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Expression of Transcriptional Factor Genes (*Oct-4*, *Nanog*, and *Sox-2*) and Embryonic Stem Cell-Like Characters in Placental Membrane of Buffalo (*Bubalus bubalis*)

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Abstract The aim of the study was to assess the expression of transcriptional factor genes and embryonic stem cell-like characters in the placental membrane of buffalo (*Bubalus bubalis*). Along with the placenta, amniotic fluid, maternal peripheral blood, and umbilical cord blood samples were taken for the future study. The isolation and culture of cells from the placental membrane was followed by the determination of RT-PCR-based markers (Oct-4, Nanog, Sox-2, alkaline phosphatase, stem cell factor, and Nestin) of these cells. Placental membrane cells also positively expressed alkaline phosphatase staining. We isolated adherent cells from trypsin–EDTA-digested placentas and examined these cells for morphology, surface markers, and differentiation potential and found that they expressed several stem cell markers. They also showed neurogenic and adipogenic differentiation potentials under appropriate guided conditions. We suggest that placenta-derived cells have multilineage differentiation potential similar to mesenchymal stem cells in terms of morphology and cell-surface antigen expression. The placenta may prove to be a useful source of mesenchymal stem cells.

Keywords Amniotic membrane · Differentiation · Placenta · Stem cells

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Introduction

Regenerative medicine based on cell therapy and tissue engineering methodologies is a newly emerging, multidisciplinary field involving biology, medicine, and genetic manipulation (Parolini and Soncini 2006). Modern improvement in the study of stem cells has unlocked new perspective for their application in cell treatment. The present resources of stem cells are embryonic stem cells and adult-type stem cells; however, their use poses both ethical and technical problems. The finding of other stem cell sources that do not raise ethical problems, that are easily accessible, and that are sufficiently numerous to be used for therapeutic purposes has been attempted (Mihu et al. 2009). A new source that meets all these requirements is placenta.

In this study, we focused on the presence of stem cell characters in the placental membrane because these are less studied in the bubaline species and are speculated to have good differentiation related to a high number of cell varieties—significantly greater than that of adult-type stem cells. Because they are located at the maternal–fetal border, they appear to have high immunological acceptance, which makes them simple to apply in the transplantation process. The aim of this study was to isolate placental membrane cells and to assess the evidence of their stem cell like properties.

The water buffalo is an important livestock species contributing significantly to dairy, agriculture, leather and meat production in several countries (Singh et al. 2009), and research is going on to enhance its production efficiency and conservation of elite and native genotypes (Gautam et al. 2008; Singh et al. 2009). Establishing stem cells for assisted reproduction and health application has been emphasized in this species (Dev et al. 2011a, b; Singh et al. 2011;

Yadav et al. 2012). In this study, we report characterization of pluripotency molecular markers and embryonic stem cells like characters in placental membrane from bubaline. Ability of prolonged cultured cells to differentiate in vitro into various cell lineages is investigated.

Materials and Methods

Chemicals and Media

All chemicals, reagents, culture media, and antibiotics used in this study were of cell culture grade and were obtained from Sigma Chemical Company (USA) unless otherwise indicated. Fetal bovine serum (FBS) was from Hyclone (Thermo Scientific, USA), and Trizol was from Invitrogen (USA). Disposable 35 × 10 mm cell culture petri dishes, six-well tissue culture plates, and centrifuge tubes were procured from Tarsons Products (India). Membrane filters were from Advanced Micro-Devices (India). The primers were synthesized by GenxBio (India). The culture media was filter sterilized (0.22 μm) before use.

Sampling and Transportation

Of all the placentas gathered, six were from natural deliveries and one was from a dead fetus. They were transported in cold Dulbecco phosphate-buffered saline (DPBS) solution in a thermally insulated container on ice. Along with the placentas, different samples from amniotic fluid and umbilical cord blood were taken. All the samples were directly processed following laboratory-standardized protocols. Some of samples were separated by centrifugation with a Ficoll density gradient and amassed in liquid nitrogen awaiting its later use. The placentas were assessed macroscopically and microscopically. Stained histologic preparations for placenta were completed.

