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Tooth, hair and claw: comparing epithelial stem cell niches of ectodermal appendages

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Abstract

The vertebrate ectoderm gives rise to organs that produce mineralized or keratinized substances, including teeth, hair, and claws. Most of these ectodermal derivatives grow continuously throughout the animal's life and have active pools of adult stem cells that generate all the necessary cell types. These organs provide powerful systems for understanding the mechanisms that enable stem cells to regenerate or renew ectodermally derived tissues, and remarkable progress in our understanding of these systems has been made in recent years using mouse models. We briefly compare what is known about stem cells and their niches in incisors, hair follicles, and claws, and we examine expression of *Gli1* as a potential example of a shared stem cell marker. We summarize some of the features, structures, and functions of the stem cell niches in these ectodermal derivatives; definition of the basic elements of the stem cell niches in these organs will provide guiding principles for identification and characterization of the niche in similar systems.

Keywords

stem cells; niche; tooth; hair; nail; claw; ectodermal derivatives; *Gli1*

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Competing interests statement

The authors declare no competing financial interests.

Several ectodermally derived follicular structures, such as teeth, hair and nails, produce mineralized or keratinized substances that are secreted throughout the animal's life. The functions of these appendages include physical protection, camouflage, thermal insulation, feeding, and sensory perception. Comparing these three organs is useful for several reasons. First, a number of human genetic diseases, notably the ectodermal dysplasias, affect teeth, hair and nails in patients. Second, abnormalities of these organs can be readily detected because of their accessible exterior location, and this accessibility also can enable experimental procedures. Third, their relatively simple physiology makes these organs convenient models for deciphering the molecular underpinnings of postnatal renewal and regeneration. This is further facilitated by being able to capture all cell types, at successive stages of differentiation, in a single histological section.

Ectodermal appendages originate from an ectoderm-derived epithelium and a neural crest or mesoderm-derived mesenchyme [1–3]. Sequential epithelial-mesenchymal interactions drive development of these organs, which occurs in three phases: initiation, morphogenesis, and differentiation [4]. An epithelial thickening appears prior to an invagination into the mesenchyme. The mesenchyme then condenses into a papilla, followed by differentiation of specific epithelial and mesenchymal cell types. The morphogenesis of tooth, hair and claw utilize many of the same signaling pathways, as reviewed elsewhere [5–9], whereas differentiation of the mineralized or keratinized components is governed by organ-specific events.

The regenerative ability of tooth, hair and claw, as well as many other adult tissues, is dependent on tissue-specific stem cell (SC) populations that self-renew to maintain stable numbers and that possess the capacity to differentiate into distinct cell lineages. Adult SCs usually reside in a niche, which is a physiologically limited microenvironment whose nature and location vary, depending on the tissue [10]. Deciphering the signaling pathways that control the delicate balance between self-renewal and differentiation is fundamental to understanding how SCs are regulated in their niches.

Here, we provide an overview and comparison of the structures and signaling pathways involved in renewal and regeneration of mouse incisors, hair follicles and claws. Additionally, to illustrate potential similarities between these systems, we have compared the expression of *Gli1*, a known dental SC marker, in tooth, hair and claw.

Incisors

Teeth are used to catch and chew food and, in some species, for defense. The dentition of mammals encompasses great diversity. In contrast to humans, who replace their deciduous teeth, mice have only one set of teeth in their lifespan, consisting of four incisors and twelve molars. In some rodents, all teeth grow continuously, but in mice only the incisors grow continuously throughout the life of the animal.

The ability of the incisor to grow depends on the presence of epithelial and mesenchymal SCs that have the capacity to self-renew and differentiate into all cell types of the adult tooth (Fig. 1A). SCs located in the labial cervical loop (laCL) contribute to a population of transit-amplifying (T-A) cells that undergo several rounds of cell division before they move distally

and differentiate into ameloblasts or stratum intermedium cells [11]. The ameloblasts secrete enamel matrix that mineralizes. The pre-odontoblasts, which are derived from SCs in the mesenchyme, are located adjacent to the inner dental epithelium and give rise to dentin-secreting odontoblasts that maintain contact with the basement membrane. Rodent incisors do not have a typical crown or root but rather possess a crown-like labial (near the lip) surface covered by enamel and a root-like lingual (near the tongue) surface where enamel is absent. Periodontal ligament and alveolar bone are derived from the mesenchyme and anchor the teeth to the bones in the jaw.

