Isolation and characterization mesenchymal stem cells from red panda (*Ailurus fulgens styani*) endometrium

Dong-Hui Wang1,2,3,†, Xue-Mei Wu2,†, Jia-Song Chen1,2,3, Zhi-Gang Cai1,2,3, Jun-Hui An1,2,3, Ming-Yue Zhang1,2,3, Yuan Li2, Fei-Ping Li1,2,3, Rong Hou1,2,3 and Yu-Liang Liu1,2,3,*

1 Chengdu Research Base of Giant Panda Breeding, 1375 Panda Road, Northern Suburb, Chengdu, 610081, Sichuan Province, China
2 Sichuan Key Laboratory of Conservation Biology for Endangered Wildlife, 1375 Panda Road, Northern Suburb, Chengdu, 610081, Sichuan Province, China
3 Sichuan Academy of Giant Panda, 1375 Panda Road, Northern Suburb, Chengdu, 610081, Sichuan Province, China

*Corresponding author: Chengdu Research Base of Giant Panda Breeding. Email: sdluyuliang@163.com
† These authors contributed equally to this work.

Endometrial mesenchymal stem cells (eMSCs) are undifferentiated endometrial cells with self-renewal, multidirectional differentiation and high proliferation potential. Nowadays, eMSCs have been found in a few species, but it has never been reported in endangered wild animals, especially the red panda. In this study, we successfully isolated and characterized the eMSCs derived from red panda. Red panda eMSCs were fibroblast-like, had a strong proliferative potential and a stable chromosome number. Pluripotency genes including *Klf4*, *Sox2* and *Thy1* were highly expressed in eMSCs. Besides, cultured eMSCs were positive for MSC markers CD44, CD49f and CD105 and negative for endothelial cell marker CD31 and haematopoietic cell marker CD34. Moreover, no reference RNA-seq was used to analyse the eMSCs transcriptional expression profile and key pathways. Compared with skin fibroblast cell group, 9104 differentially expressed genes (DEGs) were identified, among which are 5034 genes upregulated, 4070 genes downregulated and the top 20 enrichment pathways of DEGs in Gene Ontology (GO) and the Kyoto Encyclopedia of Genes Genomes (KEGG) mainly associated with G-protein coupled receptor signalling pathway, carbohydrate derivative binding, nucleoside binding, ribosome biogenesis, cell cycle, DNA replication, Ras signalling pathway and purine metabolism. Among the DEGs, some representative genes about promoting MSCs differentiation and proliferation were upregulated and promoting fibroblasts proliferation were downregulated in eMSCs group. Red panda eMSCs also had multiple differentiation ability and could differentiate into adipocytes, chondrocytes and hepatocytes. In conclusion, we, for the first time, isolated and characterized the red panda eMSCs with ability of multiplication and multilinage differentiation *in vitro*. The new multipotential stem cell could be beneficial not only for the germ plasm resources conservation of red panda, but also for basic or pre-clinical studies in the future.

Key words: red panda, mesenchymal stem cell, endometrium, cell differentiation, Cell culture

Editor: Dr. Steven Cooke

Received 25 April 2021; Revised 30 August 2021; Editorial Decision 8 January 2022; Accepted 7 February 2022

Cite as: Wang DH, Wu X-M, Chen J-S, Cai Z-G, An J-H, Zhang M-Y, Li Y, Li F-P, Hou, R, Liu Y-L. (2022) Isolation and characterization mesenchymal stem cells from red panda (*Ailurus fulgens styani*) endometrium. *Conserv Physiol* 10(1): coac004; doi:10.1093/conphys/coac004.

© The Author(s) 2022. Published by Oxford University Press and the Society for Experimental Biology. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.
Introduction

Red pandas (*Ailurus fulgens*), world-famous wild animals, are mainly distributed in the west of China, the Himalaya mountain ranges of Nepal, India, Bhutan and Myanmar (Li et al., 2005). The red pandas are recently classified as two separate species *Ailurus fulgens fulgens* and *Ailurus fulgens styani*. In China, both subspecies are found and were separated by Yalu Zangbu River (Hu et al., 2020). Due to the destructed habitats and declining population, the red panda was listed as endangered by the International Union for Conservation of Nature (IUCN) Red List of Threatened Species in 2015 (Glatston et al., 2015). Nowadays, some measures such as *ex situ* and *in situ* conservations were taken to protect red pandas. In addition, the efficient preservation and utilization of stem cell resources is also an important way to protect endangered wildlife (Stanton et al., 2019). Assisted reproduction combined with embryonic stem cell technology also played a valuable role in the effort to protect endangered species (Hildebrandt et al., 2018; Saragusty et al., 2020).

Mesenchymal stem cells (MSCs), firstly identified in the bone marrow, are a population of pluripotent cells with high proliferative rate, low immunogenicity, self-renewal and multi-directional differentiation potential throughout the entire stage of life (Friedenstein et al., 1966; Li et al., 2015; Mason et al., 2014). In humans, MSCs have been identified in many tissues including umbilical cord blood and adipose tissues (Lv et al., 2014). As for wild animals, earlier studies by our group have identified the giant panda bone marrow MSCs (Liu et al., 2013) and umbilical cord MSCs (Liu et al., 2021), as well as the red panda bone marrow MSCs for the first time (An et al., 2020).

In addition to the above-mentioned tissues, endometrium contains a small population of cells with typical MSC properties (Gargett et al., 2009). EndometrialMSCs (eMSCs) expressed cell surface markers, including CD29, CD44, CD73, CD90 and CD105 (Du et al., 2016; Gargett & Masuda, 2010; Murphy et al., 2008), had the ability to differentiate into adipocytes, osteoblasts, chondrocytes and smooth muscle cells *in vitro* (Rink et al., 2017). Moreover, eMSCs could generate endometrial stroma in xenograft assays (Masuda et al., 2010). eMSCs have been harvested and characterized from humans (Cheng et al., 2017; Queckborner et al., 2020), pigs (Miernik & Karasinski, 2012), heifers (Calle et al., 2019; de Moraes et al., 2016), horses (Cabezas et al., 2018), dogs (De Cesaris et al., 2017; Sahoo et al., 2017) and sheep (Ghobadi et al., 2018). This type of cells showed similar properties to bone marrowMSCs and may provide an available source for cell-based therapies due to their strong regenerative capacity (Gargett et al., 2009). The therapeutic potential of eMSCs has already been demonstrated in relation to premature ovarian failure (Lai et al., 2015), Parkinson’s disease (Wolff et al., 2011), pelvic organ prolapse (Emmerson & Gargett, 2016) and angiogenesis (Pence et al., 2013).

