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RESEARCH ARTICLE

STEM CELLS AND REGENERATION

Identification and characterization of putative stem cells in the adult pig ovary

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ABSTRACT

Recently, the concept of 'neo-oogenesis' has received increasing attention, since it was shown that adult mammals have a renewable source of eggs. The purpose of this study was to elucidate the origin of these eggs and to confirm whether neo-oogenesis continues throughout life in the ovaries of the adult mammal. Adult female pigs were utilized to isolate, identify and characterize, including their proliferation and differentiation capabilities, putative stem cells (PSCs) from the ovary. PSCs were found to comprise a heterogeneous population based on c-kit expression and cell size, and also express stem and germ cell markers. Analysis of PSC molecular progression during establishment showed that these cells undergo cytoplasmic-to-nuclear translocation of Oct4 in a manner reminiscent of gonadal primordial germ cells (PGCs). Hence, cells with the characteristics of early PGCs are present or are generated in the adult pig ovary. Furthermore, the *in vitro* establishment of porcine PSCs required the presence of ovarian cell-derived extracellular regulatory factors, which are also likely to direct stem cell niche interactions *in vivo*. In conclusion, the present work supports a crucial role for c-kit and kit ligand/stem cell factor in stimulating the growth, proliferation and nuclear reprogramming of porcine PSCs, and further suggests that porcine PSCs might be the culture equivalent of early PGCs.

KEY WORDS: Ovarian stem cells, Oogenesis, Kit ligand, Nuclear reprogramming, Differentiation

INTRODUCTION

The question of 'neo-oogenesis' has received renewed attention since it was shown that the mouse ovary has an unexpected ability to regenerate immature oocytes after their destruction (Johnson et al., 2004). The culture of cells attained from scrapings of the human ovarian surface epithelium (OSE) resulted in the formation of large oocyte-like cells (OLCs) expressing zona pellucida proteins (Bukovsky et al., 2005), leading the authors to suggest that putative germ cells within the OSE of the postnatal ovary differentiate from mesenchymal progenitors in the ovarian tunica albuginea. In line with this possibility, small round (2-4 μm diameter) c-kit/stage-specific embryonic antigen (SSEA)-positive cells were isolated from human OSE cells. These cells expressed early primordial germ cell (PGC) markers, including OCT4 (POU5F1), NANOG and SOX2 (Virant-Klun et al., 2008). The isolated PGCs were similar to cells termed 'very small embryonic-like (VSEL) stem cells', which have been found in a number of human and other animal adult tissues (Ratajczak et al., 2008).

More recently, female germline stem cells (FGSCs) were shown to be capable of producing oocytes, and the fertilized oocytes were in turn capable of generating offspring in mice. The FGSCs were identified at the ovarian surface as cells of ~12-20 μm diameter. These cells expressed germ cell markers but not early stem cell markers (Zou et al., 2009), raising controversy as to their true nature (Telfer et al., 2005; Zhang et al., 2012). Some stem cell biologists assert that FGSCs appear after the PGC stage but before the formation of true oogonia, and can be thus classified as 'growth-arrested oogonia' (Abban and Johnson, 2009; Notarianni, 2011). However, no evidence for the presence of oogonia was found in the human ovary after their final clearing during the first 2 years of postnatal development (Byskov et al., 2011), and therefore arguments persist as to the origin of FGSCs (De Felici, 2010; Oatley and Hunt, 2012).

White et al. (2012) confirmed that the ovaries of reproductive age adult humans possess rare, mitotically active germ cells that have the capacity to generate oocytes. Furthermore, Hayashi et al. (2012) reported that the transplantation of both female PGCs and embryonic gonadal somatic cells underneath the ovarian bursa or the kidney capsule of recipient mice resulted in the transformation of induced embryonic stem cells (ESCs) into PGC-like cells. The PGC-like cells then went on to contribute to the pool of OLCs in the reconstituted ovaries. These studies jointly indicate the possibility of reconstituting crucial aspects of human as well as murine female germline cell development *in vitro*. However, important questions remain regarding the origin, nature and potential roles of these germ cells before any serious consideration of their application to human medicine can be made.

Cell cultures derived from OSE scrapings were employed to show convincingly that VSEL stem cells exist in the adult OSE of human and other large mammals, and confirmed the *in vitro* development of OLCs from OSE tissue (Bukovsky et al., 2005; Virant-Klun et al., 2008; Parte et al., 2011). Although these data support the presence of postnatal oogenesis in adult humans and other mammals, the culture systems employed were very simple, and it remains unknown whether the cells obtained in fact constitute genuine proliferating populations.

In addition, in contrast to the wave of meiosis initiation observed in fetal mouse ovaries, a radial gradient is observed in human fetal ovaries. This suggests the existence of species-specific differences in meiosis commencement cues, with local somatic cell interactions versus diffusible signals operating in humans versus mice (Gkountela et al., 2013). The procurement of mammalian models of oogenesis other than the mouse is therefore essential for understanding such mechanisms, as some of the events in mouse oogenesis diverge widely from those in human oogenesis (Anderson et al., 2007; Zayed et al., 2007). As such, the aim of

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this study was to isolate, identify and characterize germline stem cells from the ovary of adult pigs, to elucidate their origin, and finally to investigate the regulation of their proliferation, reprogramming and differentiation *in vitro*.

RESULTS

Cell culture media

MEM-Alpha, StemPro-34 and DMEM-F12 were initially used for the optimization of putative stem cell (PSC) culture conditions. Although this study also used culture supplements, such as GDNF, bFGF (FGF2), EGF and LIF, that are essential for the maintenance of spermatogonial stem cells (Kubota et al., 2004) and FGSCs (Zou et al., 2009), these culture conditions were deemed insufficient for the establishment of porcine PSCs (supplementary material Table S3). Therefore, the utility of DMEM-F12 supplemented with 10% fetal bovine serum (FBS) or 10% Knockout Serum Replacement (KSR) (Invitrogen) was examined, as was that of DMEM supplemented with B27 (Invitrogen) or various concentrations of stem cell factor (SCF; also known as kit ligand) (0, 10, 20, 30, 40, 50 ng/ml; STEMCELL Technologies, Vancouver, Canada) (Fig. 1).

The results showed that supplementation with SCF significantly enhanced the proliferation of PSCs in a concentration-dependent manner. Supplementation with FBS stimulated the proliferation of certain, morphologically flat ovarian somatic cells, and interfered with the growth of the PSCs. Furthermore, PSCs cultured with KSR readily reaggregated with ovarian somatic cells to form clumps, also inhibiting PSC proliferation (Fig. 1A-C). Therefore, DMEM-F12 supplemented with B27 (DMEM-F12/B27) plus 40 ng/ml SCF was considered the most effective medium for PSC growth (Fig. 1D).

Ovarian cell-derived regulatory factors are crucial for the establishment of PSCs

Primary ovarian cells formed spherical colonies comprising compact clusters of small round PSCs (5-7 µm in diameter) 1 day after culture in DMEM-F12/B27 plus SCF, interspersed with a few red blood cells (RBCs) (Fig. 2Aa,b). The PSC clusters appeared dark and shiny, with constituent cells that were smaller or similar in size to RBCs (6-8 µm). The PSCs could easily be distinguished from the RBCs at 1 day because the latter were of the typical biconcave disc shape (Fig. 2Ab). The PSCs had completely round nuclei that took up almost the entire volume of the cell, as evidenced by DAPI staining (Fig. 2Ba), as has been described for VSEL stem cells in the adult human ovary (Parte et al., 2011). However, the PSCs were either not detected or only weakly detected by May-Grunwald-Giemsa staining (Fig. 2Bb).

