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Postnatal epithelium and mesenchyme stem/progenitor cells in bioengineered amelogenesis and dentinogenesis

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ABSTRACT

Rodent incisors provide a classic model for studying epithelial–mesenchymal interactions in development. However, postnatal stem/progenitor cells in rodent incisors have not been exploited for tooth regeneration. Here, we characterized postnatal rat incisor epithelium and mesenchyme stem/progenitor cells and found that they formed enamel- and dentin-like tissues *in vivo*. Epithelium and mesenchyme cells were harvested separately from the apical region of postnatal 4–5 day rat incisors. Epithelial and mesenchymal phenotypes were confirmed by immunocytochemistry, CFU assay and/or multi-lineage differentiation. CK14+, Sox2+ and Lgr5+ epithelium stem cells from the cervical loop enhanced amelogenin and ameloblastin expression upon BMP4 or FGF3 stimulation, signifying their differentiation towards ameloblast-like cells, whereas mesenchyme stem/progenitor cells upon BMP4, BMP7 and Wnt3a treatment robustly expressed Dspp, a hallmark of odontoblastic differentiation. We then control-released microencapsulated BMP4, BMP7 and Wnt3a in transplants of epithelium and mesenchyme stem/progenitor cells in the renal capsule of athymic mice *in vivo*. Enamel and dentin-like tissues were generated in two integrated layers with specific expression of amelogenin and ameloblastin in the newly formed, *de novo* enamel-like tissue, and DSP in dentin-like tissue. These findings suggest that postnatal epithelium and mesenchyme stem/progenitor cells can be primed towards bioengineered tooth regeneration.

1. Introduction

Tooth develops upon interactions between epithelium and mesenchyme cells through distinctive morphological stages [1]. Rodent incisors continuously grow throughout postnatal life and have been robustly utilized as a classic model to study tooth development. Epithelium stem cells cluster in the cervical loop in the apical region of the rodent incisor, and differentiate into ameloblasts or enamel-forming cells [2]. This apical epithelium stem cell niche in the rodent incisor is regulated by cascades of signaling pathways including BMPs, Wnt, SHH and FGFs [3], and shares similarities to the hair follicle stem cell niche [4,5]. Continuous self-renewal and differentiation of epithelium and mesenchyme stem cells in the rodent incisor replenish enamel and dentin, and sustain continuous growth and eruption of rodent incisors. However, this powerful model of self-renewing epithelium and mesenchyme stem cells in the rodent incisor has not been harnessed towards tooth regeneration.

Cell source is a central impediment for tooth regeneration in patients and has stimulated numerous investigations [6]. Mouse embryonic tooth germ cells, specifically E10 dental epithelium or E14.5 dental mesenchyme, can clearly initiate tooth morphogenesis [6]. Mouse E14.5 dental mesenchyme, when combined with oral epithelium of toothless chicks, gave rise to a developing tooth organ [7]. Reconstitution of E10 dental epithelium with postnatal bone marrow stromal cells also led to formation of a tooth organ [8]. E14.5 dental epithelium and mesenchyme cells, when reconstituted in a collagen gel, not only formed a tooth germ in organ culture, but also generated an erupted tooth when transplanted into the socket of an extracted adult tooth in the mouse [9,10]. Recently, E14.5 mouse dental mesenchyme, when reconstituted into cell sheets with iPS-like cells formed tooth-like structures [11,12]. Reconstituted mouse embryonic dental mesenchyme cells

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with human gingival epithelial cells formed developing tooth roots [13].

Despite the remarkable progress, the human equivalent to E10 to E14.5 mouse embryonic tooth germ cells, or ~3 month human embryonic tooth germ cells, are not applicable in human patients. A postnatal, somatic cell source with or without cellular programming is necessary for human applications of whole tooth regeneration, given severe safety concerns over and virtual impossibility in the application of embryonic tooth germ cells in patients [6]. Thus, a postnatal cell source that can yield amelogenesis and dentinogenesis is critically needed. Effort has been made to search for postnatal stem/progenitor cells that can be utilized in the regeneration of individual tooth structures including dentin, cementum and/or dental pulp or tooth roots [14–17]. However, little is understood about the potential for postnatal stem/progenitor cells in driving amelogenesis and odontogenesis. Thus, the objective of the present study was to investigate whether postnatal dental stem/progenitor cells can be manipulated for tooth regeneration. We hypothesized that postnatal dental stem/progenitor cells retain some of the capacity as pre-natal cells towards amelogenesis and odontogenesis.

2. Materials and methods

2.1. Isolation and culture of epithelium and mesenchyme stem/progenitor cells

Following IACUC approval, 4/5-day-old, post-natal Sprague–Dawley rats were sacrificed to isolate incisor epithelium and mesenchyme cells [18]. Briefly, the mandible was aseptically removed (Fig. 1A) and digested in 2% collagenase (Gibco, Carlsbad, CA) in Dulbecco's Modified Eagle's Medium (DMEM: Invitrogen, Carlsbad, CA) at 4 °C overnight. The epithelium layer with the cervical loop was carefully separated from dental mesenchyme under dissection microscope (Fig. 1B, C). The cervical loop, which harbors dental epithelium stem cell niche [2], was illustrated in Fig. 1B (arrowhead). The isolated dental epithelium was further digested with 0.3-mg/mL collagenase and 0.4-mg/mL dispase (Gibco) for 30 min in Hank's Balanced Salt Solution and then filtered through a 40-mm cell sieve. Single cell suspension was cultured in LHC-9, serum-free epithelium growth medium with 1% antibiotics/antimicrotics.

