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Commentary

Reawakening GDNF's regenerative past in mice and humans

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ABSTRACT

The ability of an animal to regenerate lost tissue and body parts has obviously life-saving implications. Understanding how this ability became restricted or active in specific animal lineages will help us understand our own regeneration. According to phylogenic analysis, the glial cell line-derived neurotrophic factor (GDNF) signaling pathway, but not other family members, is conserved in axolotls, a salamander with remarkable regenerative capacity. Furthermore, comparing the pro-regenerative Spiny mouse to its less regenerative descendant, the House mouse, revealed that the GDNF signaling pathway, but not other family members, was induced in regenerating Spiny mice. According to GDNF receptor expression analysis, GDNF may promote hair follicle neogenesis – an important feature of skin regeneration – by determining the fate of dermal fibroblasts as part of new hair follicles. These findings support the idea that GDNF treatment will promote skin regeneration in humans by demonstrating the GDNF signaling pathway's ancestral and cellular nature.

1. Hair follicle development and post-natal cycling

Hair is a distinguishing feature of mammals, and each hair follicle functions as a mini-organ with dynamic roles such as regulation of angiogenesis, neurogenesis, wound healing and skin regeneration [1]. Skin appendages in vertebrates, such as hair follicles and sweat glands, develop during embryonic life through a series of interactions between the surface ectoderm, which differentiates to form epithelial placodes, and the underlying mesenchymal cells [2,3] (Fig. 1A). Conserved signaling factors specify and regulate ectodermal domains, with additional pathways reinforcing morphogenesis and homeostasis of ectodermal appendages over time [4,5]. The development of hair follicles begins at embryonic day 14, when the initiating, widespread Wnt signal travels from the mesenchyme to act on the epidermis, thickening it to become the placode [6]. In turn, the placode provides the first epithelial signals, such as Fgf20, that induce the specification and condensation of local upper dermal fibroblasts into the dermal condensate, giving rise to the dermal papilla [7]. Second dermal signals then promote the proliferation of placode progenitors for down-growth into the dermis. Other signals such as Shh and Tgf-beta further promote the maturation and formation of the different hair follicle lineages in mice until morphogenesis is complete by postnatal day (P) 14[8]. Despite advances, many details about epidermal-dermal crosstalk remain elusive; the full spatiotemporal account of hair follicle morphogenesis is still incomplete [9–13].

Interestingly, mammalian hair follicles go through cycles throughout adulthood that include phases of growth (anagen), regression (catagen), and rest (telogen), which mimics several embryonic processes [14,15]. During the anagen phase, the hair follicle's own elements are regenerated, which is controlled by many of the same signaling pathways as during morphogenesis [16]. Likewise, epithelial-mesenchymal communication is essential in postnatal cycling [14]. Signals from the mesenchymal dermal

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papilla to the epithelial stem cells initiate proper anagen entry, which then controls the proliferation and spatially ordered differentiation of transit-amplifying progenitor cells [17,18]. As hair follicles mature, cells differentiate to form distinct epithelial layers organized in concentric circles around the centralized hair shaft. The hair follicle is divided into three major layered compartments: the innermost layer, which contains the medulla, cortex, and cuticle, the inner root sheath (IRS), and the outer root sheath (ORS) [18]. Together, the hair cycle and follicle serve as model systems for studying the intrinsic and extrinsic factors that control stem cell quiescence, activation, and the commitment of stem-cell precursors to differentiation, as well as apoptotic death of those cells to allow the cycle to be repeated [14,19–21].

2. GDNF: hair follicles and skin

Because the nervous system and the skin epidermis share an ectodermal origin, neurotrophic factors may play important roles in controlling skin appendage formation and homeostatic hair cycling [22–27]. Previously, the Braun and Lisse groups used cell-type specific overexpression and conditional ablation studies in mice to identify glial cell line-derived neurotrophic factor (GDNF) signaling as another important member of signaling molecules that induce anagen and thus regulate proper homeostatic cycling and maintenance of the mature hair follicle [28]. GDNF was originally isolated by heparin affinity chromatography and cloned as a neuroprotective and regenerative factor for dopaminergic neuronal subpopulations [29]. It has since been discovered to play critical roles in the maintenance of spermatogenesis within the testis, as well as in the regulation of renal organogenesis and enteric neurogenesis [30–33]. The GDNF family of ligands, including neurturin (NRTN), mediate the activation of the transmembrane REarranged during Transfection (RET) receptor tyrosine kinase via a ligand-binding receptor subunit called GDNF factor receptor alpha (GFRα) [34]. There are four GFRα family members, with GDNF and NRTN preferentially binding to GFRα1 and GFRα2, respectively, for subsequent RET activation [35]. GFRα receptors are glycosylphosphatidylinositol (GPI) anchored to a lipid-rich raft that floats on the plasma membrane. When GDNF binds to GFRα1, RET is quickly recruited to the raft to form a high affinity GDNF-GFRα1-RET complex, which initiates trans-phosphorylation of specific intracellular tyrosine residues in dimerized RET, activating

