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Stem cell properties and neural differentiation of sheep amniotic epithelial cells☆

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Abstract

This study was designed to verify the stem cell properties of sheep amniotic epithelial cells and their capacity for neural differentiation. Immunofluorescence microscopy and reverse transcription-PCR revealed that the sheep amniotic epithelial cells were positive for the embryonic stem cell marker proteins SSEA-1, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81, and the totipotency-associated genes Oct-4, Sox-2 and Rex-1, but negative for Nanog. Amniotic epithelial cells expressed β-III-tubulin, glial fibrillary acidic protein, nestin and microtubule-associated protein-2 at 28 days after induction with serum-free neurobasal-A medium containing B-27. Thus, sheep amniotic epithelial cells could differentiate into neurons expressing β-III-tubulin and microtubule-associated protein-2, and glial-like cells expressing glial fibrillary acidic protein, under specific conditions.

Key Words

neural regeneration; stem cells; sheep; amniotic epithelial cells; isolation and culture; stem cell characteristics; differentiation; differentiation potential; reverse transcription-PCR; immunofluorescence microscopy; grants-supported paper; neuroregeneration

Research Highlights

(1) Sheep amniotic epithelial cells were isolated, cultured and shown to possess stem cell properties.
(2) Sheep amniotic epithelial cells were induced to differentiate into neuron-like cells by incubation in Neurobasal-A medium containing B-27 for 28 days *in vitro*.
(3) Sheep amniotic epithelial cells have potential utility for the treatment of nervous system diseases.

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INTRODUCTION

Stem cells are of fundamental significance and value to the fields of regenerative medicine, gene therapy and pharmaceutical research. However, the clinical application of stem cells requires that they are safe for patient use, show therapeutic efficacy, and can be obtained in sufficient quantities. The main stem cell populations considered for clinical applications include embryonic stem cells, adult stem cells and induced pluripotent stem cells. Embryonic stem cells originate from the inner cell mass of preimplantation embryos. Adult stem cells originate from specific tissues and organs. Ectopic expression of four transcription factors, Oct4, Klf4, Sox2 and c-Myc, was used to reprogram somatic cells to induced pluripotent stem cells[1]. Embryonic stem cells and induced pluripotent stem cells

are considered the most promising stem cells because of their capacity for multi-lineage differentiation[2-7], but their clinical application poses safety concerns. Embryonic stem cells are subject to immunological rejection and may induce tumorigenesis following allogeneic transplantation[8-11]. Induced pluripotent stem cells are generated by reprogramming somatic cells through exogenous gene transfer, and therefore endogenous gene expression may be affected and result in tumorigenesis[12-14]. Some studies reported that the therapeutic efficacy of induced pluripotent stem cells is promising, but their long-term effects have not been verified. Furthermore, embryonic stem cells are difficult to obtain in large quantities[15-17], and their use raises ethical controversy because they are obtained by destroying early embryos. Induced pluripotent stem cells are obtained by reprogramming somatic cells, but their transfer efficiency is low (about 0.01–0.2%)[18-21]. Obtaining sufficient induced pluripotent stem cells requires much time and expense. Therefore, adult stem cells are being increasingly investigated as alternatives to embryonic and induced pluripotent stem cells.

Amniotic epithelial cells are a form of adult stem cells extracted from the lining of the inner membrane of the placenta[22]. Amniotic epithelial cells express the embryonic stem cell markers SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81, as well as the transcription factor Oct4, a key regulator of embryonic stem cell pluripotency. The highest expression of these markers is observed in amniotic epithelial cells from the middle layer of the placenta, followed by those in the bottom and top layers[23]. Amniotic epithelial cells can differentiate into various cells of the endoderm (liver, pancreas), mesoderm (myocardial cells) and ectoderm (nerve cells) lineages[23-29]. The treatment of neurodegenerative disease is currently one of the main challenges in neuroscience. The clinical application of neural stem cells offers hope for the treatment of neurodegenerative diseases, but ethical considerations regarding embryo-derived neural stem cells and the limited availability of autologous neural stem cells have restricted the clinical transplantation of neural stem cells[30-33]. Native amniotic epithelial cells can be used for allogeneic transplantation without producing immunological rejection[34]. Amniotic epithelial cells are obtained from discarded placentas after delivery, which are widely available without ethical constraints. Furthermore, the isolation and culture of amniotic epithelial cells from placenta are simple and inexpensive. Amniotic epithelial cells offer the same therapeutic advantages as stem cells, and amnion cells/tissue transplantation has been used in clinical trials in 50 cases[34-35] without causing tumorigenesis. Amniotic epithelial cell transplantation is non-oncogenic in animals with immune deficiency[36]. The use of amniotic epithelial cells for the treatment of some stubborn diseases demonstrated good therapeutic outcomes and no toxicity or side effects, including liver disease[37-38], lung disease[39-42], heart disease[43], diabetes mellitus[44], nervous system disease[45] and tendon injury[46].

As such, if amniotic epithelial cells can be induced to differentiate into nerve cells, amniotic epithelial cells would be a valuable source for cell replacement therapy. Previous studies have mainly focused on human amniotic epithelial cells. Therefore, this study was performed to characterize the stem cell properties of amniotic epithelial cells isolated and cultured from sheep amnion, including their capacity for neural differentiation *in vitro*.

