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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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Abstract

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 a 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

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Contributions

H.Z. and Y.C. designed the study. H.Z. carried out the experiments and analyzed the data. J.F. participated in the guinea pig and microarray experiments. K.S. and O.K. did the cell lineage tracing and hedgehog inhibitor experiments. S.S. and P.S. contributed to the stem cell marker study. H.Z. and Y.C. co-wrote the paper.

Competing financial interests

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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 multi-potential 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 tri-lineage 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 (Mendez-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 one 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 cyto-differentiation gradient from the immature region apically towards 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, 2012). It has been proposed that incisor MSCs are localized near the cervical loop region that can give rise to transit amplifying (TA) cells (Feng et al., 2011; Lapthanasupkul et al., 2012). TA cells can be easily identified based on their active proliferation, and they give rise to committed pre-odontoblasts 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 (Mendez-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., 2013; Knox et al., 2010). 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 a 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 (LRCs) 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 (Supplementary Figure 1). Nerves in the incisor accompany arteries to form the neurovascular bundle (NVB) (Supplementary Figure 1). 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*$^{LoxP\text{-}STOP\text{-}LoxP\text{-}tTA}$*;tetO-H2BGFP* (WTH) (Supplementary Figure 2A) to identify LRCs in the dental mesenchyme. After confirming that doxycyclin exerts stringent control over H2BGFP expression in the dental mesenchyme (Supplementary Figure 2B), we performed label retaining analysis using 4-6 week old WTH mice followed by a four-week chase period. Our experimental design was based on a time course study (Supplementary Figure 2D-2I) and the previous finding that odontoblasts and ameloblasts in mouse incisors are turned over within one month (Harada et al., 1999; Smith and Warshawsky, 1975). After complete H2BGFP labeling of the dental pulp and a four-week chase, all LRCs surround the NVB, centered around arteries and accompanying nerves but not around veins or capillaries. (Fig. 1A-C). The dental mesenchyme near the cervical loop contains fast-dividing TA cells (Lapthanasupkul et al., 2012; Parsa et al., 2010). Short-term EdU incorporation experiments indicate that LRCs and EdU-positive TA cells are adjacent to, but mutually exclusive from, each other, with LRCs near the NVB in the center surrounded by TA cells (Fig. 1D, E). Next, we injured incisors with a needle and collected samples 24 hours later. EdU was injected 2 hours 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 (Fig. 1F-H).

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 (Fig. 2A-C). Gli1 expression was also detectable in dental epithelial cells (Fig. 2A) and in the post-mitotic odontoblasts of the labial side mesenchyme. A similar Gli1+ expression pattern in the incisor was also detectable in Gli1-GFP mice (Supplementary Figure 3A). FACS analysis of incisors from Gli1-GFP mice indicated that there are around 2300 Gli1+ cells in each lower incisor, comprising less than 5% of the entire incisor mesenchyme population (Supplementary Figure 3B). To determine whether Gli1+ cells support incisor homeostasis, we generated *Gli1-Cre*^{ERT2}*;ROSA26*$^{LoxP\text{-}STOP\text{-}LoxP\text{-}ZsGreen1}$ (*Gli1-CE;Zsgreen*) mice and injected tamoxifen at 4-6 weeks of age. We detected ZsGreen+ cells near the cervical loop region 72 hours after the first injection (Fig. 2D). This ZsGreen+ population included Gli1-expressing cells and the derivatives they produced within the last 72 hours. Over a four-week period, Gli1+ cells expanded towards the tip of the incisor and eventually populated the entire

dental mesenchyme (Fig. 2D). To test whether Gli1+ cells can self-renew and continuously support mesenchyme turnover, we examined Gli1 expression at 6 months of age (Supplementary Figure 3 H). In *Gli1-CE;ZsGreen* mice induced at 6 months, a small number of ZsGreen+ cells were detectable in the cervical loop region 72 hours after the first injection and after one month the entire pulp mesenchyme was populated by Gli1+ cell derivatives (Supplementary Figure 3I, J). 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 (Supplementary Figure 3C, D).

To compare the LRC and Gli1+ populations, we generated *Gli1-LacZ;WTH* tetra-transgenic mice in which LRCs are labeled by H2BGFP and Gli1+ cells are labeled by 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 (Fig. 2E, F). Similar to LRCs, Gli1+ cells and TA cells 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 one-month-old *Gli1-LacZ* mice. Gli1 activity was not significantly changed 48 hours after injury (Supplementary Figure 3E). EdU incorporation experiments indicate that Gli1+ cells begin proliferating by 24 hours after injury (Supplementary Figure 3F). To determine whether Gli1+ cells can contribute to injury repair, we first induced one-month-old *Gli1-CE;ZsGreen* mice with tamoxifen and then injured the incisor 72 hours after induction. Two weeks after injury, reparative dentin had formed, as indicated by the distorted shape and disorganized dentin tubules (Figure 2H, asterisk), and was derived from Gli1+ cells (Figure 2I). Therefore, Gli1+ cells in the incisor are quiescent MSCs that support both homeostasis and injury repair.

Gli1 is a member of the hedgehog signaling pathway that responds to hedgehog family ligands (Jiang and Hui, 2008). To determine the role of the hedgehog pathway in regulating incisor MSCs, we fed adult mice Shh inhibitor HhAntag for one month as previously described (Seidel et al., 2010). Inhibitor administration significantly reduced Gli1 activity in the incisor (Supplementary Figure 3K, L). Treatment with inhibitor also significantly reduced dentin formation but had little effect on cell proliferation within the mesenchyme (Supplementary Figure 3M-P). The administration of inhibitor had no significant effect on mesenchymal cell apoptosis or the number of LRCs within the incisor mesenchyme, suggesting that stem cell maintenance was not affected (Supplementary Figure 2Q-U). In addition, we tested the effect of Shh on incisor MSCs *in vitro*. Shh at various concentrations had no significant effect on cell proliferation (Supplementary Figure 3V). Alizarin red staining and real-time PCR indicated that odontogenic differentiation was enhanced by the presence of Shh (Supplementary Figure 3W, X). Based on both *in vivo* and *in vitro* data, we conclude that Shh in the incisor mainly functions to regulate the odontogenic differentiation process but has little effect on stem cell maintenance or cell proliferation.

Sensory nerves in the mesenchyme provide Shh for the periarterial Gli1+ cells

A previous report suggested that Shh from the dental epithelium triggers mesenchymal Gli1 activity (Seidel et al., 2010), but this model cannot explain the specific peri-NVB Gli1+ pattern. To test whether dental epithelial Shh signaling activates Gli1 in the dental mesenchyme, we generated *K14-rtTA;tetO-Cre;Shh*$^{flox/flox}$ mice (*K;T;Shh*). Doxycyclin induction at one month of age efficiently eliminated Shh expression in the dental epithelium (Supplementary Figure 4A, B, A', B'). However, no defect was detectable in *K;T;Shh* mutant incisors one month after induction. Enamel and dentin mineralization are indistinguishable in *K;T;Shh* and control incisors based on micro CT images (Supplementary Figure 4C, D).