Recently, *in vitro* gametogenesis was proposed as the ultimate solution for infertility caused by loss or compromised function of gametes (Makar & Sasaki, 2020). Reconstitution of primordial germ cell (PGC) specification from pluripotent cells is an essential first step for *in vitro* gametogenesis. Previous study reported that PGC-like cells were successfully derived from canine adipose mesenchymal stem cells (Wei et al., 2016). Human amniotic membrane MSCs could be induced to express PGC gene markers and have enough potential to PGC specification (Alifi & Asgari, 2020). During uterine organogenesis, cell communications were closer and polypotential germ cells differentiated and grew into myometrium and endometrial layers (Makar, 2017). Moreover, endometrium is the site for embryo implantation and accompanies all stages of post-implantation embryo development and has a direct intercellular communication with the embryo (Massimiani et al., 2019). Early studies in bovine have found that eMSCs ensured the maternal immunomodulation required for embryo survival (Calle et al., 2019). Therefore, the eMSCs would have the potential to PGC specification, *in vitro* gametogenesis and embryo implantation regulation.

For endangered wild animals, cell germ plasm resources are extremely precious, which is one of the key factors to protect the genetic diversity of species. In addition, the research on the reproductive mechanism of wild animals is not well explained and the potential regulation should be further studied.

Therefore, the aim of this study was to isolate and characterize MSCs from red panda endometrium. The new type of cells will be beneficial for germ plasm resources conservation of red panda, as well as for basic or pre-clinical studies in the future.

Material and methods

Isolation of eMSCs from red pandas

The samples used in this study were obtained postmortem from six red pandas (Supplementary Table S1), which were raised in the Chengdu Research Base of Giant Panda Breeding. All eMSCs isolation performance of the six red pandas were performed as follows. Briefly, the harvested uterus samples were washed five times in phosphate-buffered saline (PBS, Gibco) with 5% antibiotic–antimycotic solution (Gibco). Uteruses were cut into 1 × 1 × 1 mm pieces without fatty tissues under sterile condition, and then washed twice in PBS. The uteruses were mechanically minced and dissociated into single cells with collagenase type IV (1 mg/ml; Gibco) for 15 min at 37°C, then centrifuged at 600 g for 3 min. Discarding the supernatant, the tissue was further digested with 0.25% trypsin (Gibco) for 15 min at 37°C. Cell suspensions were filtered through a 40-µm sieve (BD Falcon). The filtrates were centrifuged at 600 g for 5 min at room temperature. The cell pellets were resuspended and cultured...
in culture dishes (diameter, 10 cm; Corning) containing low-glucose Dulbecco’s modified Eagle’s medium (LG-DMEM, Gibco) supplemented with 10% foetal bovine serum (Gibco), 10 ng/ml basic fibroblast growth factor (Peprotech) and 1× antibiotic–antimycotic. The cells were incubated at 37°C with 5% CO₂, and the medium was changed every 2 days. At ~10 days, the primary eMSCs were passaged with 1× TrypLE Express (Gibco) when the cells reached ~80% confluence.

**Cellular proliferation assay**

Red panda eMSCs at passages 4–7 were seeded in 24-well plates at a density of 1 × 10⁴ cells per well to establish the growth curves. The numbers of cells per three wells were counted every day for eight successive days. Cells were counted by automatic cell counter (Countstar) after acridine orange (AO)/propidium Iodide (PI) staining (Countstar).

**Cell surface antigen analysis**

Red panda eMSCs at passage 4 were digested with TrypLE Express (Gibco), then washed twice and incubated in buffer with the relevant antibody or with the corresponding isotype control IgG for 40 min. Then cells were washed three times and analysed by using flow cytometry (NovoCyte, ACEA). Compensation and data analysis were performed using FlowJo software (Tree Star, Inc., Ashland, OR, USA). Corresponding antibodies used for flow cytometry analysis were listed in Table 1.

**Karyotype analysis**

For karyotype analysis, red panda eMSCs at passage 8 were exposed to 10 μg/ml colcemid (Beyotime) for 4 h, then digested and resuspended in 0.075 M KCl (Sigma) at 37°C for 40 min. After that, eMSCs were fixed in acetic acid and methanol (1:3) (Sigma). The numbers of chromosomes were counted by an inverted fluorescence microscope (BX53, Olympus) with an oil immersion objective. Chromosome images were analysed by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

**RT-PCR**

Red panda eMSCs at passage 4 were used for reverse transcription-polymerase chain reaction (RT-PCR) analysis.

Total RNA of cultured cells was extracted with the RNAprep Pure Cell Kit (TIANGEN) in accordance with the manufacturer’s instruction. Then, the samples were treated with DNase to remove possible contamination by genomic DNA and reverse transcribed into cDNA using PrimeScript RT reagent Kit (Takara). The specific primer sequences were listed in Table 2, and β-actin was used as reference gene.

**RNA-seq and differentially expressed genes enrichment analysis**

Red panda eMSCs isolated from three different individuals and corresponding red panda skin fibroblast cells (skin FCs) at passage 4 were seeded in culture dishes (diameter, 10 cm) and treated with normal growth medium. When the cells reached ~80% confluence, the cells were collected and treated with Trizol (Thermo Fisher) as manufacturer’s protocol to extract total RNA and then for RNA-seq (Novogene). The RNA-seq data were assembled and analysed as no reference genome sequences. Differential expression analysis was performed with DESeq2. For functional enrichment analysis, DEGs were mapped to terms in the GO database, and then searched for significantly enriched GO terms (P < 0.05). DEGs were mapped to the KEGG database, and searched for significantly enriched KEGG pathways (P < 0.05).

**Western blotting**

Red panda skin FCs and eMSCs at passage 4 were washed twice with PBS and resuspended in EBC 250 lysis buffer (Beyotime). All other western blotting procedures were conducted as previously reported (An et al., 2020). Briefly, protein concentration was determined by using the protein assay reagent (Bio-Rad). Then, proteins were separated on 4–5% precast gels (Bio-Rad) and electrotransferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked in 5% skim milk diluted in Tris-buffered saline containing 0.1% Tween 20 for 1 h. After blocking, the membranes were incubated overnight at 4°C with rabbit anti-SOX2 (dilution 1:1000) (ab97959, Abcam), rabbit anti-GAPDH (dilution 1:1000) (ab9485, Abcam) and rabbit anti-ALB (dilution 1:1000) (ab207327, Abcam) as primary antibodies. After washing, membranes were incubated with an anti-rabbit HRP-conjugated secondary antibody (dilution 1:5000) (111-035-003, Jackson ImmunoResearch).
Table 2: Primers used in RT-PCR

| Gene | Primer nucleotide sequence (5’ to 3’) | Product size (bp) | Annealing |
|------|--------------------------------------|-------------------|-----------|
| Klf4 | F:GTGTCGGGCTGATGTCCCTGTTG           | 153               | 60°C      |
|      | R:CCCAATCAAGCAAGAGGCA                |                   |           |
| Thy1 | F:GCCACGGAACCTCACCACC               | 130               | 60°C      |
|      | R:CTGCGTGAAGTGGATCTTGGA              |                   |           |
| Sox2 | F:AACCAGGGCAATGGACAGCTA              | 226               | 58°C      |
|      | R:CCGGATGAGCAATGGTGCTTGAGG           |                   |           |
| CD44 | F:CAATGIGCAATGGAGCGGTA               | 151               | 60°C      |
|      | R:CTTGACGGTGATCCGTCCTTC              |                   |           |
| CK18 | F:GCAGATGAGGAGAGACACACAG             | 337               | 60°C      |
|      | R:TCGCCAGGCCAGGGGTTAGGG              |                   |           |
| ALB  | F:ACGAGCCGAGCTGCTGACTG               | 363               | 60°C      |
|      | R:CTGAGGCTGGCACAAGTTC                |                   |           |
| DKK1 | F:ACAGACCGAGGTTGATGGATATTC           | 310               | 60°C      |
|      | R:TCCTGACGGTGGAGGTCTGGAGA            |                   |           |
| β-actin | F:ACGATATCTGCTGGCCCTTGTA            | 220               | 60°C      |
|      | R:ACAAATCCGTTCGTGGATGG               |                   |           |

for subsequent detection by ECL (Millipore). Band intensities were calculated using densitometry in Quantity One software (Bio-Rad).