After 1 week, the PSCs increased in number and size, and some grew to ~10-12 µm (Fig. 2Ac; supplementary material Fig. S1). Most of the PSCs were 10-12 µm in diameter after 10 days in culture, forming groups of cells that clustered around the ovarian cell colonies (Fig. 2Ca,b). At this time, the colonies and the surrounding PSCs were treated with 0.05% trypsin-EDTA for 2 min to disperse the PSCs, while leaving most of the colonies intact. Then, the cells were passed through a 40-µm filter to remove all of the remaining colonies, which contained ovarian cells such as theca stem cells and granulosa cells (Honda et al., 2007; Kossowska-Tomaszczuk et al., 2009). The filtered cells were cultured on laminin-coated dishes or on a mitomycin C-treated mouse embryonic fibroblast (MEF) feeder layer. After 1 month in culture under these conditions, with one passage per week, the proliferation of the PSCs was reduced.

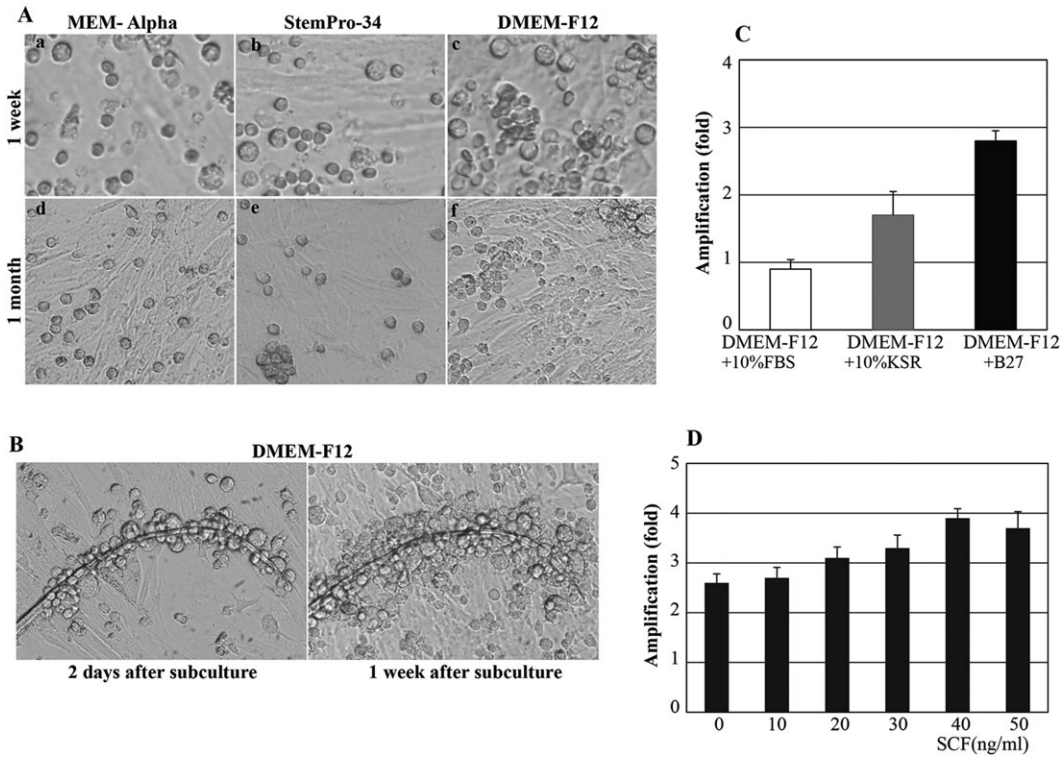

Fig. 1. Comparison of culture media and culture supplements for the establishment of PSCs. (A) Proliferation of PSCs after 1 week of culture in MEM-Alpha (a), StemPro-34 (b) and DMEM-F12 (c) medium. After 1 month in culture, DMEM-F12 exhibited a significant effect on PSC proliferation (f, compared with d,e). (B) Spontaneously differentiated oocytes appeared after subculture in DMEM-F12. (C) Effect of KSR and serum-free B27 supplementation on PSC proliferation (n=6). PSCs were cultured for 7 days on gelatin-coated dishes. Note the improved growth of PSCs in DMEM-F12 supplemented with B27 (DMEM-F12/B27) versus KSR. (D) Effect of SCF on PSC proliferation (n=6). PSCs were cultured for 7 days on gelatin-coated dishes with DMEM-F12/B27 supplemented with various concentrations of SCF (10, 20, 30, 40 or 50 ng/ml). PSC proliferation was considerably improved in the presence of 40 ng/ml SCF. Error bars indicate s.e.m.

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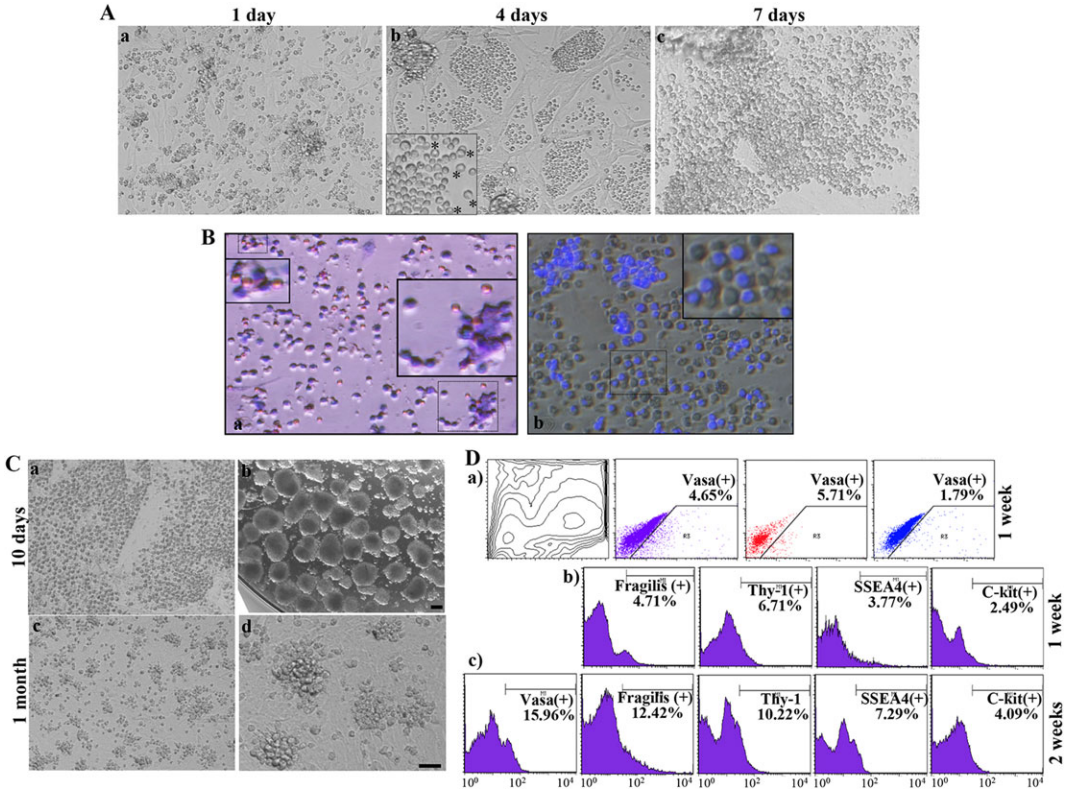