Proliferation analysis and HE staining also demonstrated no significant difference between *K;T;Shh* and control incisors (Supplementary Figure 4E-H, G', H'). Most importantly, Gli1 expression surrounding the NVB was not affected in *K;T;Shh* incisors (Supplementary Figure 4I, J). To confirm the specificity of our Gli1 antibody, we also performed immunohistochemical staining using *Gli1*$^{LacZ/LacZ}$ (*Gli1*−/−) incisors (Bai et al., 2002), which showed no signal surrounding the NVB (Supplementary Figure 4K). Therefore, we conclude that Shh derived from the incisor epithelium does not trigger Gli1 activity in the mesenchyme and that Gli1+ cells must be supplied by a different Shh source.

To identify other Shh sources, we generated *Shh-Cre*^{ERT2}*;ROSA26*$^{LoxP\text{-}STOP\text{-}LoxP\text{-}TdTomato}$ (*Shh-CE;Tdtomato*) mice in which Shh-producing structures can be labeled by TdTomato fluorescence upon induction. Three days after the first tamoxifen injection, strong Shh activity was detectable in the trigeminal ganglion (TGG) (Supplementary Figure 5A), which contains most of the neuron bodies innervating the craniofacial region. Immunohistochemical staining with sensory nerve marker CGRP indicated these Shh secreting neurons are sensory neurons. Strong Shh activity was also detectable in the incisor epithelium but not in any mesenchymal cells three days after induction. Two weeks after induction, we detected strong reporter activity in axons of the inferior alveolar nerve (IAN), which is the sole sensory nerve innervating the adult lower incisor (Figure 3A, B). The discrepancy between TGG and IAN reporter activity appearance time suggests the TdTomato protein is first synthesized in the TGG sensory neuron cell bodies and then transported along the IAN axons to the incisor mesenchyme. The incisor is also innervated by sympathetic nerves derived from the superior cervical ganglion (SCG) (Ladizesky et al., 2001). We dissected the SCG from induced *Shh-CE;Tdtomato* mice (Savastano et al., 2010) and found no Shh activity, indicating that sympathetic neurons do not produce Shh (Figure 3C). We also conducted Shh immunohistochemical staining in the incisor, which confirmed the presence of Shh protein in the dental epithelium and mesenchyme surrounding nerve fibers in the cervical loop region (Supplementary Figure 5C, C', C''). Shh protein was also present in the TGG and IAN (Supplementary Figure 5D, E).

To test whether neural Shh is the source for mesenchymal Gli1 activity, we severed the IAN in adult *Gli1-LacZ* mice. Denervation had no effect on incisor circulation or odontoblast morphology (Supplementary Figure 5G-J). No odontoblast degeneration was observed 72 hours after denervation, whereas vascular damage rapidly led to extensive odontoblast degeneration (Supplementary Figure 5K). To confirm that the nerves were completely removed, we conducted immunohistochemical staining of incisors one month after surgery and failed to detect any nerve fibers in the mesenchyme (Supplementary Figure 5L, M). One week after denervation, Shh expression was significantly reduced in the mesenchyme but not the epithelium (Supplementary Figure 5F). Gli1 activity in the mesenchyme of the denervated incisor was also significantly reduced (Fig. 3D, E, H;). Interestingly, Gli1 activity in the epithelium was also reduced after denervation (n=10) (Fig. 2E). The number of LRCs was significantly reduced one month after denervation (Figure 3F, G, I). Denervation reduced the number of proliferating cells in the incisor mesenchyme as well (Figure 3J, K), consistent with previous studies (Chiego et al., 1981). Denervated incisors turned chalky about 2-3 weeks after denervation (20/20) and many of the denervated incisors fractured one month after the procedure (14/20) (Figure 3L, M). MicroCT analysis and HE staining indicated reduced enamel and dentin formation in the denervated incisor (Figure 3N, O, P, Q, R, S), consistent with previous studies (Chiego et al., 1983; Chiego et al., 1981; Kerezoudis et al., 1995; Kubota et al., 1985).

To investigate further, we performed microarray analysis of incisors two weeks after denervation (Supplementary Figure 5S-U) and found that denervation led to extensive gene expression changes in the incisor mesenchyme. One hundred and five genes were

downregulated and 185 genes were upregulated by over 1.5 fold in denervated incisors. Denervated incisors presented a distinctive transcript profile versus the controls (Supplementary Figure 5S). We performed real-time PCR to confirm changes in the expression of several candidate genes. Amelogenin (Amg), enamelin (Enam), Dspp and Dmp, which are involved in enamel and dentin terminal differentiation, were significantly downregulated in denervated incisors, consistent with the observed phenotypes (Figure 3T). Denervation led to downregulation of the Wnt signaling pathway via reduced Wnt3a expression and increased expression of Wnt signaling inhibitor Sfrp2 (Figure 3T). Wnt inhibition may be related to reduced proliferation in the mesenchyme. Interestingly, myelin basic protein (Mbp) and S100b, which are glial cell-specific genes, were significantly upregulated upon denervation (Figure 3T), possibly related to the glial cell proliferation following nerve damage (Chen et al., 2007). This result was also confirmed with immunohistochemical staining that showed an increase in glial cells surrounding the arteries one month after denervation (Supplementary Figure 5N, O). Denervation had no effect on Gli1+ cell lineage tracing or mesenchymal cell apoptosis (Supplementary Figure 5P-R).

Guinea pig molars contain quiescent label resisting cells and Gli1+ cells surrounding the NVB

So far, our data strongly suggest that Gli1+ cells surrounding the NVB are quiescent stem cells that support incisor mesenchyme homeostasis and that nerves provide a niche to maintain Gli1+ MSCs. To test this hypothesis further, we examined guinea pigs, which have continuously growing incisors and molars (Hashimoto E, 2008). In guinea pigs injected with BrdU daily for 10 days, most of the cells in the incisor mesenchyme incorporated BrdU, except for a group of cells surrounding the NVB (Fig. 4A), which we named label-resisting cells. These cells must be either quiescent or very active in cell division. Highly active cell division is excluded because the last BrdU injection was given 2 hours before collecting samples. Gli1 expression was also detectable surrounding the incisor NVB (Fig. 4B). Similar label resisting and Gli1+ cells were detectable surrounding the NVB in molars of guinea pigs (Fig. 4C, D).

