Porcine uterus contains a population of mesenchymal stem cells

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Abstract

The uterus has a remarkable ability of cycling remodeling throughout the reproductive life of the female. Recent findings in the human and mouse indicate that adult stem/progenitor cells may play a prominent role in the maintenance of uterine endometrial and myometrial homeostasis. We aimed to characterize the prospective stem/progenitor cells in the porcine uterus and establish a new model for uterine stem cell research. In this study, we demonstrated that cells isolated from porcine uterus have capacity for *in vitro* differentiation into adipogenic and osteogenic lineages and express the mesenchymal stem cell (MSC) markers CD29, CD44, CD144, CD105, and CD140b as revealed by RT-PCR. Moreover, we showed that some cells isolated from the porcine uterus when cultured at low density produce large clones with an efficiency of 0.035%. Simultaneously, they were negative for hematopoietic stem cell markers such as CD34 and CD45. Low expression of nestin, which is specific for neural stem cells and various progenitor cells, was also detected. We conclude that the porcine uterus contains a small population of undifferentiated cells with MSC-like properties similar to human and mouse uteri.

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Introduction

The uterus is a unique reproductive organ of outstanding remodeling ability of its main tissues: the endometrium and myometrium. The human endometrium, which consists of the functionalis and basalis layers, undergoes more than 400 cycles of proliferation, differentiation, and shedding during the woman's reproductive years (Gargett *et al.* 2009, Maruyama *et al.* 2010). It regenerates 4–7 mm of tissue due to intensive proliferation and differentiation of cells located most likely in the basalis layer in every menstrual cycle. Endometrial regeneration also follows parturition, extensive surgical resection and occurs in postmenopausal women taking estrogen replacement therapy (Gargett 2007, Wolff *et al.* 2007). In non-menstruating species such as rodents and pigs there are cycles of endometrial growth and apoptosis, rather than physical shedding of the functionalis layer. Remodeling of the endometrium is regulated by the ovarian steroid hormones estrogen and progesterone in preparation for blastocyst implantation on a monthly basis in women, every 4–5 days in mice, and approximately every 21 days in pigs. The uterus must rapidly enlarge to accommodate the developing fetus during pregnancy. This event comprises massive hypertrophy and hyperplasia of myometrial smooth muscle cells and other cell types and can be repeated multiple times throughout the reproductive life of an individual.

It has been hypothesized that adult stem or progenitor cells residing in the endometrial basalis are responsible for cyclic regeneration of the functionalis compartment (reviewed in Gargett (2007) and Gargett & Masuda (2010)). It is also likely that myometrial stem/progenitor cells are involved in the dramatic enlargement of the pregnant uterus (reviewed in Maruyama *et al.* (2010)). Adult stem cells are rare, undifferentiated cells residing in various tissues and organs within the stem cell niches. Lack of a distinct stem cell marker implicates that these cells are defined rather by their functional properties, such as high proliferative potential, self-renewal through asymmetrical division, and ability to differentiate into one or more lineages (Pittenger *et al.* 1999). Indeed, since the first evidence for the existence of clonogenic cells in the human endometrium was provided by Chan *et al.* (2004), several studies have reported the presence of a variety of stem-like cells in human and mouse uterus (Chan & Gargett 2006, Cervelló *et al.* 2007, 2010, Kato *et al.* 2007, Wolff *et al.* 2007, Dimitrov *et al.* 2008, Gargett *et al.* 2009, Ono *et al.* 2010). Furthermore, emerging evidence indicates that some pathological conditions, such as endometrial cancer, endometriosis, and leiomyomata can be attributed to dysregulation of these same stem cells, or are derived from committed cells that acquire stem-like features (Sasson & Taylor 2008, Hubbard & Gargett 2010). Most studies on uterine stem cells have focused on human rather than mouse

models, the latter being the only animal model studied until now. We postulate that due to some structural and functional similarities between the human and porcine uterus, such as reproductive cycle duration, the pig may be a valuable candidate model for investigation of uterine stem cells in normal and pathological conditions. However, to our knowledge, no data is available in this case. Hence, we aimed to find out whether the pig uterus contains a population of adult stem-like cells, which demonstrate clonogenicity, *in vitro* multi-differentiation capacity and express stem cell markers.