Isolation and Culturing of Placental Membrane Cells

The placental cells were separated by chopping followed by centrifugation (5000*g*, 10 min), then washed three times with DPBS. The cells were seeded at a density of 10^3 cells/cm^2 in six-well culture plates containing cell culture medium (Dulbecco modified Eagle medium supplemented with 16 % FBS, 1 % penicillin/streptomycin, and 1 % vitamin solution) and incubated in a humidified CO_2 incubator (Lark, China) at 38.5 °C in the presence of 5 % CO_2 (Dev et al. 2010).

The placental cells were allowed to grow and were subcultured by passaging after achieving >80 % confluence. Viability of the cells was monitored by standard protocols of exclusion of trypan blue dye, and the cells were counted with a hemocytometer (Rohem, India). Morphologic features of the cells and their anchorage to culture plates were monitored and recorded regularly.

Characterization of Stem Cells

Alkaline Phosphatase (AP) Expression

The cultured placental cells were screened for embryonic stem cell-like cells and AP expression with an AP staining kit (Sigma Chemical Company, catalog no. 86C). For this, cell culture medium was removed and the cells were fixed using the fixative 157 μl citrate solution, 50 μl formaldehyde, and 406 μl acetone for 30 s. After fixation, the cells were washed three times with DPBS for 60 s, and 60 μl alkaline dye (10 μl sodium nitrate, 10 μl fast blue base alkaline, 10 μl naphthalene, and 470 μl water) was added. The cells were left at room temperature for 15 min. The treated cells were washed 8–10 times with DPBS. Natural red dye was added and removed after 30 s. The cells were observed under an inverted microscope (Radical Instruments, India).

Oct-4, Sox-2, Nanog, AP, and Nestin Expression with RT-PCR

The method proposed by Hummon et al. (2007), with minor modifications, was used for extracting total cellular RNA. RNA was extracted from approximately 0.6 × 107 cells with Trizol (Invitrogen, USA) reagent. The Trizol extract (with the cell pellet) was transferred to 2 ml centrifuge tubes and mixed with 200 μl chloroform and isoamyl alcohol (24:1). Aqueous and organic phases were mixed by gentle shaking followed by centrifugation at 12,000×*g* for 15 min at room temperature. The supernatant was collected, and 500 μl of isopropyl alcohol was added to 1 ml of Trizol extract. The contents were remixed gently and centrifuged at 9500×*g* for 15 min. The RNA pellet was washed with 500 μl of 70 % chilled ethanol, then dried at room temperature. The dried RNA pellet was dissolved in 190 μl of diethylpyrocarbonate-treated water and treated with RNase-free DNase for removing DNA, if any. The RNA concentration was measured with a spectrophotometer (ND-1000; NanoDrop Technologies, USA).

The cDNA was synthesized by reverse transcription of mRNA purified from the cultured placental cells. The reaction mixture was composed of total cellular 5 ng RNA, 0.2 μg/μl random hexamer, 7 μg/μl cDNA direct RT, 10 μM/ μl AMV reverse transcriptase, and 40 U/μl RNase inhibitor in a total volume of 20 μl. RT-PCR was carried out at 42 °C for 60 min followed by denaturation at 95 °C for 8 min. The cDNA taken was generally 5–10 ng/μL, and

Table 1 Primer sequences, size of amplification products, and annealing temperatures