Fig. 2. Development of PSCs. (A) After isolation from the ovary, PSCs in culture appeared dark and shiny and were easily distinguished from RBCs, which had a typical biconcave disc morphology (asterisks). The PSCs gathered in hollows formed by ovarian epithelial cells after 4 days in culture (b), or were trapped within the theca stem cell colonies. The PSCs increased in number and size after 1 week (c). (B) The small PSCs (5-7 μm in diameter) were similar in size to RBCs and round in appearance, but unlike RBCs they had a high nucleus-to-cytoplasm ratio, and the nuclei were stained by DAPI. PSCs were weakly detected by May-Grunwald-Giemsa staining, whereas all of the RBCs were stained red or blue. (C) PSCs grew to a uniform size (10-12 μm) after 10 days in culture, forming groups of cells that clustered around theca stem cell colonies (a,b). PSCs were maintained for 1 month on a layer of ovarian somatic cells (c,d). (D) Flow cytometric characterization of PSCs after 1 week in culture demonstrated that 25% of the cells were small (5-7 μm) and 75% were large (10-12 μm). Vasa-positive cells comprised 1.79% of the small PSCs and 5.71% of the large PSCs (a). Some PSCs were also positive for other germ and stem cell markers, such as Fragilis, Thy-1, SSEA4 and c-kit (b). After 2 weeks in culture, the PSCs became uniform in size and made up an increasing percentage of the total cell population (c). Scale bar: 50 μm.

Furthermore, the cells changed their morphology from round to adherent, and somatic cell types appeared (supplementary material Fig. S2A,B). These observations indicate that the present culture conditions were not suitable for the establishment and long-term maintenance of PSCs.

Because the PSCs tended to gather in hollows formed by the primary ovarian cells (Fig. 2A), and because extracellular secreted factors play essential roles in stem cell-niche interactions, we hypothesized that ovarian cells might provide an appropriate *in vitro* microenvironment for the establishment, maintenance and proliferation of PSCs. Thus, we generated PSC cultures containing ovarian cells. After 10 days in culture, the colonies and the surrounding PSCs were treated with 0.25% trypsin-EDTA for 3 min. This treatment dispersed most of the cells, including the ovarian cell colony-derived cells. The dispersed cells were then passed through a 40-μm filter to remove only the largest clumps of theca stem cells, followed by culture on dishes coated with gelatin (1:1 dilution).

Under these conditions, PSCs formed clusters or grew as dispersed cells on top of flat layers of epithelial and somatic ovarian cells. The cells required passage at confluence every 5-7 days, with cultures being split at a 1:2 dilution. Although the PSCs continued to grow, most of the remaining theca stem cells and the flat cell layers gradually disappeared after more than 1 month in culture (Fig. 2Cc,d). Therefore, the PSCs were transferred onto mitomycin C-treated MEF feeder layers after 1 month for long-term culture, as described in the scheme for the establishment of PSCs (supplementary material Fig. S3A).

PSCs undergo molecular progression during establishment

Flow cytometry analysis revealed abundant PSC proliferation after isolation and culture for 1 week. Of these, 4.65% of the cells were positive for the germ cell marker Vasa and some of the cells were also positive for additional germ and stem cell markers, such as Fragilis, Thy-1, SSEA4 and c-kit (Fig. 2Da,b). At this time, two populations of PSCs were observed: one with a cell diameter of 5-7 μm and one with a cell diameter of 10-12 μm (Fig. 2Da). The cells became identical in size after 2 weeks in culture, at 10-12 μm, with an increasing percentage of cells positive for germ and stem cell markers (Fig. 2Dc).

About 2.8% of all mouse testicular cells are c-kit positive (Kanatsu-Shinohara et al., 2004) and have the capacity to become multipotent germline stem cells, whereas c-kit-negative cells go on to become spermatogonial stem cells (Izadyar et al., 2008). We similarly observed two distinct subsets of cells (c-kit positive versus c-kit negative) within the PSC population. This finding was strengthened by immunofluorescence analysis showing that, after 1 month in culture, most of the PSCs expressed high levels of the reprogramming factor Oct4, whereas only 22% of the PSCs expressed high levels of c-kit (Fig. 3Aa-d,B).

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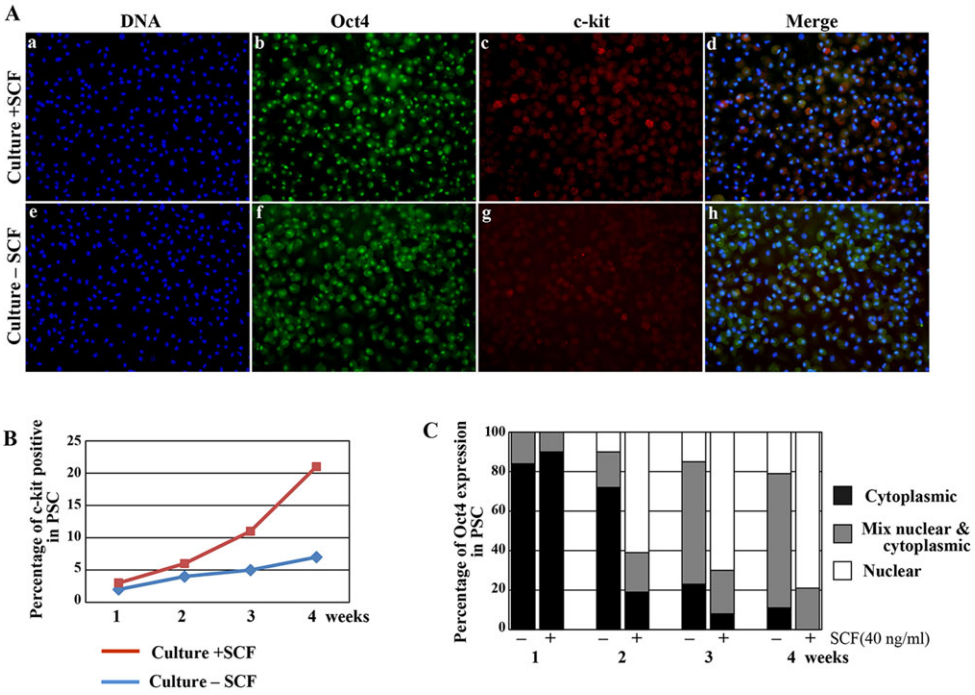

Fig. 3. SCF improves the reprogramming of porcine PSCs during establishment. PSCs were isolated and cultured in medium without and with 40 ng/ml SCF for 1, 2, 3 and 4 weeks. They were then collected for the detection of Oct4 and c-kit by immunostaining. (A) Representative immunofluorescence detection of Oct4 and c-kit expression in PSCs after 4 weeks in culture; DAPI (a,e). (B) Quantification of c-kit-positive PSCs after 1, 2, 3 and 4 weeks in culture. (C) Quantification of nuclear versus cytoplasmic localization of Oct4 in PSCs after 1, 2, 3 and 4 weeks in culture.

Interestingly, when PSCs were cultured without SCF, the percentage of c-kit-positive PSCs was significantly decreased relative to culture with SCF (Fig. 3A,B). In addition, SCF treatment significantly affected the expression of Oct4 (Fig. 3A,C). PSCs cultured in the presence of SCF exhibited intense cytoplasmic staining for Oct4 after 1 week in culture (Fig. 4B), whereas Oct4 expression was reduced in the cytoplasm and augmented in the nucleus after 2 weeks in culture (Fig. 4Ce). Furthermore, SCF treatment significantly increased the number of large PSCs expressing Oct4 in the nucleus after 1 month in culture (Fig. 3C). A similar phenomenon has been described in PGCs undergoing nuclear reprogramming over the course of fetal development in mice and humans (Anderson et al., 2007; Gkountela et al., 2013). Hence, c-kit and SCF are crucial to the nuclear reprogramming required for the establishment of porcine PSCs.