Results

Clonogenicity

Cell plates at clonal density (100 cells/cm²) proliferate and produce clones, which differ in size and number of cells. There were large clones that contained a great number of small, densely packed cells with prominent nuclei and small clones with loosely packed cells and epithelial or fibroblast-like morphology (Fig. 1). Only large clones containing more than 50 cells were counted and yielded 14 ± 4 clones per dish. Clonal efficiency (CE) was CE=0.035%. No differences between uteri from different estrus phases were observed although this was not studied in detail.

Multilineage differentiation

Cells induced to differentiate into bone lineage were stained with alkaline phosphatase (ALP) and Alizarin Red (Fig. 2). Following ALP staining there were many more violet-stained cells in the induced culture than in the non-induced negative controls after 7 and 14 days. We observed that single cells with fibroblastic phenotype had very strong staining in the induced culture whereas in the control culture there were cells with very weak staining and epithelial phenotype. Staining with Alizarin Red showed cells with calcium accumulation – small clones of intensively red-stained cells in induced

Figure 1 An example of large clones derived from single uterine cell after 14 days of culture stained with hematoxylin. Clones on the culture dish differ in the size and number of cells.

Figure 2 ALP and Alizarin Red staining of cell cultures induced for osteogenesis and control cultures. Osteogenesis was indicated by increasing in ALP (blue-stained cells) and calcium deposition (red-stained cells) and such changes were not evident in control cultures. Bar = 100 µm. ALP, alkaline phosphatase.

flasks. There were nearly no such cells in the non-induced culture after 7 and 14 days. After inducing adipogenesis, cells accumulated lipid droplets stained with Oil Red O and Nile Red, and appeared mainly rounded and bigger than cells in the control culture (Fig. 3). In the control flasks there were mostly fibroblast- and epithelial-like cells and some of them accumulated lipids. This multilineage differentiation was carried out for four porcine uteri. There were no evident differences in differentiation potential between samples harvested from the individual porcine uteri.

RT-PCR

Cells isolated from the porcine uterus and cultured *in vitro* expressed mesenchymal stem cell (MSC) markers: CD29, CD105, CD140b, CD44, and CD144 and were negative for the markers specific for hematopoietic stem cells such as CD34 and CD45 (Fig. 4). There was low signal for nestin and no signal for Oct-4. We found also the expression of endothelial cell marker CD31. Negative controls showed no bands.

Discussion

Several recent studies support the concept that tremendous uterine remodeling observed during the reproductive cycle and throughout pregnancy is based on the fine regulation of a small population of stem/progenitor cells residing in the endometrial and myometrial stem cell niches (Teixeira *et al.* 2008, Gargett & Masuda 2010, Ono *et al.* 2010). This notion is based on the investigations performed solely on human and mouse uteri that identified clonogenic cells, side-population cells, label-retaining cells, multipotent cells that can differentiate into chondrocytes or adipocytes, and cells expressing various MSC markers. We report here for the

Figure 3 Nile Red and Oil Red O staining of cell cultures induced for adipogenesis and control cultures. Adipogenesis was indicated by the accumulation of lipid droplets stained with either Nile Red or Oil Red O, which was hardly observed in the control culture. Bar = 100 μ m.

first time that the porcine uterus contains a small population of cells that form large clones *in vitro* and are able to differentiate into adipogenic and osteogenic lineages when cultured in supplemented media. Moreover, the presence of the MSC markers CD29, CD44, CD144, CD105, and CD140b and the lack of the hematopoietic stem cell markers CD34 and CD45 strongly indicate their mesenchymal origin. Our finding of undifferentiated stem-like cells persisting in the porcine uterus provide a novel model for investigating stem cell contribution to the uterine tissue maintenance and function.

We have tested the clonogenicity of porcine uterine cells as a classical approach and initial step in searching for stem or progenitor cells in a given tissue. Chan *et al.* (2004) provided the first evidence that the human endometrium contains rare clonogenic epithelial and stromal cells with high proliferative potential. Approximately 0.22% of the epithelial and 1.25% of the stromal cell populations initiated colonies when seeded at clonal density (Chan *et al.* 2004). The cloning efficiencies for