Slow-cycling SC populations have been identified using label retention experiments, either through injection with BrdU or with transgenic mice harboring a tetracycline-sensitive, histone 2B(H2B)-GFP cassette under the control of a tissue specific trans-activator [11, 12]. Label-retaining cells (LRCs) in the mouse incisor are found in the most proximal region of the mesenchyme and in the proximal halves of both the labial and lingual cervical loop. A number of genetic markers of dental SCs have been identified in recent years using *in vivo* lineage tracing. SCs expressing *Gli1* (Fig. 2A) or *Bmi1* reside in the LRC-containing regions of both epithelium and mesenchyme, whereas *Sox2* marks SCs exclusively in the laCL [12–14]. How much the epithelial SC populations marked by these three factors overlap, or whether there exists a hierarchical relationship between them, remains to be determined. Expression of *Lgr5* is found in a number of cells in the stratum intermedium of the laCL [15, 16]. In culture, these cells act like dental epithelial SCs [16]. As *Lgr5* expression does not colocalize with the slow-cycling SCs, it has been suggested that this gene marks a subpopulation of active epithelial SCs. Recently, based on *in vitro* assays and *in vivo* expression analysis, integrin $\alpha 6$ (CD49f) was also suggested to be a marker of epithelial SCs [14, 17, 18].

Several signaling pathways regulate SCs in the incisor, including the FGF, BMP/TGF- β , Notch, and Hedgehog (HH) cascades; these have been reviewed in detail elsewhere [5] and are only briefly described here. Mesenchymal FGF3 and FGF10 and epithelial FGFR1b and FGFR2b levels regulate the size and shape of the epithelial SC niche [19, 20]. Follistatin (*Fst*) regulates *Fgf3* expression [19], and deletion of *Tgfbr1* (*Alk5*) in the mesenchyme led to down-regulation of *Fgf3*, *Fgf9*, *Fgf10*, and a reduced laCL size [21]. Sprouty genes, which are FGF inhibitors, prevent the generation of ectopic ameloblasts by the lingual CL SCs [22]. Furthermore, FGF signaling regulates E-cadherin expression, cell proliferation and migration in the incisor [23] and is essential for self-renewal of the epithelial SCs [16].

Notch signaling is also an important regulator of SCs. *Notch1* and *Notch2* are expressed in the incisor epithelium and mesenchyme, whereas *Notch3* is restricted to the mesenchyme [11]. Inhibition of Notch signaling led to a reduction in the size of the laCL in explant experiments [24]. The SHH signaling pathway regulates SC progeny formation, such that the differentiating progeny of epithelial SCs express *Shh*, which signals back in a positive feedback fashion to the SCs [12]. Inhibition of HH signaling disrupts the generation of ameloblasts but not of another cell type, the stratum intermedium, from *Gli1*-positive SCs. Interestingly, the SCs do not appear to require HH signaling for survival. Finally, miRNAs play a role in the renewal and differentiation of adult SCs during SC-fueled incisor growth

[13, 25], and how miRNAs regulate SCs in the tooth is an exciting new topic that is beginning to be explored.

Hair

A defining characteristic that distinguishes mammals from other vertebrates is that their skin is covered with hair, at least in part and at some point during their lifetime. Hair serves various functions, including protection of the body and sensory organs against environmental stresses, regulation of body temperature, sensory perception, facilitation of social interactions, and camouflage.

Each hair has two main portions. The visible part is the hair shaft that projects outside the skin and consists of dead keratinized cells. The part embedded into the skin, from which the hair shaft emerges, is the hair follicle; this is the living portion of the hair. As with developing teeth, both the development and the regeneration of hair during adulthood are tightly regulated by signal interactions between ectodermal epithelium and the underlying mesenchyme. The initial development of the hair follicle occurs in successive, morphologically distinguishable stages [7, 8, 26, 27]. In the mouse, epithelial cells start to form a placode that can be observed at day 14 of embryonic development (E14). Mesenchymal cells start to condense around the epithelial placode, and cell proliferation leads to formation of the dermal papilla in the mesenchyme and down-growth of the epithelial hair germ (E15.5). Following further down-growth (E16.5), the dermal papilla is almost entirely surrounded by keratinocytes. The subsequent bulbous peg stage (E18.5) is characterized by differentiation of epithelial keratinocytes to consecutively form the concentric epithelial layers that make up the mature hair follicle. The outermost of these layers, the outer root sheath (ORS), maintains contact with the basal layer of the epidermis and, in the bulge region, houses the hair follicle stem cells (HFSC). The layers of the inner root sheath (IRS) function as a channel for the emerging hair. The first hairs of the murine coat become visible around day 4 of postnatal development.

