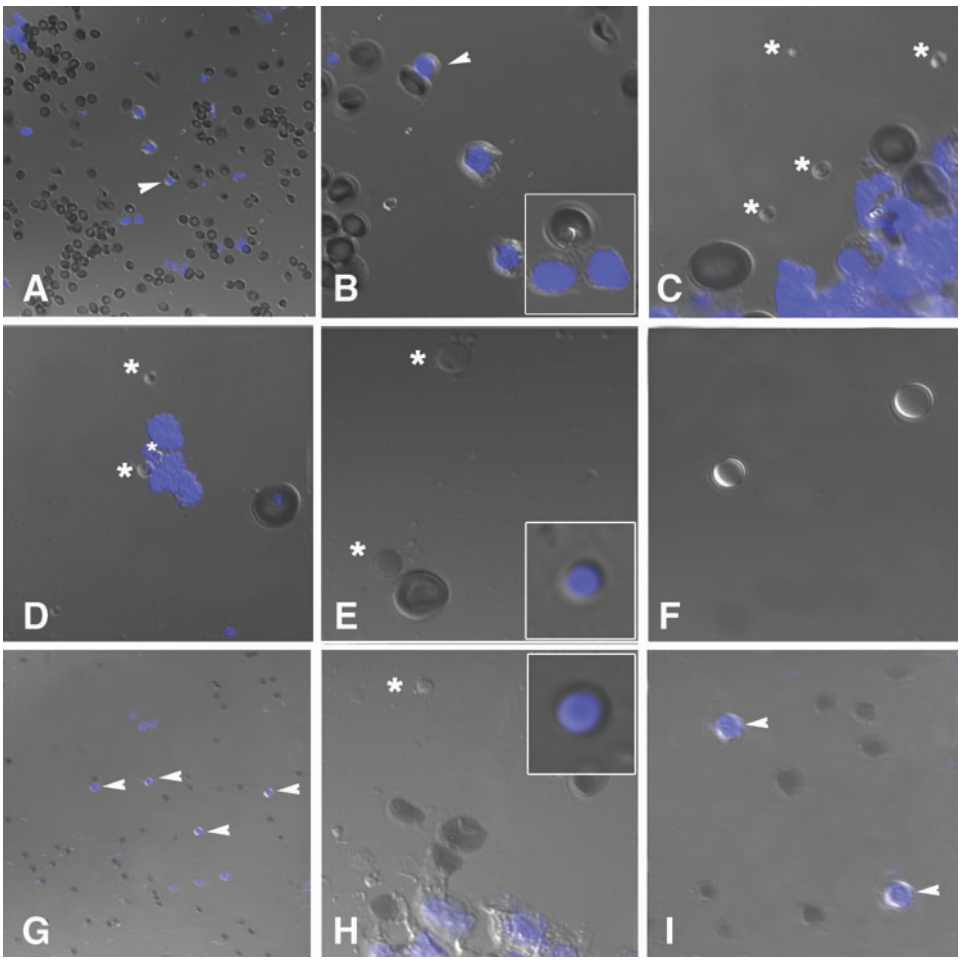
Supplementary Data

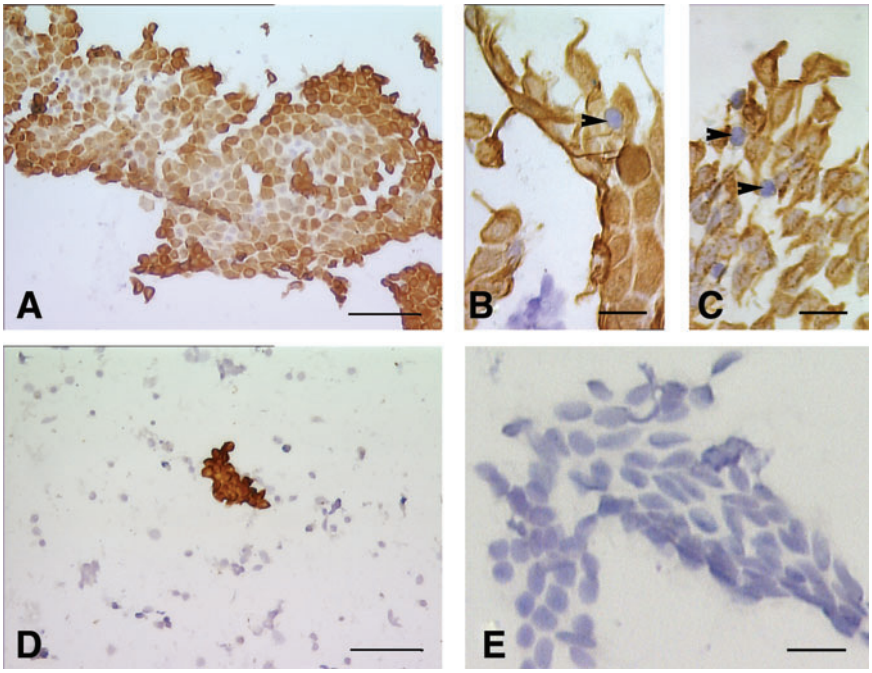
Supplementary Tables S1–S3 provide details of various studies undertaken on ovarian tissue collected from various mammalian species, details of antibodies used for characterization studies, and primer sequences for various gene transcripts used for reverse transcriptase–polymerase chain reaction (RT-PCR) and quantitative PCR.

Staining of scraped OSE cells with 4′,6-diamidino-2-phenylindole

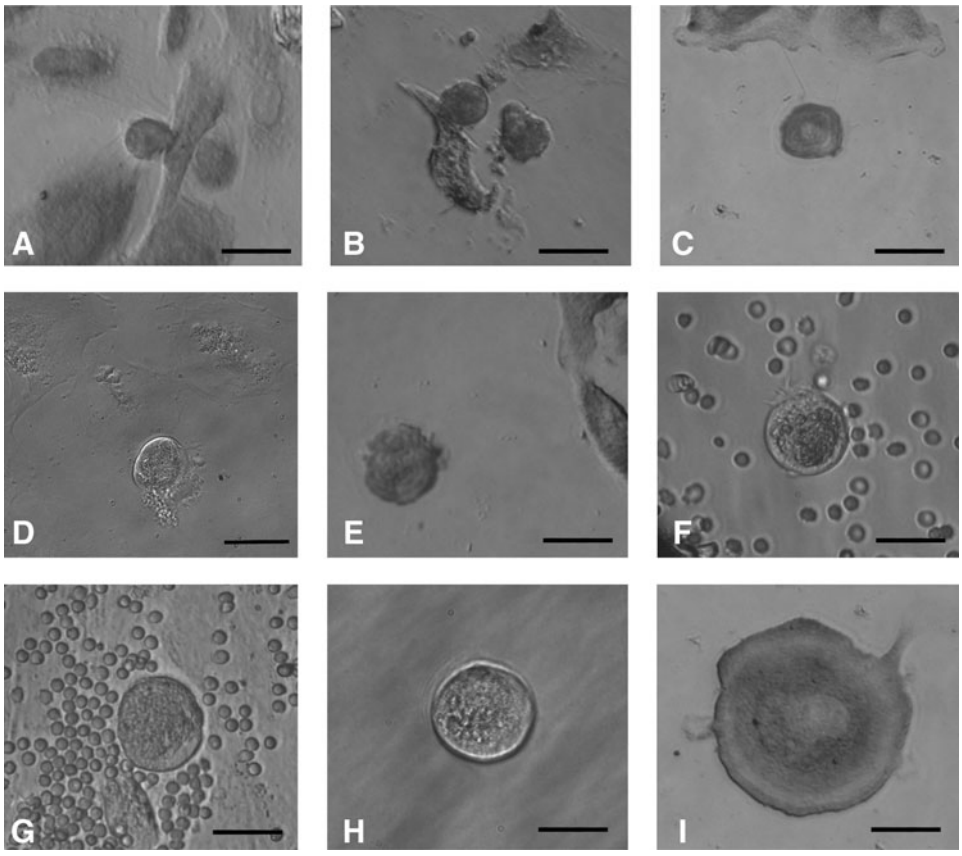
The OSE cell smears were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma; 2μg/mL; for 20 s or 20 min) and examined under confocal fluorescent microscope (LSM 510-META; ZEISS). Two distinct populations of stem cells were identified in both human (Supplementary Fig. S1A–E) and sheep (Supplementary Fig. S1G–I) OSE samples, based on their size. One group of putative stem cells was 1–3μm in diameter, distinctly smaller than RBCs and the other group of putative stem cells was 4–7μm, similar in size to the RBCs. The smaller putative stem cells remained unstained after 20 s of staining with DAPI (Supplementary Fig. S1C–E), whereas the bigger sized putative stem cells and surrounding epithelial cells stained positive with DAPI (Supplementary Fig. S1A, B, G–I). However, on staining for 20 min with DAPI, even the small putative stem cells did show pale nuclear DAPI staining (Supplementary Fig. S1E, H, inset).