After 1 week in culture, small PSCs with a cell diameter of 5-7 µm demonstrated cytoplasmic localization of the germ cell markers Vasa, Stella and SSEA4 (Fig. 4A,B; supplementary material Fig. S4A). In addition, Oct4 protein expression was found throughout entire colonies of ovarian cells, whereas Stella was only found in small PSCs gathered around the colonies (Fig. 4Be,f). This result confirmed that the ovarian cell colonies contained theca stem cells or somatic cells, as they do not express any germ cell markers (Honda et al., 2007).

After 2 weeks in culture, the PSCs became much larger and abundant in the cytoplasm, adhering loosely to the ovarian cell colonies and maintaining their expression of germ cell markers (Fig. 4Ca-d). Sohlh1 protein, which is detected in germ cell cysts, was also detected in PSCs at 2 weeks (Fig. 4Cf). Although all of the small PSCs expressed germ cell markers after 1 week (Fig. 4D), the expression levels of stem cell markers (e.g. Oct4, Nanog, Sox2, Rex1, cMyc and KLF4) showed substantial cell-to-cell variation (Fig. 4E). After 4 weeks, all of the PSCs were 10-12 µm in diameter and strongly expressed stem and germ cell markers at both the protein and mRNA level (Fig. 4Ch,i,D,E). The oocyte markers SCP3 and ZP were not detected in the cells during culture (Fig. 4D).

PSCs share characteristics with epiblast-derived PGCs

We next investigated the developmental origin of porcine PSCs. In normal development, c-kit, SSEA1 and SSEA4 are expressed by the majority of pregonadal PGCs and are progressively downregulated when PGCs enter into meiosis in the embryonic ovary (Kerr et al., 2008). By contrast, Vasa protein is detectable only when PGCs enter the gonadal ridges and remains elevated in human fetal and postnatal oocytes (Castrillon et al., 2000). VASA (DDX4)-negative VSEL stem cells (2-4 µm) isolated from the human OSE express genes typical of ESCs, such as *NANOG* and *SOX2*, thereby indicating their undifferentiated status. After culture for 3 weeks under differentiation conditions, VASA-negative cells are transformed into OLCs expressing VASA and ZP2, a marker for oocytes (Virant-Klun et al., 2008). In the present study, small Vasa-positive porcine PSCs (5-7 µm in diameter) began to reduce their expression of Nanog, Sox2 and Rex1 after 1 week in culture (Fig. 4E), indicating their transformation to a differentiating status. Previous investigations showed that Vasa-positive VSEL stem cells isolated from adult organs express several characteristic markers of early PGCs, including fetal-type alkaline phosphatase, Oct4, SSEA-1, CXCR4, Stella, Fragilis, Nobox and Hdac6 (Ratajczak et al., 2008). Because the porcine PSCs described herein similarly express a number of typical, early PGC markers (Figs 2 and 4), these findings might indicate a close association of PSCs with Vasa-positive VSELs and epiblast-derived PGCs.

In addition, the strong expression of ESC markers (e.g. Nanog, Sox2, Rex1, cMyc and KLF4) in porcine PSCs after 4 weeks in culture demonstrates that the PSCs can dedifferentiate under appropriate conditions (Fig. 4E). We have occasionally observed small, amoeboid process-bearing PSCs, which are probably counterparts to gonadal PGCs, that still retain their motile capability to wander throughout the ovarian tissue (Motta et al., 1997) (supplementary material Movie 1). Taken together with the observed molecular progression of PSCs, our results suggest that Vasa-positive cells with the characteristics of early PGCs are present or are generated in the adult pig ovary, and that these small Vasa-positive PSCs are probably derived from VSEL stem cells in the OSE.

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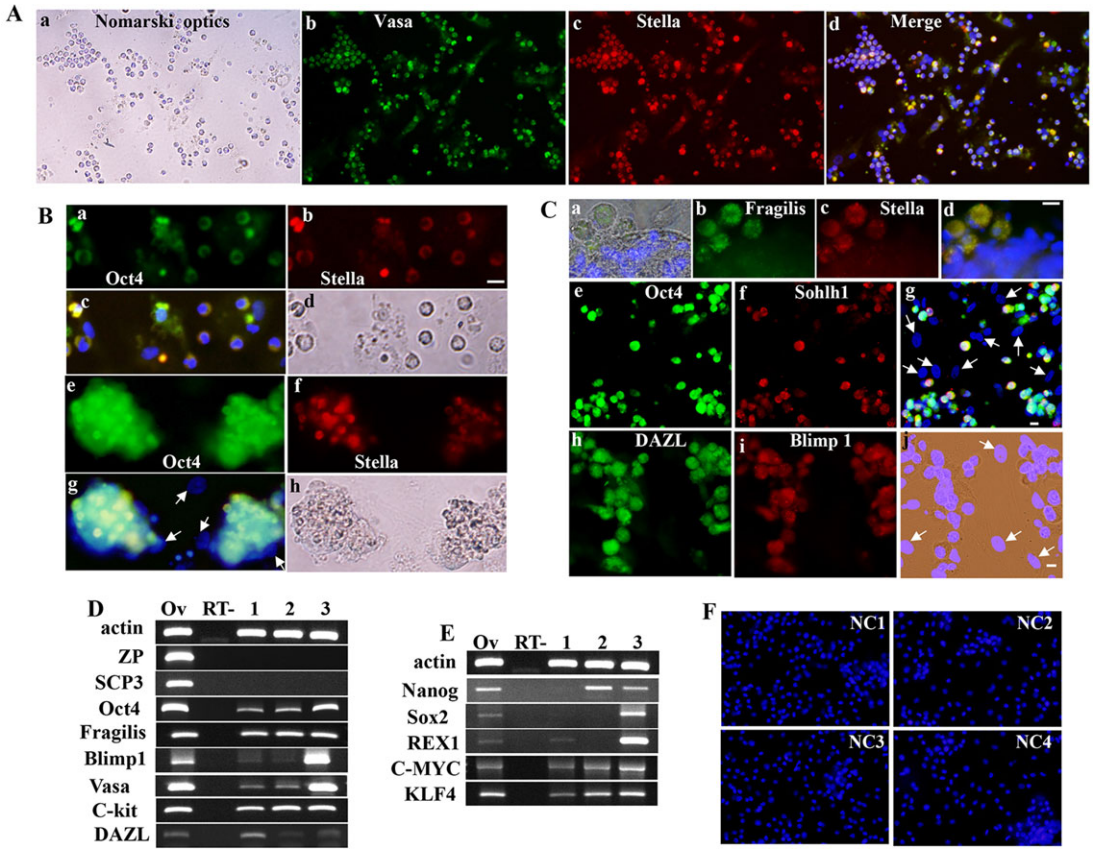

Fig. 4. PSCs undergo molecular progression during establishment. (A,B) After 1 week in culture, small PSCs showed cytoplasmic localization of Vasa, Stella and Oct4. Compact colonies were surrounded by small PSCs and contained theca stem cells or somatic ovarian cells (Bg, arrows). (C) After 2 weeks, the PSCs became larger and maintained their expression of Fragilis, Stella, Oct4 and Sohlh1. Oct4 protein expression was reduced in the cytoplasm and became localized in the nuclei of PSCs at this time (e). After 4 weeks in culture, most of the PSCs were large (10-12 μm) and maintained their expression of the germ cell markers DAZL and Blimp1. The flat layer of epithelial and somatic cells did not express any germ cell markers (g,j, arrows). (D,E) mRNA expression levels of oocyte-specific (ZP and SCP3), germ cell-specific (Fragilis, Blimp1, Vasa, c-kit and DAZL) and stem cell-specific (Oct4, Nanog, Sox2, Rex1, cMyc and KLF4) markers in PSCs. β-actin mRNA was used as the normalization control. Ov, ovarian tissue; RT-, control (water); 1, 2, small PSC samples #1 and #2 after 1 week in culture; 3, PSCs after 4 weeks in culture. (F) Alexa Fluor 488 (anti-rabbit NC1; anti-mouse NC2) and Alexa Fluor 568 (anti-rabbit NC3; anti-mouse NC4) were used as negative controls. Scale bars: 10 μm.

