2009). Donors were selected after rigorous testing according to federal regulation 21 CFR1271 of the U.S Federal Drug Administration, and MenSCs were isolated essentially as described by Meng et al. (2007) (2.1). In vitro expansion of the MenSCs allowed for obtaining 100-200 million cells after 3-4 passages. Cells that passed the release criteria (see above) were injected either intravenously or intrathecally in clinical grade saline containing 50% autologous heat inactivated serum. The 4 multiple sclerosis patients participating in the trial received between 16-30 million MenSCs applied in several discrete doses of either 3 million or 6 million cells. After a follow up of 2-12 months and monitoring by physical exam CBC/biochem panel, fecal occult blood testing, chest X-Ray and tests for PSA, CEA, and alpha fetoprotein, none of the patients described noteable events or abnormalities, with the exception of a mild self-limiting headache in one patient, which was ascribed to the lumbar puncture

procedure. Moreover, during the observation period, no disease progression was noted. While the study is limited by the small patient number, it nevertheless shows that MenSC transplantation appears to be safe if the cells are well characterized and appropriate precautions are taken.

The NIHs public registry and results database ClinicalTrials.gov lists several clinical trials related to the use of menstrual blood-derived or endometrial stem cells. While some of these trials have a research focus, aiming at obtaining new information on the properties of these cells, others are directed towards direct therapeutic applications. For example, aprospective observational study headed by Erin Wolff of the National Institutes of Health Clinical Center in Bethesda, USA, recruits normal controls, patients with rare diseases or reproductive disorders, and patients who have undergone hematopoietic stem cell transplant (planned enrollment: n=100) to investigate endometrial biopsies with the aim of a better understanding of bone marrow cell engraftment of the uterus (ClinicalTrials.gov identifier NCT01468935). A prospective observational study initiated by Carlos Simón of the IVI Valencia, Spain, recruits endometriosis patients (n=30) with the purpose of characterizing endometrial stem cells through flow cytometry and side population techniques and in vitro cellular proliferation assays to learn more about the involvement of endometrial stem cells in this disease, which is associated with reduced fertility and pain symptoms (identifier NCT01412138). As stem cell marker expression is dysregulated in endometriosis (Götte et al. 2008, Götte et al. 2011), this study follows a good rationale.

Among the therapy-oriented studies, an interventional study announced by Medistem Inc. aims at assessing the safety and feasibility of using endometrial regenerative cells / MenSCs in 15 patients with critical limb ischemia that are not eligible for surgical or catheter-based interventions (identifier NCT01558908). In this phase1/2 trial, patients are planned to be treated with increasing doses of 25 million, 50 million, or 100 million MenSCs by intramuscular injection. MenSC treatment is will be followed by assessment of treatment safety and improvement of clinical symptoms including ulcer healing, rest pain, and reduction in amputation. Moreover, the potential of MenSCs for the treatment of type 1 diabetes will be addressed in an interventional study headed by Charlie Xiang, First Affiliated Hospital of Zhejiang University, China and S-Evans Bioscsiences Co. Ltd (identifier NCT01496339). Both safety of MenSC application and improvement of diabetes mellitus-associated parameters (HbA1c, hypoglycemic events, C-peptide, blood glucose levels etc.) will be investigated in this phase 1/2 trial. Finally, an actively recruiting interventional phase 1/2 study initiated by S-Evans Biosciences Co. Ltd aims at testingthe efficacy and safety inpatients with liver cirrhosis by comparing conventional therapy plus MenSC transplantation with conventional treatment in aplacebo group(identifier NCT01483248). Taken together, the interventional studies can be expected to provide valuable information on the safety and therapeutic efficacy of MenSC transplantation in a clinical setting in the near future.

### *4. Summary and outlook*

Menstrual blood contains a pool of multipotent mesenchymal-stem-cell like cells which are characterized by the additional expression of pluripotency factors such as Oct-4. These highly proliferative cells can be obtained by simple noninvasive procedures and can be successfully expanded in vitro for prolonged periods without acquiring chromosomal aberrations. In contrast to ES cells, no ethical controversy is associated with their use in therapeutic applications. Furthermore, animal studies suggest that no increased risk of tumor formation is associated with their in vivo use, in contrast to pluripotent ES and iPS cells, which have the inherent risk of forming teratomas in vivo.

MenSCs show a low immunogenicity, are suitable for allogenic transplantation, and may even have a positive influence on the immune system of the recipient. MenSC show multilineage differentiation potential in vitro and in vivo and have been successfully employed in several animal models of human disease. Preliminary data from clinical pilot studies have demonstrated that their in vivo use in human recipients is safe. Ongoing phase 1/2 clinical trials on the therapeutic efficacy and safety on MenSC applications in human patients raise the hope that the potential of these attractive

adult stem cells can be fully assessed in the not too distant future. While MenSCs harbour the potential for autologous transplantation at least for reproductive age female patients, the majority of potential patients would greatly benefit from MenSC cell banking.

