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# Secretion of Shh by a Neurovascular Bundle Niche Supports Mesenchymal Stem Cell Homeostasis in the Adult Mouse Incisor

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## SUMMARY

**Mesenchymal stem cells (MSCs) are typically defined by their in vitro characteristics, and as a consequence the in vivo identity of MSCs and their niches are poorly understood. To address this issue, we used lineage tracing in a mouse incisor model and identified the neurovascular bundle (NVB) as an MSC niche. We found that NVB sensory nerves secrete Shh protein, which activates Gli1 expression in periarterial cells that contribute to all mesenchymal derivatives. These periarterial cells do not express classical MSC markers used to define MSCs in vitro. In contrast, NG2$^+$ pericytes represent an MSC subpopulation derived from Gli1+ cells; they express classical MSC markers and contribute little to homeostasis but are actively involved in injury repair. Likewise, incisor Gli1$^+$ cells, but not NG2$^+$ cells, exhibit typical MSC characteristics in vitro. Collectively, we demonstrate that MSCs originate from periarterial cells and are regulated by Shh secretion from an NVB.**

## INTRODUCTION

Mesenchymal stem cells (MSCs) were first identified in the bone marrow as a group of colony-forming cells with osteogenic, chondrogenic, and adipogenic potential (Friedenstein et al., 1968). MSCs have since been identified from various tissues, including skeletal muscle (Dellavalle et al., 2011), adipose tissue (Tang et al., 2008; Zuk et al., 2002), placenta (Covas et al., 2008), endometrium (Schwab and Gargett, 2007), deciduous teeth (Miura et al., 2003), and bone (Pittenger et al., 1999). Similarities between MSCs and perivascular cells have been characterized, suggesting that they may represent the same population, at least in some tissues (Covas et al., 2008; Schwab and Gargett, 2007). The best-characterized properties of MSCs include their capacity for multipotential differentiation and their immunomodulation abilities (Bernardo and Fibbe, 2013). MSCs are able to differentiate into various cell types in vitro, including osteoblasts, chondrocytes, adipocytes, or even neurons (Keating, 2012). Although MSCs have been extensively studied, their in vivo identity and supporting niche remain elusive. The definition of MSCs is based on a loose set of criteria including trilineage in vitro differentiation ability and expression of various MSC surface markers (Bianco et al., 2013; Dominici et al., 2006; Keating, 2012). To date, there are no well-defined in vivo markers or appropriate lineage analysis tools for MSCs. Similarly, although label-retaining or lineage-tracing analyses have become the gold standard for many other stem cell studies (Grompe, 2012), these techniques have rarely been applied to MSC studies (Méndez-Ferrer et al., 2010; Tang et al., 2008). Thus, at present, MSCs are defined based on their in vitro culture properties and expression profiles of multiple surface markers, with considerable controversy (Bianco et al., 2013; Keating, 2012). Based mostly on these criteria, it was proposed that the perivascular niche is an in vivo niche of MSCs and that pericytes are their in situ counterparts (Covas et al., 2008; Crisan et al., 2008; Traktuev et al., 2008). However, rigorous testing is necessary to evaluate this theory and to determine whether other sources may provide an MSC niche.

The mouse incisor provides an excellent model for MSC study because it grows continuously throughout the life of the animal. It is composed of an outer enamel surface, dentin underneath the enamel, and dental pulp in the center containing vasculature and nervous tissue. Both epithelial and mesenchymal compartments of the incisor rapidly replenish all of their cells within 1 month (Smith and Warshawsky, 1975). Self-renewal of the incisor epithelium is supported by a group of quiescent epithelial stem cells in the cervical loop region (Juuri et al., 2012; Seidel et al., 2010). Although incisor dentin is highly similar to bone, two properties that make the incisor unique from bone are its well-oriented structures and fast turnover. The odontoblasts, which form dentin, are aligned in a single layer along the inner surface of the dentin, and their arrangement displays a cytodifferentiation gradient from the immature region apically toward the tip. The vasculature and nerves of the incisor are well organized and oriented in one direction. The continuous turnover of odontoblasts is supported by stem cells within the mesenchyme, but the identity and exact localization of these stem cells in vivo remains unknown (Balic and Mina, 2010; Mao and Prockop,


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2012). It has been proposed that incisor MSCs are localized near the cervical loop region that can give rise to transit amplifying cells (TACs) (Feng et al., 2011; Lapthanasupkul et al., 2012). TACs can be easily identified based on their active proliferation, and they give rise to committed preodontoblasts and then terminal differentiated odontoblasts. This rapid turnover makes the incisor mesenchyme an excellent model for studying MSCs.

The role of nerves in the regulation of the stem cell niche remains largely unknown. The sensory nerves innervating the hair follicle regulate the response of a group of hair follicle stem cells during injury repair (Brownell et al., 2011). Sympathetic innervation regulates hematopoietic stem cell egression from the bone marrow (Katayama et al., 2006) and their emergence during embryogenesis (Fitch et al., 2012). Adrenergic nerves associate with and regulate nestin$^+$ bone marrow MSCs (Méndez-Ferrer et al., 2010). Parasympathetic nerves are essential for epithelial progenitor cells during salivary gland organogenesis and for adult gland injury repair (Knox et al., 2010, 2013). In adult tissues, nerves travel along the arteries. Together with the loose connective tissue surrounding arteries and nerves, they form a neurovascular bundle (NVB), which is a common anatomical structure found in many organs.

In this study, we use the mouse incisor as a model to determine the in vivo identity of MSCs and their corresponding niche. We show that incisor MSCs surround the arterioles and are supported by an NVB niche. These periarterial MSCs participate in both homeostasis and injury repair of incisor mesenchyme in vivo and give rise to the entire MSC population in vitro.