**Multilineage differentiation**

For adipocytic differentiation, eMSCs at passage 4 were seeded in 6-well plates and treated with adipogenic medium (Cyagen) as per the manufacturer’s protocol. The medium was changed three times per week. After 8 days, adipogenesis was evaluated by Oil red O staining (Sigma). Staining was assessed by bright-field inverted microscopy (IX73, Olympus). Red panda eMSCs cultured in normal growth medium served as control.

For chondrogenic differentiation, eMSCs at passage 4 were seeded in 6-well plates and incubated with chondrogenic differentiation medium (Cyagen) as per the manufacturer’s protocol. The medium was changed three times per week. After 21 days, chondrogenesis was detected by the staining of toluidine blue. Red panda eMSCs cultured in normal growth medium served as control.

For hepatogenic differentiation, eMSCs at passage 4 were seeded in 6-well plates and treated with hepatogenic differentiation medium (Cyagen) as per the manufacturer’s protocol. The medium was changed three times per week. After 16 days, hepatogenic differentiation was evaluated by cytokeratin 18 (CK18) (ab181597, Abcam) immunofluorescence staining, detection of CK 18, ALB and DKK1 mRNA expression, ALB protein expression, Periodic Acid-Schiff (PAS) staining and Indocyanine Green (ICG) uptake. Red panda eMSCs cultured in normal growth medium served as control.

**PAS staining**

Red panda eMSCs after hepatogenic differentiation were stained by PAS staining kit (Solarbio). Following the manufacturer’s instructions, hepatogenic differentiated cells were washed twice with PBS, then fixed with 4% paraformaldehyde (Sigma) for 15 min. Cells were incubated in 1% periodic acid for 20 min and then washed four times with distilled water. After incubating with Schiff reagent for 20 min, the cells were counterstained with Mayer haematoxylin solution for 2 min. Then, the stained cells were rinsed in distilled water and photographed under an inverted microscope (IX73, Olympus).

**ICG uptake**

Red panda eMSCs after hepatogenic differentiation were determined through detecting the cellular uptake of ICG. Briefly, hepatogenic differentiated cells were washed twice with PBS, then incubated with LG-DMEM supplemented with 1 mg/ml of ICG (Sigma), and incubated at 37°C with 5% CO₂ for 1 h. Subsequently, the cells were washed three times with PBS, and then photographed under an inverted microscope (IX73, Olympus).
Conservation Physiology • Volume 10 2022

Figure 1: Morphological characteristics, growth curves and chromosome spreads of red panda eMSCs. (A) Representative image of primary red panda eMSCs after 9 days culture. The primary cells were fibroblast like and epithelial like, presenting triangular, fusiform, ovoid or polygonal shapes. Scale bar, 200 μm. (B) Representative image of red panda eMSCs at passage 4. Fibroblast-like shape and presented polygonal or long spindle shape. Scale bar, 100 μm. (C) Representative image of red panda eMSCs at passage 7. Cells still maintain fibroblast-like and long spindle shapes. Scale bar, 100 μm. (D) The growth curves of the cells from passages 4–7. Quantified data show the mean ± SEM. (E) Chromosome spreads of red panda eMSCs at passage 8. Scale bar, 10 μm. (F) Analysis of the chromosome spreads of (E). The correct number of chromosomes in red panda is 2n = 36.

Statistical analysis

The results of growth curves were analysed by using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). Data were expressed as the mean ± SEM. All results were generated from at least three independent experiments.

Results

Isolation and culture of red panda eMSCs

After 9 days culture, the primary cells were fibroblast like and epithelial like, presenting triangular, fusiform, ovoid or polygonal shapes, and the overall percentage of eMSCs was ~50% (Fig. 1A). Red panda eMSCs at passage 4 were fibroblast like and presented polygonal or long spindle shapes (Fig. 1B). After seven generations, eMSCs also have the ability to maintain their morphological characteristics (Fig. 1C). The growth curves of red panda eMSCs from passage 4 to 7 were established. Results showed that eMSCs entered the exponential phase after 4 days culture and then reached the stationary phase after 7 days culture (Fig. 1D). After culturing eight passages, the chromosome number of red panda eMSCs are still normal (2n = 36) (Fig. 1E and F).

Surface antigens and transcription factors in red panda eMSCs

The expressions of pluripotency and MSC marker genes, including Thy1, Klf4, Sox2 and CD44, were confirmed by RT-PCR. Genes Thy1, Klf4, Sox2 and CD44 were all highly expressed in red panda eMSCs (Fig. 2A). Additionally, SOX2 protein was also detected in eMSCs, but not in skin FCs (Fig. 2B). According to the flow cytometry analysis, the red panda eMSCs were positive for MSCs phenotype CD44, CD49f and CD105 and negative for endothelial cell marker CD31 and haematopoietic cell marker CD34 (Fig. 2C).
**Figure 2:** Characterization of red panda eMSCs. (A) RT-PCR analysis of pluripotency genes in eMSCs. β-actin was used as a control for RNA sample quality. (B) The expression of SOX2 was detected in red panda eMSCs and skin FCs by western blot. GAPDH was used as the loading control. (C) Red panda eMSCs were stained with phycoerythrin (PE)-conjugated CD44, CD49f, CD105, CD34 or PE-Cyanine7 conjugated CD31 antibodies. Blue areas, signal from isotype controls; red areas, signal from the specific cell surface marker; grey areas, unstained black control.

### Differential gene expression analysis between red panda eMSCs and skin FCs

A total of 79,311 and 55,016 predicted expressed genes were identified in eMSCs and skin FCs, respectively. Of these, 47,981 genes were common expressed in eMSCs and skin FCs (Fig. 3A). Compared with skin FCs, 5,034 genes upregulated in eMSCs and 4,070 genes downregulated (Fig. 3B), and DEGs could significantly separate the samples into the eMSCs group and skin FCs (Fig. 3C). DEGs were analysed by GO annotation, and the significantly enriched GO terms mainly contained G-protein coupled receptor signalling pathway, carbohydrate derivative binding, nucleoside binding, purine nucleoside binding, ribonucleoside binding, motor activity, etc. (Fig. 3D). The signal pathways of DEGs were analysed by KEGG pathway analysis. As shown in Fig. 3E, the top 20 mainly enriched KEGG pathways were ribosome biogenesis, cell cycle, DNA replication, Ras signalling pathway, purine metabolism, etc. In addition, some representative genes about promoting MSCs differentiation and proliferation were upregulated and promoting fibroblasts proliferation were downregulated in eMSCs group when compared skin FCs. Also, all these genes significantly separated the samples into the eMSCs group and skin FCs (Fig. 3F; Table 3).