Gene	Primer sequence	Product size (bp)	Accession no.	Annealing temperature (°C)
Octamer binding-4 (*Oct-4*)	CTTCAATCGCATATTCTTTAACCA GGAGGAAGCTGACAACAACG	314	FI907061	58.0
Nanog	GCCCCTTAGTAAGCTGCTTTT GGGGTGGTGGAAATCAGTAA	317	DQ487022	58.0
Sox-2	AACCAAGACGCTCATGAAGAA GTACTGCAGGGCGCTCAC	277	EU627692	61.0
Alkaline phosphatase (*AP*)	ACCAATGGCAACCTGCTGTA CTCCTCCAGGATCTTGGCTA	180	X93604	60.0
Stem cell factor (*SCF*)	TCCCTGCTACCATCCCTATG GCTTCCCAAATCTGGATCAT	216	AY667192	59.5
Nestin	ACC TGC TGT ACA TCG GCT TT GAGGATGGTGAAGACGGAGA	307	X93604	60.0

ultrapure water instead of standard DNA was taken as negative control. The final volume of the PCR reaction consisted of 60 ng cDNA, 20 pmol of each primer (GenxBio, India), 10 mM dNTPs mixture, 25 mM of $MgCl_2$, and 3 U of Taq polymerase (all from Bangalore GeNei, India). The primer sequences used were for Oct-4, Sox-2, Nanog, AP, stem cell factor, and Nestin (Table 1). The PCR conditions were the same except for the annealing temperature (Table 1): 94 °C for 5 min for initial denaturation, denaturation at 94 °C for 30 s, elongation at 72 °C for 1 min (35 cycles), and final extension at 72 °C for 10 min. The amplified DNA fragments were resolved on a 2 % agarose gel containing 10 mg/ml ethidium bromide.

Karyotyping

Standard protocols were used to investigate the chromosomal profiles of the fibroblastic cells at different passages. The actively growing cells were incubated with colchicine (0.1 µg/ml) for 4 h at 37 °C. The treated cultures were washed twice with DPBS and trypsinized (as above). The cells were suspended and incubated in a hypotonic solution (68 mM KCl) for 20 min at 37 °C. The cells were collected by centrifugation and fixed in a chilled fixative (methanol and glacial acetic acid, 3:1) for 10 min. The cell pellet was obtained and suspended in 5 ml of chilled fixative for another 10 min. The metaphase spreads were prepared by dropping the cell suspension onto prechilled glass slides. The air-dried cell spreads were stained with Giemsa stain and observed under oil immersion. Additionally, the cells were also examined for appearance of micronuclei as indicators of genetic abnormalities during culturing (Thomas et al. 2009).

Results

Isolation and Culturing of Placental Membrane Cells

After collection, all the cells were in network form and were similar in size and shape (Fig. 1). Some of the cells were rounded and some were edge-pointed after 5 days of culturing. All the cells displayed anchorage after 9 days of culturing. After day 14, there was <80 % confluence with morphologically similar cells (fibroblastic cells).

Instead of forming uniform cell monolayers, cell clumps were also observed. Initially the cells reached 70–80 % confluence after 2 weeks. However, the passaged cells exhibited a higher growth rate, reaching 90–100 % confluence after day 16 of culturing.

Characterization of Placental Membrane Cells by AP Staining

The placental cells were found to expand extensively in fibroblast appearance. The cells were found to stain positive for AP. Cells showed embryonic stem cell-like cells properties by AP staining. Whereas the other cell (Pinna)-cultured fibroblasts did not acquire stain, the cultured cells stained red and were considered positive for AP expression (Fig. 2).

Karyotyping

The cells had a standard karyotype at different passages. No noticeable genomic aberrations (e.g., appearance of micronuclei, chromosomal fragmentation) were observed (Fig. 3).