SUPPLEMENTARY FIG. S1. Confocal micrographs of DAPI-stained human **(A–E)** and sheep **(G–I)** OSE cells for 20 s. Abundant unstained RBCs and few DAPI-stained cells were observed in human OSE **(A–C)**. The putative stem cells (PSCs) similar in size to RBCs were round in appearance, had high nucleo-cytoplasmic ratio, and DAPI-stained nuclei (*arrowhead*) **(B)**. Inset shows the DAPI staining pattern of epithelial cells. In addition, a distinct population of stem cells of very small size, stained negatively for DAPI (*asterix*) was also observed **(C–E)**. Inset shows pale DAPI signals in these cells after staining for 20 min **(E)**. These small, round cells were easily distinguished from air bubbles **(F)**. Similarly, in sheep OSE, 2 distinct populations of spherical stem cells, including small cells that stained negatively for DAPI (*asterix*), were observed **(H)** and those similar in size to RBCs (*arrowhead*) that stained positively for DAPI were also observed **(I)**. Inset shows pale DAPI signals in these cells after staining for 20 min **(H)**. The epithelial cells exhibited abundant cytoplasm as compared to the PSCs **(H)**. Note a difference in the size and shape of human versus sheep unstained RBCs. Magnification in **A** and **G**, ×520; **B–F, H,** and **I**, ×520 with 5× optical zoom. Merged image of DAPI and DIC. OSE, ovarian surface epithelium; RBC, red blood cell; DAPI, 4′,6-diamidino-2-phenylindole.



SUPPLEMENTARY FIG. S2. Immuno-localization of an epithelial cell marker cytokeratin-18 (CK-18) in sheep OSE. Majority of the cells stain positive for CK-18, when the OSE is gently scraped **(A–C)**. PSCs (*arrowhead*), clearly visible trapped in the OSE cells, stain negative for CK-18 and have a characteristic large nucleus surrounded by a thin rim of cytoplasm **(B, C)**. The cell preparation obtained by scraping the surface with greater pressure showed a mix of cluster of epithelial cells stained positive for CK-18 along with CK-18-negative cortical cells **(D)**. The cortical, unstained cells were easily distinguished from the PSCs on the basis of their size and shape. Negative control **(E)**. Scale bar = 20 μm in **(A)** and = 10 μm in **(B–E)**.



SUPPLEMENTARY FIG. S3. Oocyte-like structures of different sizes observed in human OSE cultures. Human OSE cultures were dynamic and oocyte-like structures **(A–I)** in different stages of growth and development were observed in association with mesenchymal cells in vicinity. Scale bar = 20 μm.