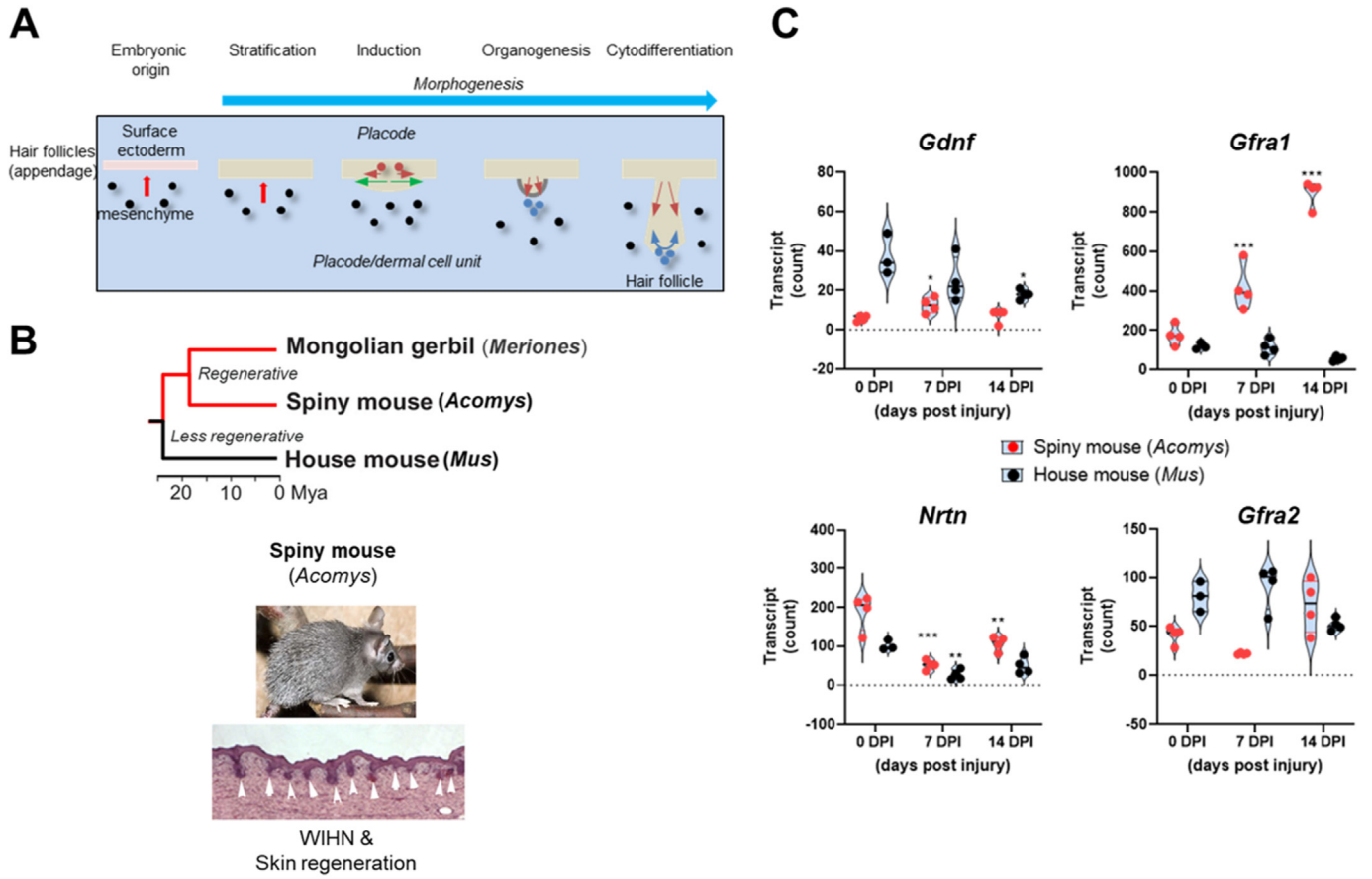

Fig. 1. Spiny mice can regenerate their skin and hair follicles with concomitant upregulation of both *Gdnf* and *Gfra1*. A) Key events and regulators of hair follicle morphogenesis. Initial signals (red upward arrows) that arise from cells in the mesenchyme (black dots) directs the undifferentiated surface ectoderm (pink bar) toward epidermal stratification and differentiation (cream bar). Placode formation is regulated by both positive signals (red arrows; WNT/beta catenin/EdaR) and inhibitory signals (green arrows; DKKs/BMPs) that target the epithelium to proliferate and grow into the dermis (red dots are proliferating cells; Shh). Signals from the developing placode (red downward arrows; FGF) induce the aggregation of mesenchymal cells to form a dermal condensate (blue dots). The dermal condensate then signals (blue upward arrows; HGF) to the follicular epithelium to continue proliferation and downward growth further into the dermis. Dermal condensates become part of the hair follicle to form the dermal papilla signaling center. B) Muridae phylogeny and divergence times of the Spiny mouse, Mongolian gerbil, and House mouse with their associated regenerative potentials (Adapted from the TimeTree resource publicly available online at http://www.timetree.org; Kumar et al., 2017 [87]). Spiny mouse and WIHN images taken from https://commons.wikimedia.org/wiki/File:Acomys.cahirinus.cahirinus.6872.jpg, and https://www.openaccessgovernment.org/the-amazing-spiny-mouse-the-champion-of-mammalian-regeneration/44150/. C) Re-analysis of comparative RNAseq gene expression studies during skin regeneration in *Mus* and *Acomys*. Data are presented as mean ± SEM error bars (n = 4); *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ (two-way ANOVA with Tukey's multiple comparisons test). Data derived from Brant et al., 2019 [71], and data repository in the Gene Expression Omnibus (GEO) (Accession GSE113081). DPI (days post-injury).

downstream signaling pathways [36]. The Ras/MAP kinase, PI3 kinase/AKT, and phospholipase C-g (PLCg) signaling pathways are all downstream of RET activation and can carry out a variety of functions such as proliferation, differentiation, cell survival, and apoptosis [37]. RET signaling can also be mediated by a soluble form of GFRα1, which is cleaved from the membrane surface by membrane phospolipases. This "trans-signaling" can occur in cells that express RET but do not express GFRα1 endogenously [38]. Alternatively, in cells lacking RET, neural adhesion molecule (NCAM), a glycoprotein that mediates cell–cell or cell–matrix adhesion, can directly interact with GDNF-GFRα1 to regulate cell–cell communication and neurite outgrowth [39].