Periarterial Gli1+ cells do not express classical MSC markers but give rise to NG2+ pericytes that express these markers

Based on our above data, we identified Gli1+ cells as the MSCs that support the homeostasis and injury repair of incisor mesenchyme *in vivo*. Conventionally, MSCs *in vitro* are defined based on various surface markers (Bianco et al., 2008; Dominici et al., 2006). Therefore, we conducted surface marker analysis of incisor MSCs. Surprisingly, the majority of LRCs or Gli1+ cells do not express the typical MSC markers CD105 and CD73, or other MSC-related markers NG2, CD146, CD44 and Sca1 (Figure 5A-J; Supplementary Figure 6A-C). We also analyzed the expression of nestin, which was previously proposed to be a surface marker for bone marrow MSCs (Mendez-Ferrer et al., 2010). We detected nestin expression in differentiated odontoblasts of the incisors but not in Gli1+ cells (Supplementary Figure 6D). CD34 has also been proposed to be a surface marker for a group of MSCs derived from the tunica adventitia of large arteries (Corselli et al., 2012). We failed to detect expression of CD34 in the incisor (Supplementary Figure 6E). Most CD44+ cells and CD146+ cells are Gli1+ LRCs, but the majority of Gli1+ LRCs are negative for CD44 and CD146 (Supplementary Figure 6F, G).

NG2+ pericytes have been proposed to represent a population of MSCs in the dental mesenchyme (Feng et al., 2011). We examined NG2 expression in the incisor using *NG2-DsRed* mice. NG2+ cells are pericytes immediately surrounding the CD31+ endothelium (Figure 5K). In some tissues, such as gut mesentery, NG2+ cells are distributed preferentially surrounding arterioles (Murfee et al., 2005), but in other tissues such as retina

and brain they are detectable surrounding all types of vasculature (Chan-Ling and Hughes, 2005; Zhu et al., 2008). We found that NG2+ cells are detectable in the incisor mesenchyme surrounding arterioles, veins and capillaries (Supplementary Figure 7D). These NG2+ pericytes express typical MSC markers including CD146, Sca1 and CD105 (Figure 5L-O). We analyzed the contribution of NG2+ cells to homeostasis using *NG2-Cre;ROSA26*$^{LoxP\text{-}STOP\text{-}LoxP\text{-}ZsGreen1}$ (*NG2-Cre;ZsGreen*) mice. In the incisor mesenchyme, NG2+ cells contribute mainly to the vasculature, making little contribution to the pulp mesenchyme or odontoblasts (Fig. 5P), as shown in a previous study (Feng et al., 2011). Upon injury, however, the contribution of NG2+ cells to odontoblasts significantly increased (Fig. 5Q, R).

Based on these results, we investigated the relationship between Gli1+ and NG2+ cells. It is apparent that NG2+ cells do not give rise to all Gli1+ cells (Figure 5P). In contrast, lineage tracing experiments indicated that Gli1+ cells give rise to the entire incisor mesenchyme except the CD31+ endothelium (Figure 5S, T). Specifically, Gli1+ cells give rise to all NG2+ or CD146+ perivascular cells (Figure 5U, V).

We also investigated Gli1+ and NG2+ cells in mouse molars. In contrast to incisors, adult mouse molars do not grow continuously. Adult molars do not contain Gli1+ cells (Supplementary Figure 7A), or LRCs surrounding the arteries (Supplementary Figure 7B, C). NG2+ cells are also found as pericytes surrounding all vasculature in the molars (Supplementary Figure 7E, F), and they express typical MSC markers CD146, CD105 and Sca1 (Supplementary Figure 7G-J). NG2+ cells contribute mainly to the vasculature of molars with little contribution to odontoblasts in *NG2-Cre;ZsGreen* mice (Supplementary Figure 7K). Upon injury, NG2+ cells were actively involved in reparative dentin formation (Supplementary Figure 7L, M).

Incisor MSCs are typical MSCs *in vitro* and are all derived from Gli1+ cells but not NG2+ cells

Next, we analyzed the incisor MSCs *in vitro*. Cells were obtained from the incisor mesenchyme and cultured under standard conditions. FACS analysis was performed on cultured P0 or P1 cells. These cells strongly express typical MSC markers including CD105 (79%), CD146 (52%), Sca1 (80%), CD73 (93%), CD44 (88%) and nestin (90%) (Figure 6A). They are negative for CD34 (3.8%), CD45 (3.7%), CD130 (2.5%) and CD271 (2%). Under appropriate conditions, incisor mesenchymal cells were able to differentiate into calcified tissue (Figure 6B), adipose tissue (Figure 6C) and chondrocytes (Figure 6D, E). Therefore, based on their surface marker expression profile and tri-lineage differentiation ability, incisor MSCs can be considered typical MSCs.

Using cells derived from *Gli1-LacZ* mouse incisors, we determined that incisor MSCs lost Gli1 expression rapidly after migration out of the tissue block. This reduction is probably due to loss of the *in vivo* NVB niche. Therefore, to test the contribution of Gli1+ cells to the MSCs *in vitro*, we induced *Gli1-CE;Tdtomato* mice. Mesenchymal cells harvested from the incisors of these mice were plated on a culture dish 72 hours after the first induction. Although only a small percentage of cells were labelled 72 hours after induction (Figure 2D), nearly all the cells (95%, n=3) on the culture dish were positively labelled 10 days after plating, indicating that Gli1+ cell derivatives ultimately populated the entire culture dish (Figure 6H-J). Immunohistochemical staining indicated that all CD146, CD105, Sca1 and CD73 positive cells were derived from Gli1+ cells (Figure 6K-N).

For comparison, we also evaluated the contribution of NG2+ cells to the MSCs *in vitro*. Cells were obtained from adult *NG2-Cre*^{ER}*;ROSA26*$^{LoxP\text{-}STOP\text{-}LoxP\text{-}Tdtomato}$ (*NG2-CE;Tdtomato*) mouse incisors 72 hours after induction. Although a few positive colonies

could be detected 10 days after plating, NG2+ cell derivatives only comprised a small percentage (<10%, *n*=3) of the MSC population in the culture dish (Figure 6O, P). Therefore, in agreement with our in vivo experiments, our *in vitro* data demonstrate that periarterial Gli1+ cells give rise to the entire MSC population *in vitro* and that NG2+ pericytes only represent an MSC subpopulation.

DISCUSSION

Two fundamental questions in the study of MSCs are centered on where the MSC niche is located and what the *bona fide* identity of MSCs is *in vivo*. Using the mouse incisor as a model, our study provides definitive answers to both questions.