Throughout life, hair follicles undergo degeneration and regeneration in a cyclic manner. The hair follicle bulge, the distally adjacent, narrower region of the isthmus, and the infundibulum, the portion of the hair follicle located above the opening of the sebaceous duct, comprise the permanent portion of the hair follicle (Fig. 1B). During anagen, the growth phase, HFSCs give rise to highly proliferative T-A matrix cells, which in turn produce the differentiated cells that make up the new hair shaft and circumjacent IRS layers. Epithelial cells encompass the dermal papilla, and the entire hair follicle is dramatically elongated. During the following catagen phase, the proximally extended epithelium undergoes apoptosis and regresses, and the dermal papilla becomes re-positioned close to the permanent part of the hair follicle. Before re-entering anagen, the permanent portion of the hair follicle rests in relative quiescence in telogen stage.

HFSCs were initially discovered as LRCs in the bulge [28–30], and subsequent studies showed that these cells self-renew, are multipotent, and are able to reconstitute hair follicles when grafted together with dermis cells [31, 32]. Based on their expression within the bulge, integrin $\alpha 6$ and CD34 were proposed as SC markers, and these have been widely used for

sorting HFSCs by flow cytometry [33]. Lineage tracing studies using tamoxifen-inducible Cre lines identified *Krt14* [34], *Krt15* [35], *Lgr5* [36], and *Gli1* [37] as markers expressed in HFSCs. Furthermore, hair follicle cells are derived from cells that express *Shh* or *Sox9* during embryonic development [38, 39]. Cells within the bulge show non-overlapping patterns of expression of the proposed SC markers, and *Lgr5* marks cycling cells rather than LRCs, which points to the existence of sub-populations of cells that may be heterogeneous with regard to their regeneration potential [36, 40]. HFSCs are located in the mid-region of the permanent portion of the hair follicle during active phases, and in closer proximity to the dermal papilla during the quiescent telogen phase.

Cyclic regeneration of hair follicles in the adult skin is tightly regulated by the interplay of numerous signaling pathways. This process involves extensive interactions between the SCs in the bulge and the surrounding mesenchymal compartment. Most of these signaling interactions are very similar to the ones that guide hair follicle morphogenesis [32]. Wnt signaling plays a dominant role during induction, patterning and morphogenesis of the hair follicle and is thought to constitute the first inductive signal for hair follicle formation. Numerous gain and loss of function studies have identified Wnt signaling as a driving force for HFSC activation and anagen entry [41]. Both overexpression of Wnt signaling and upregulation of HH signaling cause early anagen entry, hyperproliferation and formation of hair follicle tumors ([41, 42] and references therein). In the normal hair follicle, HH activity is dramatically increased during anagen and is required for regulating hair follicle re-growth [43, 44]. In contrast, BMP signals from the dermis and underlying adipocytes repress HFSC activation [45, 46]. During the hair cycle, BMP2 and BMP4 expression occurs periodically but out-of-phase with peaks of Wnt/ β -catenin activation. The balance between both signaling pathways is critical and divides telogen into a refractory phase (BMP high, Wnt low) and a phase competent for hair regeneration (BMP low, Wnt high) [46, 47]. Furthermore, transient TGF-beta activation during late telogen through signals from the dermal papilla antagonizes BMP signaling in the HFSCs [48]. FGF7 and FGF10 also promote the shift to hair follicle regeneration that has been proposed to occur in two phases, involving activation of hair germ cells during the initial stage [49]. FGF18, in contrast, is crucial for maintaining the telogen phase and has repressive effects on the SCs in the bulge [32, 50].

Claws

Nails and claws provide protection to fingertips and toes and are used for climbing, self-defense, and manipulating food. Despite being easily accessible, these small organs have not been the focus of much study to date. However, as evidence has emerged pointing to a requirement for partial retention of the nail organ for mammalian digit regeneration [51, 52], the mechanisms underlying nail renewal have attracted more attention [53].