RESULTS

In vitro growth characterization of sheep amniotic epithelial cells

Sheep amniotic epithelial cells grew adherently in culture as polygonal, spherical or elliptical cells in a paving stone arrangement (Figure 1). On top of the attached cells, round brilliant cells and a few cell spheroids with strong refraction were visible. These cells contained a large nucleus and scant cytoplasm, and were smaller than the adherent cells. The number of floating round brilliant cells was low, but their refraction was strong (Figure 1).

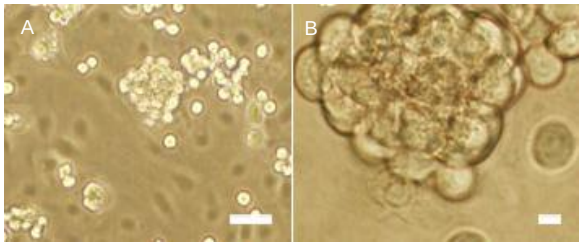

Figure 1 Morphology of passage 2 sheep amniotic epithelial cells (phase contrast microscope).

(A) Adherent cells on the bottom of the wells were polygonal and gray, with round single cells and cell aggregates (spheroids, white) present above them. (B) High magnification image of the morphology of spheroids. Scale bars: (A) 50 μm; (B) 10 μm.

Cells at passages 2–5 exhibited a morphology identical to primary cells. The cells from passage 6 proliferated

slowly and became large and deformed. The cells from passage 8 detached from the wells, died, and could not be subcultured.

Stem cell properties of sheep amniotic epithelial cells

Immunofluorescence microscopy revealed that sheep amniotic epithelial cells expressed the embryonic stem cell marker proteins, Oct-4, SSEA-1, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81, to different degrees (Figure 2).

Reverse transcription-PCR showed that sheep amniotic epithelial cells expressed Oct-4, Sox-2 and Rex-1, but did not express Nanog. Sheep fibroblasts served as negative controls (Figure 3). Therefore, genes that control the multi-directional differentiation of stem cells were expressed in cultured sheep amniotic epithelial cells.

Morphological changes in amniotic epithelial cells after induced differentiation

After preinduction with 1 mmol/L 2-mercaptoethanol, a small number of adherent cells died and the round brilliant cells in the top layer remained unchanged. The cells grew slowly after induction. After 3 days of differentiation, the round brilliant cell bodies became enlarged. After 14 days, the cells were further enlarged and some cells displayed processes. After 21 days, the cell bodies had further enlarged and visible processes were present. Some cells died during differentiation. Neuron- and glial-like cells are shown at 28 days in Figure 4.

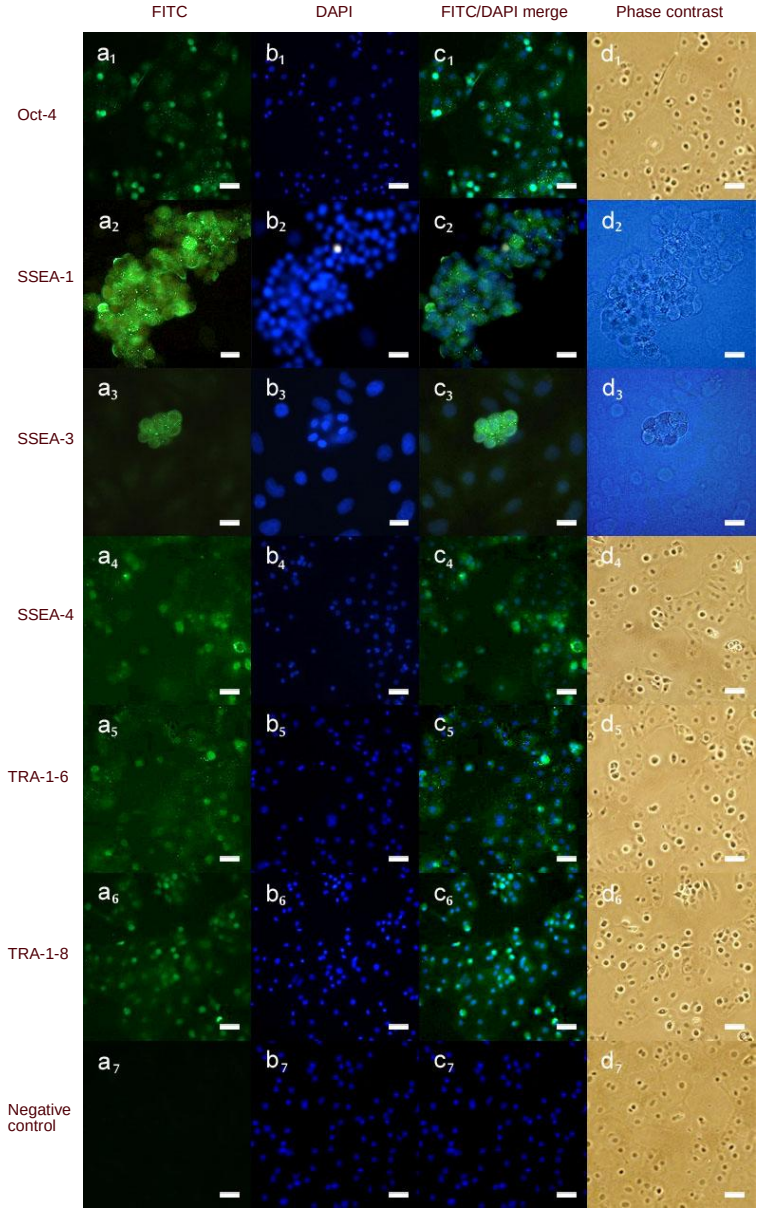

Figure 2 Expression of marker proteins by sheep amniotic epithelial cells.