cells derived from other tissues varied between 0.01% for colon to about 5% for epithelial cells from the epidermis, prostate, and mammary glands (Chan *et al.* 2004). Moreover, Schwab *et al.* (2005) demonstrated that clonogenicity of epithelial and stromal cells did not vary between proliferative, secretory, and inactive human endometrium, suggesting that ovarian steroid hormones do not maintain the clonogenic potential of these cells. Contrary to the above, other studies indicate that proportion of side-population cells isolated from human endometrium differ according to the phase of the menstrual cycle. The highest proportion of side-population cells isolated from the human endometrium was observed in the early proliferative phase (Masuda *et al.* 2010) or shortly after menstruation (Kato *et al.* 2007, Tsuji *et al.* 2008). In our experiments, we did not observe any differences between cells isolated from uteri at the preovulatory or secretory phases, although this was not studied in detail. We report here that 0.035% of cells isolated from the porcine uterus formed large clones containing more than 50 cells when seeded at low density. Hence, a small population of cells of high proliferative ability resides in the porcine uterus.

A functional property of MSCs is the ability to differentiate into adipocytes, osteocytes, chondrocytes, and myocytes, when cultured *in vitro* in inducing media (Pittenger *et al.* 1999, Colter *et al.* 2000, Deans & Moseley 2000, Zuk *et al.* 2002). We demonstrated in our study that cells derived from the porcine uterus are potent to differentiate into osteogenic and adipogenic lineages. This evidence indicates that the porcine uterus contains a population of multipotent cells and is in agreement with a study of human myometrial progenitor cells, which possess the ability to differentiate into these two lineages (Ono *et al.* 2007). Another study showed that endometrial-derived cells could differentiate into a chondrocyte lineage, which was demonstrated by the expression of articular cartilage markers, sulfated glycosaminoglycans, and type II collagen (Wolff *et al.* 2007). Other studies of human endometrial cells reported phenotypic differentiation into four mesenchymal lineages (Schwab & Gargett 2007).

We conducted RT-PCR assays on cells isolated from porcine uterus before and after the first passage

Figure 4 RT-PCR analysis of undifferentiated, mesenchymal, hematopoietic, and endothelial cell markers on cells isolated from a porcine uterus cultured *in vitro*. Positive control for nestin are murine bone marrow cells after second passage. Positive and negative controls are indicated by + and – respectively.

(at ~7 and at 21 days of culture) but we did not examine the effect of long-term culture on expression patterns of stem cell markers.

The transcription factor Oct-4 (POU5F1) is involved in the self-renewal of undifferentiated pluripotent stem cells (Nichols *et al.* 1998). Matthai *et al.* (2006) provided the first evidence for cells expressing the stem cell marker Oct-4 in the human endometrium. Recently, Bentz *et al.* (2010) reported that endometrial Oct-4 was not differentially expressed during the menstrual cycle and was not involved or modulated by hormone-induced cyclic changes in the endometrium. We have failed to detect the presence of Oct-4 in primary cell culture harvested from the porcine uterus, although our method may not be sensitive enough. Interestingly Ono *et al.* (2010) reported that expression of Oct-4 decreases during *in vitro* culture and at the mRNA level became undetectable after 14 days of culture.

On the other hand, however, our cell culture expressed an intermediate filament protein, nestin, which is characteristic of immature neurons and other undifferentiated cells (Wiese *et al.* 2004). Moreover, experiments performed on adipose-derived stem cells (ADSCs) revealed that expression of nestin decreases at later passage (Wan Safwani *et al.* 2011).

Our study indicates the absence of the hematopoietic stem cell markers CD34 and CD45 on porcine uterine cells, although others reported the presence of such cells in the human endometrium (Lynch *et al.* 2007) and showed that they have vasculogenic potential in the murine uterus (Onodera *et al.* 2006). Bone marrow-derived cells may contribute to stromal and epithelial regeneration in the murine endometrium (Du & Taylor 2007), although it is unclear whether they were hematopoietic or MSCs.