Consequently, there is a need for defining unified preparation procedures and safety requirements regarding the the clinical use of these cells, and for providing an informational infrastructure that allows to locate matching donor cells for a specific patient in need of MenSC therapy. As similar procedures and infrastructures are already well-established in other areas of stem cell therapy, a successful translation of the exciting recent research findings on MenSCs and endometrial stem cells into clinics should be feasible within a short timeframe.

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**Table I. Stemness-related marker expression of menstrual blood-derived stem cells**

| Marker | Reference |
|---|---|
| Oct-4 | Patel et al. 2008, Borlongan et al. 2010, Allickson et al. 2011, Nikoo et al. 2012 |
| SSEA-4 | Patel et al. 2008, Borlongan et al. 2010, Zemel'ko et al. 2011, Allickson et al. 2011 |
| Nanog | Borlongan CV et al. 2010, Allickson et al. 2011 |
| CD 9 | Meng et al. 2007, Nikoo et al. 2012 |
| CD10 (CALLA) | Hida et al. 2008 |
| CD13 | Cui et al. 2007, Zemel'ko et al. 2011, Allickson et al. 2011 |
| CD29 (integrin ?1) | Meng et al. 2007, Cui et al. 2007, Hida et al. 2008, Zemel'ko et al. 2011, Nikoo et al. 2012 |
| CD41a | Meng et al. 2007 |
| CD44 (hyaluronan receptor) | Meng et al. 2007, Cui et al. 2007, Hida et al. 2008, Allickson et al. 2011, Zemel'ko et al. 2011, Nikoo et al. 2012 |
| CD49f (integrin ?6) | Allickson et al. 2011 |
| CD54 (ICAM-1) | Cui et al. 2007 |
| CD55 (decay accelerating factor) | Cui et al. 2007, Hida et al. 2008 |
| CD59 | Meng et al. 2007, Cui et al. 2007, Hida et al. 2008 |
| CD73 (ecto-5'-nucleotidase) | Cui et al. 2007, Allickson et al. 2011, Zemel'ko et al. 2011, Nikoo et al. 2012 |
| CD90 | Meng et al. 2007, Hida et al. 2008, Allickson et al. 2011, |

| | |
|---|---|
| | Zemel'ko et al. 2011 |
| CD105 (endoglin) | Cui et al. 2007, Zemel'ko et al. 2011, Nikoo et al. 2012 |
| CD117 (c-kit) | Cho et al. 2004, Patel et al. 2008, Allickson et al. 2011 |
| CD166 (ALCAM) | Hida et al. 2008, Allickson et al. 2011 |
| TERT/Telomerase | Meng et al. 2007, Patel et al. 2008, Allickson et al. 2011 |

**Table II. Therapeutic application of MenSCs and endometrial stem cells**

| Disease | Stem cell source | Application | Reference |
|---|---|---|---|
| Myocardial infarction | MenSCs and immortalized endometrium-derived cells | use of *in vitro* differentiated cardiomyocytes in nude rat model of myocardial infarction | Hida et al. 2008 |
| Diabetes (type 1) | endometrial stroma-derived cells | use of *in vitro* differentiated insulin-producing cells in mouse model of diabetes | Santamaria et al. 2011 |
| Diabetes (type 1) | endometrial cells (hysterectomy) | use of *in vitro* differentiated insulin-producing cells in mouse model of diabetes | Li et al. 2010 |
| Diabetes (type 1) | MenSCs | human phase 1/2 clinical trial announced | Clinicaltrials.org identifier NCT01496339 |
| Parkinson's disease | endometrial stroma cells (curettage) | use of *in vitro* transdifferentiated cells in mouse model of Parkinson's disease | Wolff et al. 2011 |

| | | | |
|---|---|---|---|
| Multiple sclerosis | MenSCs | safety study in four human MS patients by intravenous and intrathecal injection | Zhong et al. 2009 |
| Stroke | MenSCs | use of *in vitro* in rat model of stroke | Borlongan et al. 2010 |
| Duchenne muscular dystrophy | MenSCs | use of myogenically *in vitro* differentiated MenSCs in *mdx* mouse model | Cui et al. 2007 |
| Critical limb ischemia | MenSCs | use in mouse model of critical limb ischemia | Murphy et al. 2008 |
| Critical limb ischemia | MenSCs | human phase 1/2 clinical trial announced | Clinicaltrials.org identifier NCT01558908 |
| Liver cirrhosis | MenSCs | actively recruiting human phase 1/2 clinical trial | Clinicaltrials.org identifier NCT01483248 |