Oct-4 is present in the nuclei, and SSEA-1, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 are present in the cell membrane. Fluorescein isothiocyanate (FITC)-labeled cells exhibited a green color. 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei exhibited a blue color. Scale bars: 100 μm for all images with the exception of 50 μm for a_2–d_2, a_3–d_3.

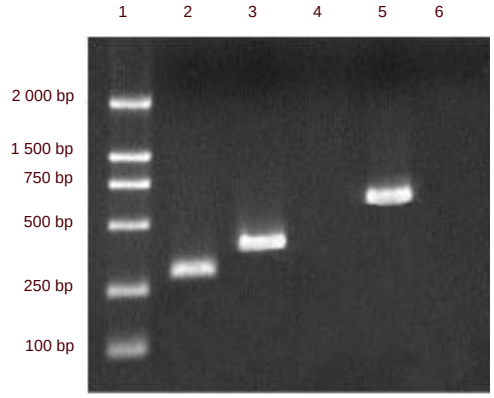

Figure 3 Expression of totipotency-associated genes in sheep amniotic epithelial cells (reverse transcription-PCR).

Sheep amniotic epithelial cells were positive for Oct-4, Sox-2 and Rex-1, but negative for Nanog. 1: DL2000 DNA marker; 2: Rex-1 (297 bp); 3: Sox-2 (341 bp); 4: Nanog (498 bp); 5: Oct-4 (571 bp); 6: negative control.

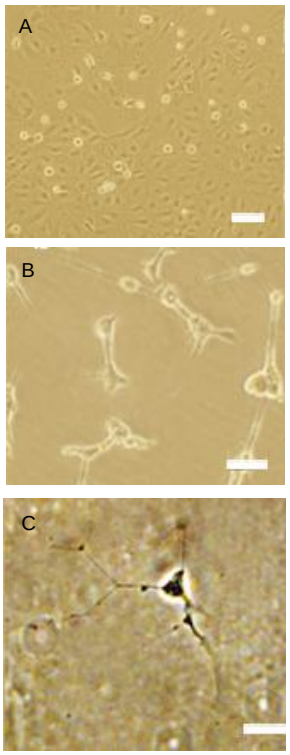

Figure 4 Induced neural differentiation of sheep amniotic epithelial cells (phase contrast images).

(A) Sheep amniotic epithelial cells at passage 2 before induction. Adherent cells on the bottom of the wells were polygonal, and round brilliant cells were present above them.

(B) Sheep amniotic epithelial cells displayed processes and the cell bodies increased in size at 28 days after induction.

(C) Single neurons exhibited clear thin neurites at 28 days after induction.

Scale bars: (A) 100 μm; (B, C) 50 μm.

Expression of specific marker proteins following induced differentiation

Immunofluorescence microscopy demonstrated that β-III-tubulin-positive cells were visible after 28 days of differentiation (Figure 5a_1). These cells included neuronal cells with one axon and two dendrites (a_1: 1, 3) or one axon and many dendrites (a_1: 2), and neurons with other morphologies (a_1: 3). Glial fibrillary acidic protein-positive cells were visible (Figure 5a_2). Glial-like cells without a typical astrocyte morphology were present (a_2: 1, 2, 3).

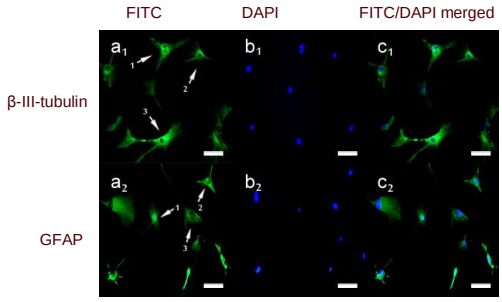

Figure 5 Sheep amniotic epithelial cells after induced differentiation (fluorescence micrographs).

β-III-tubulin-positive cells and glial fibrillary acidic protein (GFAP)-positive cells were detectable in the medium at 28 days. Fluorescein isothiocyanate (FITC)-labeled cells exhibited a green color, and 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei exhibited a blue color. Arrows 1, 2, 3 showed the neuronal cells with one axon and two dendrites or one axon and many dendrites. Scale bars: 50 μm.

Expression of specific marker genes following induced differentiation

On day 0, nestin and β-III-tubulin expression was observed, but microtubule-associated protein-2 expression was not detected. After 28 days of differentiation, nestin, β-III-tubulin and microtubule-associated protein-2 expression was visible (Figure 6). Sheep fibroblasts served as negative controls.

DISCUSSION

Amniotic epithelial cells can be induced to differentiate into nerve cells/tissues using different methods. Human amniotic epithelial cells were induced to differentiate primarily into glial cells with a few neurons by culture in standard medium supplemented with all-trans retinoic acid and fibroblast growth factor 4[47]. Dulbecco's modified Eagle's medium/F12 (DMEM/F12) supplemented with dimethyl sulfoxide, butyl hydroxyanisole, KCl, valproic acid, forskolin, hydrocortisone and insulin was used to induce the differentiation of rat amniotic epithelial cells into

immature neurons[48]. In accordance with a previously published method[49], serum-free neurobasal-A medium containing B-27 was used to induce neural differentiation in sheep amniotic epithelial cells. B-27 contains vitamin E, superoxide dismutase, selenium and glutathione, and maintains the survival and growth of nerve cells *in vitro* [50].