It is known that culture conditions may change the expression of some antigens. Some markers, e.g. CD34, which are present on freshly isolated MSCs from bone marrow, disappear during cell culturing (Fibbe & Noort 2003). Changes in adhesion molecules expression are supposed to be connected with the process of cell maturation or differentiation (Bruder *et al.* 1998). Moreover, ADSCs maintain or even increase the expression of MSC markers such as CD29, CD44, and CD90 during long-term cell culture suggesting that ADSCs became a more uniform population (Park & Patel 2010). Alternatively, some pluripotent genes (e.g. *NANOG*, *Oct4* (POU5F1), and *Rex1* (ZFP42)) are detected only in cells from early passages. Regarding the endometrial stem/progenitor cells, it was reported that a primary heterogeneous population of cells isolated from the human endometrium with each passage became more homogenous. High expression of MSC markers (CD73, CD29, and CD90) but not hematopoietic markers (CD45, CD34, CD14, CD3, CD19, and CD16/56) was observed after four passages and remains stable during long-term culture (Dimitrov *et al.* 2008). Cells isolated

from menstrual blood retain the expression of MSC markers (e.g. CD9, CD29, CD41a, CD59, CD73, CD90, and CD105) after culturing for more than 68 doublings (Meng *et al.* 2007). Clonal cultures obtained from cells isolated from human uterine biopsy showed high expression of the MSC markers CD90, CD73, CD105, and CD146 after long-term cultures. Simultaneously, cells were negative for markers of endothelial and primitive hematopoietic cells as well as monocytes and macrophages (Schüring *et al.* 2011).

We conclude that the porcine uterus harbors a population of prospective stem or progenitor cells that are clonogenic, able to differentiate into osteogenic and adipogenic lineages, and express a panel of undifferentiated and MSC markers such as nestin and CD29, CD44, CD105, CD140b, and CD144.

Materials and Methods

Cell isolation

Porcine (*Sus scrofa*) uteri ($n=5$) were obtained from the local abattoirs or from the Department of Animal Reproduction Biotechnology, National Research Institute of Animal Production at Balice near Krakow. The uteri were obtained from adult animals at preovulatory or secretory phases of the estrus cycle. The approximate stage of the estrous cycle was determined by examination of ovarian morphology, the follicle size, and appearance of the corpora lutea (Akins & Morrisette 1968). The uterine horns were opened lengthwise and washed with sterile PBS. Small pieces of the uterus containing endometrial and myometrial tissues were cut off and then minced finely into 1 mm³ fragments with scissors. Tissue samples were digested with sterile 0.2% collagenase (Sigma–Aldrich Chemie GmbH) in PBS for 2 h at a temperature of 37 °C as described previously (Karasinski *et al.* 2000). Tissue homogenate was centrifuged at 100 *g* for 10 min and cell suspension was filtered through a nylon membrane to remove undigested tissue fragments. Cells were plated in DMEM (Sigma–Aldrich Chemie GmbH) with 10% fetal bovine serum (FBS; Sigma–Aldrich Chemie GmbH), 100 U penicillin and 100 µg streptomycin/ml and 1% L-glutamine (Sigma–Aldrich Chemie GmbH) in plastic 25 cm² culture flasks (standard conditions). Cells were cultured in a humidified chamber with 5% CO₂ and 37 °C. The medium was changed every 3–4 days until cells reached confluence. Cell cultures were routinely examined with an inverted light microscope Zeiss Axiovert 40 CFL and pictures were captured with Zeiss AxioCam MRc 5.

Clonogenicity

To examine the ability to form clones, cells from the culture were first gently treated with 0.25% trypsin containing 0.02% EDTA (Sigma–Aldrich Chemie GmbH) to get a single cell suspension. After centrifugation, cells were plated in 60 mm plates at clonal density of 100 cells/cm² and cultured for 21 days in standard medium, which was changed every 3–4 days. Plates were microscopically examined to make sure that each clone originated from a single cell. After 3 weeks,

Table 1 Primer sequences, product length, and temperature for PCR amplification.