The perivasculature has been proposed to be the niche for various types of MSCs and many other stem cells (Crisan et al., 2008; Kokovay et al., 2010; Krautler et al., 2012; Tang et al., 2008). Nevertheless, it remains largely unknown how the vasculature regulates MSCs and whether arteries, veins and capillaries comprise different MSC niches. A recent study suggested that CD34+ periarterial adventitial cells may represent a different group of MSCs than the pericytes surrounding the capillaries and that cells obtained from the adventitia can differentiate into pericytes in vitro (Corselli et al., 2012). Our results indicate that the incisor MSCs are localized surrounding arterioles and are regulated by the NVB niche. They do not express typical MSC markers or CD34+ and comprise less than 5% of the entire incisor mesenchyme. It is noteworthy that Gli1+ cells or LRCs surround only arterioles accompanied by nerves, not all arterioles (see Figure 1C, 2C), consistent with an essential role for the nerve in the MSC niche.

The mouse incisor is innervated by sensory and sympathetic nerves (Ishizuka and Hiura, 1992; Johansson et al., 1992; Tabata et al., 1998; Zhang et al., 1998) but is devoid of parasympathetic nerves (Olgart, 1996; Sasano et al., 1995). Nerve fibers accompanying the arteries are located within the periarterial region, which is similar to the tunica adventitia of free arteries. These nerves are unmyelinated fibers surrounded by S100+ glial cells (Ishizuka and Hiura, 1992; Zhang et al., 1998). We show here that Shh produced by sensory neurons of the TGG is transported through the IAN and activates Gli1 expression in adjacent periarterial mesenchymal cells (Figure 7A). The hedgehog signaling pathway is critical for the commitment of the osteoblast lineage (Rodda and McMahon, 2006). In the incisor, it is possible that the Shh signal from the sensory nerve also regulates the odontogenic commitment of incisor MSCs. Crosstalk between sensory nerves and arteries regulates the formation of the NVB during development (Lawson et al., 2002; Li et al., 2013; Mukouyama et al., 2005; Mukouyama et al., 2002). Our study indicates that this crosstalk continues into adulthood. Disruption of the NVB environment alters MSC homeostasis, as demonstrated by our denervation experiment. It remains unknown how artery components regulate the MSCs. Gli1+ cells surrounding arteries have been observed throughout the body and have been proposed to be the stem cells for the arterial walls (Majesky et al., 2012; Passman et al., 2008). Although the NVB is a common anatomical structure throughout the body, it will require further study to determine whether the NVB also functions as an MSC niche in other organs. In a previous study, Shh regulating hair bulge stem cells was provided by CGRP-;Runx3+ propioceptive sensory neurons of the dorsal root ganglion (DRG) (Brownell et al., 2011). In our study, Shh was provided by CGRP+ neurons of the TGG. Such a difference might be due to the distinct developmental origins of the DRG and TGG and the lack of proprioceptive sensory neurons in the TGG (Senzaki et al., 2010). Shh from the dental epithelium has been proposed to regulate the differentiation of dental mesenchymal cells (Seidel et al., 2010). Although our data from *K14-rtTA;tetO-Cre;Shh*$^{flox/flox}$ mice do not support this hypothesis, we do not rule out the possibility that

Shh derived from dental epithelium may regulate the dental MSCs during embryonic development.

The NVB contains more than just nerves and arteries. Glial cells and endothelium may also play roles in the MSC niche. Intriguingly, our immunostaining and real-time PCR data showed that the number of glial cells and expression of myelination-related genes increased after denervation. In addition, Wnt signaling appeared to be inhibited after denervation. Wnt inhibition may be related to the reduced proliferation in the mesenchyme. None of these effects are clearly related to Shh signaling. Denervation not only interrupts the Shh signaling pathway but also many other signals, consistent with the extensive gene expression changes shown by our microarray results. In addition, the phenotypes in the incisor after denervation or Shh inhibitor administration are similar but not identical. Although both procedures led to reduced dentin formation, the Shh inhibitor did not alter mesenchymal cell proliferation or stem cell maintenance, whereas denervation did. Therefore, Shh cannot be the only molecule secreted from the nerve that regulates MSCs. It remains unknown whether other factors, such as VEGF, also regulate MSC function (Lawson et al., 2002; Mukouyama et al., 2002). It also remains unknown whether cellular components such as glial cells or endothelium participate in niche regulation. The NVB is an intricate environment composed of multiple molecular and cellular components, and it will require future studies to elucidate their contribution to the MSC niche.

Defining MSCs has been difficult and controversial. While the definitions of most other stem cell types are based on their *in vivo* abilities to support homeostasis or injury repair (Grompe, 2012), the definition of MSCs currently relies mostly on unreliable *in vitro* assays and surface marker analysis. In addition, stem cells in other organs usually comprise a very small percentage of the entire population, but the MSC definition has included the majority of the cell population on the culture dish based on their high expression of classical MSC markers including CD73, CD105, Sca1 and others (Bianco et al., 2013; Covas et al., 2008; Crisan et al., 2008; Dominici et al., 2006; Traktuev et al., 2008).

Here we identified Gli1 as an *in vivo* MSC marker that fulfills both the rigorous *in vivo* criteria established in many other stem cell studies and the *in vitro* criteria used in traditional MSC studies. Our results indicate that the classical MSC markers may not be appropriate markers to identify MSCs *in vivo*. In the incisor, these markers define pericytes that surround all vasculature *in vivo*, but the pericytes are derived from more primitive Gli1+ MSCs that do not express these markers (see Figure 7B). The pericytes mainly function in injury repair but not homeostasis. It is possible that the pericytes are a subpopulation of MSCs that participate in emergency responses such as injury repair and their intimate association with all vasculature enables them to respond immediately to tissue injury. These observations also suggest that injury repair should be considered distinct from physiological homeostasis. These processes might be regulated by different activation mechanisms and supported by different stem cell populations. Interestingly, the differential contribution of stem cells to homeostasis and repair has also been shown in the hair follicle (Ito et al., 2005). This difference may also raise questions as to the utility of evaluating stem cell properties with transplantation assays, because transplantation is more similar to injury repair than to homeostasis and is not a physiological process.

Our study also highlights the incisor as an excellent model for studying MSCs. The significance of the mouse incisor as a stem cell model has long been overlooked likely because continuously-growing incisors are unique to rodents and adult human teeth do not grow continuously. With the establishment of Gli1 as an *in vivo* marker, we will be able to target MSCs specifically and precisely to inactivate a specific gene in order to test its *in vivo* function in regulating MSCs.

In summary, our study identifies an unexpected function for the neurovascular bundle, as an MSC niche, and clarifies the identity and functions of MSCs *in vivo* (Figure 7A, B). Using the incisor as a model, we show that MSCs originate from periarterial cells *in vivo* and are supported by the NVB niche. These periarterial cells support incisor homeostasis and give rise to the entire MSC population *in vitro*. In contrast, conventional MSC surface markers define pericytes that make only a minor contribution to incisor homeostasis *in vivo* or to the MSC population *in vitro*. These pericytes contribute mainly to injury repair but not to homeostasis. Collectively, our discoveries will have an important impact on the definition and identification of MSCs *in vivo*.