GDNF is expressed in the overlying epidermal layer of cells in both embryonic [40] and adult skin [28,41], as well as in a subset of hair follicle stem cells in adult mice [42]. This suggests the presence of tissue specific GDNF gradients, which may govern the formation and maintenance of hair follicles and wound repair. Previous research linked variable *Gdnf* mRNA expression in mice to different stages of the natural hair cycle [25,43]. *Gdnf* mRNA levels peak during anagen and then decrease during the anagen-catagen transition, indicating that GDNF-GFRα1 signaling may promote the survival of cells that regulate hair follicle growth. In support of this notion, heterozygous loss of *Gfra1* function results in increased regression of hair follicles demonstrating that GDNF can control the murine hair cycle [43]. Our group discovered that *Gfra1* is specifically expressed by dermal papillary (DP) cells and bulge stem cells (BSC) of hair follicles using a *Gfra1* gene reporter mouse line [5,28]. Furthermore, conditional ablation of both *Ret* and *Gfra1* within BSCs in mice results in impaired hair follicle formation and growth, respectively [5,28]. DP cells are the mesenchymal component of hair follicles that control the activation of adult BSCs at rest and the differentiation of actively proliferating progenitor cells committed to the hair follicle lineage [44]. Furthermore, DP cells can induce epithelia to form hair follicles and may have stem cell-like properties because they can reconstitute the skin dermis [44]. BSCs are multipotent, and when activated, they can transform into epidermal, hair follicle, and sebaceous gland cells – an important function for correcting any imbalances that may occur due to injury and/or disease [45]. It is unknown whether *Gfra1* and *Ret* signaling can specify a lineage in BSCs. Furthermore, whether *Gfra1* and *Ret* signaling can target dermal fibroblasts, including DP cells, to reconstitute and repattern damaged skin is unknown from a wound healing and regenerative standpoint. Answering these questions is a significant step forward in regenerative biology, as homeostasis and regeneration of skin is impaired without the proper contribution of both hair follicle stem cells and dermal fibroblasts.

3. Skin regeneration and wound-induced hair neogenesis

Several species, including zebrafish and axolotls, can overcome scarring via epimorphic regeneration, which is a process similar to embryonic tissue development in which less differentiated blastemal cells arise and retain positional memory to form new tissues [46,47]. Mammalian species, on the other hand, do not

