Isolation, Characterization, and Differentiation of Dental Pulp Stem Cells in Ferrets

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Abstract

Introduction: The ferret canine tooth has been introduced as a suitable model for studying dental pulp regeneration. The aim of this study was to isolate and characterize ferret dental pulp stem cells (fDPSCs) and their differentiation potential. **Methods:** Dental pulp stem cells were isolated from freshly extracted ferret canine teeth. The cells were examined for the expression of stem cell markers STRO-1, CD90, CD105, and CD146. The osteo/odontogenic and adipogenic differentiation potential of fDPSCs was evaluated. Osteogenic and odontogenic marker genes were evaluated using quantitative real-time polymerase chain reaction (qRT-PCR) on days 1, 4, and 8 after osteo/odontogenic induction of fDPSCs including dentin sialophosphoprotein (DSPP), dentin matrix protein-1, osteopontin, and alkaline phosphatase. Human dental pulp cells were used as the control. The results were analyzed using 3-way analysis of variance. **Results:** fDPSCs were positive for STRO1, CD90, and CD105 and negative for CD146 markers with immunohistochemistry. fDPSCs showed strong osteogenic and weak adipogenic potential. The overall expression of DSPP was not significantly different between fDPSCs and human dental pulp cells. The expression of DSPP in osteo/odontogenic media was significantly higher in fDPSCs on day 4 ($P < .01$). The overall expression of dentin matrix protein-1, osteopontin, and alkaline phosphatase was significantly higher in fDPSCs ($P = .0005$). **Conclusions:** fDPSCs were positive for several markers of dental pulp stem cells resembling human DPSCs and appeared to show a stronger potential to differentiate to osteoblastic rather than odontoblastic lineage. *(J Endod 2016;42:418–424)*

Key Words

Dairy cattle, *in vitro* fertilization, microfluidic sperm sorter, sex-sorted embryos, sex-sorted sperm

Regenerative endodontic treatment has recently gained attention as a preferred option to treat teeth with immature apices (1–3). The optimal result in regenerative endodontic treatment is to regenerate the pulp-dentin complex to restore pulpal vitality and function in previously infected immature teeth (2, 4). Although current clinical regenerative procedures show reasonable outcomes regarding healing of periapical disease (5, 6), formation of the new vital tissue leading to root development is not as predictable (7, 8), and the newly formed tissue might not be a pulp tissue (9, 10).

It is generally recognized that tissue regeneration requires an interaction of stem cells and growth factors in a bioactive scaffold (11). The contemporary protocol for regenerative endodontic treatment recommends using the blood clot as a scaffold containing growth factors and stem cells. A clinical study showed that the concentration of stem cell markers in the blood clot induced in the root canal space is significantly higher than peripheral blood (12). Clinically, the types and numbers of stem cells that are attracted into the root canal space cannot be adequately controlled. Tissue engineering strategies give the clinician the opportunity to control the source of stem cells and their fate (13).

Dental pulp stem cells (DPSCs) have the capability of self-renewal and the capacity of multilineage differentiation. They are also capable of differentiating to adipocytes and neural-like cells (14). DPSCs are the precursors of odontoblastlike cells (15). As shown previously, DPSCs are capable of regenerating pulplike tissue *in vivo* in a controlled sterile environment (16). Huang et al (17) examined DPSCs or stem cells of the apical papilla (SCAP) mixed with a scaffold inserted into human root slices. The results showed formation of a pulplike tissue with a well-established vascularity, a functional layer of odontoblastlike cells on the dentinal walls (17). Therefore, DPSCs are considered as a potent stem cell source for regeneration of the pulp-dentin complex.

The ferret model has been suggested as an appropriate model for studying pulp regeneration (18). However, information on DPSCs in the ferret has not been reported. Also, the characteristics and differentiation potential of ferret stem cells need to be determined in order to be used as part of a tissue engineering strategy in dental pulp regeneration. Therefore, the aim of this study was to isolate, characterize, and determine the differentiation potential of ferret DPSCs (fDPSCs).