EXPERIMENTAL PROCEDURES

Animals and tamoxifen administration

All animal models (sixteen different transgenic lines), their source of origin (e.g. JAX ID#), and original references describing each of these sixteen transgenic lines are listed in Supplemental Table 1. All mouse experiments were conducted in accordance with protocols approved by the Department of Animal Resources and the Institutional Animal Care and Use Committee of the University of Southern California. Tamoxifen (TM) (Sigma) was dissolved in corn oil (20 mg/ml) and injected intraperitoneally (i.p. 10 mg daily for 3 days). EdU (200 mg/kg) was injected i.p. 2 hr prior to sacrifice.

X-gal staining

Samples from mice were fixed in 0.2% glutaraldehyde and decalcified with 20% EDTA for 2 weeks. Frozen sections of 12 μm thickness were cut prior to X-gal staining. Detection of β-gal activity in tissue sections was carried out as per standard protocol.

Immunofluorescent staining and in situ hybridization

The following antibodies were used in our study: αSMA (Abcam ab5694 1:100), β3-Tubulin (Abcam ab78078 1:1000), S100b (Abcam ab868 1:1000), Shh (Abcam ab73958 1:100), Nestin (Abcam ab6142 1:200), Th (Abcam ab6211 1:1000), CGRP (Abcam ab4901 1:1000), CD31 (BD Bioscience 550274 1:25), β-Gal (MP Biomedical NBP1-78259, 1:50), Gli1 (Novus Biological NBP1-78259, 1:25), NG2 (Millipore MAB5384, 1:200), CD146 (BD Biosciences 562230, 1:100), CD105 (BD Biosciences 555690, 1:100), Sca1 (BD Biosciences 558162, 1:100), PDGFrβ (eBiosciences 14-1402-82, 1:100), CD44 (BD Biosciences 553134 1:100), CD130 (BD Biosciences 555757 1:100), GFP (Abcam ab1218, 1:100), BrdU (Invitrogen 93-3944, ready to use). Staining was performed according to standard procedures. Shh exon2 probe was kindly provided by Andrew McMahon (Dassule et al., 2000). In situ hybridization was performed according to standard procedures.

Injury assay

The incisor injury protocol was adapted from a previous study (Feng et al., 2011). For the molar injury assay, molars were drilled with size 25 endodontic files to expose the pulp cavity and then covered with dental cement. For vasculature injury, a 28G needle was used to puncture through the mandibular foramen to damage the inferior alveolar artery.

Denervation surgery

The inferior alveolar nerve was severed using microsurgery as previously described (Chiego et al., 1981). A sham operation was performed on the other side of the same animal following the same surgical procedures except for resection of the nerve to create a control. The denervation procedure had no impact on food or water uptake or the general health condition of the mice.

Label resisting analysis

Six- to twelve-week-old guinea pigs were given i.p. injections with BrdU (150mg/kg) for 10 consecutive days. Samples were collected 2 hours after the last injection and processed for further analysis.

Incisor mesenchymal cell culture and differentiation

Incisor pulp was obtained from mouse lower incisors and the dental epithelium was carefully removed with fine forceps. The pulp tissue was minced into 0.5 mm pieces and the tissue blocks were transferred into a T25 culture flask (Corning) and incubated with αMEM +20% FBS (Gibco) containing ascorbic acid and glutamate (Gibco) at 37°C in 5% CO_2. Osteogenic, adipogenic or chondrogenic differentiation was performed as previously described (Chung et al., 2009).

Statistical analysis

SPSS13.0 was used for statistical analysis. Significance was assessed by independent two-tailed Student's *t*-test or analysis of variance (ANOVA). A *p* value of less than 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- The neurovascular bundle represents an in vivo niche for MSCs
- Sensory nerves secrete Shh to regulate MSCs surrounding the arterioles
- Classical MSC markers define pericytes, which represent an MSC subpopulation in vivo
- Pericytes contribute mainly to injury repair but not homeostasis

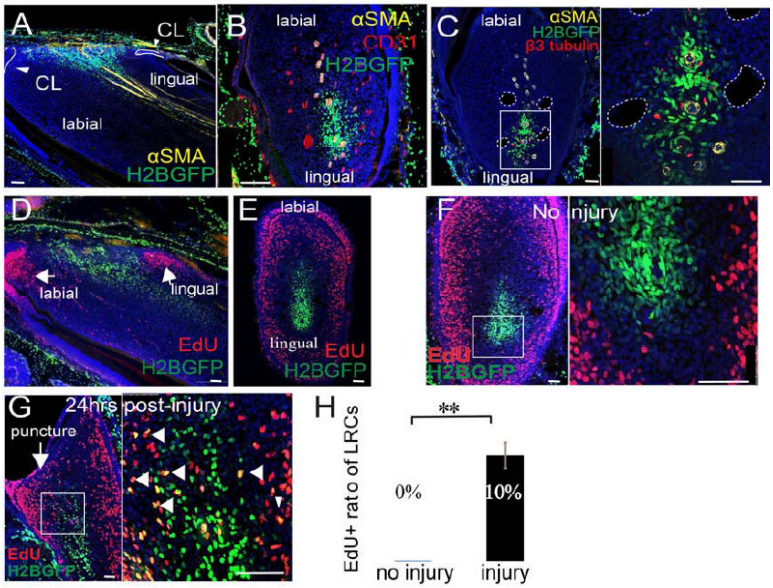

Figure 1. The neurovascular bundle (NVB) provides a niche for quiescent stem cells

A-B. Sagittal (**A**) and cross sections (**B**) of *WTH* mouse incisors chased for one month (LRCs appear green due to H2BGFP), after αSMA (yellow) and CD31 (red) immunohistochemical staining. In the sagittal sections, the apical region of the incisor is oriented to the left side. In the cross sections, the labial side of the incisor is oriented to the top. αSMA labels arteries. CD31 labels all vasculature. CL, cervical loop. **C.** β3-tubulin (red) and αSMA (yellow) staining of cross sections of chased *WTH* mouse incisors. β3-tubulin labels nerves. Boxed area is shown magnified to the right. Dotted white lines outline veins. **D-E.** Sagittal (**D**) and cross (**E**) sections of chased adult *WTH* mouse incisors treated with EdU (red). **F-G.** Chased *WTH* mouse incisors treated with EdU (red) without (**F**) or with (**G**) injury to the tooth. Arrow indicates injury site (puncture). Arrowheads indicate double labeling (yellow) of LRCs and EdU incorporation. Boxed areas are shown magnified to the right. Nuclear DAPI staining is in blue. **H.** Quantification of LRCs incorporating EdU before and after injury, as shown in **F** and **G**. Values are plotted as mean ±SEM (*n*=5, at least 500 cells were counted in each sample; **, $p<0.01$. *n*=4). Scale bars, 100μm.