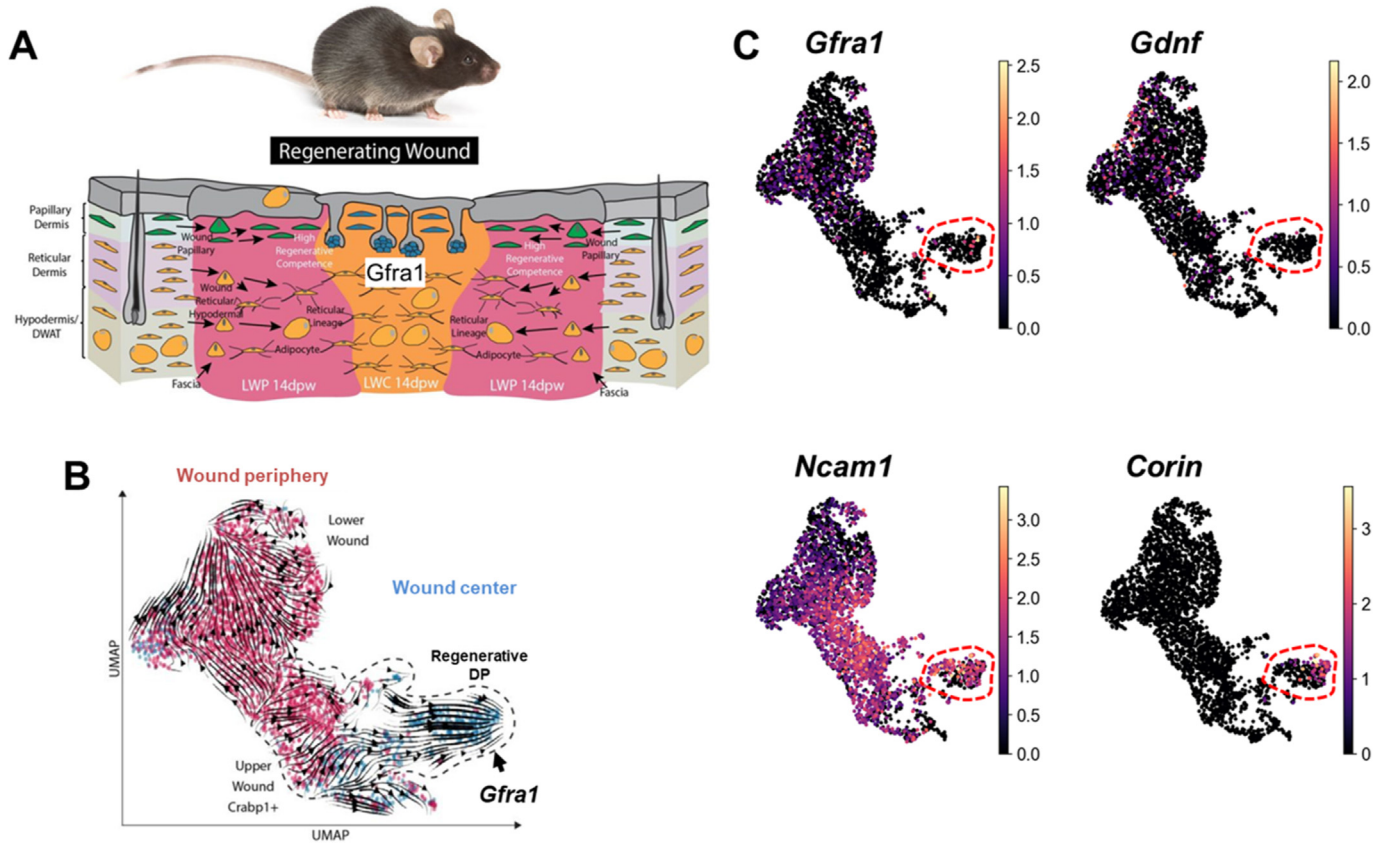

Fig. 2. Expression of *Gfra1* and *Gdnf* in distinct dermal cell populations in large regenerating wounds of older mice. A) Schema of regenerating large wounds after 14 days post wounding (dpw) in the House mouse (1.5 cm × 1.5 cm). Schema derived from Phan QM et al., 2020 [67], an open access article distributed under the terms of the Creative Commons CC BY license. B) Regenerating wounds have distinct populations of upper wound fibroblasts with higher regenerative potential compared to the lower wound fibroblast populations. Population clusters were identified in Uniform Manifold Approximation and Projection (UMAP) utilizing signature markers such as *Crabp1* (i.e., to mark upper wound population). C) Quantification of cellular expression of various transcripts within different populations. Regenerative dermal papillae (DP) population is encircled by red dashes. *Corin* is a signature of regenerative DP cells. Data derived from Phan QM et al., 2020 [67]. Large wound periphery (LWP), large wound center (LWC).