Gene	Primer sequences	Length (bp)	Temperature (°C)
<i>Oct-4/POU5F1</i>	F: 5'-AGAACATGTGTAAGCTGCGGCC-3' R: 5'-ACTCTTCTCAGCCCRAGCTG-3'	232	54
<i>Nestin</i>	F: 5'-GAAAAGTTCCAGCTGGCTGTG-3' R: 5'-AGGGACATCTTGAGGTGYGC-3'	112	54
<i>CD29</i>	F: 5'-GGACGCTTACTGCAGGAAAGAG-3' R: 5'-ACAGTCACAKGCRCTGCCAGTG-3'	241	56
<i>CD31</i>	F: 5'-CACCRGGTGCTGTCTATAAGG-3' R: 5'-CCAGTGTCACCTGCGGTTGG-3'	202	54
<i>CD34</i>	F: 5'-GGAGCCACCAGAGCTAYTCC-3' R: 5'-CCTGGCCTCCACRRTTCTCC-3'	172	54/56
<i>CD44</i>	F: 5'-AACCGYGATGGYACYCGCTAC-3' R: 5'-GGAYGCCARGATGATSAGCCATTCT-3'	377	54
<i>CD45</i>	F: 5'-GCATCCATCCTCGTCCACTGC-3' R: 5'-GATAGATGCTGGCGATGATGTC-3'	180	54
<i>CD105</i>	F: 5'-CGCTTCAGCTTCTCCTCCG-3' R: 5'-CACCACGGGCTCCCCGCTTG-3'	281	56
<i>CD140b</i>	F: 5'-TACGTGCCCATGYTGGACATG-3' R: 5'-TGGTAGCTGAAGCCCACGAG-3'	175	54
<i>CD144</i>	F: 5'-TGCAACGAGCRGGGCGAGTT-3' R: 5'-CRCCGCCCTCCTCRTCGTA-3'	220	54/56

cells were fixed with 2% paraformaldehyde (PFA) and stained with hematoxylin to visualize clones, washed with tap water and left to dry. Colonies containing more than 50 cells (where clones were also macroscopically visible) were counted. CE was obtained by formula (number of clonal cells/number of inoculated cells) × 100% (Chan *et al.* 2004). We analyzed 20 plates from five individuals.

Multilineage differentiation

Cells from porcine uteri were cultured in standard conditions until they reached ~90% confluence. Then cultures were treated with differentiating media to induce adipogenic or osteogenic differentiation according to Pittenger *et al.* (1999). Briefly, to induce osteogenesis cells were cultured in DMEM, 10% FBS, 10^{-7} M dexamethasone, and 100 µg/ml ascorbate (Sigma-Aldrich Chemie GmbH) for 7–14 days and the medium was replaced with fresh one every 2–3 days. Following incubation, cells were fixed with 2% PFA and stained with ALP staining solution or Alizarin Red to detect the expression of ALP and cell mineralization (calcium deposition) respectively. Cells cultured only in standard medium, which served as control group, were stained in the same way. Adipogenesis was induced by treatment of cell culture with adipogenic inducing medium (AIM) composed of DMEM, 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 100 µM indomethacin, and 10 µg/ml insulin or adipogenic maintaining medium (AMM) containing DMEM, 10% FBS, and 10 µg/ml insulin (according to Pittenger *et al.* 1999) for 19 days according to the following scheme. On days 1, 3, 7, 9, 13, and 15 cultures were treated with AIM medium while on days 5, 11, and 17 this medium was replaced with AMM medium. Then, cells were fixed with 4% PFA and stained with Nile Red (Molecular Probes, Inc., Eugene, OR, USA) or Oil Red O (Sigma-Aldrich Co.) to visualize the accumulation of lipid droplets. Cells stained with Nile Red were examined with a confocal

microscope the Zeiss Axiovert 200M LSM 510 META, and cells treated with Oil Red O by light microscopy. Control cells were cultured in the standard medium in the same humidified chamber and stained as experimental cultures.

RT-PCR

For RT-PCR analysis were used cells before and after the first passage (at ~7 and 21 days of culture). The total RNA of cultured cells was extracted with the RNagents Total RNA Isolation System (Promega Corporation). Cells were detached from the culture flask with 0.1% trypsin in 0.02% EDTA and centrifuged at 100 *g* for 10 min. Pellet cells were treated with denaturing solutions and RNA was extracted in phenol: chloroform:isoamyl alcohol. The concentration of isolated RNA was measured with NanoDrop (ND-1000 Spectrophotometer). RNA quality was assessed by spectrophotometry and the nucleotide to protein ratio (260:280) was within the acceptable range (1.8–2.1). Then DNA was transcribed from RNA with DNA polymerase (Promega Corporation) and PCR was performed with the primer sequences as shown in Table 1. Electrophoresis was carried out with 3% agarose gel containing ethidium bromide for 20 min. Positive control for nestin were murine bone marrow cells after second passage cultured in plastic flasks in DMEM LG, 20% FBS, and 10 ng/ml basic fibroblast growth factor (bFGF). Cells were obtained from the Department of Medical Biotechnology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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