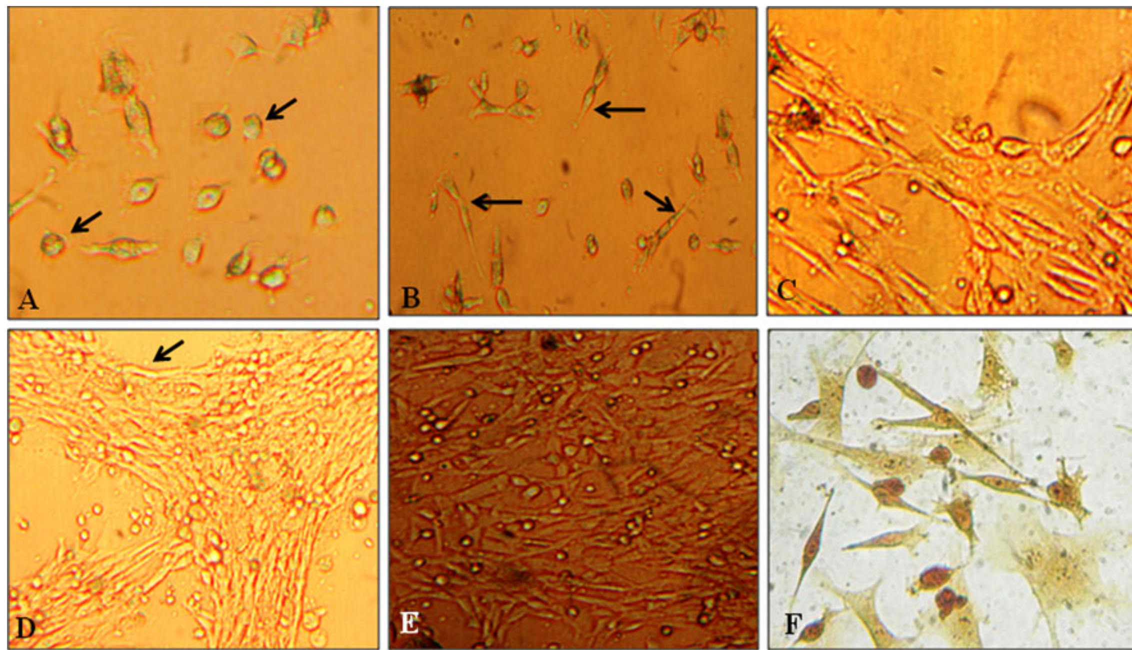

Fig. 1 Culturing of placental membrane-derived cells. **a** All the cells appear rounded on day 2. **b** Edge and connective bridge formation start on day 3. **c** Cells start to regularly proliferate in the whirlpool on day 5. **d** About 50 % confluence take place on day 8 of culturing. **e** Less than 90 % confluence is reached on day 14. **f** Cells after the second day of the trypsin–EDTA step. After each 5-day interval, cells were in need of feeding with the same concentration and composition of supplements as the last cultured cells

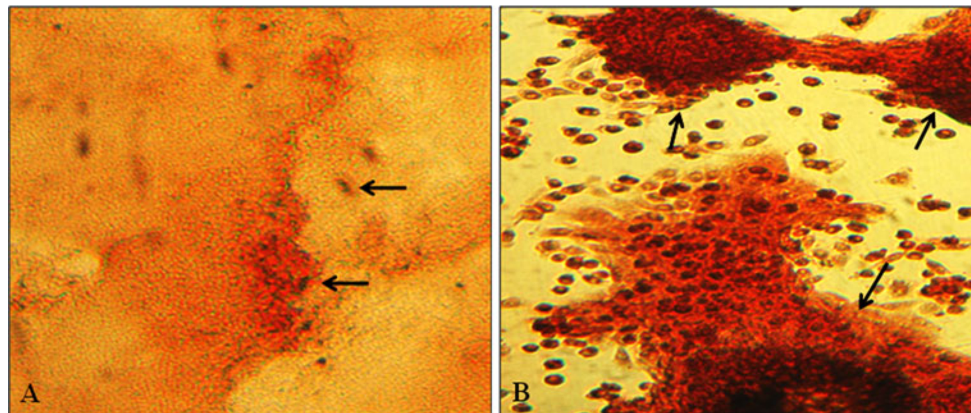

Fig. 2 Expression of strong positive alkaline phosphatase staining at passages 3 and 5. Placental membrane cells revealed a strong positive staining (*red*), showing that cells have an embryonic cell-like character. **a** Placental membrane cells at low magnification appear as clumps (×100). **b** A clear embryonic stem cell-like colony formation appears at high magnification (×250)

Characterization of Placental Membrane Cells for Oct-4-, Sox-2-, Nanog-, AP-, and Nestin-positive RT-PCR Expression

At passages 2–4, cells were collected to analyze different gene expressions. RT-PCR-based studies showed strong positive expression for *Oct-4*, *Sox-2*, *Nanog*, *AP*, and *Nestin* genes. Agarose gel electrophoresis of RT-PCR product revealed PCR amplicons of 314, 277, 317, 180, and 307, respectively (Fig. 4).