Maintenance of PSCs *in vitro* and induced differentiation into OLCs

Newly established PSCs were expanded *in vitro* for at least 6 months and passaged 30 times without loss of proliferative potential (Fig. 5A). Moreover, the cells maintained expression of the identifying germline markers (Fig. 5B; supplementary material Fig. S4B). The estimated cell doubling time was 48-72 h (Fig. 5C). After that, although differentiated cells increased among PSCs after long-term culture, they retained high proliferation as shown by large numbers of PSCs double positive for BrdU and Oct4 or Vasa (Fig. 5D,E). Live cell imaging showed that the germinal granules were equally separated into daughter PSCs after cell division (Fig. 5F, arrows). These cytoplasmic structures are characteristically observed in germline cells, becoming discernible at later stages of germ cell differentiation (Chuma et al., 2009). These results demonstrate that live PSCs undergo mitosis in culture, providing the clearest evidence of *in vitro* oogenesis.

In addition, the PSCs showed positive alkaline phosphatase staining, and the intensity of the staining was stronger in the germinal granules than in any other region of the cell (Fig. 5G). Cytogenetic analysis also showed that the PSCs had a normal karyotype of 38, XX (Fig. 5H). Transplantation of PSCs into immunodeficient mice failed to result in teratoma formation, indicating that these cells are not pluripotent stem cells (Fig. 5I).

To confirm the presence of *in vitro* oogenesis, we transduced a transgene encoding EGFP into porcine PSCs that had been cultured for more than 6 months to create EGFP-PSCs. The EGFP-PSCs reaggregated with dispersed adult pig ovarian cortical tissue (OCT) cells at a ratio of one EGFP-PSC to five OCT cells (Fig. 6Aa). After 2 days in culture, numerous clumps of aggregated cells formed that contained both EGFP-PSCs and OCT cells (Fig. 6Ab). After 2 weeks in culture, many primordial OLCs were observed that consisted of both EGFP-positive OLCs derived from the EGFP-PSCs, and EGFP-negative OLCs derived from the OCT cells (Fig. 6Ac,d). Hence, OLCs were spontaneously generated from PSCs reaggregated with ovarian tissues, consistent with earlier reports from mouse and human models (Pacchiarotti et al., 2010; White et al., 2012).

To study the differentiation potential of OLCs further, the PSCs, after 3 weeks of isolation (supplementary material Fig. S4C), were cultured under differentiation conditions for 4 weeks. During this time, some of the PSCs grew large in size (~50 μm in diameter) and aggregated with others to form oocyte-cumulus complex (OCC)-like structures (Fig. 6Bb, arrows). Although all of the PSCs were exposed to the same culture medium, only ~0.1% developed into OCC-like structures (supplementary material Fig. S5A). This is similar to the situation in the ovary, where a high somatic cell to oocyte ratio is required to provide the requisite microenvironment for oocyte growth and differentiation.

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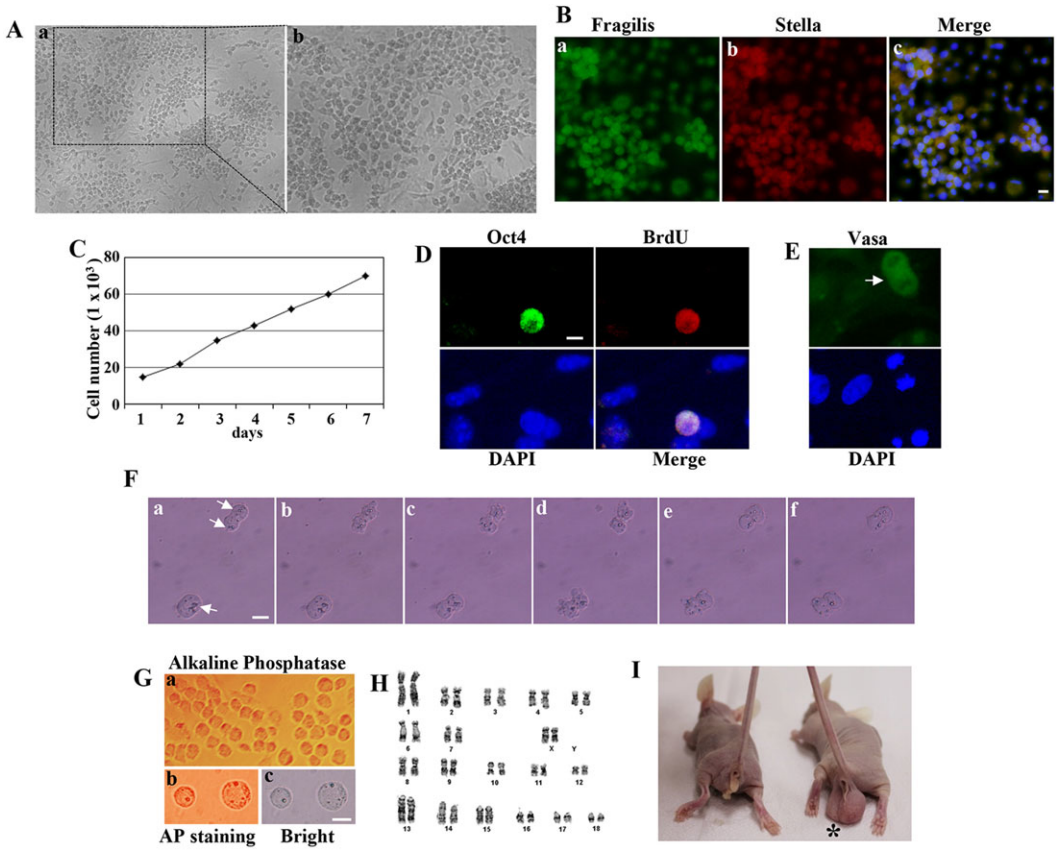

Fig. 5. Characterization and maintenance of PSCs. (A) Maintenance of PSCs after long-term culture on MEF feeder cells. (B) PSCs could be expanded *in vitro* for months without the loss of germ cell markers. (C) Selected cell lines were frozen/thawed and propagated for at least 6 months, with an estimated cell doubling time of 48-72 h. (D,E) BrdU incorporation together with Oct4 (D) and Vasa (E) expression was detected in the PSCs after long-term culture, whereas the feeder cells were negative for these markers (see merge with DAPI image). Arrow indicates a dividing PSC. (F) The presence of actively dividing PSCs was demonstrated by live cell imaging [with photos taken from the beginning (a) until the end of cell division (f)]. Arrows indicate germinal granules. (G) PSCs stained positive for alkaline phosphatase (a). High magnifications (b,c) show PSCs in M phase (large; right) and in S phase (small; left). (H) PSCs showed a normal karyotype (38, XX). (I) Teratoma formation was assessed after the transplantation of PSCs into the testes of immunodeficient mice. No tumors were found at 5 months after PSC transplantation, whereas control murine ESCs formed tumors at 1 month after transplantation (asterisk). Scale bars: 10 μm.