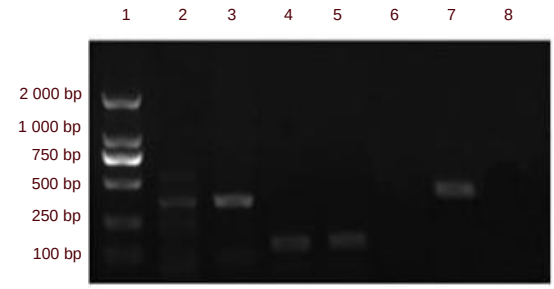

Figure 6 Expression of nestin, β-III-tubulin and microtubule-associated protein-2 in sheep amniotic epithelial cells after 28 days of differentiation (reverse transcription-PCR).

Nestin and β-III-tubulin expression was detected before and 28 days after induction. Expression of microtubule-associated protein-2 was not detected before induction, but could be detected after 28 days of induction. 1: DL2000 DNA marker; 2, 4, 6: prior to induction; 3, 5, 7: 28 days after induction; 8: negative control; 2, 3: nestin (361 bp); 4, 5: β-III-tubulin (152 bp); 6, 7: microtubule-associated protein-2 (442 bp).

Our method included a modification to preinduce the cells with DMEM/F12 containing 10% dimethyl sulfoxide for 24 hours. This was performed to accelerate the differentiation of sheep amniotic epithelial cells, but the effect was not significant. Sheep amnion was digested with the trypsin substitute, TrypLE™ Select, to isolate sheep amniotic epithelial cells. Miki *et al* [11, 23] found that human amniotic epithelial cells exhibit gradational growth, with round bright cells and cell spheroids being observed on top of a layer of adherent cells. Cultured sheep amniotic epithelial cells also exhibited gradational growth: cells adhering to the bottom of the culture flask were polygonal, round or elliptic, large in size and arranged like paving stones. Round brilliant cells and a few cell spheroids with strong refraction were visible among the attached cells. These latter cells were characterized by large nuclei and scant cytoplasm, and were small in size and scattered. A small number of suspended round bright cells in the medium exhibited strong refraction. These cells were easily lost during medium replacement and are therefore difficult to detect. Cultured sheep amniotic epithelial cells included a lower layer of adherent cells, a middle layer of round or spheroid cells, and a few round bright cells suspended in the medium with strong refraction. Morphologically, the sheep amniotic epithelial cells were consistent with human amniotic epithelial cells. Immunofluorescence microscopy and reverse transcription-PCR confirmed that the round bright cells and cell spheroids were positive for Oct-4, SSEA-1, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, Oct-4, Sox-2 and Rex-1, suggesting that sheep amniotic epithelial cells with multi-lineage differentiation potential were obtained.

In vitro neural differentiation of sheep amniotic epithelial cells was induced using neurobasal-A medium. After 28 days of induction, β-III-tubulin, glial fibrillary acidic protein, nestin and microtubule-associated protein-2 were expressed. Of these proteins, β-III-tubulin expression is highest in neurons[51]. β-III-tubulin expression is mainly detected in immature neurons and some tumor cells, but not in glial cells and most non-neuronal cells[52]. Microtubule-associated protein-2 is necessary for neuron dendrite development; its expression is high in mature neurons and it is used as a marker for neuron identification[53]. Glial fibrillary acidic protein is a specific intermediate filament of the astrocyte cytoskeleton and is used as a specific marker of astrocytes[54]. Nestin is an intermediate filament protein that is known as a neural stem/progenitor cell marker[55]. Nestin is associated with the multi-lineage differentiation potential of progenitor cells during embryonic development and the regeneration of adult tissues[56]. In this study, sheep amniotic epithelial cells were successfully induced to differentiate into β-III-tubulin-positive immature neurons, microtubule-associated protein-2-positive mature neurons, and glial fibrillary acidic protein-positive glial cells. Although the cells differentiated from sheep amniotic epithelial cells expressed glial fibrillary acidic protein, no glial fibrillary acidic protein-positive cells with a typical astrocyte morphology were observed. This may be because cells remain in the early differentiation stage, suggesting that sheep amniotic epithelial cells have potential for the treatment of nervous system diseases.

This study used serum-free neurobasal-A medium containing B-27 to induce cell differentiation. To verify whether sheep amniotic epithelial cells have the potential for neural differentiation, the medium was supplemented with only B-27 rather than other factors such as β-fibroblast growth factor and N23. B-27 contains vitamin E, superoxide dismutase, selenium and glutathione, and maintains the survival and growth of nerve cells *in vitro* [57]. This induction medium only contains two reagents, is simple to use, and is inexpensive. A previous study demonstrated that the expression of amniotic epithelial cell surface markers was affected by

noggin, basic fibroblast growth factor and retinoic acid[58]. The addition of retinoic acid and fibroblast growth factor-4 to the medium induced the differentiation of human amniotic epithelial cells into glial cells and neurons[23]. The addition of noggin alone, retinoic acid alone or both together to the medium also induced the differentiation of human amniotic epithelial cells into nerve cells[59]. Many investigators have verified the neural differentiation of amniotic epithelial cells[45, 58-59]. The effects of other induction methods and medium supplements on the neural differentiation of sheep amniotic epithelial cells require further investigation. Clinical experiments suggested that amniotic epithelial cell transplantation could improve the symptoms of some typical neuropathies. Transplanted human amniotic epithelial cells lessened the symptoms of Parkinson's disease in a mouse model created by excising the nerve controlling dopamine secretion[60]. Transplanted human amniotic epithelial cells migrated to the ischemic region to reduce the infarct volume in rat models of stroke[61]. Neuron-like cells were observed at 5 weeks after mouse amniotic epithelial cell transplantation into ischemic hippocampi of adult gerbil[62]. It remains poorly understood whether sheep amniotic epithelial cells can be used to repair host neurons in the treatment of brain injury and neurodegenerative disease.