### Differentiation of red panda eMSCs

For adipocytic differentiation, cells were cultured in adipocytic induction medium. In the control group, no positive staining signal of Oil-red O was detected (Fig. 4A). In the induced group, many lipid droplet accumulations were detected by the staining of Oil-red O (Fig. 4B).

For chondrogenic differentiation, cells were cultured in chondrogenesis differentiation medium to form chondrogenic pellets. The toluidine blue staining was used to evaluate the
sulfated proteoglycans of the cartilage matrices. The results showed positive staining signals (Fig. 4C).

To determine whether red panda eMSCs have the ability to endoderm differentiation, cells were cultured in hepatogenic differentiation medium. As shown in Fig. 5A, the cells after differentiating became flat, presenting polygonal morphology when compared with the control group. Immunofluorescence staining revealed strong positive signals of the hepatogenic marker CK18 in the hepatogenic-induced group, but no positive staining was detected in the control group (Fig. 5B). The results of PAS staining showed that extensive cytoplasmic positive staining signalling (red to purple) were only observed in the hepatogenic-induced group (Fig. 5C). The hepatogenic differentiated group successfully showed an indocyanine green uptake staining and the control group could not uptake ICG (Fig. 5D). In addition, the results of RT-PCR further showed that the hepatic and liver progenitor marker genes CK18, ALB and DKK1 were highly expressed in the hepatogenic-induced group when compared with the control.
Table 3: Representation of 10 upregulated genes and 10 downregulated genes in red panda eMSCs compared with skin FCs

| Gene name | Expression | log₂FC | q value | Gene description |
|-----------|------------|--------|---------|------------------|
| PDGFRA    | Upregulated| 3.013  | 0.000   | Directs eMSCs differentiation towards chondrocyte progenitor fate (Bartoletti et al., 2020) |
| TBX3      | Upregulated| 1.298  | 0.000   | Plays an important role in osteogenic differentiation and proliferation of human mesenchymal stem cells derived from adipose tissue (Lee et al., 2007) |
| CXCR7     | Upregulated| 3.584  | 0.000   | Critical regular for MSC-mediated vasculogenesis (Wei et al., 2020) |
| EDNRB     | Upregulated| 5.457  | 0.000   | Direct bone marrow-derived hMSCs for osteo- and chondro-lineage differentiation (Lee et al., 2019) |
| MAPK1     | Upregulated| 1.023  | 0.000   | Promote osteogenesis and angiogenesis (Bian et al., 2019) |
| GDNFRA    | Upregulated| 2.600  | 0.000   | Improve the efficiency of MSC in the recovery of kidney (Esmaeilizadeh et al., 2020) |
| SFRP1     | Upregulated| 6.128  | 0.000   | Regulate the proliferation and differentiation of bone mesenchymal stem cells (Tang et al., 2019) |
| FN1       | Upregulated| 1.602  | 0.000   | Regulate myofibroblast differentiation of endometrial MSCs (Zhang et al., 2019) |
| LTBP2     | Upregulated| 6.092  | 0.000   | Expressed primarily in cell types of mesenchymal origin, particularly osteoblasts and chondrocytes (Davis et al., 2014) |
| HAND2     | Upregulated| 4.547  | 0.000   | Establishing distinct mesenchymal compartments (Osterwalder et al., 2014) |
| SERPINE1  | Downregulated| −6.749 | 0.000   | Contribute to epithelial dedifferentiation, G2/M proliferative arrest, fibrogenesis (Gifford et al., 2021) |
| PDGFC     | Downregulated| −1.678 | 0.000   | Induce progressive fibrosis (Wright et al., 2014) |
| FBLN1     | Downregulated| −4.985 | 0.000   | Associate with lung fibrosis in both humans and mice and stabilizes collagen formation (Liu et al., 2019) |
| THBS25    | Downregulated| −1.455 | 0.000   | A key regulator of fibrosis (Reinecke et al., 2013) |
| CTGF      | Downregulated| −3.648 | 0.000   | Associate with atrial fibrosis (Chen et al., 2019) |
| JAGGED    | Downregulated| −2.487 | 0.000   | Play a key role in tissue fibrosis (Zhao et al., 2018) |
| PTPN11    | Downregulated| −2.242 | 0.000   | Associate with fibrosis promotion during the early stages of HCC development (Kang et al., 2017) |
| ITGB1     | Downregulated| −1.811 | 0.000   | Associate with proliferation and migration of fibroblasts (Baszanska et al., 2021) |
| VCAN      | Downregulated| −1.766 | 0.000   | Affect phenotype of cultured human dermal fibroblasts (Merrilees et al., 2016) |
| FBLN5     | Downregulated| −4.630 | 0.000   | Affect adhesion and proliferation of human fibroblast-like cells (Fu et al., 2016) |

Discussion

In the present study, we successfully isolated eMSCs in red panda endometrium. The primary cells were fibroblast like and epithelial like, presenting triangular, fusiform, ovoid or polygonal shapes, because primary cells were a mixed-cell group, containing epithelial cells, basal layer cells and eMSCs. After passage cultivations, fibroblast-like eMSCs gradually became the major cells, suggesting that the currently used culture method would greatly benefit eMSCs growth and reproduction. During proliferation in eight successive days, eMSCs at passage 4 experienced a short lag phase from Days 1 to 4 and subsequently a logarithmic rise from Day 4, then reached the stationary phase at Day 7 (Fig. 1). However, eMSCs at passages 5–7 did not reach the plateau phase at Day 8. It may be that the cells did not reach the maximum growth density. The eMSCs at passage 7 still had a strong proliferative potential, suggesting eMSCs had strong proliferation stability. These results were similar to the growth curves of mesenchymal stem cells isolated from red panda bone marrow (An et al., 2020). Red panda eMSCs at passage 8 showed normal
diploid karyotype ($2n = 36$), suggesting that this type of cell had a stable and normal growth and reproduction, as well as further confirmed that the currently used culture method was appropriate.

To characterize the red panda eMSCs isolated in present study, we examined some MSCs biomarkers, such as CD44, CD49f, CD90 and CD105. The red panda eMSCs highly expressed CD44, CD49f, CD105, but not expressed CD31 and CD34 when compared with the isotype control. The results of RT-PCR also confirmed CD90 (Thy1) was highly expressed in red panda eMSCs. These results suggested that the red panda eMSCs were neither endothelial cell nor haematopoeitic cell and was in accord with the mesenchymal cells standards of the International Society for Cell Therapy (Dominici et al., 2006). Pluripotency genes Sox2 and Klf4 also expressed in red panda eMSCs. However, other markers THY1 and OCT4 were not detected with western blot analysis, this may be because there are no specific antibodies available for red panda or the expression of these markers in red panda eMSCs is too low to be detected. In the future studies, more specific antibodies to recognize red panda should be selected and further supplement the current study.