Immunocytochemistry for Cytokeratin 18

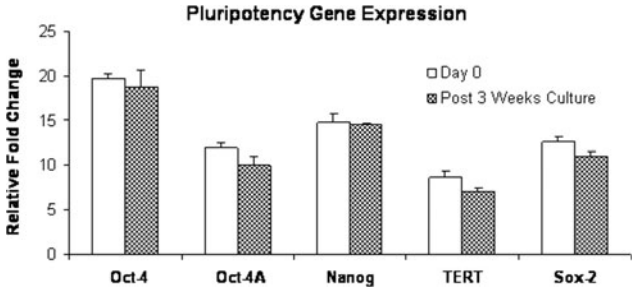
To ensure proper scraping of OSE without contamination with the underlying cortical cells, sheep ovarian tissue was used as it was available in plenty from the slaughter house by immunostaining for Cytokeratin 18 (Ck-18), an epithelial cell marker [1].

Sheep OSE and cortical cells, collected separately by gentle scraping and by applying more pressure, respectively, were immuno-stained for Ck-18. The fixed cell smears were brought to room temperature, rinsed with Dulbecco's phosphate-buffered saline (DPBS) and incubated for an hour in 3% BSA in DPBS prior to overnight incubation at 4°C with 1:100 dilution of Ck-18 primary antibody (Sigma). Next day, after DPBS wash, 30 min incubation with secondary antibody using Super Sensitive Polymer HRP kit, (BioGenex), and DPBS wash, the color reaction was detected using 0.05% diaminobenzidine and 0.05% hydrogen peroxide in DPBS. The reaction was terminated after 3–5 min depending on color development by dipping the slides in tap water. The cell smears were counterstained with hemotoxylin, dehydrated in subsequent alcohol grades as per standard histological procedures, cover slipped, and mounted in DPX (Qualigens Fine Chemicals). The slides were examined under upright microscope (90i, NIKON) and representative images were recorded.

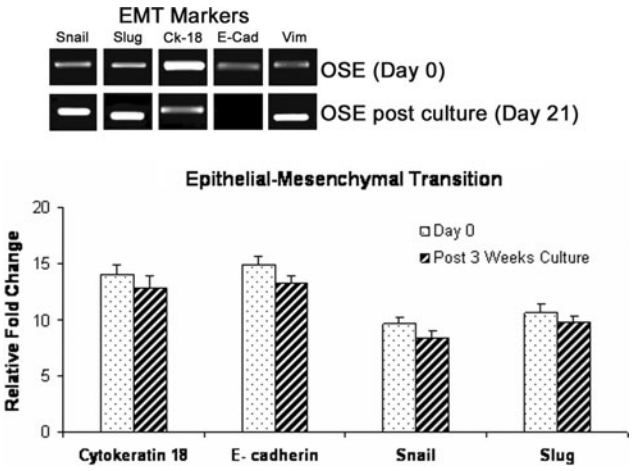
The OSE cells collected by gentle scraping of sheep ovaries stained positive for CK-18 (Supplementary Fig. S2A–C). A few round and Ck-18-negative stem cells were occasionally observed trapped within the epithelial cells cluster (Supplementary Fig. S2B, C). The cell preparation made by applying more pressure during scraping of OSE demonstrated the presence of a mixture of large number of CK-18-negative cortical cells and cluster of epithelial cells that stained positive (Supplementary Fig. S2D).

Oocyte-like structures of different sizes observed in human OSE cultures

Oocyte-like structures in different stages of growth and development with obvious difference in size were observed in association with mesenchymal cells in vicinity (Supplementary Fig. S3).