## RESULTS

### Label-Retaining Cells Surround the NVB

In the mouse incisor, major arteries and veins are arranged in parallel along the long axis with the arterial branches aligned on the midline bisecting the incisor (Figure S1 available online). Nerves in the incisor accompany arteries to form the NVB (Figure S1). To investigate the in vivo mechanism of MSC-supported incisor mesenchyme homeostasis, we performed label-retaining analysis. H2BGFP-based label-retaining analysis has been used for identifying stem cells in various tissues (Foudi et al., 2009; Tang et al., 2008; Tumbar et al., 2004). We generated triple-transgenic mice, *Wnt1-Cre;ROSA26$^{LoxP-STOP-LoxP-tTA}$; tetO-H2BGFP* (WTH) (Figure S2A), to identify label-retaining cells (LRCs) in the dental mesenchyme. After confirming that doxycycline exerts stringent control over H2BGFP expression in the dental mesenchyme (Figure S2B), we performed label-retaining analysis using 4- to 6-week-old WTH mice followed by a 4-week chase period. Our experimental design was based on a time course study (Figures S2D–S2I) and the previous finding that odontoblasts and ameloblasts in mouse incisors are turned over within 1 month (Harada et al., 1999; Smith and Warshawsky, 1975). After complete H2BGFP labeling of the dental pulp and a 4-week chase, all LRCs surround the NVB, centered on arteries and accompanying nerves, but not on veins or capillaries. (Figures 1A–1C). The dental mesenchyme near the cervical loop contains fast-dividing TACs (Lapthanasupkul et al., 2012; Parsa et al., 2010). Short-term 5-ethynyl-2′-deoxyuridine (EdU) incorporation experiments indicate that LRCs and EdU-positive TACs are adjacent to, but mutually exclusive from, each other, with LRCs near the NVB in the center surrounded by TACs (Figures 1D and 1E). Next, we injured incisors with a needle and collected samples 24 hr later. EdU was injected 2 hr before sacrifice. In injured incisors, approximately 10% of H2BGFP LRCs incorporated EdU, indicating that the normally slow-cycling mesenchymal cells (LRCs) were stimulated by injury to proliferate (Figures 1F–1H).

### Gli1$^+$ Cells Surrounding the NVB Are MSCs Supporting the Homeostasis and Injury Repair of Incisor Mesenchyme

Previous results have suggested that Gli1 may be a dental epithelial stem cell marker (Seidel et al., 2010). We hypothesized that Gli1 might also be a marker for incisor MSCs. We analyzed the Gli1 expression pattern in incisors using *Gli1-LacZ* mice. We detected Gli1 expression in the mesenchyme surrounding the NVB, centered on arteries and accompanying nerves, but not veins or capillaries (Figures 2A–2C). Gli1 expression was also detectable in dental epithelial cells (Figure 2A) and in the postmitotic odontoblasts of the labial side mesenchyme. A similar Gli1$^+$ expression pattern in the incisor was also detectable in Gli1-GFP mice (Figure S3A). Fluorescence-activated cell sorting (FACS) analysis of incisors from Gli1-GFP mice indicated that there are around 2,300 Gli1$^+$ cells in each lower incisor, comprising less than 5% of the entire incisor mesenchyme population (Figure S3B). To determine whether Gli1$^+$ cells support incisor homeostasis, we generated *Gli1-Cre$^{ERT2}$; ROSA26$^{LoxP-STOP-LoxP-ZsGreen1}$* (*Gli1-CE;Zsgreen*) mice and injected tamoxifen at 4–6 weeks of age. We detected ZsGreen$^+$ cells near the cervical loop region 72 hr after the first injection (Figure 2D). This ZsGreen$^+$ population included Gli1-expressing cells and the derivatives they produced within the last 72 hr. Over a 4-week period, Gli1$^+$ cells expanded toward the tip of the incisor and eventually populated the entire dental mesenchyme (Figure 2D). To test whether Gli1$^+$ cells can self-renew and continuously support mesenchyme turnover, we examined Gli1 expression at 6 months of age (Figure S3H). In *Gli1-CE; ZsGreen* mice induced at 6 months, a small number of ZsGreen$^+$ cells were detectable in the cervical loop region 72 hr after the first injection and after 1 month the entire pulp mesenchyme was populated by Gli1$^+$ cell derivatives (Figures S3I and S3J). Moreover, we assessed *Gli1-CE;ZsGreen* incisor samples at 4 and 17.5 months after induction and found that the entire mesenchyme was still populated with Gli1$^+$ cell derivatives (Figures S3C and S3D).

To compare the LRC and Gli1$^+$ populations, we generated *Gli1-LacZ;WTH* tetra-transgenic mice in which LRCs are labeled with H2BGFP and Gli1$^+$ cells are labeled with β-galactosidase (β-gal). Colocalization of β-gal and LRC signals indicate that around 95% ± 0.06% of Gli1$^+$ cells are quiescent LRCs and 80% ± 0.09% of LRCs are Gli1$^+$, suggesting heterogeneity of both the Gli1$^+$ and LRC populations (Figures 2E and 2F). Similar to LRCs, Gli1$^+$ cells and TACs are adjacent to but mutually exclusive from each other, further supporting our conclusion that Gli1$^+$ cells are MSCs in the incisor mesenchyme (Figure 2G).

To test whether Gli1$^+$ cells can be activated upon injury, we injured incisors of 1-month-old *Gli1-LacZ* mice. Gli1 activity was not significantly changed 48 hr after injury (Figure S3E). EdU incorporation experiments indicate that Gli1$^+$ cells begin

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