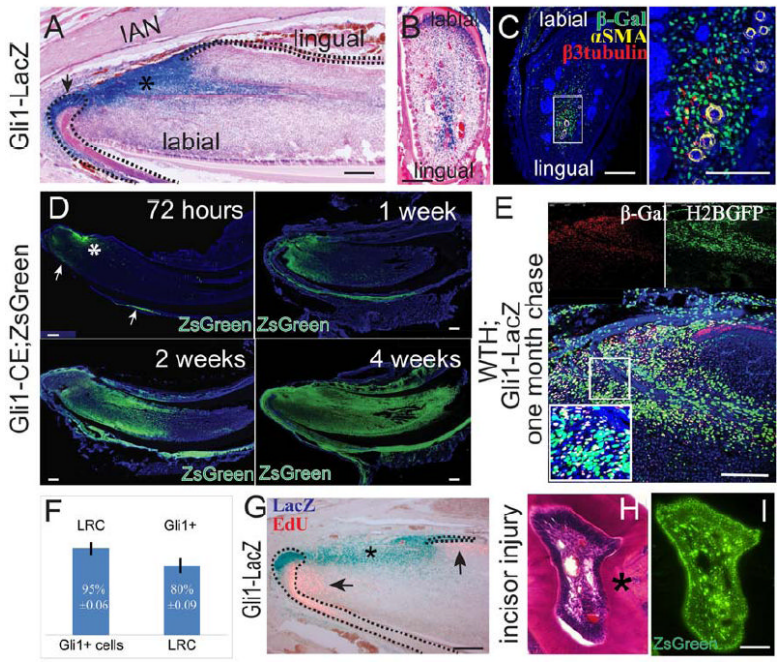

Figure 2. Gli1+ cells surrounding the NVB support tissue homeostasis and injury repair

A-C. LacZ staining (blue) of sagittal (**A**) and cross (**B**) sections; β-gal (green), αSMA (yellow), β3-tubulin (red) and DAPI (blue) immunohistochemical staining of adult *Gli1-lacZ* incisor (**C**). αSMA and β3-tubulin label arteries and nerves, respectively. Boxed area in C is shown magnified to the right. Asterisk indicates Gli1 activity in the mesenchyme. Arrow indicates Gli1 activity in the epithelium. Dotted lines outline cervical loop dental epithelium. **D.** Time course of Gli1+ cell lineage tracing (green) in adult *Gli1-CE;ZsGreen*^{flox} mice after tamoxifen induction. Asterisk indicates incisor mesenchyme derived from Gli1+ cells. Arrows indicate Gli+ cell derivatives in the epithelium. **E.** β-gal staining in chased adult *WTH;Gli1-LacZ* tetra-transgenic mouse incisors shows Gli1+ and LRC colocalization. Red β-gal staining indicates Gli1 expression. LRCs appear green from H2BGFP. The boxed area is enlarged in the inset. Yellow cells are double stained for Gli1+/LRCs. **F.** Quantification of results from panel **E**. 95%±0.06 of Gli1+ cells are LRCs, whereas 80%±0.09 of LRCs are Gli1+. Values are plotted as mean ±SEM (*n*=4). **G.** LacZ (blue) and EdU (pink) staining of incisors from 6-week-old *Gli1-LacZ* mice. EdU was injected two hours before collecting samples. Dotted lines outline cervical loop dental epithelium. **H-I**. H&E staining (H) and fluorescent image (I) of incisor cross sections from 4- to 6-week-old *Gli1-CE;ZsGreen* mice after incisor injury. Asterisk indicates reparative dentin formation. Images from adjacent sections show the contribution of Gli1+ cells to reparative dentin formation. Scale bars, 100 μm

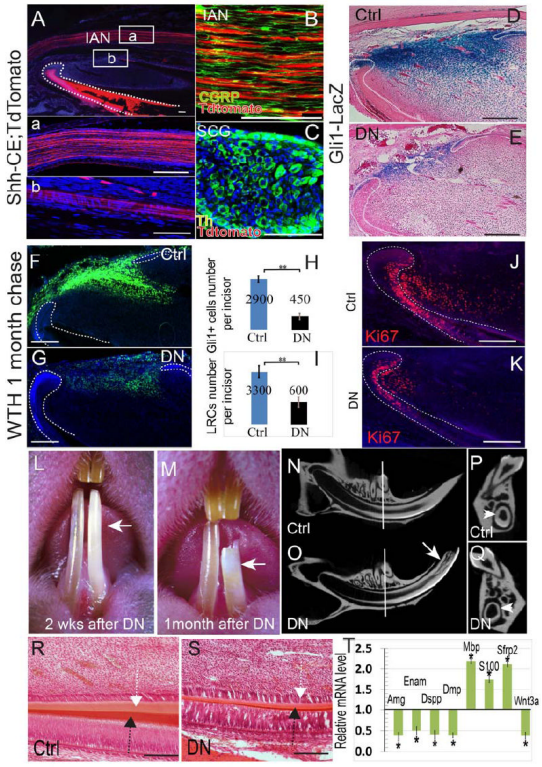

Figure 3. Sensory nerves provide Shh to Gli1+ cells. Denervation disrupts the incisor MSC niche and causes abnormal phenotypes

A. Adult *Shh-CE;TdTomato* mouse incisors 2 weeks after tamoxifen induction. Boxed areas (a) and (b) are shown magnified below. Note the Shh expression (red) in the inferior alveolar nerve (IAN) (a) and nerve bundles accompanying the artery (b). Dotted lines outline the dental epithelium. DAPI staining is in blue. **B.** Sensory nerve marker CGRP staining (green) of the IAN in adult *Shh-CE;TdTomato* mouse incisors. **C.** Th staining (green) of the superior cervical ganglion (SCG) in adult *Shh-CE;TdTomato* mouse incisors. The SCG is negative for Shh activity (red). Th staining labels sympathetic neurons. **D- E.** LacZ staining (blue) of control (Ctrl) or denervated (DN) *Gli1-lacZ* incisors indicates significantly reduced Gli1 activity after denervation. Dotted lines indicate the cervical loop epithelium. **F-G.** LRC (green) of control (Ctrl) or denervated (DN) *WTH* incisors one month after chasing. Dotted lines outline the dental epithelium. **H-I.** Quantification of results from panels D-E (H) and F-G (I). Values are plotted as mean ±SEM (**, $p<0.01$; $n=5$). **J-K.** Ki67 staining shows fewer proliferating cells in the mesenchyme of denervated incisors (K) as compared with control (sham-operated) incisors (J). **L-M.** Denervated incisors (arrow) turn chalky white 2 weeks after surgery (L; $n=20$) and fracture within a month (M; $n=14$). **N-Q**. Longitudinal micro-CT images of sham-operated (control) incisor (N) and denervated incisor (O). Arrow indicates the fracture site. Cross sections (P, Q) were sampled at comparable positions, indicated by white lines in N-O. Arrowheads indicate the dentin wall of the incisors. **R-S**. HE staining of control (Ctrl) and denervated (DN) incisor longitudinal sections after one month. Note the reduced thickness of the enamel (black arrow) and dentin (white arrow). **T.** Real-time PCR data of indicated genes in denervated incisors compared to control incisors. Values are plotted as mean ±SEM (*, $p<0.05$; $n=4$). Scale bars, 100 μm.