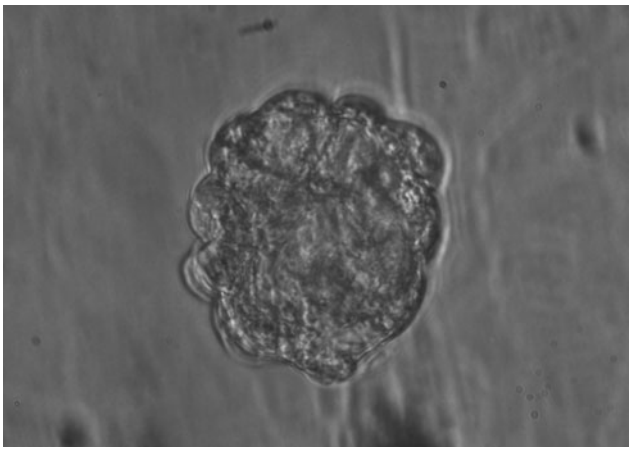
SUPPLEMENTARY FIG. S4. Real-Time Quantitative PCR analysis of pluripotent gene transcripts in human OSE cells (*empty bars*) and postculture (*solid bars*) ($n=6$). Graph depicts mean ± SE for expression of Oct-4, Oct-4A, Nanog, TERT, and Sox-2. Note the presence of both Oct-4 and Oct-4A transcripts in OSE. However, expression of these markers did not show significant reduction postculture, possibly because of the presence of ES cell-like colonies and embryoid body-like structures—thus making any conclusion difficult.



SUPPLEMENTARY FIG. S5. Reverse transcriptase–polymerase chain reaction analysis to study epithelial mesenchymal transition (EMT) where OSE cells were positive for transcripts of epithelial marker cytokeratin (Ck-18) and E-cadherin, mesenchymal marker vimentin, and showed weak expression of transcription factors Snail and Slug. Note the relative down regulation of Ck-18, loss of E-cadherin, and marked upregulation of vimentin, Snail, and Slug. This gene transcript expression pattern suggests the phenomenon of EMT in OSE postculture. Real-Time Quantitative PCR analysis of genes related to EMT expression in human OSE cells (*dotted bars*) and postculture (*solid bars*) ($n=3$). Graph depicts mean ± SE for EMT markers CK-18, E-cadherin, Snail, and Slug in OSE cells and cells postculture. No significant variation was noted, unlike the reverse transcriptase–polymerase chain reaction data, possibly because of inherent biological differences and heterogeneity in starting cells from which RNA was extracted for the studies. The results reflect the importance of carrying out such studies on carefully selected cell population to generate meaningful data.

Quantitative PCR

These studies were carried out to quantitatively assess the regulation of pluripotent and EMT markers in human OSE and post 3 weeks culture. Quantitative assessment of expression patterns for pluripotent genes and EMT markers was



SUPPLEMENTARY VIDEO S1. Video of sheep embryo-like structure exhibiting rolling movement in culture dish (×400).

Supplementary Table S1. Experiments Conducted on Ovarian Tissue Obtained from Different Mammalian Species

Studies undertaken	*Human*	*Sheep*	*Monkey*	*Rabbit*
H&E staining of paraffin-embedded sections and scraped OSE cells	✓	✓	Data not shown	Data not shown
OSE studied under Hoffman optics for presence of stem cells	✓	✓	✓	✓
DAPI staining of OSE cells	✓	✓	Data not shown	Data not shown
Three weeks' culture of OSE cells	✓	✓	✓	✓
To ensure proper scraping of OSE	ND	Cytokeratin 18 immunostaining	ND	ND
Immunolocalization of pluripotent markers	Oct-4 and SSEA-4	Oct-4 and SSEA-4	ND	ND
Immunolocalization of germ cell markers	c-Kit, DAZL, GDF-9, VASA and ZP4	ND	ND	ND
RT-PCR for pluripotent markers	Oct-4A, Oct-4, Nanog, Sox-2 and TERT	Oct-4, Nanog, Sox-2 and Stat-3	ND	ND
RT-PCR for germ cell markers	Oct-4 and c-Kit	ND	ND	ND
RT-PCR for EMT markers	Cytokeratin 18, vimentin, E-cadherin, Snail and Slug	ND	ND	ND
Real Time PCR studies	Oct-4, Oct-4A, Nanog, Sox-2, TERT	ND	ND	ND
To study alkaline phosphatase activity of ES-like colonies that appear in 3 weeks culture	Alkaline phosphatase staining	ND	ND	ND