Gene expression analysis showed that OLCs expressed many of the same germ cell markers as PSCs (Fig. 6C). However, the oocyte markers ZP, ZPC, SCP3 and GDF9 were only found in OLCs after 2 weeks of differentiation. After 3-4 weeks of differentiation, these oocyte markers reached expression levels in OLCs that were similar to those in normal germinal vesicle (GV)-stage oocytes (Fig. 6C), as summarized in the procedure for the differentiation of PSCs (supplementary material Fig. S3B).

Immunostaining clearly showed that the germ cell markers Blimp1 and DAZL were expressed in all of the PSCs, whereas the OLCs alone exhibited positive staining for the oocyte markers GDF9 and LHX8 (Fig. 7Aa-c; supplementary material Fig. S5B). In addition, the OLCs exhibited positive staining for Vasa, c-kit, DAZL, Stella, SCP3 and GDF9, whereas the adjacent somatic cells were negative, indicating specific expression of these germ cell markers in OLCs (Fig. 7A,B). As with normal primordial oocytes, the PSC-generated OLCs contained many cytoplasmic germinal granules (Fig. 7C). After 2 weeks in culture, ~10% of the PSCs grew sufficiently large to approximate the size of fully grown oocytes (>100 μm; Fig. 7D). The cells also expressed oocyte and germ cell markers (supplementary material Fig. S5C,D).

To elucidate whether the oocytes generated were truly derived from mitotically active PSCs, and did not instead represent oocytes derived from primary ovarian cells, we isolated and purified PSCs by SSEA4-based magnetic bead sorting, as small SSEA4-positive cells from human ovarian cell cultures are reportedly related to ESCs and cells of the germinal lineage (Virant-Klun et al., 2013), and small porcine PSCs showed cytoplasmic expression of SSEA4 (supplementary material Fig. S4A). Cell sorting resulted in the collection of 759±46 (s.e.m. for three replicate experiments) cells from ten different ovaries. The SSEA4-positive cells were then transfected with EGFP. Owing to the important role of ovarian cell-derived regulatory factors in the establishment of porcine PSCs, the GFP-positive SSEA cells were aggregated with dispersed adult pig OCT cells as described above and cultured for more than 1 month.

Finally, EGFP-positive SSEA cells were differentiated into OLCs *in vitro* and transplanted into immunodeficient female mice. The further *in vitro* differentiation of OLCs provided direct evidence for EGFP-positive live oocytes (Fig. 7E). The dual immunofluorescence-based detection of EGFP *in vivo*, along with detection of either the oocyte-specific transcription factor LHX8 or the early ovarian follicle-specific growth and differentiation factor GDF9, identified many GFP/LHX8 or GFP/GDF9 double-positive cells distributed throughout the xenograft (Fig. 7F, arrows). These results convincingly demonstrate the differentiation capacity of PSCs into oocytes, both *in vitro* and *in vivo*.

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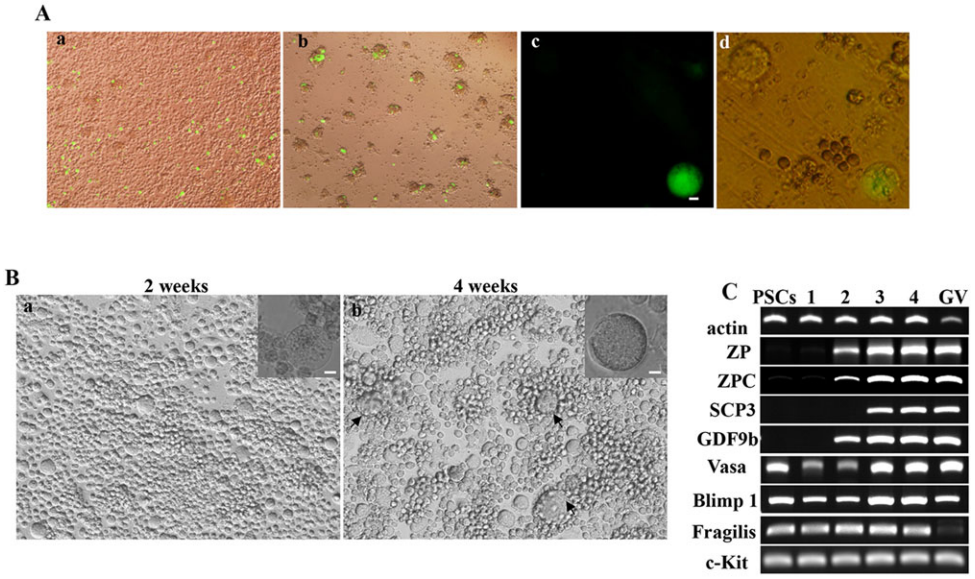

Fig. 6. Induced differentiation of PSCs into OLCs. (A) Expression of EGFP-positive cells was observed throughout the clumps of PSCs reaggregated with dispersed adult pig OCT cells (a,b). Primordial EGFP-positive OLCs derived from EGFP-positive PSCs and EGFP-negative OLCs derived from EGFP- negative OCT cells were both observed after 2 weeks in culture (c,d). (B) After culture under differentiation conditions for 2-4 weeks, some of the PSCs formed primordial OLCs (30-35 μm in diameter; a, inset), and some of the PSCs proceeded to form OLCs (50 μm in diameter; b, inset) or OCC-like structures (b, arrows). (C) mRNA expression levels of oocyte-specific (ZP, ZPC, SCP3 and GDF9b) and germ cell-specific (Vasa, Blimp1, Fragilis and c-kit) markers in differentiated cells. β-actin mRNA was used as the normalization control. PSCs, control PSCs at 3 weeks after isolation; 1, 2, 3, 4, PSCs that differentiated into OLCs after 1, 2, 3 and 4 weeks, respectively; GV, oocyte derived from pig ovary. Scale bars: 10 μm.

DISCUSSION

The current study has shown that cells with characteristics of early PGCs are present or are generated in the adult pig ovary. Moreover, porcine PGC-like PSCs continue to maintain their germ stem cell identity *in vitro* and can differentiate into OLCs under appropriate culture conditions. In addition, experimental evidence showed that PGC-like PSCs are probably generated from Vasa-positive VSEL stem cells *in vitro*. Finally, we demonstrated the important role of ovarian cell-derived regulatory factors and the proximal stem cell niche in the establishment of porcine PSCs.

Our results are consistent with those of other investigators suggesting that PSCs in the OSE originate from VSELs, and that PSCs might support neo-oogenesis. However, whether VSELs can proliferate *in vitro* or *in vivo* has yet to be elucidated. The self-renewal and differentiation of stem cells in the body must be properly controlled by the specialized microenvironment of the stem cell niche (Morrison and Spradling, 2008), and secreted factors (e.g. extracellular matrix molecules, cytokines) produced by niche cells are known to play essential roles in stem cell-niche interactions. However, the biological, molecular and functional nature of the OSC niche remains largely unknown.

The present study suggests that co-culture with ovarian cells is necessary for the establishment of PGC-like PSCs. Communication between germline and somatic cells is indispensable for stem cell maintenance, as well as for germ cell proliferation and differentiation. Importantly, human and bovine OSE-derived cells co-express SCF and c-kit, implying that SCF can act as an autocrine factor in the normal OSE (Parrott et al., 2000). Interestingly, we demonstrated that SCF increased not only the proliferation of PSCs, but also the proportion of c-kit-positive PSCs. SCF also mediated alterations in the cytoplasmic-to-nuclear translocation of Oct4 after 2 weeks in culture. Therefore, SCF stimulated the growth, proliferation and nuclear reprogramming of porcine PSCs.