Following sample collection, culture and passage, very few amniotic mesenchymal cells were visible. In this study, we did not attempt to identify different cell types within the cultures.

In summary, in this study we characterized the stem cell properties and neural cell differentiation potential of sheep amniotic epithelial cells, thus demonstrating the potential clinical application of amniotic epithelial cells. Clinical trials are essential for identifying new medicines, and the sheep is similar to human in terms of its size and physiological characteristics[63]. Sheep amniotic epithelial cells provide a novel cell source, and the sheep is a good animal model for clinical and safety studies with human amniotic epithelial cells, suggesting the potential for allogeneic transplantation between species. Sheep amniotic epithelial cells have potential utility for the study of cattle, horse and swine amniotic epithelial cells *in vitro*.

MATERIALS AND METHODS

Design

A cell characterization study.

Time and setting

Experiments were performed at the Experimental Center, Veterinary Medicine College of Inner Mongolia Agricultural University, China from June 2011 to March 2012.

Materials

Five placentas were aseptically obtained from sheep after delivery at the Sheep Farm, Lingeer County, Inner Mongolia, China, placed in an ice box, and delivered to the laboratory within 2 hours. All protocols were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[64].

Methods

Isolation and culture of sheep amniotic epithelial cells

Amnion was obtained using a mechanical method, washed with Dulbecco's PBS and weighed. The amnion was treated with pre-warmed TrypLE (1 mL/g) (TrypLE™ Select, Invitrogen, Carlsbad, CA, USA) at 37°C and 100 r/min for 10 minutes. The supernatant was discarded, and the amnion was digested with TrypLE (1 mL/g) at 37°C and 250 r/min for 30 minutes, before adding 10% fetal bovine serum to terminate the digestion. The digestion was repeated twice. The supernatants from the three digestions were pooled, filtered through a 200-mesh filter and centrifuged at 1 500 r/min for 5 minutes. The supernatant was removed. The specimens were suspended and precipitated using DMEM/F12 (Invitrogen) supplemented with 1% penicillin-streptomycin (Invitrogen), 1% insulin, transferrin-selenium (Invitrogen), 1% minimum essential medium with non-essential amino acids (Invitrogen), and 15% fetal bovine serum (Invitrogen). The cells were counted and cell viability was measured by trypan blue staining (Invitrogen). Cells were plated at 1×10^6/flask and incubated at 37°C in 5% CO_2 and saturated humidity.

Subculture of sheep amniotic epithelial cells

The cells were subcultured when they reached 80–90% confluence at a ratio of 1: 3. The culture conditions were identical to those described above. The medium was replaced every 2–3 days.

Stem cell properties of sheep amniotic epithelial cells

Immunofluorescence microscopy was used to detect the embryonic stem cell marker proteins, Oct-4, SSEA-1, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81, in sheep amniotic epithelial cells. Passage 2 sheep amniotic epithelial cells (1×10^5/mL) were incubated on glass slides

overnight. After removing the medium, the cells were washed in PBS, fixed in 4% paraformaldehyde at room temperature for 20 minutes, and washed with PBS. For the detection of Oct-4 (Yili Biological Technology Co., Ltd., Shanghai, China), the cells were washed with 0.35% Triton X-100, and then with PBS. Subsequently, the cells were blocked with 4% goat serum at room temperature for 30 minutes. The serum was discarded and primary antibodies for Oct-4, SSEA-1, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 (1 mg/mL; mouse anti-sheep monoclonal antibodies, Millipore, Bedford, MA, USA) were added. As a negative control, the cells were incubated with PBS instead of primary antibody at room temperature for 1 hour, and then washed with PBS. Secondary FITC-labeled goat anti-mouse antibody (1: 50; KPL, Gaithersburg, MA, USA) was added at room temperature for 40 minutes. After washing with PBS, the specimens were stained with 4′,6-diamidino-2-phenylindole (DAPI, Beijing Seajet Scientific, Beijing, China), washed with PBS, mounted, and then observed using a fluorescence microscope (Olympus, Tokyo, Japan).

For reverse transcription-PCR analysis of totipotency-associated gene expression, Oct-4, Sox-2, Nanog and Rex-1 were selected. Total RNA was extracted using the RNAfast200 Total RNA Extraction Kit (Fastagen, Shanghai, China), in accordance with the kit instructions. 5 μL of RNA was electrophoresed on a 1% agarose gel (100 V, 30 minutes) to examine the integrity, content and purity. Conserved regions in the human, cattle, swine and sheep mRNAs were selected for primer design. The primer sequences are listed in Table 1.