In order to analyse the red panda eMSCs characters in gene expression profile, RNA-seq analysis between eMSCs and skin FCs was performed. To date, the complete gene expression profile of red panda has not yet been established, thus the present study adopted a no reference transcriptome analysis, which might lead to an incomplete functional genes annotation. We used Trinity software (Grabherr et al., 2011) to identify 86 346 unigenes (genes), and the number of genes was different from referenced mammalian animals. We identified 9104 genes that were differentially expressed. Moreover, the top 20 enrichment pathways of DEGs in GO and the KEGG mainly associated with G-protein coupled receptor signalling pathway, carbohydrate derivative binding, nucleoside binding, ribosome biogenesis, cell cycle, DNA replication, Ras signalling pathway, purine metabolism and cell cycle. These results suggested that eMSCs had a high frequency of cellular activity and proliferative capacity. Among the DEGs, some genes about promoting MSCs differentiation and proliferation were upregulated and promoting fibroblasts proliferation were downregulated in eMSCs group (Table 3), which further confirmed the eMSCs pluripotency.

MSCs have the ability to multiple differentiation, and earlier studies had reported that MSCs could differentiate into adipocytes, osteoblasts, chondrocytes, neural cells and smooth muscle cells (Liechty et al., 2000; Wu et al., 2007). In the present study, we confirmed the red panda eMSCs could be differentiated into adipocytes and chondrocytes, from which were mesoderms. Additionally, the red panda eMSCs also had the ability to differentiate into hepatocytes, from which were endoderms. This result revealed that the red

Figure 4: Adipocytic and chondrogenic differentiation of red panda eMSCs. (A) Red panda eMSCs were cultured in the adipocytic induction medium for 8 days, and then evaluated by Oil-red O staining to reveal lipid vacuoles. Untreated cells were used as control. Scale bar, 50 μm. (B) Red panda eMSCs were cultured in the chondrogenic differentiation medium for 21 days, and then evaluated by toluidine blue staining to reveal the sulfated proteoglycans of the cartilage matrices. Scale bar, 50 μm.
panda eMSCs have the capacity for differentiation potential across embryonic lineage boundaries. Interestingly, previous research had shown that MSCs from bone marrow could differentiate into hepatocytes in vivo (Schwartz et al., 2002). Moreover, some studies revealed that eMSCs are likely to be derived from bone marrow (Cervello et al., 2012; Cervello et al., 2015), but the real origin of red panda eMSCs needs further studies.

The red panda eMSCs with high proliferation in vitro, stable karyotype and multipotential differentiation have more potential applications in wildlife protection and future research and/or clinical practice. Firstly, somatic cell nuclear transfer (SCNT) or interspecies SCNT (iSCNT) may be a potential tool for aiding the conservation of endangered animal species, although accompanied with low efficiency and mitochondrial heterogeneity, which could compromise the energy-making process of embryo, leading to its death (Loi et al., 2011). To date, iSCNT has been successfully performed in many endangered wild animals, such as African wildcat (Felis lybica) (Gomez et al., 2004), Bactrian camel (Camelus bactrianus) (Wani et al., 2017) and even the extinct species Pyrenean ibex (Capra pyrenaica pyrenaica) (Folch et al., 2009). A previous study tried to construct interspecies cloned embryos by using red panda fibroblasts and rabbit enucleated oocytes (Tao et al., 2009). It is well known that fibroblasts were common donor cells used in SCNT, but MSCs were also suitable. In 2018, horse MSCs from bone marrow were used as nuclear donor cells and produced healthier cloned horses compared with fibroblasts (Olivera et al., 2018). Red panda eMSCs derived from endometrium, which directly interacted with early embryos, may be more suitable and further increase the SCNT embryo development. Secondly, to date, embryonic

Figure 5: Hepatogenic differentiation of red panda eMSCs. (A) Bright field images of red panda eMSCs cultured in hepatogenic differentiation medium for 16 days, and eMSCs were changed to flat polygonal morphology. Untreated cells were used as control. Scale bar, 200 μm. (B) Red panda eMSCs after hepatogenic differentiation were stained with anti-CK18 (hepatogenic marker, green), and the nucleus were stained with DAPI (blue). Untreated cells were used as control. Scale bar, 50 μm. (C) Red panda eMSCs after hepatogenic differentiation were stained with PAS staining (purple signal). Untreated cells were used as control. Scale bar, 100 μm. (D) Red panda eMSCs after hepatogenic differentiation were detected by ICG uptake (green signal). Untreated cells were used as control. Scale bar, 100 μm. (E) The expressions of liver-specific genes in hepatogenic-induced (H) and control (C) red panda eMSCs were detected by using RT-PCR. β-actin was used as a reference gene. (F) The expressionsof ALB in hepatogenic-induced (H) and control (C) red panda eMSCs were detected by using western blot. GAPDH was used as the loading control.
diapause occurs in over 130 species of mammals (Deng et al., 2018) including red panda (Macdonald et al., 2010; Miles et al., 1979), but the potential mechanism is still unclear. Embryo implantation is a key step in the establishment of pregnancy, and the eMSCs is a great cell model for studying embryonic diapause. On one hand, endometrium of mammal secretes cytokines and growth factors that influence the development of early embryo (Cha et al., 2012). It is likely that some of growth factors control the arrested growth that occurs in diapause (Renfree & Fenelon, 2017). It is also clear that MSCs secrete a variety of cytokines and growth factors, such as insulin-like growth factor-1, vascular endothelial growth factor, epidermal growth factor, fibroblast growth factor, interleukin-6, leukaemia inhibitory factor and transforming growth factor-β (Feng et al., 2009). Therefore, it will be a new angle to study embryonic diapause from the eMSCs secretion. On the other hand, during embryo implantation, there is intercellular communication between the embryo and maternal eMSCs and peripheral blood MSCs (pbMSCs), which could chemotax to embryonic trophectoderm secretome (Calle et al., 2021a, Calle et al., 2021b). In bovine embryo implantation, the migratory capacity of eMSCs was increased towards an inflammatory niche and then reduced by the expression of implantation cytokine by the embryo, which are necessary for immunorepression to prevent embryo rejection by the maternal organism (Calle et al., 2019). Therefore, deeply to study the communication between eMSCs and embryo will be beneficial for clarifying the regulation mechanism of embryo implantation. Finally, early embryo mainly develops in the maternal oviduct and uterus, which contains a large number of factors that can promote early embryo development (Kolle et al., 2020). Recent studies found that coculture of mouse embryos and mesenchymal stem/stromal cells derived from menstrual blood enriched the embryonic microenvironment and promoted embryo development (Goncalves et al., 2020). Coculture with extracellular vesicles from endometrial-derived MSCs could increase the quality of aged mouse embryos and presumably by modulating the expression of antioxidant enzymes and promoting pluripotent activity (Marinaro et al., 2019). Therefore, paracrine regulation of eMSCs may be a feasible way to promote in vitro embryo development, which will benefit endangered wild animals like the red panda.

