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Self-renewal and multilineage differentiation of mouse dental epithelial stem cells

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Abstract Understanding the cellular and molecular mechanisms underlying the self-renewal and differentiation of dental epithelial stem cells (DESCs) that support the unlimited growth potential of mouse incisors is critical for developing novel tooth regenerative therapies and unraveling the pathogenesis of odontogenic tumors. However, analysis of DESC properties and regulation has been limited by the lack of an *in vitro* assay system and well-documented DESC markers. Here, we describe an *in vitro* sphere culture system to isolate the DESCs from postnatal mouse incisor cervical loops (CLs) where the DESCs are thought to reside. The dissociated cells from CLs were able to expand and form spheres for multiple generations in the culture system. Lineage tracing indicated that DESC within the spheres were epithelial in origin as evident by lineage tracing. Upon stimulation, the sphere cells differentiated into cytokeratin 14- and amelogenin-expressing and mineral material-producing cells. Compared to the CL tissue, sphere cells expressed high levels of expression of Sca-1, CD49f (also designated as integrin α6), and CD44. Fluorescence-activated cell sorting (FACS) analyses of mouse incisor CL cells further showed that the CD49f^{Bright} population was enriched in sphere-forming cells. In addition, the CD49f^{Bright} population includes both slow-cycling and Lgr5^{+} DESCs. The *in vitro* sphere culture system and identification of CD49f^{Bright} as a DESC marker provide a novel platform for enriching DESCs, interrogating how maintenance, cell fate determination, and differentiation of DESCs are regulated, and developing tooth regenerative therapies.

Abbreviations: FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; DESC, dental epithelial stem cell; CL, cervical loop; SC, stem cell; OEE, outer enamel epithelium; IEE, inner enamel epithelium; SR, stellate reticulum; SI, stratum intermedium; Alp, alkaline phosphatase

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Introduction

Stem cells (SCs) that have the capacity to self-renew and to give rise to differentiated progeny are of broad interest because of their potential in regenerative therapy and their purported role in tumor initiation and relapse. The ability to identify and isolate SCs is essential for understanding molecular mechanisms underlying SC self-renewal and expansion, as well as their roles in tumorigenesis. Several approaches have been used to isolate or define SCs from a variety of organs. These include cell sorting based on SC surface markers (Stingl et al., 2006; Lawson et al., 2007; Spangrude et al., 1988), bromodeoxyuridine (BrdU) or other long-term label retention for slow cycling activities (Bickenbach and Chism, 1998; Tumbar et al., 2004), sphere-forming assays for the self-renewal property (Reynolds and Rietze, 2005; Dontu et al., 2003; Xin et al., 2007), and lineage tracing for their progeny (Snippert and Clevers, 2011). Emerging evidence shows that two types of SCs exist in various tissues, in separate yet adjoining locations. The slow-cycling SCs can be identified by long-term label retention and active SCs that do not retain labels due to rapid cell divisions can be identified by expression of Lgr5 (Leucine-rich repeat-containing G protein-coupled receptor5) (Snippert and Clevers, 2011; Barker et al., 2007, 2012; Huch et al., 2013).

Teeth are highly mineralized organs derived from the dental epithelium and the underlying mesenchymal cells originally from neural crest, which undergo a series of sequential and tightly regulated processes to form a tooth (Grobstein, 1967; Thesleff and Hurmerinta, 1981; Thesleff et al., 1995). Five different lines of dental stromal cells that possess SC properties have been established from developing or mature human teeth (Gronthos et al., 2002; Miura et al., 2003; Sonoyama et al., 2008; Seo et al., 2004; Morsczeck et al., 2005). However, progressing in characterizing dental epithelial stem cells (DESCs) has been slow. It has been proposed that adult human teeth do not have DESCs since ameloblasts, the terminally differentiated dental epithelial cells, shed after tooth eruption in humans. Moreover, the lack of culture systems and well-accepted surface markers for DESCs further impede this research. Rodent incisors grow continuously throughout life, which is made possible by the existence of DESCs in the cervical loop (CL) region (Tummers and Thesleff, 2003; Yokohama-Tamaki et al., 2006; Harada et al., 1999; Harada and Ohshima, 2004). The presence of DESCs in the CL region is evidenced by the gradual differentiation of ameloblast-lineage cells apical to the incisal direction (Mitsiadis et al., 2007), the directional cell migration demonstrated by vital carbocyanine dye DiI tracking, long-term BrdU retention, cell cycle kinetics studies (Harada et al., 1999), and in vivo lineage trace of Sox2 expressing cells (Juuri et al., 2012).