Table 1 Primers used for the PCR amplification of totipotency-associated genes

Gene	Primer sequence	Product size (bp)	Annealing temperature (°C)
Oct-4	F: 5′-GCG CCG CAG GTT GGA GTG G -3′ R: 5′-GCT GCT GGG CGA TGT GGC TAA T-3′	571	60
Sox-2	F: 5′ -CAT GAA CGG CTC GCC CAC CTA CAG-3′ R: 5′-TCT CCC CCG CCC CCT CCA GTT CAC-3′	341	65
Nanog	F: 5′-TTC CTT CCT CCA TGG ATC TG-3′ R: 5’-ACC AGT GGT TGC TCC AAG AC-3′	498	60
Rex-1	F: 5′-ACA GCC CCG GCC CGT CCT CTA CCC -3′ R: 5′-ACC CCC GCA CCC TCC ACC CAC AA-3′	297	62

F: Forward; R: reverse.

Primers were synthesized by Invitrogen Corporation, Shanghai Representative Office, Shanghai, China. The reaction solution comprised: 5 × Prime Script Buffer 2 μL, Prime Script RT Enzyme Mix 0.5 μL, Oligo dT Primer (50 μmol/L) 0.5 μL, random 6 mers (100 μmol/L) 0.5 μL, total RNA 1 μL (≤ 500 ng), RNase-free dH_2O 5.5 μL. Reverse transcription was performed at 37°C for 15 minutes. The PCR reaction mix comprised: 50 μL: TaKaRa Taq (5 U/μL) 0.25 μL, 10 × PCR buffer 5 μL, dNTP mixture (each 2.5 mmol/L) 4 μL, template DNA 2.5 ng, upstream primer (20 μmol/L) 1 μL, downstream primer (20 μmol/L) 1 μL, sterile distilled water 36.75 μL. The PCR reaction conditions were: 30 cycles of 94°C for 1 minute, 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, followed by 72°C for 7 minutes, 4°C termination. Sheep fibroblasts (Sheep Farm, Lingeer County, Inner Mongolia, China) served as negative controls for PCR. Gels were photographed and analyzed using the gel image analysis system (Tanon Science & Technology Co., Ltd., Shanghai, China).

Neural differentiation of sheep amniotic epithelial cells

Passage 2 sheep amniotic epithelial cells (1×10^4/mL) were seeded on lysine-coated glass slides in a 12-well plate. 1.5 mL of growth medium was added to each well. When the cells reached 60% confluence, they were washed with Hank’s balanced salt solution, preinduced with 1 mmol/L 2-mercaptoethanol for 24 hours, washed with Hank’s balanced salt solution, incubated with 500 mL serum-free neurobasal-A medium supplemented with 2% (v/v) B-27 (Invitrogen), and then observed under the microscope. The medium was replaced once every 3 days. The induction was conducted for 28 consecutive days. In the control group, passage 2 sheep amniotic epithelial cells were not induced and the growth medium was replaced once every 3 days.

Identification of differentiated cells following induction

Immunofluorescence analysis of glial fibrillary acidic protein expression following neural differentiation: at 28 days of induction, the glass slides were placed in a 35 mm petri dish, washed with PBS, fixed with 4% paraformaldehyde for 30 minutes, washed with PBS, permeabilized with 0.35% Triton X-100 for 30 minutes, washed with PBS, treated with 0.6% H_2O_2 for 30 minutes, washed with PBS, and blocked with 4% goat serum for 30 minutes. The serum was discarded. Primary rabbit anti-β-III-tubulin polyclonal antibody (1:100; Sigma, St. Louis, MO, USA) and rabbit anti-glial fibrillary acidic protein polyclonal antibody (1:100; Sigma) were added at 4°C overnight. After washing with PBS, FITC-labeled

goat anti-rabbit secondary antibody (1:50; Sigma) was added in the dark for 30 minutes. After washing with PBS, the cells were stained with DAPI (Biotium, Hayward, CA, USA) for 15 minutes, washed with PBS, mounted, and observed with a fluorescence microscope.

Reverse transcription-PCR analysis of specific marker gene expression following neural differentiation: the expression of nestin, β-III-tubulin and microtubule-associated protein-2 was measured by reverse transcription-PCR at 0 and 28 days following induction. Fibroblasts served as the negative control. The primer sequences are shown in Table 2.

Table 2 Primers used for the PCR amplification of totipotency-associated genes

Gene	Primer sequence	Product size (bp)	Annealing temperature (°C)
Nestin	F: 5′-ACA CCT GTG CCA ACC TTT C -3′ R: 5′- CAC TGG GTT CTC CAT CTT TG -3′	361	62
MAP-2	F: 5′-GAC CTC GCC CCC TCA TCC AC -3′ R: 5′-CTT CAG GCA ACG GCT CGG TAA C -3′	442	60
β-III-tubulin	F: 5′-GCC TTT GGG CAC CTC TTC -3′ R: 5′- AGG CAG TCG CAG TTC TCG -3′	152	60

F: Forward; R: reverse; MAP-2: microtubule-associated protein-2.

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Author contributions: Xuemin Zhu and Xiumei Wang designed the study, acquired the data and wrote the manuscript. Guifang Cao obtained the funding, provided technical support, and was in charge of manuscript authorization. Fengjun Liu, Yinfeng Yang, Junping Liu, Yuling Zhang, Xiaonan Li and Yan Mi, Lingli Zhang performed the statistical analysis. All authors read and approved the final version of the submitted manuscript.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animal Ethics Committee, Inner Mongolia Agricultural University, China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