Materials and Methods

Isolation of fDPSCs

The study protocol was approved by the University of Maryland's Institutional Animal Care and Use Committee (protocol nos. 0206006 and 0812001). One descended female and 1 male ferret were purchased for the purpose of retrieving fDPSCs (Marshall BioResources, North Rose, NY). At the time of surgery, the female

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ferret was ~66 days old, and the male ferret was 80 days old. All procedures were conducted under aseptic protocols. General anesthesia was administered for all procedures. In the female ferret, the pulp tissues from the 4 canines were extirpated and digested for fDPSCs. In the male ferret, 3 sound canine teeth were extracted and sectioned, and the pulp tissues were carefully removed under magnification and processed to isolate DPSCs. The tissues were washed 3 times with phosphate-buffered saline (PBS), minced, and digested (700 U/mL collagenase type I and 100 U/mL penicillin/streptomycin in PBS) for 1 hour at 37°C. Cells and the remaining tissues were washed 3 times in PBS and suspended in the DPSC medium (Dulbecco modified Eagle medium [DMEM]/low glucose [16, 19] and 100 U/mL penicillin/streptomycin with recombinant mouse basic fibroblast growth factor [bFGF] [Sigma-Aldrich, St Louis, MO]). bFGF was not added to the fDPCS cultures from the female. The following day nonadherent cells and tissues were removed and fresh medium added. Six days after seeding, the cell colonies were observed and passaged (Fig. 1). Additionally, some cells were frozen in CryoStor CS10 (Sigma-Aldrich) for freeze/thaw studies.

Preparation of Human Dental Pulp Cells

Human dental pulp tissues were obtained from 3 freshly extracted intact third molars. Briefly, freshly extracted teeth were cut 3 mm below the cementoenamel junction, and the pulp tissue was gently removed from the pulp chamber using sterile tissue forceps. The pulp tissue was minced and incubated in 0.05% trypsin-EDTA (Gibco-Life Technologies, Grand Island, NY) in 37°C for 5 minutes. Trypsinized pieces were cultured in DMEM (Gibco-Life Technologies) (16, 19) supplemented with 10% fetal bovine serum (FBS) (Gibco-Life Technologies) and 1% antibiotic-antimycotic supplement (Gibco-Life Technologies) containing penicillin, streptomycin, and amphotericin B in a CO_2 incubator (Nuaire, Plymouth, MN) at 37°C. The medium was changed every 3 days.

Immunocytofluorescence

The following stem cell markers were tested on the ferret cells: STRO-1, CD90, CD105, and CD146. Ferret-specific antibodies were not commercially available; therefore, monoclonal and/or polyclonal antibodies against other species were selected based on genetic homology (Novus Biologicals, Littleton, CO) to explore cross-reactivity with ferret stem cell markers (Table 1). Primary antibodies and isotype controls were tested for immunostaining at dilutions of 1:50, 1:200, and 1:500. A follow-up experiment included 1:20, 1:1000, and 1:2000 dilutions based on previous results along with cross-reactivity studies. Cells at the third passage were used for immunostaining. Briefly, cells were seeded in 48-well plates and cultured until they reached 70% confluency. Then, they were washed with PBS, fixed with 4% paraformaldehyde (30 minutes at 22°C), and washed 3 times in PBS and blocked in 7.5% bovine serum albumin in PBS for 30 minutes at 22°C. A primary antibody was then added and incubated for 1 hour at 22°C followed by washing 3 times in PBS and the addition of a secondary antibody with incubation for 1 hour at 22°C. After which, cultures were washed 3 times in PBS, stained with DAPI (4′,6-diamidino-2-phenylindole), and imaged under a fluorescence microscope (EVOS FL; Life Technologies, Grand Island, NY).