The nail unit [54], including the proximal nail fold, nail matrix, nail bed, and hyponychium, produces the nail plate (Fig. 1C). In the adult mouse, the ventral matrix consists of multiple layers of proliferating keratinocytes. These express epithelial keratins and eventually undergo apoptosis, migrating distally to produce keratinized layers of the nail plate [55]. The nail bed, which is more distal, consists of only 2 to 3 cell layers. A granular epithelium

(hyponychium) starts after the nail plate detachment and ends at the distal groove. On the opposite dorsal side, the ventral matrix reflects outward to cover the proximal nail plate. The apical matrix becomes a granular ventral epithelium (eponychium), which joins the digit epidermis and produces the cuticle.

The nail stem cell (NSC) niche has not yet been well-defined. In BrdU pulse-chase experiments in mice, LRCs were described in the basal cell layer of the distal nail matrix adjacent to the nail bed [56]. In human fetal nails, which have a tissue architecture that is similar to the mouse claw [57], NSCs were suggested to be located in the ventral proximal nail fold [58] based on expression of follicular SC markers (KRT15, KRT19 and PHLDA1). Recently *in vivo* lineage tracing of *Krt14*-expressing cells provided further evidence that self-renewing SCs that sustain tissue growth are located in the proximal matrix [53].

Surprisingly, these cells are highly proliferative and express Ki67 [53], similar to *Lgr5*-expressing SCs in the intestine [59]. The *Krt14*-expressing cells in the proximal nail matrix are also positive for KRT17, a marker not expressed in the distal matrix, and double positive cells showed the highest regeneration potential based on their ability to form colonies *in vitro* [53].

Whether progenitors that normally facilitate nail growth also play a role in the regeneration of mammalian digits after amputation is a question of major interest. Conditional inactivation of β -catenin or Wntless in *Krt14*-expressing cells revealed an essential role for Wnt signaling in nail differentiation [53]. These mutants not only failed to produce a nail structure, but also were unable to regenerate bone after amputation of the digit tip distal to the SC niche. High levels of Wnt pathway activation can be observed in the distal nail matrix, but not in the SC-containing proximal part. Wnt pathway activation has an important role in the distal nail matrix in attracting the nerves required for promoting mesenchymal blastema growth.

Discussion

Almost 200 years ago, the naturalist de Blainville established his theory of the vertebrate “phanere” (phaneros in ancient Greek meaning “apparent”) as a “follicular organ...wherein the produced solid portion is excreted... and constantly remains on the surface of the animal” [60]. He was the first to consider teeth as components of the integumentary system, similar to hair, nail, claw, hoof, feather, horn and antlers, all of which were believed to be inorganic secretions [61, 62]. Today we know that, in addition to their externally visible, keratinized or mineralized acellular portion, teeth, hair, and nails are in fact very much alive, and they undergo stem-cell fuelled, directional growth as part of their normal homeostasis. This growth is a continuous renewal, in the case of the murine incisors and claws, whereas hair follicles show cyclic regeneration. By comparing the SCs and their niches in the mouse incisor, hair follicle, and claw, some common features, structures, and functions can be identified.

Location of the stem cells

Incisors, hair follicles and nails share a common tissue origin as ectodermal derivatives, and they undergo similar morphogenetic programs [5, 8, 26, 63]. These developmental parallels

likely account for some of the similarities in their SC niches. In both tooth and nail, which continuously renew, SCs are located at the most proximal end, with the forming progeny progressing towards the distal aspect (Fig. 1A, C). In contrast, in the active hair follicle, the slowly cycling, label-retaining bulge stem cells are concentrated mostly in the mid-region of the permanent portion of the hair follicle (Fig. 1B). During the quiescent phase, these HFSCs are located in closer proximity to the mesenchymal dermal papilla. When activated, the organ first becomes extended in a proximal direction, from where a pool of T-A cells gives rise to differentiating cells that start moving distally to form a new hair shaft.

Some similarities in the components of the niches of these three organs have been identified. Epithelial (E)-cadherin is a cell-cell junctional protein essential in promoting morphogenesis and maintaining skin and its appendages [64, 65]. E-cadherin has recently been described as an important factor for the maintenance of incisor SCs as well [23]. Similarly, integrin alpha-6 marks incisor SCs [17, 18], and this protein is also expressed around the hair bulge [32] and in the basal layer of both nail matrix and epidermis [66].