REFERENCES

[1] Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;126(4):663-676.
[2] Raya A, Rodríguez-Pizà I, Guenechea G, et al. Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. Nature. 2009; 460(7251):53-59.
[3] Wernig M, Zhao JP, Pruszak J, et al. Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease. Proc Natl Acad Sci U S A. 2008; 105(15):5856-5861.
[4] Narazaki G, Uosaki H, Teranishi M, et al. Directed and systematic differentiation of cardiovascular cells from mouse induced pluripotent stem cells. Circulation. 2008; 118(5):498-506.
[5] Park TS, Galic Z, Conway AE, et al. Derivation of primordial germ cells from human embryonic and induced pluripotent stem cells is significantly improved by coculture with human fetal gonadal cells. Stem Cells. 2009;27(4):783-795.
[6] Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998;282(5391):1145-1147.
[7] Amit M, Carpenter MK, Inokuma MS, et al. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol. 2000;227(2):271-278.
[8] Reubinoff BE, Pera MF, Fong CY, et al. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol. 2000;18(4):399-404.
[9] Draper JS, Pigott C, Thomson JA, et al. Surface antigens of human embryonic stem cells: changes upon differentiation in culture. J Anat. 2002;200(Pt 3): 249-258.
[10] Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A. 1981;78(12):7634-7638.
[11] Matsui Y, Zsebo K, Hogan BL. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell. 1992;70(5):841-847.
[12] Miura K, Okada Y, Aoi T, et al. Variation in the safety of induced pluripotent stem cell lines. Nat Biotechnol. 2009; 27(8):743-745.
[13] Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007;131(5):861-872.
[14] Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. Nature. 2007; 448(7151):313-317.
[15] Smith AG. Embryo-derived stem cells: of mice and men. Annu Rev Cell Dev Biol. 2001;17:435-462.

[16] Lanzendorf SE, Boyd CA, Wright DL, et al. Use of human gametes obtained from anonymous donors for the production of human embryonic stem cell lines. Fertil Steril. 2001;76(1):132-137.
[17] Kaji EH, Leiden JM. Gene and stem cell therapies. JAMA. 2001;285(5):545-550.
[18] Ou L, Wang X, Zou F. Is iPS cell the panacea? IUBMB Life. 2010;62(3):170-175.
[19] Hong H, Takahashi K, Ichisaka T, et al. Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. Nature. 2009;460(7259):1132-1135.
[20] Kawamura T, Suzuki J, Wang YV, et al. Linking the p53 tumour suppressor pathway to somatic cell reprogramming. Nature. 2009;460(7259):1140-1144.
[21] Li H, Collado M, Villasante A, et al. The Ink4/Arf locus is a barrier for iPS cell reprogramming. Nature. 2009; 460(7259):1136-1139.
[22] Miki T. Amnion-derived stem cells: in quest of clinical applications. Stem Cell Res Ther. 2011;2(3):25.
[23] Miki T, Lehmann T, Cai H, et al. Stem cell characteristics of amniotic epithelial cells. Stem Cells. 2005;23(10): 1549-1559.
[24] Sakuragawa N, Thangavel R, Mizuguchi M, et al. Expression of markers for both neuronal and glial cells in human amniotic epithelial cells. Neurosci Lett. 1996; 209(1):9-12.
[25] Sakuragawa N, Enosawa S, Ishii T, et al. Human amniotic epithelial cells are promising transgene carriers for allogeneic cell transplantation into liver. J Hum Genet. 2000;45(3):171-176.
[26] Nakajima T, Enosawa S, Mitani T, et al. Cytological examination of rat amniotic epithelial cells and cell transplantation to the liver. Cell Transplant. 2001;10(4-5): 423-427.
[27] Fang CH, Jin J, Joe JH, et al. In vivo differentiation of human amniotic epithelial cells into cardiomyocyte-like cells and cell transplantation effect on myocardial infarction in rats: comparison with cord blood and adipose tissue-derived mesenchymal stem cells. Cell Transplant. 2012;21(8):1687-1696.
[28] Zhao YJ, Fang N, Chen DX, et al. Study on inducing differentiation of human amniotic epithelial cells into insulin secreting cells in vitro. Zhongguo Ying Yong Sheng Li Xue Za Zhi. 2012;28(2):139-143.
[29] Lange-Consiglio A, Corradetti B, Bizzaro D, et al. Characterization and potential applications of progenitor-like cells isolated from horse amniotic membrane. J Tissue Eng Regen Med. 2012;6(8):622-635.
[30] Wadman M. Embryonic stem-cell research exempt from ban, NIH is told. Nature. 1999;397(6716):185-186.
[31] Holden C, Vogel G. Stem cell lines. 'Show us the cells', U.S. researchers say. Science. 2002;297(5583):923-925.
[32] Suckiel E. Human embryonic stem cell research: a critical survey of the ethical issues. Adv Pediatr. 2008;55:79-96.
[33] Hanley J, Rastegarlari G, Nathwani AC. An introduction to induced pluripotent stem cells. Br J Haematol. 2010; 151(1):16-24.
[34] Akle CA, Adinolfi M, Welsh KI, et al. Immunogenicity of human amniotic epithelial cells after transplantation into volunteers. Lancet. 1981;2(8254):1003-1005.
[35] Sakuragawa N, Yoshikawa H, Sasaki M. Amniotic tissue transplantation: clinical and biochemical evaluations for some lysosomal storage diseases. Brain Dev. 1992;14(1): 7-11.
[36] Ilancheran S, Michalska A, Peh G, et al. Stem cells derived from human fetal membranes display multilineage differentiation potential. Biol Reprod. 2007;77(3):577-588.
[37] Luo HW, Huang XJ, Huang FZ, et al. Preliminary study on transdifferentiation of human amniotic epithelial cells and its intrasplenic transplantation. Zhongguo Xiufu Chongjian Waike Zazhi. 2011;25(2):139-143.
[38] Manuelpillai U, Tchongue J, Lourensz D, et al. Transplantation of human amnion epithelial cells reduces hepatic fibrosis in immunocompetent CCl_4-treated mice. Cell Transplant. 2010;19(9):1157-1168.
[39] Moodley Y, Ilancheran S, Samuel C, et al. Human amnion epithelial cell transplantation abrogates lung fibrosis and augments repair. Am J Respir Crit Care Med. 2010;182(5): 643-651.
[40] Hodges RJ, Jenkin G, Hooper SB, et al. Human amnion epithelial cells reduce ventilation-induced preterm lung injury in fetal sheep. Am J Obstet Gynecol. 2012;206(5): 448.e8-15.
[41] Das S, Ismail NA. Effect of human amnion epithelial cells on pulmonary development. Am J Obstet Gynecol. 2012; 206(4):e4-5; author reply e5.
[42] Murphy SV, Shiyun SC, Tan JL, et al. Human amnion epithelial cells do not abrogate pulmonary fibrosis in mice with impaired macrophage function. Cell Transplant. in press.
[43] Dong W, Chen H, Yang X, et al. Treatment of intracerebral haemorrhage in rats with intraventricular transplantation of human amniotic epithelial cells. Cell Biol Int. 2010;34(6): 573-577.
[44] Qureshi KM, Oliver RJ, Paget MB, et al. Human amniotic epithelial cells induce localized cell-mediated immune privilege in vitro: implications for pancreatic islet transplantation. Cell Transplant. 2011;20(4):523-534.
[45] Yang X, Song L, Wu N, et al. An experimental study on intracerebroventricular transplantation of human amniotic epithelial cells in a rat model of Parkinson's disease. Neurol Res. 2010;32(10):1054-1059.
[46] Barboni B, Curini V, Russo V, et al. Indirect co-culture with tendons or tenocytes can program amniotic epithelial cells towards stepwise tenogenic differentiation. PLoS One. 2012;7(2):e30974.
[47] Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981; 292(5819):154-156.
[48] Shinya M, Komuro H, Saihara R, et al. Neural differentiation potential of rat amniotic epithelial cells. Fetal Pediatr Pathol. 2010;29(3):133-143.