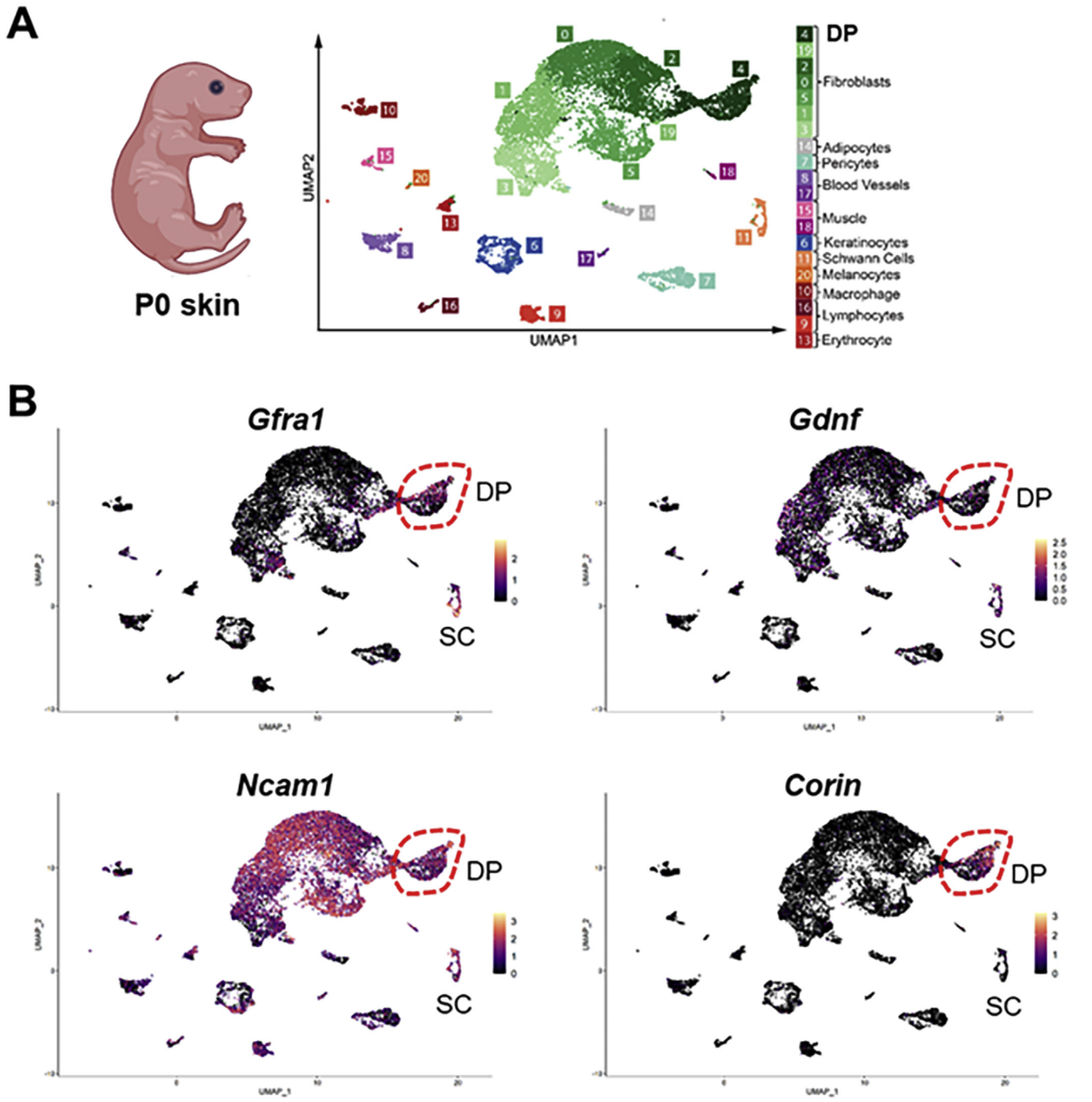

Fig. 3. Expression of Gfra1 and Gdnf in distinct dermal cell populations in early post-natal mice. A) Schema of various cell populations in early post-natal (P0) skin of the House mouse. Distinct population clusters were identified in Uniform Manifold Approximation and Projection (UMAP) utilizing signature markers. Schema derived from Phan QM et al., 2020 [75], an open access article distributed under the terms of the Creative Commons CC BY license. B) Quantification of cellular expression of various transcripts within different populations. Regenerative dermal papillae (DP) population is encircled by red dashes. *Corin* is a signature of regenerative DP cells. Data derived from Phan QM et al., 2020 [75]. SC (Schwann cell).

regenerate lost/damaged cutaneous tissue and instead replace it with a dense fibrotic scar [48,49]. Given the presence of conserved genes in species with high and low regenerative capacity, it is likely that less-regenerative organisms can regenerate tissues and organs if provided with the appropriate lineage-specific factors and induction of conserved gene regulatory programs [50,51]. Interestingly, some mammalian species, such as the African/Egyptian Spiny mouse (genus *Acomys*) [52], have retained the ability to regenerate skin appendages without scarring after injury, in contrast to the house mouse (*Mus musculus*), a more recent descendant of the Old-World mouse lineage (Fig. 1B). Spiny mice are notable for their ability to regenerate skin through non-lethal predation involving wound-induced hair neogenesis (WIHN). During WIHN, a progeny of interfollicular, epidermal, and dermal cells become “embryonic-like” to restore early epithelial-mesenchymal interactions, resulting in the regeneration