Discussion

The mesenchymal cells of the placental membrane can represent an important source of cells with pluripotential characteristics (Benirschke and Kaufmann 2000; Bieback et al. 2004; In 'T Anker et al. 2004; Matikainen and Laine 2005; Strom and Miki 2003; Yen et al. 2005). During this study, we succeeded in isolating, culturing, and characterizing these cells by RT-PCR and by AP staining. To our knowledge, this provides the first evidence of RT-PCR- and

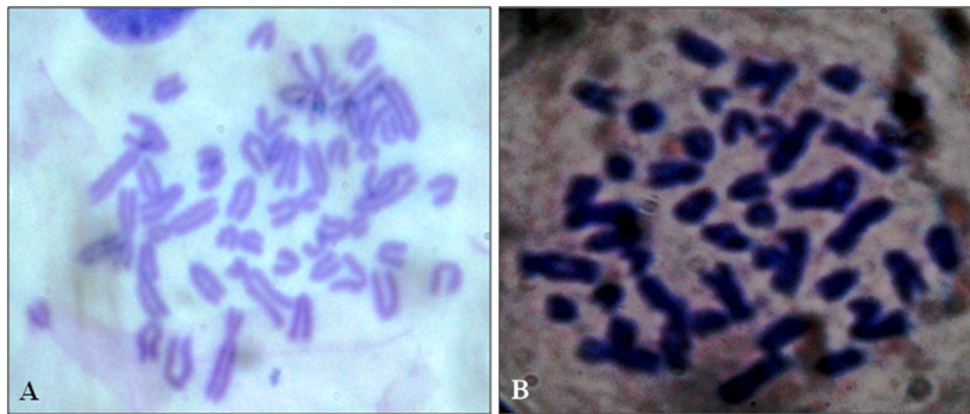

Fig. 3 Cells were found to have a normal karyotype during the different passages. **a** Passage 2 (×1000). **b** Passage 5 (×1000)

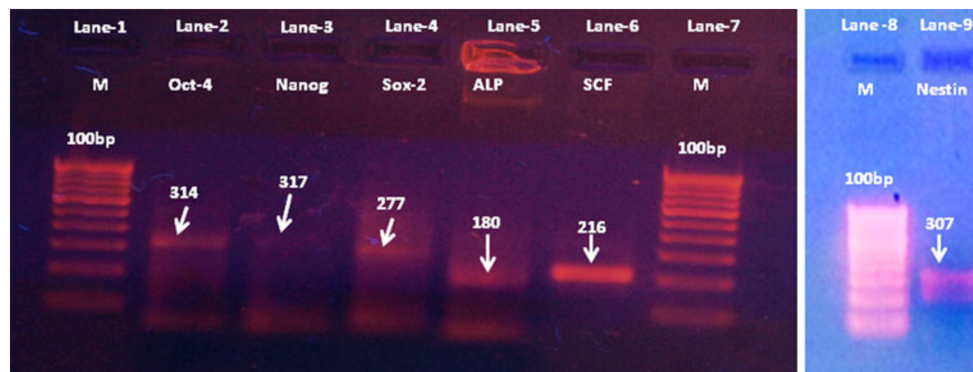

Fig. 4 RT-PCR-based studies showed the strong positive expression for *Oct-4, Nanog, Sox-2,* alkaline phosphatase (*ALP*), stem cell factor (*SCF*), and *Nestin* genes. Agarose gel electrophoresis of analysis of RT-PCR product revealed PCR amplicons of 314, 317, 277, 180, 216, and 307, respectively