OSE, ovarian surface epithelium; ES, embryonic stem; EMT, epithelial-mesenchymal transition; H&E, hematoxylin and eosin; RT-PCR, reverse transcriptase–polymerase chain reaction; ND, not done.

Supplementary Table S2. Antibodies Used for Immuno-Localization Studies

Sl. No.	*Antibody*	*Nature*	*Antibody source*	*Localization*	*Detection system*	*Reference*
1	Oct -4	Stem Cell Marker	Polyclonal antibody Abcam, UK	Nuclear and cytoplasmic	Immuno flourescence Staining ALEXA FLOUR 488 (Molecular Probes, Invitrogen, CA)	1
2	SSEA-4	″	Monoclonal antibody Millipore	Cell surface	″	2
3	c- Kit	Germ Cell Marker	Polyclonal DAKO	Cytoplasmic	Super Sensitive Polymer HRP Kit, BioGenex	**3**
4	DAZL	″	Polyclonal Abcam, UK	″	″	4
5	GDF-9	″	Polyclonal Abcam, UK	″	″	5
6	VASA	″	Polyclonal R & D Systems, USA	″	Immunocruz Kit, Santa Cruz Biotechnology Inc.	6
7	ZP4	″	Monoclonal, Gift- Dr SK Gupta, NII, New Delhi, India	″	Super Sensitive Polymer HRP Kit, BioGenex	7
8	Cytokeratin -18	Epithelial Cell Marker	Monoclonal Sigma, USA	″	″	8

Supplementary Table S3. Details of Primers Used for Reverse Transcriptase–Polymerase Chain Reaction Analysis

Gene		*Primer sequence*	*Product size (bp)*	*References*
		Stem cell markers (Human)		
Oct-4[a]	F	GAAGGTATTCAGCCAAACGAC	315	9
	R	GTTACAGAACCACACTCGGA		
Oct-4 A	F	AGCCCTCATTTCACCAGGCC	448	1
	R	TGGGACTCCTCCGGGTTTTG		
Nanog	F	TGCAAATGTCTTCTGCTGAGAT	285	9
	R	GTTCAGGATGTTGGAGAGTTC		
Sox-2	F	ATGCACCGCTACGACGTGA	437	10
	R	CTTTTGCACCCCTCCCATTT		
TERT	F	AGCTATGCCCGGACCTCCAT	185	11
	R	GCCTGCAGCAGGAGGATCTT		
		Stem cell markers (Sheep)		
Oct -4[a]	F	CAATTTGCCAAGCTCCTAAA	290	12
	R	TTGCCTCTCACTTGGTTCTC		
Nanog	F	TTCCCTCCTCCATGGATCTG	501	12
	R	AGGAGTGGTTGCTCCAAGAC		
Sox-2	F	TGATACGGTAGGAGCTTTGC	362	12
	R	CTTTTGCCCCTTTAGAGACC		
Stat-3	F	TGGACAACATCATTGACCTG	239	12
	R	CTGCTGCTTGGTGTAAGGTT		
		Germ cell markers (Human)		
c-Kit	F	AAGGACTTGAGGTTTATTCCT	345	13
	R	CTGACGTTCATAATTGAAGTC		
		EMT markers (Human)		
CK-18	F	CTGGAGACCGAGAACCGGA	447	14
	R	TCCGAGCCAGCTCGTCAT′		
E Cad	F	TTCCTCCCAATACATCTCCCTTCACAGCAG	280	14
	R	CGAAGAAACAGCAAGAGCAGCAGAATCAGA		
Vimentin	F	GTCCTCGTCCTCCTACCGCA 3′	546	14
	R	GTTTTCGGCTTCCTCTCTCT 3′		
Snail	F	TTCCAGCAGCCCTACGACCAG	640	14
	R	CGGACTCTTGGTGCTTGTGGA		
Slug	F	AAGCATTTCAACGCCTCCAA	489	14
	R	AAGGTAATGTGTGGGTCCGA		
		Housekeeping genes		
Human	F	GTCAGTGCTGGACCTGACCT	255	2
GAPDH	R	CACCACCATGTTGCTGTAGC		
Sheep	F	GCC CAG AAC ATC ATC CCT G	232	15
GAPDH	R	GGT CCT CAG TGT AGC CTA G		