The relationship between nerves and SCs in these organs is intriguing, as nerves are known to regulate the niche of certain SCs in mammals [67]. It has recently been shown that digit tip regeneration depends on nerves and mesenchymal SCs beneath the nail bed [53]. In the hair, the arrector pili muscle, inserted on the bulge, is stimulated by the sympathetic nervous system. The upper domain of the hair follicle bulge is *Gli1* positive (Fig. 2), and this expression has been proposed to be due to SHH produced by nerves [37]. In the case of the tooth, the role of the nerves as potential sources of SHH is an area of active investigation.

Cycling status

SC pools with different cycling properties may be present in all three organs. In the incisor, so far only a group of slow cycling SCs in the proximal cervical loop has been identified, although as discussed above, hints that subpopulations of SCs may exist are beginning to emerge. In contrast, the SCs in the claw appear to turn over rapidly [53], and the role of the LRCs identified further distally in the nail bed remains unclear at present. In the case of hair, both slow and fast cycling SCs have been identified [28, 35, 36, 68]. At the beginning of anagen, hair germ cells are the first to be activated, and these start proliferating prior to activation of their progenitors in the bulge to regenerate the hair follicle [48, 49]. Early stem cell descendants cycle slower than further committed progeny, and both were shown to re-enter the bulge, as stem and niche cells respectively, at the end of the cycle [69]. A recent study using intravital microscopy for *in vivo* lineage tracing elegantly demonstrated the influence of spatial location within the niche on stem cell fate [70]. Furthermore, ablation experiments revealed that stem cells in the HF bulge are dispensable for HF regeneration and demonstrated a remarkable plasticity of epithelial HF cells, which are able to regenerate the missing SC population. Similar dynamics may exist in the incisor and nail systems but have not been studied to date.

Signaling and markers

The major signaling pathways that are responsible for orchestrating development and regeneration throughout the body, including FGF, HH, Wnt, BMP/TGF- β , and Notch, are

either known or presumed to be active in the organs under discussion here. *Gli1* is an important marker that has been demonstrated to label SCs in both the incisor and the hair follicle. Interestingly, in both systems there are multiple, physically or compartmentally separated pools of cells that express *Gli1*. In the incisor, expression is found in the LRC-containing regions of both labial and lingual epithelial SC niches as well as in the proximal mesenchyme. In the telogen hair follicle, cells in the upper bulge, the hair germ and the mesenchymal dermal papilla are *Gli1*-positive. This co-expression in (1) slowly cycling SCs, (2) cells with a capacity to quickly respond to anagen-inducing cues [49], and (3) cells that constitute a signaling center crucial for regulating cyclic growth is intriguing and points to a role for HH signaling in coordinating SC-regulating signals across the entire organ.

To investigate *Gli1* as a potential marker for SCs in the nail system, we analyzed expression in the adult murine claw (Fig. 2). Interestingly, *Gli1* marks a small group of cells near the proximal nail matrix where the fast cycling, *Krt14*-expressing, KRT17-positive SCs are housed [53], pointing to potential conservation of *Gli1* as a marker for SCs in all three systems. Genetic lineage tracing studies in the nail will be required in the future to definitively determine if *Gli1* marks SCs in this organ as well. While *Gli1* itself is dispensable [71], HH signalling is required for tooth and hair follicle morphogenesis [72–74], as well as for generation of ameloblasts during adult renewal of the incisor and for anagen entry during the hair cycle and maintenance of molecular identity of the hair follicle stem cells [12, 37]. Expression of *Gli1*, which marks cells that respond to HH signalling [75], in the stem cell-containing region of the nail unit suggests a role for the HH pathway also during renewal of the nail. The precise role of HH signaling in this organ, and whether HH regulation directs a conserved function of the stem cells in all three organs, remains to be elucidated. In contrast to HH signaling, which appears to be active in all three organs, Wnt signaling is active in hair and nail, but at least based on the existing data, does not seem to be active in the incisor epithelium. It is interesting to note that the Wnt target gene *Lgr5*, which marks cycling SCs in the hair follicle, has been reported to be expressed in the laCL epithelium [15, 16]. However, lineage tracing from *Lgr5*-positive cells has not yet been performed in either the incisor or the claw. Comparison of the role of Wnt and HH signaling, as well as of other signaling pathways such as FGF and BMP, will likely reveal additional similarities between tooth, nail and hair epithelial SCs in the future.

In summary, the three organs discussed here have many similarities in terms of SC organization and function, as well as some important differences. By comparing SCs and their niches in these related yet distinct organs, we will obtain further insight into the myriad ways that renewal and regeneration are regulated.