[49] Murphy S, Rosli S, Acharya R, et al. Amnion epithelial cell isolation and characterization for clinical use. Curr Protoc Stem Cell Biol. 2010;Chapter 1:Unit 1E.6.
[50] Marcus AJ, Coyne TM, Black IB, et al. Fate of amnion-derived stem cells transplanted to the fetal rat brain: migration, survival and differentiation. J Cell Mol Med. 2008;12(4):1256-1264.
[51] Sun L, Lee J, Fine HA. Neuronally expressed stem cell factor induces neural stem cell migration to areas of brain injury. J Clin Invest. 2004;113(9):1364-1374.
[52] Nagai A, Kim WK, Lee HJ, et al. Multilineage potential of stable human mesenchymal stem cell line derived from fetal marrow. PLoS One. 2007;2(12):e1272.
[53] Kim HJ, Hida H, Jung CG, et al. Treatment with deferoxamine increases neurons from neural stem/progenitor cells. Brain Res. 2006;1092(1):1-15.
[54] Hu JL, Jiang XD, Zou YX, et al. Culture and identification of neural stem cells derived from the subventricular zone of adult mice. Nan Fang Yi Ke Da Xue Xue Bao. 2008; 28(11):1942-1946.
[55] Lendahl U, Zimmerman LB, McKay RD. CNS stem cells express a new class of intermediate filament protein. Cell. 1990;60(4):585-595.
[56] Wiese C, Rolletschek A, Kania G, et al. Nestin expression-a property of multi-lineage progenitor cells? Cell Mol Life Sci. 2004;61(19-20):2510-2522.
[57] Brewer GJ, Torricelli JR, Evege EK, et al. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J Neurosci Res. 1993;35(5):567-576.
[58] Marcus AJ, Coyne TM, Black IB, et al. Fate of amnion-derived stem cells transplanted to the fetal rat brain: migration, survival and differentiation. J Cell Mol Med. 2008;12(4):1256-1264.
[59] Niknejad H, Peirovi H, Ahmadiani A, et al. Differentiation factors that influence neuronal markers expression in vitro from human amniotic epithelial cells. Eur Cell Mater. 2010; 19:22-29.
[60] Kakishita K, Nakao N, Sakuragawa N, et al. Implantation of human amniotic epithelial cells prevents the degeneration of nigral dopamine neurons in rats with 6-hydroxydopamine lesions. Brain Res. 2003;980(1): 48-56.
[61] Liu T, Wu J, Huang Q, et al. Human amniotic epithelial cells ameliorate behavioral dysfunction and reduce infarct size in the rat middle cerebral artery occlusion model. Shock. 2008;29(5):603-611.
[62] Okawa H, Okuda O, Arai H, et al. Amniotic epithelial cells transform into neuron-like cells in the ischemic brain. Neuroreport. 2001;12(18):4003-4007.
[63] Mauro A, Turriani M, Ioannoni A, et al. Isolation, characterization, and in vitro differentiation of ovine amniotic stem cells. Vet Res Commun. 2010;34 Suppl 1:S25-28.
[64] The Ministry of Science and Technology of the People's Republic of China. Guidance Suggestions for the Care and Use of Laboratory Animals. 2006-09-30.

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