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Characterisation and differentiation of porcine ovarian theca-derived multipotent stem cells ☆

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ABSTRACT

In this study, the cellular properties and in vitro differentiation capacity of porcine ovarian theca-derived multipotent stem cells (TSCs) were examined. Isolated TSCs were expanded into a homogeneous population that had a typical fibroblast-shaped morphology and was positive for alkaline phosphatase activity. Cell cycle analysis indicated that TSCs had high proliferative potential. Flow cytometry analysis demonstrated expression of mesenchymal cell surface markers (CD29, CD44 and CD90) on TSCs. Among three pluripotent markers tested (OCT4, NANOG and SOX2), only SOX2 was expressed in TSCs at protein and mRNA levels. Cytochemical staining demonstrated that TSCs differentiated in vitro into osteocytes and adipocytes. Lineage specific transcripts expressed by differentiated osteocytes including osteonectin, osteocalcin and RUNX2. Lineage specific transcripts expressed by differentiated adipocytes included adipocyte fatty acid binding protein-2 (aP2) and peroxisome proliferator-activated receptor-γ2. Following induction in oogenesis media, TSCs exhibited sequential changes in morphology, resembling oocyte-like cells (OLCs), and expressed transcription factors (OCT4, NANOG and SOX2), oocyte-specific marker genes (GDF9B, C-MOS, DAZL, VASA, ZPC, SCP3 and STELLA) and the folliculogenesis marker follicular stimulating hormone receptor. These results indicated that TSCs derived from ovarian follicles are capable of differentiating into mesenchymal lineages and OLCs.

Introduction

Several studies have demonstrated the ability of embryonic stem cells (ESCs) to differentiate in vitro into oocytes or germ cells (Hubner et al., 2003; Toyooka et al., 2003; Clark et al., 2004; Geijsen et al., 2004). Germline stem cells (GSCs) derived from mouse ovaries are able to sustain oocytes and follicle production (Johnson et al., 2004). Following transplantation into ovaries of infertile mice, GSCs are able to undergo oogenesis, resulting in the birth of live offspring (Zou et al., 2009). Neo-oogenesis is also exhibited by cells of extra-gonadal origin, such as bone marrow and peripheral blood (Johnson et al., 2005); such cells are able to rescue long term fertility in mice after bone marrow transplantation (Lee et al., 2007). These results suggest that, in addition to ESCs, GSCs and somatic stem cells have the potential to differentiate into germ cells.

Primordial germ cell (PGC)-like cells and oocyte-like cells (OLCs) can be generated in vitro from porcine skin-derived stem cells (Dyce et al., 2006, 2011; Linher et al., 2009), pancreatic stem cells (Danner et al., 2007) and muscle-derived stem cells (Lv et al., 2012). We have also demonstrated that skin, adipose and ovarian stroma-derived stem cells possessed the ability to differentiate in vitro into OLCs (Song et al., 2011). The multipotency of luteinising granulosa cells derived from mature ovarian follicles has been demonstrated by differentiation into three distinct lineages (Kossowska-Tomaszczuk et al., 2009). In mice, purified putative thecal stem cells can be expanded in vitro and, after stimulation, are able to differentiate into early precursors and steroidogenic cells (Honda et al., 2007). These reports support the existence of multipotent stem cells in ovarian tissues and their ability to form different kinds of cells under suitable culture conditions.

Thecal cells are essential components of the ovarian follicle, providing both structural integrity and the androgen substrate for granulosa cells to produce oestrogen, thus playing an important role in folliculogenesis (Young and McNeilly, 2010). These cells

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arise from fibroblast-like precursor cells within the ovarian stroma and exhibit properties of self-renewal and differentiation in mice (Honda et al., 2007). The present study characterised porcine ovarian theca-derived multipotent stem cells (TSCs) by determining their alkaline phosphatase (AP) activity, cell cycle status, expression of cell surface and pluripotency-related markers, and ability to differentiate in vitro into osteocytes, adipocytes and OLCs.