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Highlights

- Mouse incisor, hair and claw utilize stem cell-based continuous growth and have similar embryonic origins and architectures
- Comparing the stem cell niches of these organs will improve our understanding of the mechanisms that enable renewal or regeneration in mammals
- Gli1 is potentially a common stem cell marker for mouse tooth, hair and nail

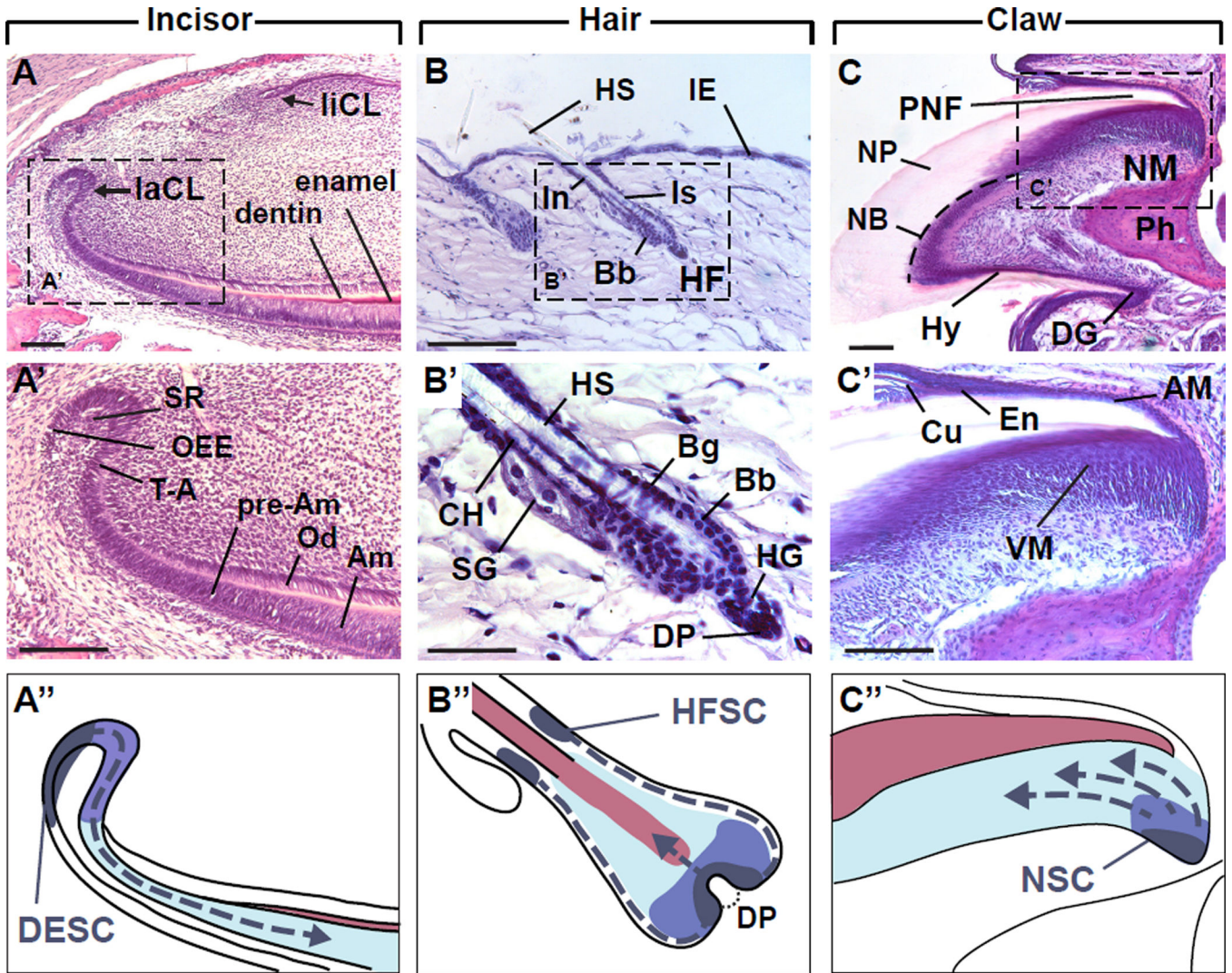


Figure 1. Stem cell location and direction of progeny movement in adult mouse incisor, hair and claw