The function of the OSE during the mammalian postnatal period remains elusive. Whether germline stem cells exist in the adult mammalian ovary and, if they do exist, whether they can generate oocytes, need to be precisely addressed. A recent study indicated that oogonia fail to stain with pluripotent immunohistochemical markers after 2 years of age in human (Byskov et al., 2011). However, these findings do not rule out the possibility of *de novo* transformation of OSE cells into multipotent stem-like cells in the postnatal human ovary. On the other hand, Kerr et al. (2012) found no evidence for the regeneration of primordial follicles after chemical- or γ-radiation-mediated depletion. We demonstrated in an earlier study that busulfan treatment is cytotoxic to murine oocytes, stimulating follicular apoptosis and disrupting folliculogenesis (Park et al., 2013). Nonetheless, the finite number of oocytes formed during the fetal period does not rule out the possibility of neo-folliculogenesis.

In an effort to ascertain the existence of FGSCs in postnatal mouse ovaries, adult mouse ovaries were recently shown to be capable of supporting the formation of new follicles when provided with transplanted premeiotic female PGCs and companion pre-follicular cells. The transplanted PGCs were, however, only able to form follicles with their own pre-follicular cells, and the transplanted pre-follicular cells could only form follicles with the transplanted PGCs (Zhang et al., 2012). Although the authors concluded that neo-oogenesis does not normally occur in adult mouse ovaries, these results nevertheless provide an answer to the important question of whether the adult ovary can support neo-oogenesis from transplanted PGCs. Taken together, we suggest that germline stem cells per se might not persist in postnatal and adult mammalian ovaries, but that progenitor cells/small PSCs in the ovary can instead differentiate into germline stem cells under appropriate conditions.

Notably, our observations indicate that early PGC-like PSCs are found in the adult pig ovary. These PGC-like PSCs might correspond to PGCs that survive into adulthood, rather than to the large (~15-20 μm) migrating PCGs. Although PGC reprogramming has not yet

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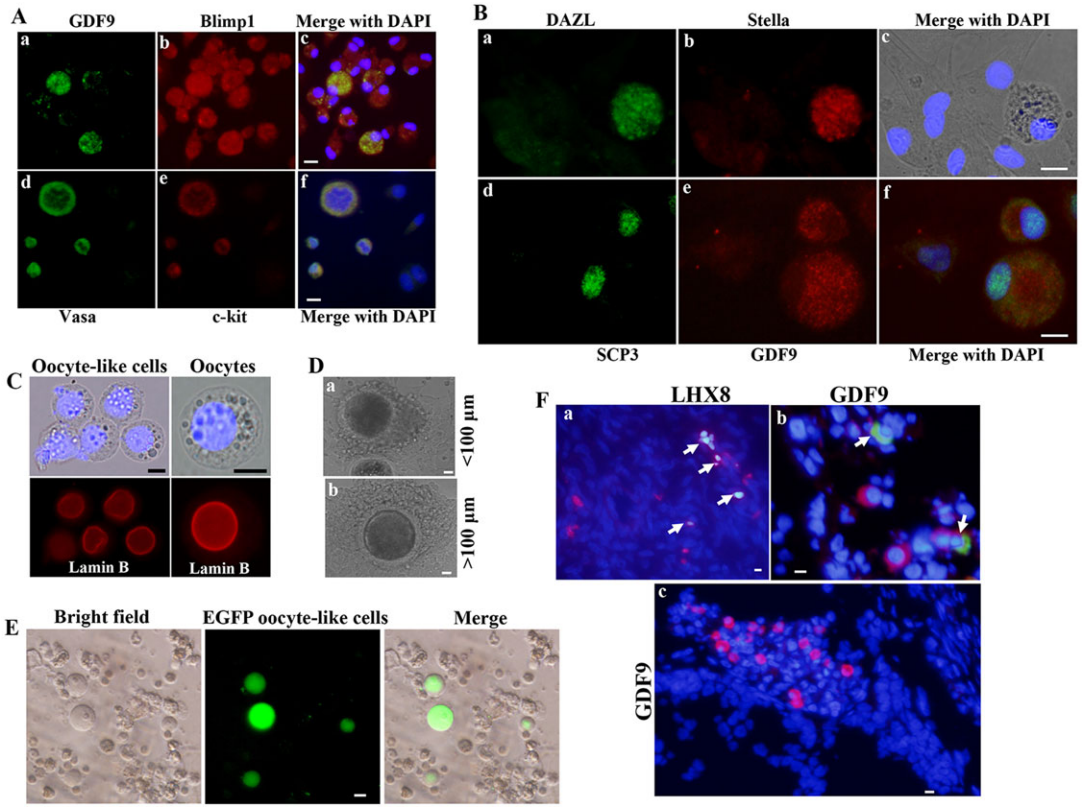

Fig. 7. Characteristics of OLCs generated from PSCs. (A,B) OLCs exhibited positive staining for GDF9, Blimp1, Vasa, c-kit, DAZL, Stella and SCP3, whereas the adjacent somatic cells were negative for these markers (see in merged image c,f). (C) As with normal primordial oocytes, the PSC-generated OLCs contained many cytoplasmic germinal granules. (D) Under differentiation, OLCs grew as large as growing oocyte-like cells (a) or fully grown oocyte-like cells (b). (E) *In vitro* differentiation of OLCs provided direct evidence for EGFP-positive living oocyte-like cells. (F) Dual immunofluorescence analysis of EGFP expression (green) and either LHX8 or GDF9 expression (red) in murine xenografts following EGFP-PSC injection for 2 weeks (a,b). EGFP-positive oocytes were not detected in the pig ovarian tissue in control xenografts, whereas GDF9 was detected in all oocytes (c). Arrows indicate injected EGFP-PSCs in the OCT. Scale bars: 20 µm.

been reported in the pig, studies on PGC reprogramming in the human fetal ovary and the testis showed nuclear localization of Oct4 during the first trimester, with intense cytoplasmic expression during the second trimester. At week 17 of fetal development, Oct4 is again identified in the nucleus (Bhartiya et al., 2010; Gkountela et al., 2013). We also found that PSCs undergo similar cytoplasmic-to-nuclear reprogramming of Oct4 expression, with localization of Oct4 detected in the nucleus of large PSCs. Although the significance of cytoplasmic Oct4 expression is unknown, it is notably coincident with major global epigenetic changes, such as the wholesale epigenetic loss of H3K27me3 and H2A.Z in PGCs, followed by the expression of Oct4 in the cytoplasm (Gkountela et al., 2013).

Why porcine PGCs should be maintained in the postnatal ovary is still a matter of controversy. Recent investigations suggest the presence of two distinct PGC populations in human fetal gonads. While Vasa-positive PGCs enter meiosis in the fetal ovary, the fate of c-kit-positive PGCs remains unclear (Gkountela et al., 2013). The authors propose that c-kit-positive PGCs persisting in the second trimester gonad represent a more primitive PGC population than Vasa-positive cells, an idea supported by their maintenance of a core germ cell gene expression signature at the single-cell level. The work of Gkountela and colleagues also raises questions about the lineage relationships and fates of the c-kit-positive cells. As Laird (2013) discusses, will they be culled in a wave of apoptosis or, as their transcriptome suggests, will they enter meiosis and be conserved in the ovary? Although these issues require further investigation, we maintain that the adult mammalian ovary contains a small number of undifferentiated cells with stem cell characteristics, which, under suitable conditions, can undergo proliferation and differentiation.