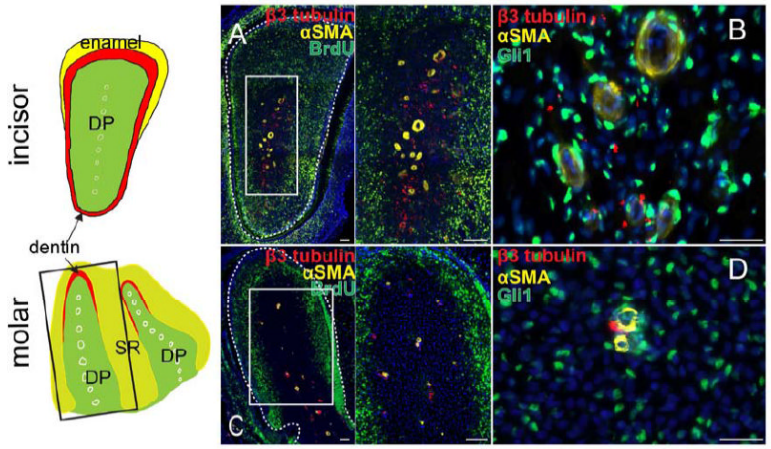

Figure 4. Guinea pig molars contain quiescent label resisting cells and Gli1+ cells surrounding the NVB

Schematic drawing depicts a cross section of the anatomy of the guinea pig incisors (top) and a horizontal section of the anatomy of the guinea pig molars (bottom). White circles outline arteries in the dental pulp (DP, green). Box indicates the approximate position of panel C. Enamel, yellow. Dentin, red. SR, stellate reticulum (light green). **A**. Cross section of guinea pig incisor injected continuously with BrdU (green) for 10 days and stained with αSMA (yellow) and β3-tubulin (red). Boxed area is shown magnified to the right. **B**. Immunohistochemistry for αSMA (yellow), β3-tubulin (red) and Gli1 (green) in guinea pig incisor. **C**. Horizontal section of guinea pig molar injected continuously with BrdU (green) for 10 days and stained with αSMA (yellow) and β3-tubulin (red). Boxed area is shown magnified to the right. **D**. Immunohistochemistry for αSMA (yellow), β3-tubulin (red) and Gli1 (green) in guinea pig molars. Dotted lines outline dental pulp. Scale bars, 100 μm.

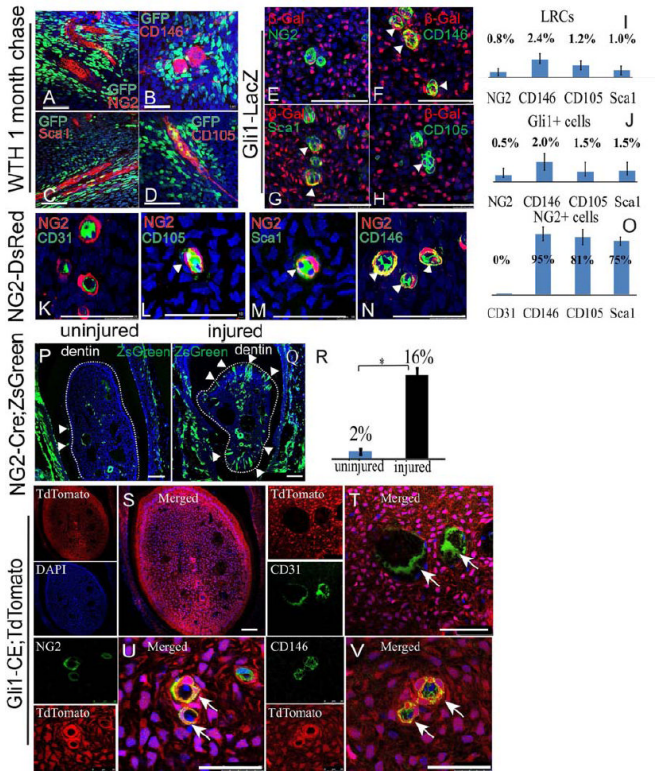

Figure 5. Gli1+ cells do not express classical MSC markers. NG2+ cells are pericytes derived from Gli1+ cells that express classical MSC markers and contribute mainly to injury repair but not homeostasis

A-D. Immunohistochemical staining of CD146, CD105, Sca1 and NG2 (red) in chased adult *WTH* mouse incisors (LRCs appear green due to H2BGFP). **E-H** Co-immunohistochemical staining of β-Gal (red) with MSC markers (green) NG2, CD146, Sca1 and CD105 in *Gli1-LacZ* mouse incisors at 1 month of age. Colocalization appears yellow, indicated by arrowheads. **I-J.** Quantification of results from A-H indicates that the majority of LRCs and Gli1+ cells are negative for MSC markers. Values are plotted as mean ±SEM. **K-N.** Immunohistochemical staining of CD31 and MSC markers (green) including CD105, Sca1 and CD146 in the incisor mesenchyme of *NG2-DsRed* mice. Yellow indicates coexpression (arrowheads). DAPI is in blue. **O.** Quantification of results from K-N indicates that NG+ cells are pericytes expressing MSC markers. Values are plotted as mean ±SEM. **P-Q.** NG2 derived cells (green) in the incisor mesenchyme of *NG2-Cre;ZsGreen* mice untreated (**P**) or 3 weeks after injury (**Q**) (*n*=6). Arrowheads indicate odontoblasts derived from NG2+ cells. Dotted line outlines pulp chamber. **R.** Quantification of the percentage of NG2 derived odontoblasts from P-Q. Values are plotted as mean ±SEM. (*, $p<0.05$, *n*=6). **S.** Single and merged staining of cross section of *Gli-CE;TdTomato* incisor one month after tamoxifen induction. TdTomato fluorescence (red) marks cells derived from Gli1+ cells. DAPI is blue. **T**. Endothelium marker CD31 (green) immunostaining of incisors from *Gli-CE;TdTomato* mice 1 month after tamoxifen induction. Arrows indicate CD31+ endothelium is not derived from Gli1+ cells. **U-V**. Immunohistochemical staining (green) of NG2 (**U**) and CD146 (**V**) in *Gli-CE;TdTomato* mouse incisor 1 month after induction indicates that NG2+ and CD146+ cells are derived from Gli1+ cells. Colocalization appears yellow, indicated with arrows. Scale bars, 100 μm.