(A) In the proximal part of the incisor, the labial cervical loop (laCL) gives rise to enamel-producing ameloblasts, whereas on the lingual cervical loop (liCL) side, dentin is the only mineralized tissue. (A') Stem cells in the laCL produce T-A cells, which differentiate into pre-ameloblasts (pre-Am). Subsequently, functional ameloblasts (Am) produce enamel, while the mesenchymal odontoblasts (Od) produce dentin. The SCs are housed in the stellate reticulum (SR) and outer enamel epithelium (OEE). (A'') The dental epithelial stem cell (DESC) niche is located in the proximal part of the laCL, and the progeny cells differentiate as they move distally. (B) The hair shaft, which protrudes from the interfollicular epidermis (IE), is produced by the hair follicle (HF) which consists of several portions: infundibulum (In), isthmus (Is), and bulb. (B') Telogen hair follicle (HF) with sebaceous gland (SG) and club hair (CH). The bulb (Bb), hair germ (HG) and mesenchymal dermal papillae (DP) are located below the hair follicle bulge (Bg). (B'') The hair follicle stem cells (HFSC) are located in the outer root sheath in the bulge region. During anagen, the T-A matrix cells generate a new HS and surrounding cell layers. (C) The nail unit consists of a proximal nail

fold (PNF), nail matrix (NM), nail bed (NB, dashed line), and hyponychium (Hn), and produces the nail plate (NP). The nail epithelium ends at the distal groove (DG). The terminal phalanx bone (Ph) supports the nail unit. (C') The nail matrix consists of a ventral matrix (VM) and an apical matrix (AM). The eponychium (En) covers the distal part of the NP. The cuticle (Cu) is made of dead cells. (C'') The nail stem cells (NSC) appear to be located in the proximal part of the VM and provide differentiating cells that migrate distally to produce keratinized layers of the nail plate. A' B' and C' depict hematoxylin and eosin stained tissue sections from 6 week old wild-type mice. In A'', B'', and C'', the SCs (dark blue), transit-amplifying cells (T-A, purple), differentiating/differentiated cells (light blue), and the mineralized or keratinized secretion (pink) are diagrammed. Scale bars: (A, A', C, C') 100 μm ; (B, B') 30 μm . Tissue processing and staining was performed as previously reported [12].

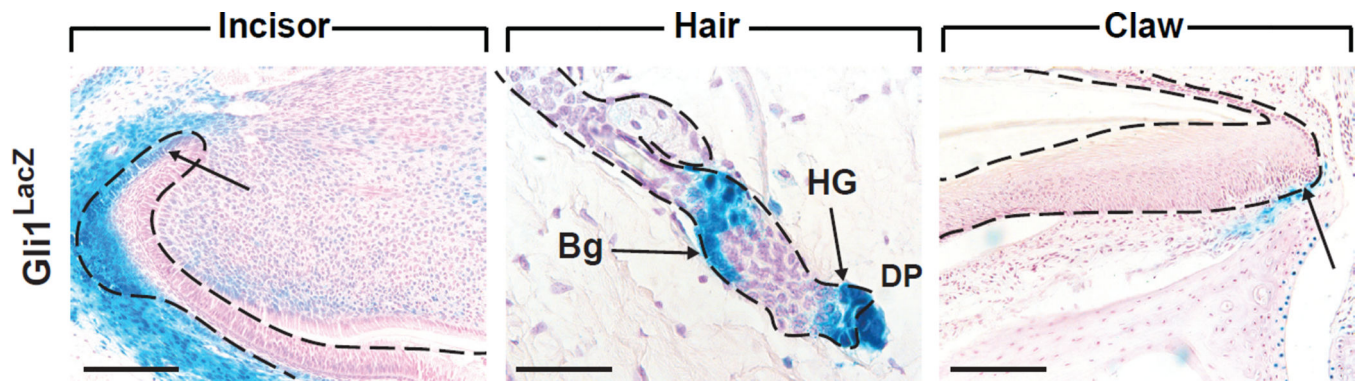


Figure 2. *Gli1* expression in mouse incisor, hair and claw

(A) In the incisor, *Gli1* is expressed in the labial cervical loop epithelium proximal to the T-A area (arrow), as well as in the mesenchyme. (B) In the telogen phase hair follicle, cells in the bulge (Bg), hair germ (HG) and mesenchymal dermal papilla (DP) express *Gli1*. (C) In the nail unit, a small number of cells near the basal layer of the proximal ventral matrix are stained. Scale bars: (A, C) 100 μm ; (B) 30 μm . X-Gal staining on *Gli1*^{lacZ/+} mice was performed as previously described [12].