**Conclusion**

In this study, we, for the first time, isolated and characterized the red panda eMSCs in endometrium. Red panda eMSCs were fibroblast-like and highly expressed the pluripotency genes including Thy1, Klf4 and Sox2. Additionally, red panda eMSCs were positive for MSC markers CD44, CD49f and CD105 and negative for endothelial cell marker CD31 and haematopoietic cell marker CD34. The red panda eMSCs also had the pluripotent differentiation capacities of adipocytes, chondrocytes and hepatocytes. Using RNA-seq, significant DEGs were identified, which further demonstrated the eMSC gene expression characters. The new multipotent stem cell could not only benefit the germ plasm resources conservation of red panda, but also basic or pre-clinical studies in the future.

**Funding**

This work was supported by the Sichuan Science and Technology Program (2020JDQ0074); the Chengdu Research Base of Giant Panda Breeding (2020CPB-B07); and the Chengdu Giant Panda Breeding Research Foundation (CPF2017–16).

**Supplementary material**

Supplementary material is available at Conservation Physiology online.

**Conflict of interest**

The authors declare no conflict of interest.

**Authorship contribution statement**

Dong-Hui Wang: conceptualization, methodology, writing (original draft), review and editing. Xue-Mei Wu: visualization, investigation and writing (original draft). Jia-Song Chen, Yuan Li and Fei-Ping Li: investigation. Zhi-Gang Cai, Jun-Hui An and Ming-Yue Zhang: formal analysis. Rong Hou: project administration. Yu-Liang Liu: conceptualization, methodology, review and editing.

**Data availability**

The data underlying this article are available in the article and in its online supplementary material.

**References**

Alifi F, Asgari HR (2020) Alteration in expression of primordial germ cell (PGC) markers during induction of human amniotic mesenchymal stem cells (hAMSCs). J Reprod Infertil 21: 59–64.

An JH, Li FP, He P, Chen JS, Cai ZG, Liu SR, Yue CJ, Liu YL, Hou R (2020) Characteristics of mesenchymal stem cells isolated from the bone marrow of red panda. Zoology (Jena) 140: 125775.

Bartoletti G, Dong C, Umar M, He F (2020) Pdgfra regulates multipotent cell differentiation towards chondrocytes via inhibiting Wnt9a/beta-catenin pathway during chondrocranial cartilage development. Dev Biol 466: 36–46.

Baszansowka W, Misiura M, Osciolskowa I, Palka J, Miltyk W (2021) Extracellular proline (PEPD) induces anabolic processes through EGFR,
Research article

Conservation Physiology • Volume 10 2022

beta1-integrin, and IGF-1R signaling pathways in an experimental model of wounded fibroblasts. *Int J Mol Sci* 22: 942.

Biyan Y, Du Y, Wang R, Chen N, Du X, Wang Y, Yuan H (2019) A comparative study of HAMSCs/HBMSCs transwell and mixed coculture systems. *JUBMB Life* 71: 1048–1055.

Cabezas J, Rojas D, Navarrete F, Ortiz R, Rivera G, Saravia F, Rodriguez-Alvarez L, Castro FO (2018) Equine mesenchymal stem cells derived from endometrial or adipose tissue share significant biological properties, but have distinctive pattern of surface markers and migration. *Theriogenology* 106: 93–102.

Calle A, Gutiérrez-Reinoso MA, Re M, Blanco J, De la Fuente J, Monguí- Tortajada M, Borràs FE, Yáñez-Mó M, Ramírez MA (2021) Bovine peripheral blood MSCs chemotax towards inflammation and embryo implantation stimuli. *J Cell Physiol* 236: 1054–1067.

Calle A, López-Martín S, Monguí-Tortajada M, Borràs FE, Yáñez-Mó M, Ramírez MA (2019) Bovine endometrial MSC: mesenchymal to epithelial transition during luteolysis and tropism to implantation niche for immunomodulation. *Cell Stem Res Ther* 10: 23.

Calle A, Toribio V, Yáñez-Mó M, Ramírez MA (2021) Embryonic trophoectoderm secretomics reveals chemotactic migration and intercellular communication of endometrial and circulating MSCs in embryonic implantation. *Int J Mol Sci* 22: 5638.

Cervello I, Gil-Sanchis C, Mas A, Faus A, Sanz J, Moscardo F, Higuerares G, Sanz MA, Pellicer A, Simon C (2012) Bone marrow-derived cells from male donors do not contribute to the endometrial side population of the recipient. *Plos One* 7: e030260.

Cervello I, Gil-Sanchis C, Santamaria X, Cabanillas S, Diaz A, Faus A, Pellicer A, Simon C (2015) Human CD133(+) bone marrow-derived stem cells promote endometrial proliferation in a murine model of Asherman syndrome. *Fertil Steril* 104: 1552–1560.e3.

Cha J, Sun X, Dey SK (2012) Mechanisms of implantation: strategies for successful pregnancy. *Nat Med* 18: 1754–1767.

Chen JQ, Guo YS, Chen Q, Cheng XL, Xiang GJ, Chen MY, Wu HL, Huang QL, Zhu PL, Zhang JC (2019) TGFbeta1 and HGF regulate CTGF expression in human uterine fibroblasts and are involved in uterine remodelling in patients with rheumatic heart disease. *J Cell Mol Med* 23: 3032–3039.

Cheng Y, Li L, Wang D, Guo Q, He Y, Liang T, Sun L, Wang X, Cheng Y, Zhang G (2017) Characteristics of human endometrium-derived mesenchymal stem cells and their tropism to endometriosis. *Stem Cells Int* 2017: 1.

Davis MR, Andersson R, Severin J, de Hoon M, Bertin N, Baillie JK, Kawaji H, Sandelin A, Forrest AR, Summers KM et al. (2014) Transcriptional profiling of the human fibrillin/LTBP gene family: key regulators of mesenchymal cell functions. *Mol Genet Metab* 112: 73–83.

De Cesaris V, Grolli S, Bresciani C, Conti V, Basini G, Parmigiani E, Biagiardi E (2017) Isolation, proliferation and characterization of endometrial canine stem cells. *Reprod Domest Anim* 52: 235–242.

Deng L, Li C, Chen L, Liu Y, Hou R, Zhou X (2018) Research advances on embryonic diapause in mammals. *Anim Reprod Sci* 198: 1–10.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prokop D, Horwitz E (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. *Cytotherapy* 8: 315–317.

Du X, Yuan Q, Qu Y, Zhou Y, Bei J (2016) Endometrial mesenchymal stem cells isolated from menstrual blood by adherence. *Stem Cells Int* 2016: 1.

Emmerson SJ, Gargett CE (2016) Endometrial mesenchymal stem cells as a cell based therapy for pelvic organ prolapse. *World J Stem Cells* 8: 202–215.

Esmaeilzadeh Z, Mohammad B, Rajabibazl M, Ghaderian SMH, Omran MD, Fazeli Z (2020) Expression analysis of GDNF/RET signaling pathway in human AD-MSCs grown in HEK 293 conditioned medium (HEK293-CM). *Cell Biochem Biophys* 78: 531–539.

Feng DQ, Zhou Y, Ling B, Gao T, Shi YY, Wei HM, Tian ZG (2009) Effects of the conditioned medium of mesenchymal stem cells on mouse oocyte activation and development. *Braz J Med Biol Res* 42: 506–514.