AP staining-based characterization of placental membrane cells in in vitro bubaline species. Even though stem cells and their potential applications have emerged as a potential oasis in the field of disease treatment, there is a difference of opinion among scientists even in the definition of the term *stem cell*, which is still clearly an evolving concept (Parker and Cotanche 2004). The main characteristic of stem cells is their pluripotency and their self-regeneration capacity, properties conferred by the presence of some cell transcription factors, of which the most studied are Oct-3/4 and Nanog. Oct-3/4 and Nanog are expressed in embryonic stem cells, tumor germ cells, and adult stem cells. They are essential in the formation of the internal cell mass and are necessary for the self-regeneration function (Barry et al. 2005; Matikainen and Laine 2005). AP, or TRA-2-39, is also a glycoprotein expressed by nondifferentiated cells and teratocarcinoma cells (Erices et al. 2000; Niyibizi et al. 2004; Thellin et al. 2000; Zhang et al. 2004a, b).

Various approaches have been reported to isolate cells with stem and progenitor cell characteristics from placental tissues, as summarized in a recent review by Matikainen and Laine (2005). Such cells have been isolated from the intact human term placenta (Yen et al. 2005), fetal and the maternal portions of the placenta (In 'T Anker et al. 2004), internal regions of the placental lobules (Fukuchi et al. 2004), amniochorionic fetal membrane (Bailo et al. 2004), amniotic epithelium (Sakuragawa et al. 1996), and amniotic mesenchymal cells (Sakuragawa et al. 1997). Mesenchymal cells have also been isolated from human (Elwan and Sakuragawa 1997) and porcine amniotic fluid (Ishii et al. 1999).

Efforts are being made to study various types of pluripotent stem cells (Huang et al. 2010; Sritanaudomchai et al. 2007; Verma et al. 2007) in buffalo (*Bubalus bubalis*), the mainstay of the dairy and meat industries in many countries. The present study is a preliminary effort to investigate whether the cells in bubaline placental membrane cells can be cultured and whether they exhibit stem cell-like attributes. Our results show that placental membrane cells strongly expressed Oct-4 during the earlier passages, then displayed poor expression as the cell differentiation passed away. Mihu et al. (2009) found Oct-4 expression in placental membrane cells; our finding similarly supports Oct-4 expression. Pluripotency markers, AP,

Oct-3/4, and SSEA-4 had an intensely positive expression and SOX-2 and Nanog a weakly positive expression (In 'T Anker et al. 2004; Mihu et al. 2009; Pittenger et al. 1999). Our result also support the same finding that the placental membrane strongly expresses Oct-4 and AP, and weakly positively expresses Sox-2 and Nanog.

In conclusion, placental membrane cells may be isolated and maintained in vitro for prolonged periods of time. Many important features of pluripotent stem cells, including Oct-4, Sox-2, Nanog, AP, stem cell factor, cyclin A, Nestin, and FGF-5, indicating their pluripotency and the capacity for in vitro differentiation, were demonstrated. This analysis showed that the placenta-derived mesenchymal stem cell-like cells could be simply isolated and prolonged without morphologic and quality changes in medium supplemented only with FBS. Therefore, the placenta may prove to be an attractive and rich source of mesenchymal stem cells. The presence of stem cells in the placenta may be quite useful. Amniotic membrane transplantation has been successfully used in a variety of ocular surface conditions. Further studies are required to better understand the precise nature of placenta-derived cells and to explore their potential clinical applications.

This membrane has unique properties such as promotion of normal epithelialization and suppression of inflammation and scar formation. These properties are beneficial for treatment of various conjunctival and corneal disorders such as retinal pigment epithelial (RPE) detachment, neurotrophic ulcers, shield ulcers, chemical injuries, pterygium surgery, and conjunctival surface reconstruction.

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