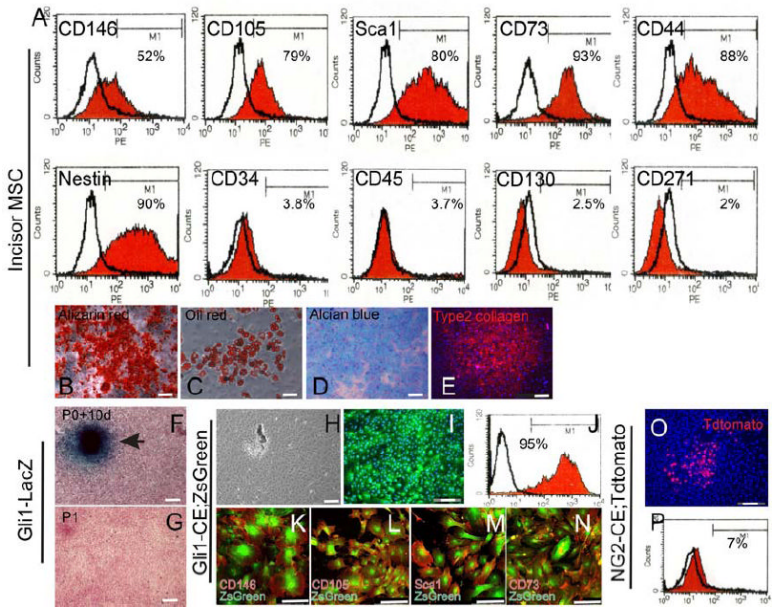

Figure 6. Incisor MSCs derived from Gli1+ cells are typical MSCs *in vitro*

A. FACS analysis of cells obtained from cultured incisor mesenchymal cells indicates they strongly express CD146, CD105, Sca1, CD73, CD44 and nestin, but are negative for CD34, CD45, CD130 and CD271. **B**. Alizarin red staining of cultured incisor mesenchymal cells 2 weeks after osteogenic induction. **C**. Oil red staining of cultured incisor mesenchymal cells 2 weeks after adipogenic induction. **D-E.** Alcian blue staining (**D**) and type II collagen staining (**E**) of incisor mesenchymal cells 1 month after chondrogenic induction. **F, G**. LacZ staining of cultured cells obtained from *Gli1-LacZ* mouse incisors at 10 days after plating (**F**) or at P1 (**G**). Cells were counterstained with nuclear fast red. **H-J**. Phase contrast (**H**) and fluorescent images (**I**) of cultured incisor mesenchymal cells from adult *Gli1-CE;ZsGreen* mice. Cultures were analyzed 10 days after plating. ZsGreen fluorescence indicates cells derived from Gli1+ cells. FACS analysis indicates that approximately 95% of cells on the culture dish are derived from Gli1+ cells (**J**). **K-N.** Immunohistochemical staining of MSC markers CD146, CD105, Sca1 and CD73 (red) in cultured incisor mesenchymal cells from adult *Gli1-CE;ZsGreen* mice. Cells expressing these MSC markers are all derived Gli1+ cells. **O-P.** Cultured incisor mesenchymal cells from *NG2-CE;TdTomato* mouse incisors 72 hours after induction. Cultures were analyzed 10 days after plating. TdTomato fluorescence indicates cells derived from NG2+ cells (**O**). FACS analysis indicates that approximately 7% of cells on the culture dish are derived from NG2+ cells (**P**).

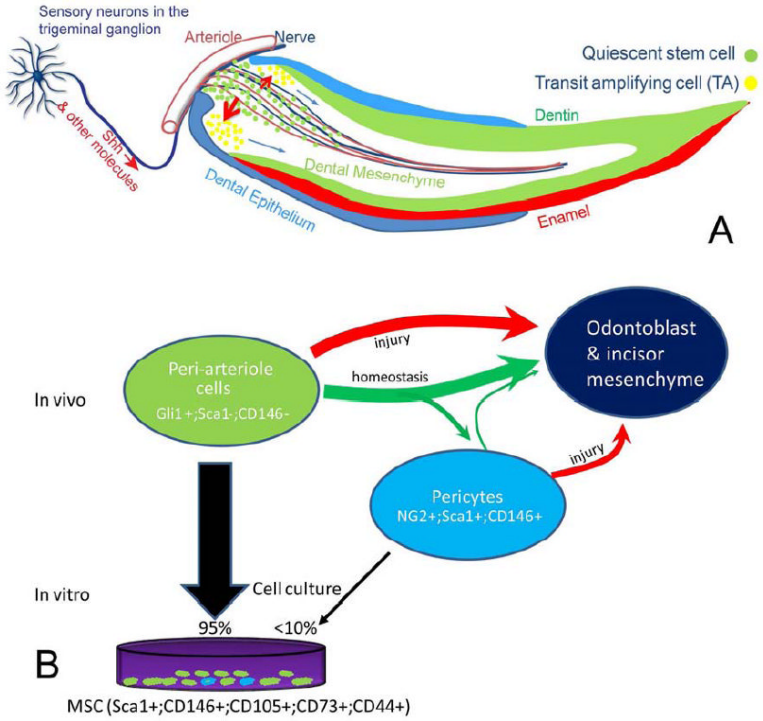

Figure 7. Schematic diagram of our model of the NVB niche and the *in vivo* origin of incisor MSCs

A. The NVB provides a niche to support the continuous turnover of incisor mesenchyme. Sensory neurons in the trigeminal ganglion produce Shh, which is transported through axons into the incisor mesenchyme. Shh activates Gli1 expression in the quiescent stem cells surrounding the arterioles near the cervical loop region and regulates the odontogenic differentiation process. These quiescent stem cells continuously give rise to actively dividing TA cells, which then differentiate into odontoblasts and all other dental mesenchymal derivatives to support the rapid cellular turnover of the incisor. **B.** *In vivo* origin of the incisor MSCs. The Gli1+ cells surrounding the NVB are the most primitive MSC population. They continuously give rise to odontoblasts under both homeostasis and injury repair situations. The majority of Gli1+ cells do not express classical MSC markers including CD146, CD105 and Sca1. NG2+ cells are pericytes surrounding all vasculature and express classical MSC markers. NG2+ cells are an MSC subpopulation derived from Gli1+ cells. They mainly function in injury repair but not in homeostasis. Incisor MSCs on the culture dish are entirely derived from periarterial Gli1+ cells but only a few are derived from NG2+ cells. In summary, incisor MSCs originate from periarterial cells *in vivo* and are supported by the NVB niche.