It has been proposed that slow cycling DESCs within the CL mainly reside in the stellate reticulum (SR) (Harada et al., 1999; Harada and Ohshima, 2004; Suomalainen and Thesleff, 2010). Recently, however, by tracing the long-term retention of H2B-GFP fusion protein (Tumbar et al., 2004), it has been shown that slow cycling DESCs are mainly located in the outer enamel epithelium (OEE) (Seidel et al., 2010). Interestingly, similar to rapidly renewing adult tissues, such as intestine and hair follicle, expression of the active SC marker Lgr5 has been found in the SR region of the CL (Suomalainen and Thesleff, 2010). Several other SC markers, such as Bmi-1, Oct 3/4, and Sox2 have recently been reported to be expressed in the CL (Li et al., 2011; Juuri et al., 2012). In vivo lineage tracing experiments further shows that the Sox2 positive DESCs give rise to multiple lineages of tooth epithelial cells (Juuri et al., 2012). However, to date the isolation of DESCs is still problematic because of the lack of DESC surface markers and the paucity of *in vitro* assay systems for isolating and testing DESC properties.

To address these critical issues, we established an *in vitro* sphere culture system for DESCs isolated from the CL. Thorough analyses indicated that the DESC sphere cells displayed SC properties and expressed high levels of SC markers, including Sca-1, CD49f (also designated as integrin α6), and CD44. Furthermore, it was demonstrated that CD49f^{Bright} was a suitable cell surface marker for identifying and isolating DESCs. Therefore, our study establishes a foundation for enriching and expanding DESCs for dental regenerative treatments and for understanding DESC-related pathogenesis.

Materials and methods

Animals and isolation of tissues

All animals were housed in the Program of Animal Resources of the Institute of Biosciences and Technology, Texas A&M Health Science Center, and were handled in accordance with the principles and procedure of the *Guide for the Care and Use of Laboratory Animals*. All experimental procedures were approved by the Institutional Animal Care and Use Committee. Mice carrying the *Nkx3.1*^{Cre} knock-in alleles (Lin et al., 2007), ROSA26^{LacZ} (Soriano, 1999), ROSA26^{EYFP} (Srinivas et al., 2001) reporter alleles, K5rtTA (Diamond et al., 2000), H2B-GFP (Tumbar et al., 2004), Lgr5^{LacZ}, Lgr5$^{EGFP\text{-}ires\text{-}CreERT2}$ (Barker et al., 2007), and Lgr4^{LacZ} (Weng et al., 2008) transgenes were maintained and genotyped as described elsewhere. Inducible K5rtTA-H2BGFP expression was achieved by administration of regular chow containing 0.0625% doxycycline (Harlan Teklad).

Dissociation of the CL epithelial cells for DESC sphere culture

The CL regions defined as the apical tissue distal to the tooth mineralized portion (Fig. 1A) were dissected from postnatal day (P) 7 mice unless otherwise indicated. The dissected tissue was first incubated in a solution containing 1 mg/ml dispase and 1 mg/ml collagenase I (Life Technologies, Grand Island, NY) for 30 min at 37 °C. Tissues were further dissociated by incubation in 0.005% trypsin for 25 min at 37 °C with gentle pipetting. Cells were sieved through a 40 μm cell strainer (Falcon) to obtain a single-cell suspension. The cells were suspended in 50 μl oral epithelial progenitor medium (CnT-24) (Cellntec Advanced cell systems, Switzerland), and mixed with Matrigel (BD Biosciences) at a 1:1 ratio at a density of 50,000 cells/ml in primary cultures and 10,000 cells/ml in subsequent