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**Figure legends:**

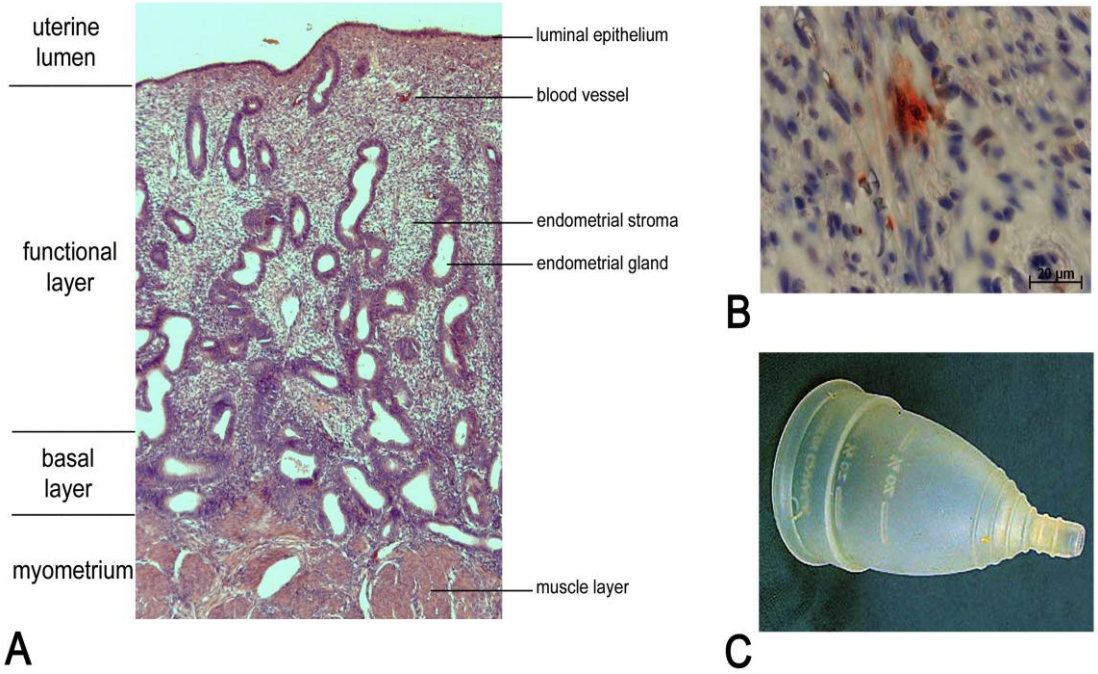


**Figure 1: Stem cells in the human endometrium**. A) Histological architecture of the human endometrium. Compared to the functional layer of the endometrium, which is shed during menstruation, the basal layer is characterized by a dense stroma. The muscular layer of the myometrium can be clearly distinguished from the endometrium. B) Putative adult stem cells displaying positive immunostaining (red) for the RNA-binding protein Musashi-1 in the endometrial stroma. C) The menstrual cup is a device frequently used to collect menstrual blood for MenSC isolation procedures.

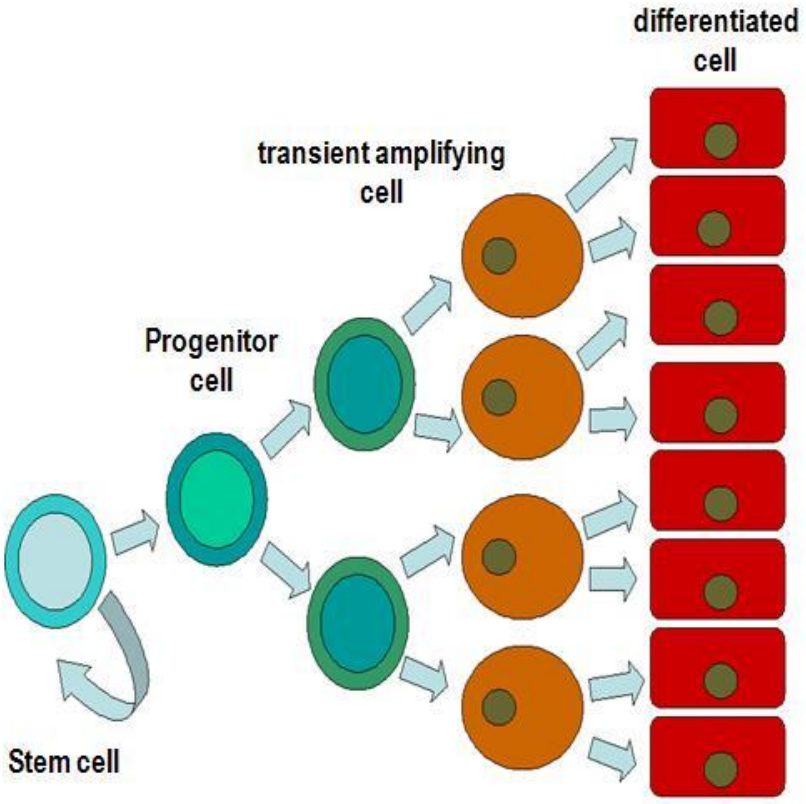


**Figure 2:Hierarchy of adult stem cells**. The adult stem cell is multipotent and characterized by asymmetric cell division. The stem cell resides within a niche (not shown) which keeps the stem cell in an undifferentiated, slow cycling state. Cell division results in self renewal and generation of a committed precursor cell, which is also called progenitor cell. Transient amplifying cells are progenitor cells characterized by high proliferative activity as a prerequisite for efficient tissue regeneration. Successive acquisition of differentiation markers and associated functional changes ultimately generate terminally differentiated cells which fulfill a specialized function within the human body.

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