VSELs isolated from adult tissues might epitomize an 'all-powerful' stem cell for regenerative medicine applications, as suggested by Ratajczak et al. (2008). Like ESCs, VSELs are pluripotent with maximum regenerative potential, but unlike ECSs they do not form teratomas. The question of whether pluripotent stem cells that appear during the culture of mammalian ovarian tissue originate from unipotent germ stem cells will probably be resolved in due course, but perhaps more important are our findings showing that it is in fact possible to derive and expand autologous stem cells from ovarian tissue. The isolation and characterization of human PSCs will contribute considerably to the prospect of using stem cells to produce developmentally competent oocytes *in vitro*, with clear clinical potential. Our work also supports further inquiry into a myriad of health parameters in premenopausal woman, with applications in tissue repair and restoration.

MATERIALS AND METHODS

Ethics statement

The treatment of the pigs used in this research followed guidelines of the Institutional Animal Care and Use Committee of the National Institute of Animal Science, Suwon, South Korea (approval no. 2009-004, D-grade).

Isolation and purification of PSCs

Ovaries (10-12 for each experiment) were collected from prepubertal gilts at a local slaughterhouse. Cortical slices (0.1-0.5 mm thick) were cut from the ovarian surface using a surgical blade (No. 21, Feather Safety Razor, Osaka,

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Japan) (Bui et al., 2007) and dissociated by mincing, followed by a two-step enzymatic digestion involving a 15 min incubation with 1 mg/ml collagenase (type IV, Sigma-Aldrich) dissolved in Hank's Balanced Salt Solution (HBSS) and 10 min with 0.25% trypsin-EDTA at 38.5°C. Trypsin was neutralized by adding 10% fetal bovine serum (FBS), and tissues dispersed into single cells by gentle pipetting. The dispersed cells were passed through a 40-µm filter and the dissociated cells were allocated to 60 mm gelatin-coated tissue culture dishes and incubated overnight.

To prepare the primary ovarian cells, fibroblasts were allowed to attach to the bottom of a gelatin-coated culture plate, while the floating cells were passaged onto a secondary culture plate after vigorous pipetting. The cells were maintained at 38.5°C in an atmosphere of 5% CO_2 in air. After selection, 1-2×10^4 cells were plated in one well of a 24-well gelatin-coated plate (Corning). Half of the culture medium was changed every other day, and the primary ovarian cells were passaged further as described in the Results.

PSCs were then isolated based on their expression of SSEA4 via magnetic bead sorting. After a two-step enzymatic digestion, the ovarian cells were incubated with anti-SSEA4 antibody for 30 min on ice. After rinsing and resuspending in HBSS, mouse anti-IgG magnetic beads (Miltenyi Biotec) were added to the cell suspension and incubated for a further 30 min on ice. After one additional wash, the cell preparations were loaded onto MACS Cell Separation columns and separated according to the manufacturer's specifications (Miltenyi Biotec). Small (5-7 µm diameter) SSEA4-positive PSCs were obtained and transfected with enhanced green fluorescent protein (EGFP) as described below.

Transduction of the EGFP transgene into PSCs

An HIV-1-based self-inactivating lentiviral vector plasmid (pLV-EGFP) was constructed as described (Ikawa et al., 2003). For lentiviral vector transduction, a single-cell suspension of PSCs (1-2×10^6 cells) was mixed with the lentiviral vector in 100 ml for 6 h (10^7 U final concentration). After washing with PSC culture medium, transduced cells were cultured on a layer of MEF feeder cells.

Immunohistochemistry

Cells and tissues were fixed and treated, and then quantitative analysis was conducted as described (Bui et al., 2010). Antibodies and the dilutions employed are summarized in supplementary material Table S1.

Bromodeoxyuridine (BrdU) incorporation assay

PSCs were cultured in medium containing BrdU (50 µg/ml; Sigma-Aldrich) for 5 days. Detection of DNA synthesis was performed as described previously (Bui et al., 2010).

Flow cytometry and reverse transcription PCR (RT-PCR)

Cells were prepared and treated as described previously (Bui et al., 2012). Synthesized cDNAs were subjected to RT-PCR using the specific primers listed in supplementary material Table S2.

Differentiation of PSCs into OLCs

A two-stage culture system was established for (1) PSC differentiation and (2) PSC growth. First, PSCs were plated at 1×10^4 cells per well of a 24-well tissue culture plate (Corning) that was treated with poly-D-lysine (0.05 mg/ml; Sigma-Aldrich) and laminin (0.005 mg/ml; Sigma-Aldrich). Cells were maintained at 38.5°C in an atmosphere of 5% CO_2 in air in differentiation medium containing DMEM (Invitrogen), penicillin/streptomycin (Invitrogen), 5% FBS (Invitrogen), 5% porcine follicular fluid (Sigma-Aldrich), 0.23 mM sodium pyruvate (Sigma-Aldrich), 0.1 mM non-essential amino acids (Invitrogen), 2 mM L-glutamine (Millipore) and 0.1 mM β-mercaptoethanol (Millipore). One half of the culture medium was replaced every 2-3 days. A number of aggregates containing large cells formed after 3-4 weeks.

Next, for PSC growth, the aggregates were collected and transferred to growth medium containing TCM199 (Invitrogen), 3 mg/ml BSA (Sigma-Aldrich), 5 µl/ml insulin/transferrin/selenium A (Invitrogen), 0.23 mM sodium pyruvate (Sigma-Aldrich), 1 mg/ml fetuin (Sigma-Aldrich), 1 ng/ml EGF, 0.05 IU follicle-stimulating hormone (Sigma-Aldrich), 0.03 IU luteinizing hormone (Sigma-Aldrich), 0.01 mM dibutyryl cAMP (Sigma-Aldrich) (Cayo-Colca et al., 2011) and 1% polyvinylpyrrolidone (PVP) 360 (Sigma-Aldrich) (Hashimoto et al., 2007). The aggregated cells were cultured for 2 weeks, replacing half the medium every 2-3 days.

Intraovarian PSC injection and xenografting

Twenty-four pig OCT pieces (2×2×1 mm) were individually injected with ~1×10^3 EGFP-PSCs using a 10 µl NanoFil syringe with a 35-gauge bevelled needle (World Precision Instruments). Recipient nude female mice were anesthetized and a small incision was made along the dorsal flank for subcutaneous insertion of the pig ovarian tissue (four grafts per mouse). Xenografts were removed 1-2 weeks after transplantation, fixed in 4% paraformaldehyde, paraffin embedded and serially sectioned (6 µm) for immunohistochemical analysis using a mouse monoclonal antibody against GFP. High-temperature antigen retrieval was first performed using 0.01 M sodium citrate buffer (pH 6.0). After cooling, sections were incubated for 10 min with 3% hydrogen peroxide in methanol to block endogenous peroxidase activity as per the manufacturer's protocol (Vector Laboratories). Sections were then blocked for 1 h using 1% normal goat serum and incubated with GFP antibody for immunostaining. Negative controls (the xenografted tissues that received vehicle injections) were run in parallel and did not show a positive signal. To confirm and extend these observations, dual immunofluorescence-based detection of GFP and either GDF9 or LHX8 in xenografted human ovarian tissues was performed with DAPI counterstaining.

Karyotyping and teratoma formation

Cells were prepared and treated as described previously (Bui et al., 2012).

Statistical analysis

Each experiment was repeated at least five times. More than 50 immunostained samples were examined in each group. Results are presented as mean±s.e.m. Data were analyzed by applying Student's *t*-test.

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Competing interests

The authors declare no competing financial interests.

Author contributions

H.-T.B., N.V.T. and J.-H.K. designed the experiments, analyzed and discussed the results. H.-T.B. and D.-N.K. performed the experiments. T.K. provided GFP transgenes for FGSCs. Y.-J.C., M.-H.K. and J.-W.H. contributed new reagents/analytic tools. H.-T.B. wrote the manuscript.

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Supplementary material

Supplementary material available online at
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