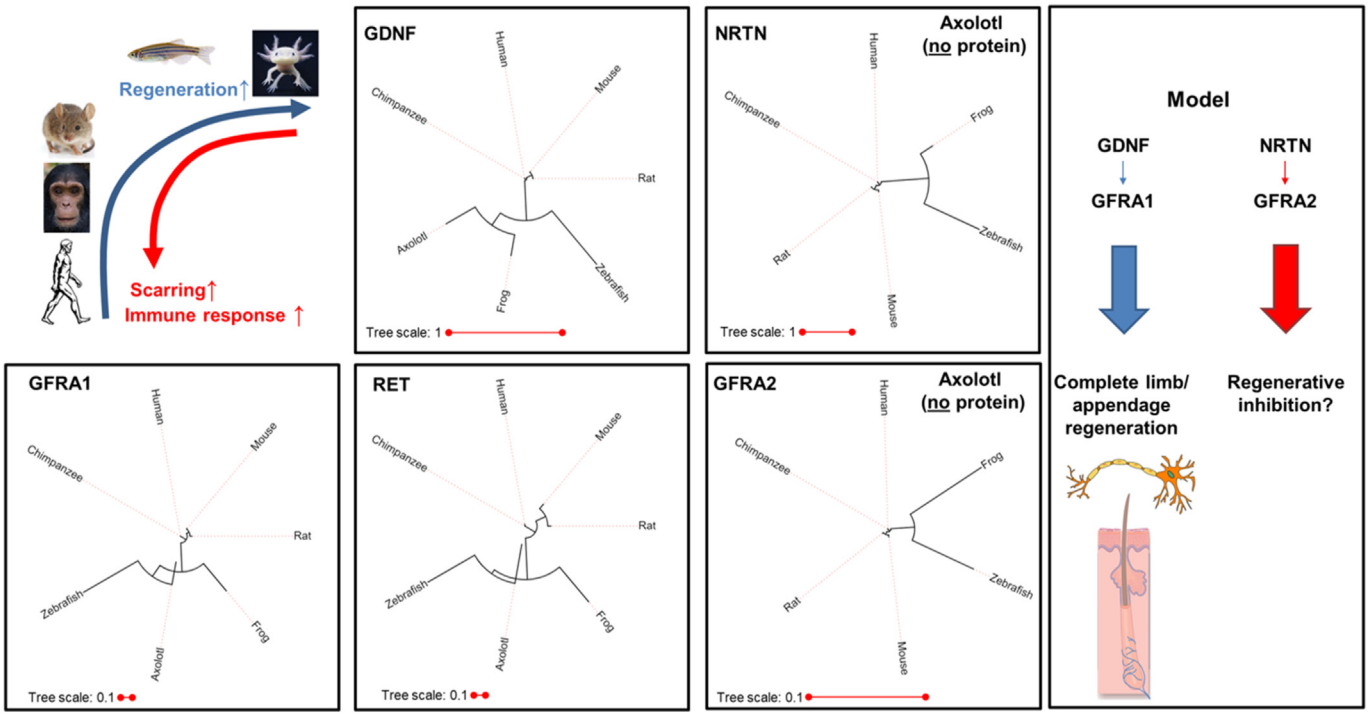

Fig. 4. The ancestral character of the GDNF-GFRα1-RET signaling pathway during regeneration. Left panel: Scar formation is reduced by suppression of inflammation, which is an innate feature of animals with high regenerative capacity such as zebrafish and axolotls geared towards complete limb/appendage regeneration. Center panels: Phylogenetic trees of GDNF and NRTN signaling components using reference sequences from the Protein Information Resource (PIR) from the National Center for Biotechnology Information (NCBI). Unrooted trees were constructed by PHYLIP 3.6 using the input sequences with 1000 bootstrap replicates. The scale bar (branch lengths) represents 0.1–1.0 expected amino acid replacement per site estimated by the PRODIST program of PHYLIP 3.6 package. Right panel: Model for the ancestral nature of the GDNF signaling pro-regenerative pathway. NRTN-GFRα2 may contribute to regenerative inhibition. Human (Homo sapien), chimpanzee (*Pan troglodytes*), rat (*Rattus norvegicus*), mouse (*M. musculus*), frog (*Xenopus laevis*), zebrafish (*Danio rerio*).

of hair follicles, fat, and arrector pili muscles [53]. Interestingly, some find WIHN to be akin to the blastema formation that occurs in axolotls, for example, that may entail similar signaling regulatory networks. Although the progeny of pre-existing hair follicle BSCs change identity and migrate into the wound neoepidermis, which can be modulated by GDNF [28], they appear to contribute to transient skin repair and are insufficient to regenerate hair follicles [54]. Instead, neogenic hair follicles are thought to develop from non-bulge epithelial and dermal fibroblasts, which exhibit greater lineage plasticity and commitment after wounding [55–60]. Skin regeneration and WIHN occurs in the House mouse, but to a lesser extent than in the Spiny mouse [56,61–64], and only under large wound conditions [61,65].