Dental mesenchyme was isolated under dissection microscope by a surgical cut in the apical region (dashed line in Fig. 1C) at a location directly posterior to the newly formed dentin. The isolated dental mesenchyme was minced and digested with 0.3-mg/mL collagenase and 0.4-mg/mL dispase for 30 min in DMEM, filtered through a 40-mm cell sieve with single cells cultured in DMEM with 10% FBS and 1% antibiotics/antimicrotics.

2.2. Immunocytochemistry

Dental epithelium cells were cultured 5 days and fixed in 4% paraformaldehyde. Fixed cells were treated with 0.1% Triton X-100 (Sigma–Aldrich, St. Louis, MO) for 5 min, incubated with blocking buffer for 60 min (Oddesay, Lincoln, NE), and further incubated with primary antibodies at 4 °C overnight, including anti-cytokeratin 14 (CK14) mouse monoclonal IgG (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Sox2 rabbit polyclonal IgG (1:200, Cell Signaling, Danvers, MA), anti-Lgr5 mouse monoclonal IgG (1:100, Origene, Rockville, MD), anti-Notch 1 rabbit polyclonal IgG (1:400, Millipore, Billerica, MA), anti-alkaline phosphatase rabbit polyclonal IgG (1:400, Abcam, Cambridge, MA, US), anti-ameloblastin rabbit polyclonal IgG (1:500, Santa Cruz Biotechnology) and anti-amelogenin mouse monoclonal IgG (1:500, Santa Cruz Biotechnology). Alexa Fluor® 555 Donkey Anti-Mouse IgG (H + L), Alexa Fluor® 488 Goat Anti-Mouse IgG (H + L) and FITC-conjugated goat anti-rabbit IgG (1:1000) were applied for 60 min. Cells were sealed with Vecta shield mounting

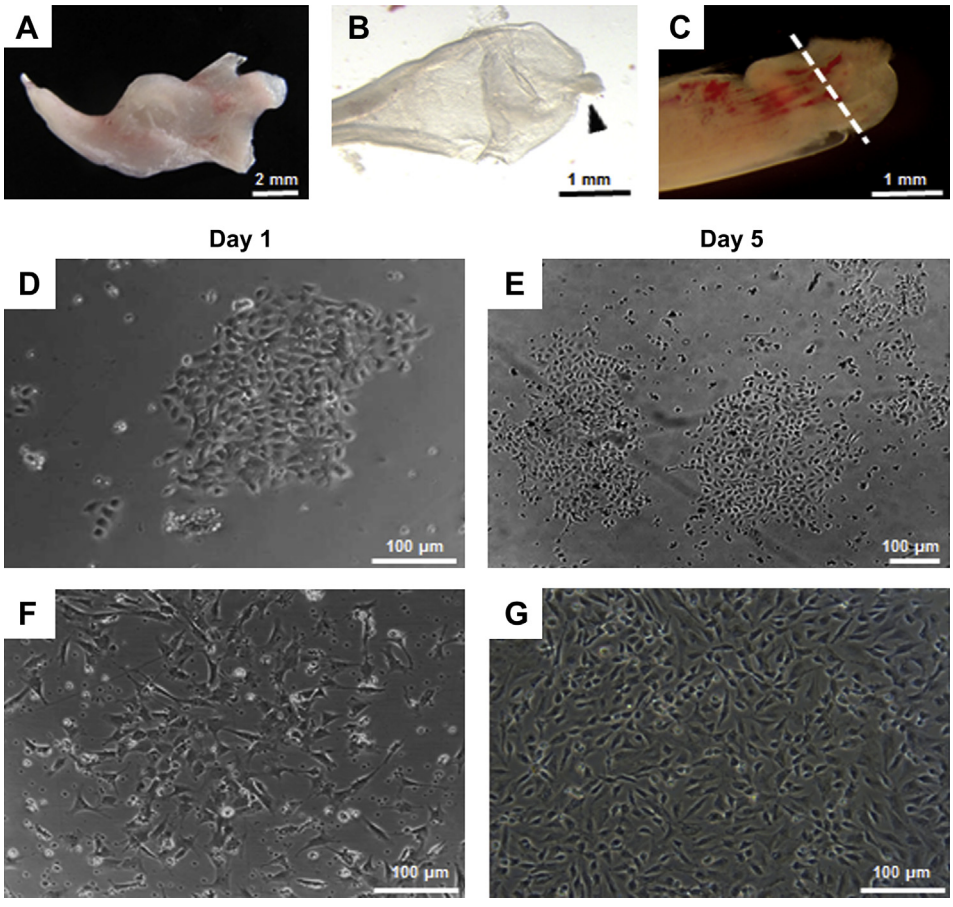

Fig. 1. Microdissection and subculture of rat dental epithelium and mesenchyme cells from 4 to 5 day postnatal rat incisors. A) Surgically removed mandible from a Sprague–Dawley rat showing erupted incisor. B) The dental epithelium layer with the cervical loop was carefully separated from dental mesenchyme under dissection microscope. The arrowhead shows distinctive structure of the cervical loop, which harbors dental epithelium stem cell niche. C) Dental mesenchyme was isolated with a surgical cut in the apical regions (dashed line) immediately posterior to the newly formed dentin. D, E) Dental epithelium stem cells grew into typical pebble-like colonies and propagated in LHC-9, serum-free medium in five days. F, G) Dental mesenchyme stem cells showed typical spindle shape, fibroblast-like morphology during passaging.