Differentiation Studies

Cells were plated in culture plates and incubated in the following media:

1. Osteo/odontogenic differentiation medium containing DMEM/low glucose plus 10 nmol/L dexamethasone, 10 mmol/L β-glycerophosphate, 50 μg/mL ascorbate phosphate, 10 nmol/L 1,25-dihydroxyvitamin D3, and 10% FBS. At 2 time points (4 weeks and 8 weeks), cultures were fixed in 60% isopropanol, and extracellular matrix mineralization was determined by staining with 1% alizarin red S.

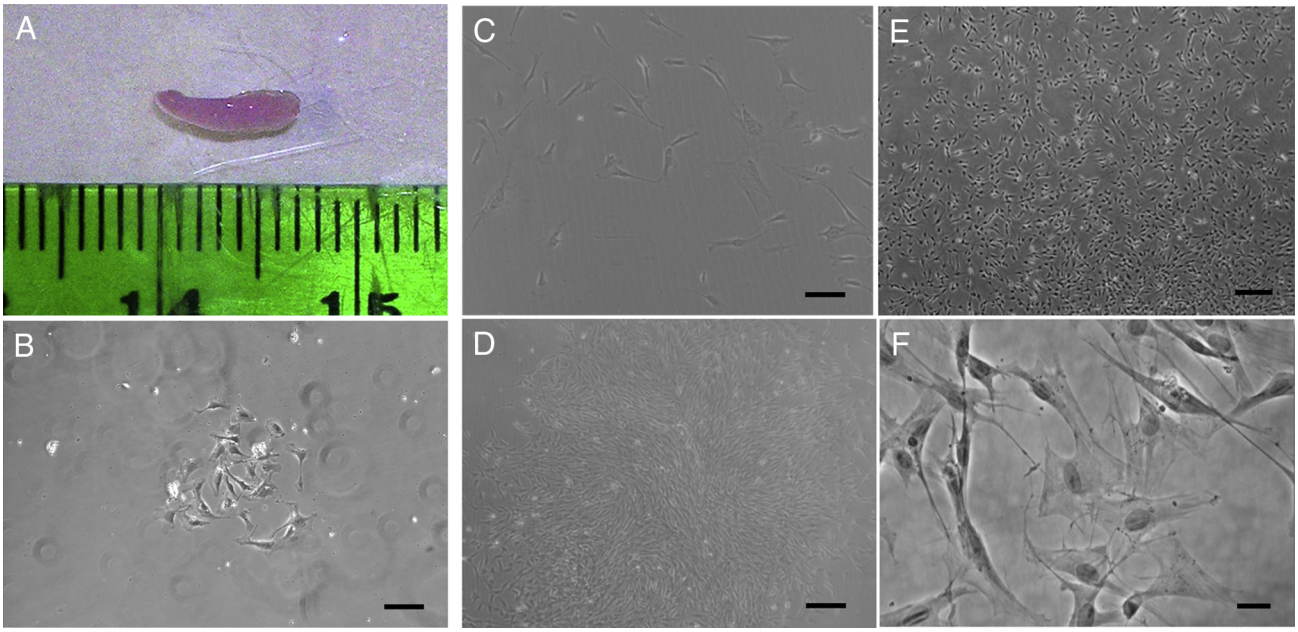

Figure 1. Isolation and culturing of fDPSCs. (*A*) Freshly isolated pulp tissue from the ferret canine. (*B*) fDPSCs on day 6 at passage 0 (from female ferret without FGF). (*C*) fDPSCs on day 2 at passage 0. (*D*) Formation of a colony-forming unit fibroblast of fDPSCs on day 4 at passage 0 (×40). (*E*) fDPSCs on day 3 at passage 4. (*F*) Higher magnification of the cells at passage 4 showing the fibroblastlike stellate shape of the cells. *A* and *B* are from the first pilot ferret, and *C*–*F* are from the second experimental ferret. Scale bar: (*A*) smallest increment is mm, (*B* and *C*) 100 μm, (*D* and *E*) 250 μm, and (*F*) 25 μm.