There are two types of fibroblasts: upper dermal (papillary) fibroblasts that cause WIHN and lower dermal (reticular) fibroblasts that produce extracellular matrix (ECM) for wound healing [66]. Lower wound fibroblasts, once activated, can transdifferentiate into collagen-secreting smooth muscle actin (SMA)-expressing myofibroblasts to support wound tissue architecture [10,67,68]. Furthermore, myofibroblasts can transdifferentiate into fat cells, which promote scar-free wound healing [61]. The upper subset of wound fibroblasts (also called neogenic condensates) is designated to produce DP cells of neogenic hair follicles, which promote scarless wound healing via growth factors and proteolytic enzymes produced by newly formed hair follicles [56,61,67,69,70]. Despite these findings, much remains unknown about the heterogeneity of wound dermal fibroblasts and their capacity for cellular reprogramming. It is worth noting that the roles of neurotrophic factors in WIHN and skin regeneration have yet to be investigated.

4. GDNF signal activation and skin regeneration in spiny mice

Spiny mice are unique in that they encounter both structural (i.e., hair follicle) and tissue (i.e., layers of skin) regeneration after wounding. The Maden group's gene expression data comparing the skin injury responses of House and Egyptian Spiny mice (*Acomys cahirinus*) was re-examined to identify novel signaling systems that may play a role in regeneration [71] (Fig. 1C). According to the original characterization, adult Spiny mice exhibit a Wnt-mediated dermal fibroblast response after wounding, which is the most studied pathway in the field. However, at 7- and 14-days post-injury (DPI) in Spiny mice, a previously unknown statistically significant 5-fold increase in *Gdnf* and *Gfra1* mRNA expression was discovered. Because cells release GFRα1 after injury, a proportion of soluble GFRα1 may also modulate regeneration in distant cells. Less-regenerative House mice, on the other hand, showed a decrease in *Gdnf* mRNA levels at 14 DPI but no change in *Gfra1* levels (Fig. 1C). Furthermore, the NRTN-GFRα2 pathway, which is known to play a role in skin as well [26,43], was decreased after wounding in both House and Spiny mice (Fig. 1C). *Gfra2* mRNA levels did not significantly change after injury in both House and Spiny mice (Fig. 1C), as well as the remaining GFRα family members *Gfra3* and *Gfra4* (not shown). Finally, *Ncam1* was significantly induced in both House and Spiny mice after wounding (5.5–3.7 fold, respectively, adjusted *p* value 0.0001), suggesting that *Gfra1* and *Ncam1* co-induction may be required for the regenerative phenotype in Spiny mice [71]. Overall, these results suggest that GDNF-GFRα1 signaling plays a functional and conserved role in skin regeneration and WIHN.

5. GDNF signal activation across dermal fibroblast subpopulations in regenerating, embryonic and early post-natal skin of House mice

Recent findings from single-cell RNA sequencing (scRNAseq) of large regenerative wounds and early post-natal skin of House mice provide a framework for understanding GDNF signaling during regeneration and development (Figs. 2A and 3A). In large wounds of adult House mice, *Gfra1* is expressed by both upper-regeneration- and lower-repair-competent dermal fibroblast subpopulations [63,67,69] (Fig. 2B and C). *Gfra1* and *Ncam1* are both co-expressed by regeneration-competent fibroblasts, suggesting that GDNF could play a functional role in the development of neogenic hair follicles [67,69]. *Gdnf* was primarily expressed by lower dermal fibroblasts with repair competence, suggesting the formation of a GDNF-rich substratum to aid wound healing and skin regeneration [67] (Fig. 2C). This phenomenon may be similar to gut development, where GFRα1-positive enteric neuron precursors migrate down the gut mesenchyme, which produces GDNF [72,73]. Nonetheless, a few cells in the upper dermal papillary compartment express *Gdnf*, which may be sufficient to stimulate neogenic hair formation after wounding. *Gdnf* is also upregulated in wound-associated mesenchymal stem cells (MSCs), implying yet another potential source of the neurotrophic factor [74]. Overall, the findings suggest that GDNF may aid wound healing and hair follicle regeneration by targeting specific skin cell types at wound sites.