Materials and methods

Isolation and culture of theca-derived multipotent stem cells

Porcine ovaries collected at an abattoir were transferred into phosphate buffered saline (PBS, Sigma). Ovarian follicles (>4 mm size) were collected using fine forceps and a scalpel blade under a microscope and the interstitial tissue was removed. Follicles were cut in half in a dish containing Dulbecco's-PBS (D-PBS, Sigma) and the granulosa cells were removed by gentle scraping. The remaining portions of the follicles comprising thecal layers were dissected manually and incubated in 0.1% collagenase (Sigma) for 30 min, followed by inactivation with 30% fetal bovine serum (FBS, Gibco) for 5 min at room temperature (RT). Isolated cells were filtered through 100 and 40 μm cell strainers (BD Falcon) and washed twice in D-PBS supplemented with 1 mg/mL polyvinyl alcohol (Sigma), 100 U/mL penicillin G (Sigma) and 100 μg/mL streptomycin (Sigma) by centrifugation at 500 *g* for 5 min. Cells were cultured in advanced-Dulbecco's modified Eagle's medium (A-DMEM, Gibco) supplemented with 10% FBS, 2 mM glutamine (Sigma), 100 U/mL penicillin G and 100 μg/mL streptomycin at 38.5 °C in a 5% CO_2 incubator. When they reached confluence, TSCs were dissociated in 0.25% trypsin–ethylene diamine tetraacetic acid (EDTA) (Gibco) and centrifuged at 500 *g* for 5 min to form a pellet. Cells at passage 3 were used for further experiments.

Alkaline phosphatase activity

Cells were fixed with 3.7% formaldehyde for 1 h, stained for AP using the AP chromogen kit (BCIP/NBT; Promega) for 30 min at RT and evaluated under a microscope. Porcine adult skin fibroblasts (AFs) were used as a negative control. Adult ear skin was sliced into 1–2 mm^2 pieces, plated onto a cell culture dish and cultured in media as described for TSCs. On day 3, when migration of fibroblasts from the margin of the skin was demonstrated, the ear tissue was gently removed from culture dishes. The culture medium was changed every 3 days. AFs at passage 3 were used for further analysis.

Cell cycle and cell surface markers

TSCs at 80% confluence were analysed for cell cycle status and surface marker expression by fluorescence-activated cell sorting (FACS, Becton Dickinson), as described previously (Song et al., 2011). For cell cycle analysis, TSCs were fixed in 70% ethanol (Sigma) and stained with 10 μg/mL propidium iodide (Sigma). A total of 1×10^4 cells were analysed for DNA content in triplicate. Fluorescein isothiocyanate (FITC)-conjugated antibodies against CD44 (catalogue number 553133, clone IM7, monoclonal, rat anti-mouse CD44, 1:100; BD Pharmingen), CD90 (catalogue number 555595, clone 5E10, monoclonal, mouse anti-human CD90, 1:100; BD Pharmingen) and CD45 (catalogue number 340664, clone 2D1, monoclonal, mouse anti-human CD45, 1:100; BD Pharmingen) were used for cell surface marker expression. FITC-conjugated goat anti-mouse IgG (catalogue number 554001, polyclonal, 1:100; BD Pharmingen) was used to visualise unlabelled mouse anti-porcine CD29 (catalogue number 552369, clone NaM 160-1A3, monoclonal, mouse anti-pig CD29, 1:100; BD Pharmingen). Isotype-matched controls were analysed in parallel in each run. Analysis was performed for 1×10^5 events using FACScalibur and CellQuest software (Becton Dickinson).

Immunofluorescence staining

TSCs were incubated with primary antibodies: OCT4 (catalogue number sc-8628, goat polyclonal, 1:200), NANOG (catalogue number sc-30331, goat polyclonal, 1:200) and SOX2 (catalogue number sc-20088, rabbit polyclonal, 1:200) (Santa Cruz Biotechnology) overnight at 4 °C. Incubation with FITC-conjugated donkey anti-goat IgG (catalogue number 705-095-147, 1:200) or donkey anti-rabbit IgG (catalogue number 711-095-152, 1:200) (Jackson IR Laboratories) was performed for 45 min at 38.5 °C. Nuclei were counterstained with 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI, Sigma) for 5 min and negative controls were performed with secondary antibodies alone. Images were taken with a fluorescence microscope (Leica).

Osteogenic and adipogenic differentiation

Osteogenic and adipogenic differentiation of TSCs was induced using previously published methods (Pittenger et al., 1999; Vacanti et al., 2005). Osteogenesis was induced in DMEM (Gibco) containing 10% FBS with 0.1 μM dexamethasone (Sigma), 50 μM ascorbate-2-phosphate (Sigma) and 10 mM β-glycerol phosphate (Sigma) for 4 weeks. Differentiated cells were stained with von Kossa and alizarin red S (Sigma). Adipogenesis was induced in DMEM containing 10% FBS with 1 μM dexamethasone, 10 μM insulin (Sigma) and 200 μM indomethacin (Sigma) for 4 weeks. Differentiation was confirmed by oil-red O staining (Sigma).