Folch J, Cocero MJ, Chesne P, Alabart JL, Dominguez V, Cognie Y, Roche A, Fernandez-Arias A, Marti J, Sanchez P et al. (2009) First birth of an animal from an extinct subspecies (*Capra pyrenaica pyrenaica*) by cloning. *Theriogenology* 71: 1026–1034.

Friedenstein AJ, Piatetsky S II, Petratkovka KV (1966) Osteogenesis in transplants of bone marrow cells. *J Embryol Exp Morphol* 16: 381–390.

Furie N, Stheyberg D, Elkhaitib R, Perry L, Ullmann Y, Feferman Y, Preis M, Flugelman MY, Tzchori I (2016) Fibulin-5 regulates keloid-derived fibroblast-like cells through integrin beta-1. *Int J Cosmet Sci* 38: 35–40.

Gargett CE, Masuda H (2010) Adult stem cells in the endometrium. *Mol Hum Reprod* 16: 818–834.

Gargett CE, Schwab KE, Zillwood RM, Nguyen HP, Wu D (2009) Isolation and culture of epithelial progenitors and mesenchymal stem cells from human endometrium. *Biol Reprod* 80: 1136–1145.

Ghobadi F, Rahmanifar F, Mehrabani D, Tamadon A, Dianatpour M, Zare S, Razeghaz Jahromi I (2018) Endometrial mesenchymal stem stromal cells in mature and immature sheep: an in vitro study. *Int J Reprod Biomed* 16: 83–92.

Gifford CC, Lian F, Tang J, Costello A, Goldschmeding R, Samarakoon R, Higgins PJ (2021) PAI-1 induction during kidney injury promotes fibrotic epithelial dysfunction via deregulation of klotho, p53, and TGF-beta1-receptor signaling. *FASEB J* 35: e21725.

Glennon J, Wei F, Zaw T, Sherpa A (2015) The IUCN red list of threatened species: *Ailurus fulgens*. https://www.iucnredlist.org/species/714/110023718.

Gomez MC, Pope CE, Giraldo A, Lyons LA, Harris RF, King AL, Cole A, Godke RA, Dresser BL (2004) Birth of African wildcat cloned kittens born from domestic cats. *Cloning Stem Cells* 6: 247–258.
Goncalves MF, Asensi KD, Nascimento ALL, de Barros JHO, Santos RA, Andrade CBV, Kasai-Brunswick TH, Frajlait M, Ortiga-Carvalho TM, Goldenberg R (2020) Human menstrual blood-derived mesenchymal cells improve mouse embryonic development. *Tissue Eng Part A* 26: 769–779.

Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q et al. (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat Biotechnol* 29: 644–652.

Hildebrandt TB, Hermes R, Colleoni S, Diecke S, Holtze S, Renfree MB, Stejskal J, Hayashi K, Drukker M, Loi P et al. (2018) Embryos and embryonic stem cells from the white rhinoceros. *Nat Commun* 9: 2589.

Hu Y, Thapa A, Fan H, Ma T, Wu Q, Ma S, Zhang D, Wang B, Li M, Yan L et al. (2020) Genomic evidence for two phylogenetic species and long-term population bottlenecks in red pandas. *Sci Adv* 6: eaax5751.

Kang HJ, Chung DH, Sung CO, Yoo SH, Yu E, Kim N, Lee SH, Song JY, Kim CJ, Choi J (2017) SHP2 is induced by the HbxB NF-kappaB pathway and contributes to fibrosis during human early hepatocellular carcinoma development. *Oncotarget* 8: 27263–27276.

Kolle S, Hughes B, Steele H (2020) Early embryo-maternal communication in the oviduct: a review. *Mol Reprod Dev* 87: 650–662.

Lai D, Wang F, Yao X, Zhang Q, Wu X, Xiang C (2015) Human endometrial mesenchymal stem cells restore ovarian function through improving the renewal of germinal stem cells in a mouse model of premature ovarian failure. *J Transl Med* 13: 155.

Lee HS, Cho HH, Kim HK, Bae YC, Baik HS, Jung JS (2007) Tbx3, a transcriptional factor, involves in proliferation and osteogenic differentiation of human adipose stromal cells. *Mol Cell Biochem* 296: 129–136.

Lee MS, Wang J, Yuan H, Jiao T, Tsai TL, Squire MW, Li WJ (2019) Endothelin-1 differentially directs lineage specification of adipose- and bone marrow-derived mesenchymal stem cells. *FASEB J* 33: 996–1007.

Li L, Wang D, Zhou J, Cheng Y, Liang T, Zhang G (2015) Characteristics of human amniotic fluid mesenchymal stem cells and their tropism to human ovarian cancer. *Plos One* 10: e0123350.

Li M, Wei F, Goossens B, Feng Z, Tamate HB, Bruford MW, Funk SM (2005) Mitochondrial phylogeography and subspecific variation in the red panda (*Ailurus fulgens*): implications for conservation. *Mol Phylogenet Evol* 36: 78–89.

Liechty KW, MacKenzie TC, Shaaban AF, Radu A, Moseley AM, Deans R, Marshak DR, Flake AW (2000) Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep. *Nat Med* 6: 1282–1286.

Liu G, Cooley MA, Jarnicki AG, Borghuis T, Nair PM, Tjin G, Hsu AC, Haw TJ, Fricker M, Harrison CL et al. (2019) Fibulin-1c regulates transforming growth factor-beta activation in pulmonary tissue fibrosis. *JCI Insight* 5: e124529.

Liu Y, Li F, Cai Z, Wang D, Hou R, Zhang H, Zhang M, Yie S, Wu K, Zeng C et al. (2021) Isolation and characterization of mesenchymal stem cells from umbilical cord of giant panda. *Tissue Cell* 71: 101518.

Liu Y, Liu Y, Yie S, Lan J, Pi J, Zhang Z, Huang H, Cai Z, Zhang M, Cai K et al. (2013) Characteristics of mesenchymal stem cells isolated from bone marrow of giant panda. *Stem Cells Dev* 22: 2394–2401.

Loi P, Modlinski JA, Ptak G (2011) Interspecies somatic cell nuclear transfer: a salvage tool seeking first aid. *Theriogenology* 76: 217–228.

Lv FJ, Tuan RS, Cheung KM, Leung VY (2014) Concise review: the surface markers and identity of human mesenchymal stem cells. *Stem Cells* 32: 1408–1419.

Macdonald EA, Northrop LE, Czekala NMZB (2005) Pregnancy detection from fecal progestin concentrations in the red panda (*Ailurus fulgens*). *Zoo Biol* 24: 419–429.

Makar K, Sasaki K (2020) Roadmap of germline development and in vitro gametogenesis from pluripotent stem cells. *Andrology* 8: 842–851.

Makiyan Z (2017) Endometriosis origin from primordial germ cells. *Organogenesis* 13: 95–102.

Marinaro F, Macias-Garcia B, Sanchez-Margallo FM, Blazquez R, Alvarez V, Matilla E, Hernandez N, Gomez-Serrano M, Jorge I, Vazquez J et al. (2019) Extracellular vesicles derived from endometrial human mesenchymal stem cells enhance embryo yield and quality in an aged murine model. *Dagger*. *Biol Reprod* 100: 1180–1192.