Furthermore, in early post-natal skin (P0), neuronal-like cells and dermal fibroblasts express *Gdnf* and *Gfra1*, implying that GDNF may be sourced by glial cells to facilitate WIHN (Fig. 3) [75–77]. In P0 mouse skin, scRNAseq data reveal widespread fibroblastic *Gdnf* expression, whereas *Gfra1* expression was limited to the Corin-positive regenerative-competent DP cells and the surrounding dermal sheath (Fig. 3B). *Ncam1* was expressed in nearly every cell type in P0 skin. In both large adult regenerative wounds and P0 skin, *Ret* is expressed in epidermal and follicular keratinocytes, as well as in Schwann cells (not shown) [63,67,69,75–77]. These findings suggest that GDNF may signal by both “trans” and “alternative” ways in large regenerative skin wounds in adults, as well as during the early stages of morphogenesis. According to a recent transcriptomic analysis of developing embryonic skin, *Gdnf* and *Gfra1* were also expressed by the dermal condensate and sheath population of cells [5,78], suggesting a role in embryonic hair formation. Interestingly, comparative scRNAseq analysis of wounded skin revealed that *Gfra1* mRNA expression within dermal fibroblasts decreased as mice got older, suggesting that the GDNF dermal response, i.e., the skin's overall regenerative capacity, decreases as mice age [75]. Thus, adult skin may rely on a GDNF-GFRα1-dependent early fibroblastic reprogramming strategy to promote regeneration when stimulated.

6. Conservation and evolution of the core GDNF-GFRα1-RET signaling pathway during tissue regeneration.

Over time, tissue regeneration and wound healing have been suppressed across and within phyla, raising questions about the nature and origin of the key signaling systems [79,80]. To gain a better understanding of the relationships between the GDNF and NRTN signaling pathways, as well as their potential for regeneration across species, phylogenic tree analysis was performed (Fig. 4). There was less GDNF divergence between amphibians and more between zebrafish and land vertebrates based on branch lengths. This observation could be due to clade-specific features, as axolotls and frogs are tetrapods with body plans similar to humans [81]. This pattern was also found for both GDNF receptors, GFRα1 and RET, among the species studied, indicating similar ligand:receptor evolution and association to regeneration. The NRTN pathway, on the other hand, demonstrated the greatest divergence among axolotls, the most regenerative species in our comparison. An NCBI Taxonomy Browser search for the NRTN and GFR2 proteins in axolotls (*Ambystoma mexicanum*, Taxonomy ID: 8296) yielded no annotated proteins. A Megablast search of the recently sequenced axolotl genome (GenBank assembly GCA 002915635.3) using human *NRTN* and *GFRA2* sequences revealed no similar sequences as well [82]. These findings imply that the GDNF-GFRα1 signaling pathway, which is conserved in axolotls, plays a role in complete limb/appendage regeneration in contrast to the NRTN-GFRα2 signaling pathway. Because axolotls lack both NRTN and its main receptor, GFRα2, the findings suggest that this network may either contribute to regeneration inhibition or the acquisition of ancillary roles in other species and tissue systems.

These findings support the previously held belief that skin tissue and structural regeneration capacity are ancestral, and that the GDNF-GFRα1-RET pathway may be overshadowed or “switched off” by unknown mechanisms in certain conditions or phyla [79,80]. Recent studies have highlighted the role of nerves in axolotl limb regeneration [83], implying that nerve tissue may be the common denominator of the GDNF-GFRα1-RET signaling pathway, which was originally identified as a potent nerve growth and survival factor [29]. Along these lines, Spiny mice have been shown to have a greater capacity to regenerate the spinal cord and functional recovery following injury than House mice, owing to decreased scar formation, which creates a more permissible environment for axon regeneration [84]. The extracellular matrix was post-translationally “rewired” in Spiny mice following injury, which may account for the increase in regenerative capacity, according to the same study. Interestingly, Spiny mice had higher levels of β-1,3-N-acetylglucosaminyltransferase 7 (β3GNT7), an enzyme required for keratin sulphate proteoglycan (KSPG) synthesis, as well as higher levels of KSPG deposition at the injury site. GDNF and receptor status are currently unknown in the context of spinal cord injury in Spiny mice compared to House mice. Furthermore, whether the increase in KSPG can act as a matrix component to harbor GDNF to influence its bioactivity and bioavailability given its proteoglycan binding capacity and known ability to improve spinal cord repair after injury [29,85], is unknown.

In contrast to axolotls, as animal organs and tissues became more complex, the GDNF-GFRα1-RET signaling pathway may have begun to function in cell types other than nerve cells to promote different aspects of regeneration [28,86]. Activating the GDNF-GFRα1-RET signaling pathway may thus be a method of promoting complete limb and skin regeneration in less regenerative animals like humans, opening the door to exciting future research into our own survival. The elucidation of GDNF's non-classical regenerative roles is already becoming a reality, thanks to the introduction of new molecular, genomic, and epigenomic technologies to provide a more refined understanding of regenerative medicine and biology.

Declaration of competing interest

The authors have no conflict interests to declare.

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