Reverse transcriptase PCR

Total RNA was extracted using the RNeasy mini kit (Qiagen). cDNA synthesis was performed from 1 μg RNA using the Omniscript Reverse Transcription Kit (Qiagen) at 55 °C for 30 min. PCR was performed using Maxime-PCR Premix (iNtRON Biotechnology) (Song et al., 2011); oligonucleotide primers used are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as a reference gene.

Differentiation into oocyte-like cells

In vitro differentiation of TSCs into OLCs was induced according to the protocol of Dyce et al. (2006), with minor modifications. Oogenesis was induced in DMEM Nutrient Mixture F12 (DMEM/F12, 1:1, Gibco) supplemented with 5% FBS, 5% porcine follicular fluid, 0.23 mM sodium pyruvate (Sigma), 0.1 mM non-essential amino acids (Gibco), 2 mM L-glutamine and 0.1 mM β-mercaptoethanol (Sigma) for 48 days; the medium was changed twice each week. To assess development, at day 48 of differentiation the cell aggregates were cultured in TCM-199 (Gibco) growth medium supplemented with 3 mg/mL BSA (Sigma), 5 μg/mL insulin, 2.75 μg/mL transferrin, 2.5 μg/mL selenium mix (ITS+3, Sigma), 0.005 U/mL follicle stimulating hormone (Sigma), 0.0025 U/mL luteinising hormone (Sigma), 0.11 mg/mL sodium pyruvate and 1 ng/mL epidermal growth factor (Sigma) for 15 days; the medium was changed every 3 days. Induced OLCs were evaluated for morphological changes under a fluorescence microscope (Nikon) after being stained with 1 μg/mL bisbenzimide (Sigma) for 5 min.

Chromosomal analysis

Blastocyst-like structures in TCM-199 were treated with 0.01 μg/mL demecolcine (Gibco) for 8 h. Following dissociation with 0.25% trypsin–EDTA, the cells were harvested and incubated in 75 mM KCl solution (Sigma) for 30 min at RT, then fixed

Table 1
Primer sequences and product size for reverse transcriptase PCR.

Gene	Primer sequence (5′–3′)	Product size (base pairs)
OCT4	AGGTGTTCAGCCAAACGACC TGATCGTTTGCCCTTCTGGC	335
SOX2	GCCTGGGCGCCGAGTGGA GGGCGAGCCGTTCATGTAGGTCTG	443
NANOG	ATCCAGCTTGTCCCCAAAG ATTTCATTCGCTGGTTCTGG	438
Osteonectin (ON)	TCCGGATCTTTCCTTTGGTTTCTA CCTTCACATCGTGGCAAGAGTTTG	187
Osteocalcin (OC)	CTGGACCAACATCTTGAGCA ACCCCTTTGGTGGTGTTGTA	205
RUNX2	CAGACCAGCAGCACTCCATA AACGCCATCGTTCTGGTTAG	171
aP2	AACCCAACCTGATCATCACTG TCTTTCCATCCCACTTCTGC	192
PPARγ2	GCGCCCTGGCAAAGCACT TCCACGGAGCGAAACTGACAC	238
GDF9B	GGATCCAGAAAAGCACAACC AGTGTCCAGGGATGAAATGC	227
C-MOS	AAATCAGCGACTTTGGTTGC CTGACGCTCCCCTGAGTAAG	200
DAZL	CCTCCAACCATGATGAATCC GGGCAAAATATCAGCTCCTG	222
VASA	TTGCAGGACGAGATTTGATG CCAATTCTCGAGTTGGTGT	165
FSHR	CTCACCTACCCCAGCCACT CTCAGGGGAGCAAGTCACAT	243
ZPC	TGGTGTACAGCACCTTCCTG ATCAGGCGCAGAGAGAACAC	202
SCP3	AGCCGTCTGTGGAAGATCAG AACTCCAACTCCTTCCAGCA	197
STELLA	TTAATCCAACCCGGACTCAG TGGTTGAGGTGGATATTCTGG	173
GAPDH	GGGCATGAACCATGAGAAGT AAGCAGGGATGATGTTCTGG	230