[a]Oct-4 is a pluripotency and germ cell marker (Supplementary Ref. 16).
F, forward; R, reverse.

carried out in human OSE cells and post 3 weeks culture. Total RNA was isolated from OSE cells using TRIZOL (Invitrogen) at day 0 and post 3 weeks in culture. About 50 ng of RNA was reverse transcribed into cDNA by Sensiscript RT kit (Qiagen). Real-time PCR analysis was performed using 2.5 μL of cDNA and 10 pmol primers (for details refer Supplementary Table S3). The comparative threshold cycle (Ct) value for pluripotent and EMT transcripts in OSE cells and cells cultured for 3 weeks were quantitated using CFX 96 Real-Time PCR system (Bio-Rad) using SYBR Green chemistry (Bio-Rad). The amplification conditions comprised of an initial denaturation at 95°C for 3 min, followed by 45 cycles of denaturation at 95°C for 30 s, annealing for 30 s at respective temperature for each gene, and elongation at 72°C for 1 min. The fluorescence emitted at each cycle was captured and the melt curve analysis was performed at the end of 45 cycles to determine the homogeneity of the amplified products. All reactions were carried out in duplicates and the experiments were repeated at least thrice to determine the reproducibility. The Ct values generated by CFX manager software (Bio-Rad) were normalized with the housekeeping gene 18S for all samples, and the ΔCt values for each gene transcript at each time point were computed. Statistical analysis was performed to calculate mean and standard error values and corresponding graph was plotted to assess the gene expression levels.

Transcripts for Oct-4 and Oct-4A both were detected in OSE and postcultured cells. However, expression of the pluripotent markers Oct-4, Oct-4A, Nanog, TERT, and Sox-2 did not show significant reduction postculture, possibly because of the presence of ES cell-like colonies and embryoid body-like structures, thus making any conclusion difficult (Supplementary Fig. S4). Similarly, expression of EMT markers CK-18, E-cadherin, Snail, and Slug were also detected in OSE cells and cells postculture. No significant variation was noted possibly because of inherent biological differences and heterogeneity in

postcultured cells from which RNA was extracted for the studies (Supplementary Fig. S5).

Molecular characterization of epithelial–mesenchymal transition

The transcripts for intermediate filaments, namely, cytokeratin (Ck-18), vimentin, adherens junction molecule E-cadherin, and transcription factors Snail and Slug were detected in initial human OSE cells. Expression of Ck-18 transcript was found to be downregulated with the loss of epithelial marker E-cadherin in cells collected postculture. This was accompanied by a relative concomitant upregulation of mesenchymal marker vimentin and transcription factors Snail and Slug in cells obtained postculture as assessed by RT-PCR study (Supplementary Fig. S5).