Mason S, Tarle SA, Osbin W, Kinfu Y, Kaigler D (2014) Standardization and safety of alveolar bone-derived stem cell isolation. *J Dent Res* 93: 55–61.

Massimiani M, Lacconi V, La Civita F, Ticconi C, Rago R, Campagnolo L (2020) Molecular signaling regulating endometrium-blastocyst crosstalk. *Int J Mol Sci* 21: 23.

Masuda H, Matsuzaki Y, Hiratsu E, Ono M, Nagashima T, Kajitani T, Arase T, Oda H, Uchida H, Asada H et al. (2010) Stem cell-like properties of the endometrial side population: implication in endometrial regeneration. *Plos One* 5: e10387.

Merrilees MJ, Zuo N, Evanko SP, Day AJ, Wight TN (2016) G1 domain of versican regulates hyaluronan organization and the phenotype of cultured human dermal fibroblasts. *J Histochem Cytochem* 64: 353–363.

Mierink K, Karasinski J (2012) Porcine uterus contains a population of mesenchymal stem cells. *Reproduction* 143: 203–209.

Miles S, Roberts DS, KJoo Z (1979) Reproduction in red pandas, *Ailurus fulgens* (carnivora: Ailuropodidae). *J Zool* 188: 235–249.

de Moraes CN, Maia L, Dias MC, Dell'Aqua CP, da Mota LS, Chapwanya A, Landim-Alvarenga FD, Oba E (2016) Bovine endometrial cells: a source of mesenchymal stem/progenitor cells. *Cell Biol Int* 40: 1332–1339.

Murphy MP, Wang H, Patel AN, Kambhampti S, Angle N, Chan K, Marleau AM, Pyszniaik A, Carrier E, Ichim TE et al. (2008) Allogeneic endometrial regenerative cells: an “off the shelf solution” for critical limb ischemia? *J Transl Med* 6: 45.
Olivera R, Moro LN, Jordan R, Pallarols N, Guglielminetti A, Luzzani C, Miruoka SG, Vichera G (2018) Bone marrow mesenchymal stem cells as nuclear donors improve viability and health of cloned horses. *Stem Cells Cloning* 11: 13–22.

Osterwalder M, Speziale D, Shoukry M, Mohan R, Ivanek R, Kohler M, Beisel C, Wen X, Scales SJ, Christoffels VM et al. (2014) Hand2 targets define a network of transcriptional regulators that compartmentalize the early limb bud mesenchyme. *Dev Cell* 31: 345–357.

Pence JC, Clancy KB, Harley BA (2015) The induction of pro-angiogenic processes within a collagen scaffold via exogenous estradiol and endometrial epithelial cells. *Biotechnol Bioeng* 112: 2185–2194.

Queckborner S, Syk Lundberg E, Gemzell-Danielsson K, Davies LC (2020) Endometrial stromal cells exhibit a distinct phenotypic and immunomodulatory profile. *Stem Cell Res Ther* 11: 15.

Reinecke H, Robey TE, Mignone JL, Muskheli V, Bornstein P, Murry CE (2013) Lack of thrombospondin-2 reduces fibrosis and increases vascularity around cardiac cell grafts. *Cardiovasc Pathol* 22: 91–95.

Renfree MB, Fenelon JC (2017) The enigma of embryonic diapause. *Development* 144: 3199–3210.

Rink BE, Amilon KR, Esteves CL, French HM, Watson E, Aurich C, Donadeu FX (2017) Isolation and characterization of equine endometrial mesenchymal stromal cells. *Stem Cell Res Ther* 8: 166.

Sahoo AK, Das JK, Nayak S (2017) Isolation, culture, characterization, and osteogenic differentiation of calf endometrial mesenchymal stem cell. *Vet World* 10: 1533–1541.

Saragusty J, Ajmone-Marsan P, Sampino S, Modlinski JA (2020) Reproductive biotechnology and critically endangered species: merging in vitro gametogenesis with inner cell mass transfer. *Theriogenology* 155: 176–184.

Schwartz RE, Reyes M, Koodie L, Jiang Y, Blackstad M, Lund T, Lenvik T, Johnson S, Hu WS, Verfaillie CM (2002) Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. *J Clin Invest* 109: 1291–1302.

Stanton MM, Tatzalos E, Donne M, Kolundzic N, Helgason I, Ilic D (2019) Prospects for the use of induced pluripotent stem cells in animal conservation and environmental protection. *Stem Cells Transl Med* 8: 7–13.

Tang L, Lu W, Huang J, Tang X, Zhang H, Liu S (2019) miR144 promotes the proliferation and differentiation of bone mesenchymal stem cells by downregulating the expression of SFPR1. *Mol Med Rep* 20: 270–280.

Tao Y, Liu J, Zhang Y, Zhang M, Fang J, Han W, Zhang Z, Liu Y, Ding J, Zhang X (2009) Fibroblast cell line establishment, cryopreservation and interspecies embryos reconstruction in red panda (*Ailurus fulgens*). *Zygote* 17: 117–124.

Wani NA, Vettical BS, Hong SB (2017) First cloned bactrian camel (*Camelus bactrianus*) calf produced by interspecies somatic cell nuclear transfer: a step towards preserving the critically endangered wild bactrian camels. *PLoS One* 12: e0177800.

Wei ST, Huang YC, Hsieh ML, Lin YJ, Shyu WC, Chen HC, Hsieh CH (2020) Atypical chemokine receptor ACKR3/CXCR7 controls postnatal vasculogenesis and arterial specification by mesenchymal stem cells via Notch signaling. *Cell Death Dis* 11: 307.

Wei Y, Fang J, Cai S, Lv C, Zhang S, Hua J (2016) Primordial germ cell-like cells derived from canine adipose mesenchymal stem cells. *Cell Prolif* 49: 503–511.

Wolff EF, Gao XB, Yao KV, Andrews ZB, Du H, Elsworth JD, Taylor HS (2011) Endometrial stem cell transplantation restores dopamine production in a Parkinson’s disease model. *J Cell Mol Med* 15: 747–755.

Wright JH, Johnson MM, Shimizu-Albergine M, Bauer RL, Hayes BJ, Surapisitchat J, Hudkins KL, Riehle KJ, Johnson SC, Yeh MM et al. (2014) Paracrine activation of hepatic stellate cells in platelet-derived growth factor C transgenic mice: evidence for stromal induction of hepatocellular carcinoma. *Int J Cancer* 134: 778–788.

Wu Y, Chen L, Scott PG, Tredget EE (2007) Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. *Stem Cells* 25: 2648–2659.

Zhang Z, Wang J, Chen Y, Luo L, Chen H, Zhu L, Wan G, Han X (2019) Activin A promotes myofibroblast differentiation of endometrial mesenchymal stem cells via STAT3-dependent Smad/CTGF pathway. *Cell Commun Signal* 17: 45.

Zhao S, Xiao X, Sun S, Li D, Wang W, Fu Y, Fan F (2018) MicroRNA-30d/JAG1 axis modulates pulmonary fibrosis through Notch signaling pathway. *Pathol Res Pract* 214: 1315–1323.