References

1. Bhartiya D, S Kasiviswanathan, SK Unni, P Pethe, JV Dhabalia, S Patwardhan and HB Tongaonkar. (2010). Newer insights into pre-meiotic development of germ cells in adult human testis using Oct-4 as a stem cell marker. J Histochem Cytochem 58:1093–1106.
2. Kumar N, I Hinduja, P Nagvenkar, L Pillai, K Zaveri, L Mukadam, J Telang, S Desai, V Mangoli, R Mangoli, S Padgaonkar, G Kaur, C Puri and D Bhartiya. (2009). Derivation and characterization of two genetically unique human embryonic stem cell lines on in-house-derived human feeders. Stem Cells Dev 18:67–77.
3. Carlsson IB, MPE Laitinen, JE Scott, H Louhio, L Velentzis, T Tuuri, J Aaltonen, O Ritvos, RML Winston and Hovatta. (2006). Kit ligand and c-Kit are expressed during early human ovarian follicular development and their interaction is required for the survival of follicles in long-term culture. Reproduction 131:641–649.
4. Anderson RA, N Fulton, G Cowan, S Coutts and PTK Saunders. (2007). Conserved and divergent patterns of expression of DAZL, VASA and OCT-4 in the germ cells of the human fetal ovary and testis. BMC Dev Biol 7:136
5. Orisaka M, K Tajima, BK Tsang and F Kotsuji. (2009). Oocyte-granulosa-theca cell interactions during preantral follicular development. J Ovarian Res 2:9.
6. Castrillon DH, BJ Quade, TY Wang, C Quigley and CP Crum. (2000). The human VASA gene is specifically expressed in the germ cell lineage. PNAS 97:9585–9590.
7. Bukovsky A, SK Gupta, P Bansal, S Chakravarty, M Chaudhary, M Svetlikova, RS White, P Copas, NB Upadhyaya, SE Van Meter and MR Caudle. (2008). Production of monoclonal antibodies against recombinant human zona pellucida glycoproteins: utility in immunolocalization of respective zona proteins in ovarian follicles. J Reprod Immunol 78:102–114.
8. Bukovsky A, M Svetlikova and MR Caudle. (2005). Oogenesis in cultures derived from adult human ovaries. Reprod Biol Endocrinol 3:17–30.
9. Klimanskaya I, Y Chung, S Becker, SJ Lu and R Lanza. (2006). Human embryonic stem cell lines derived from single blastomere. Nature 444:481–485.
10. Bhattacharya B, J Cai, Y Luo, T Miura, J Mejido, SN Brimble, X Zeng, TC Schulz, MS Rao and RK Puri. (2005). Comparison of the gene expression profile of undifferentiated human embryonic stem cell lines and differentiating embryoid bodies. BMC Dev Biol 5:5–22.
11. Chen HF, HC Kuo, CL Chien, CT Shun, YL Yao, PL Ip, CY Chuang, CC Wang, YS Yang and HN Ho. (2007). Derivation, characterization and differentiation of human embryonic stem cells: comparing serum-containing versus serum-free media and evidence of germ cell differentiation. Hum Reprod 22:567–577.
12. Sanna D, A Sanna, L Mara, S Pilichi, A Mastinu, F Chessa, L Pani and M Dattena. (2009). Oct4 expression in *in vitro* produced sheep blastocysts and embryonic stem-like cells. Cell Biol Int 34:53–60.
13. Tanikawa M, T Harad, M Mitsunari, Y Onohara, T Iwabe and N Terakawa. (1998). Expression of c-kit messenger ribonucleic acid in human oocyte and presence of soluble c-kit in follicular fluid. J Clin Endocrinol Metab 83:1239–1242.
14. Bapat SA, AM Mali, CB Koppikar and NK Kurrey. (2005). Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer. Cancer Res 65:3025–3029.
15. Muruvi W, HM Picton, RG Rodway and IM Joyce. (2005). *In vitro* growth of oocytes from primordial follicles isolated from frozen-thawed lamb ovaries. Theriogenology 64:1357–1370.
16. Pesce M, X Wang, DJ Wolgemuth, and H Scholer. (1998). Differential expression of the Oct-4 transcription factor during mouse germ cell differentiation. Mech Dev 71:89–98.