Perfecting the Imperfect: Introducing dorsoventral patterning into adult regenerating lizard tails with gene-edited embryonic neural stem cells.

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2 gene-edited embryonic neural stem cells.
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SUMMARY

Most regenerative organisms regrow replacement appendages that closely resemble their original counterparts in both development and final outcome.^{1,2} Lizards are the only amniotes able to regrow amputated tails, but regenerated lizard tails lack the dorsoventral patterning established during embryonic tail development.^{3,4} The absence of tissue patterning most obviously manifests in regenerated tail skeletons, which take the form of cartilage tubes rather than patterned vertebrae of embryonic tails.⁵ Skeletal formation in both regenerated and embryonic lizard tails is induced by sonic hedgehog (Shh) signals produced by tail neural stem cells (NSCs).^{3,5} However, the differences between regenerated and embryonic tail NSCs that account for divergent skeletal patterning during tail development and regrowth are unknown. Here we show that deviant NSC differentiation capacities drive divergent skeletal patterning between regenerated and embryonic tails and that supplementing adult tail populations with gene-edited embryonic NSCs leads to recapitulation of developmental patterning processes and outcomes during tail regrowth. We found that embryonic lizard NSCs are able to differentiate into Shh- roof plate and lateral domain lineages, while regenerated tail NSCs are restricted to Shh⁺ floor plate identities. Using a novel gene manipulation strategy in unique, asexually reproducing lizard species that allows for allogeneic cell transplantations without rejection, we showed that embryonic NSCs implanted into adult lizards contribute to regenerated tails but only retain roof plate identities when hedgehog signaling is inhibited. CRISPR/Cas9 gene editing was used to knock out (KO) the hedgehog regulator Smo and create embryonic NSC lines unresponsive to Shh stimulation. Smo KO embryonic NSC populations introduced into regenerated lizard tails differentiate into roof plate lineages and result in patterned skeletons. Our findings demonstrate the feasibility of improving fidelities of regenerative outcomes, specifically treating the “imperfectly” regenerating lizard with therapeutically-modified embryonic NSC populations to regrow nature’s first dorsoventrally patterned amniote tails.

1 **ABSTRACT**

2 Lizards are able to regrow amputated tails, but the lizard tail regenerative process fails to recapitulate the dorsoventral
3 patterning achieved during embryonic tail development. Regenerated lizard tails form ependymal tubes (ETs) that, like
4 embryonic tail neural tubes (NTs), induce cartilage differentiation in surrounding cells via sonic hedgehog (Shh)
5 signaling. Embryonic NTs are, themselves, dorsoventrally patterned, with Pax7⁺ Shh⁻ dorsal roof plate domains that
6 restrict cartilage skeletal formation induced by Pax7⁻ Shh⁺ floor domains to ventral tail regions. However, adult
7 regenerated tail ETs lack characteristically roof plate-associated structures and express Shh throughout their
8 circumferences, resulting in the formation of unpatterned cartilage tube skeletons. Both NTs and ETs contain populations
9 of neural stem cells (NSCs), but only embryonic NSC populations are able to differentiate into roof plate identities and
10 neurons. Embryonic NSCs transplanted into regenerated tail ETs retain the capacity to form roof domains but are
11 ultimately ventralized by the unchecked hedgehog signaling of regenerated lizard tail environments. We hypothesized that
12 only the simultaneous repression of hedgehog signaling and enhancement of NCS roof plate differentiation capacity
13 would induce patterning in lizard ETs and, hence, regenerated cartilage. This was tested through the use of a novel genetic
14 engineering process in which NSCs are isolated from embryos of the parthenogenetic lizard *Lepidodactylus lugubris*,
15 gene-edited in vivo, and implanted back into clonally-identical adults to regulate tail regeneration. Embryonic lizard NSC
16 lines unresponsive to hedgehog stimulation were generated through the use of CRISPR/Cas9 technologies to knockout
17 (KO) the signaling regulator smoothened (Smo). Exogenous Smo KO NSCs were injected into adult tail spinal cords,
18 where they engrafted to endogenous ependymal cell populations and contributed to dorsal domains in regenerated tail
19 ETs. Embryonic Smo KO NSCs maintained roof plate identities in vivo, and lizards treated with edited NSCs regrew tails
20 that lacked cartilage in dorsal regions. These studies represent an important milestone in the creation of the first
21 regenerated lizard tails with dorsoventrally patterned ETs and skeletal tissues.

24 **MAIN**

25 Vertebrate regeneration is often studied through the lens of embryonic development, and many of the most intensely
26 studied models of adult appendage regeneration closely follow embryonic programs to replace lost tissues.⁶ For example,
27 during both appendage development and regeneration, specialized structures act as signaling centers that direct
28 differentiation of surrounding tissues. During embryonic development, tail tissues form from tail buds, collections of
29 mesodermal cells that respond to patterning signals generated by secondary neural tubes (NTs).⁷⁻⁹ Tail NTs exhibit distinct
30 molecular differences between dorsal roof plate domains and ventral floor plate domains.¹⁰ Roof plates express distinct
31 sets of markers, including Pax7, Msx2, BMPs, and Wnts, while floor plates express Shh and FoxA2. Intermediate zones
32 between roof and floor plates are referred to as lateral domains and are marked by Pax6 expression. This separation of
33 distinct signaling molecules into dorsal and ventral domains has direct consequences on dorsoventral patterning of a range
34 of tissues, including axial skeletons. For example, Shh produced by NT floor plates induce skeletogenesis in tail bud
35 notochords and sclerotome but are antagonized by BMP signals from roof plates, thereby limiting early skeletal formation
36 to ventral regions.¹¹ Early embryonic skeletons are made up of cartilage marked by expression of collagen type II (Col2)

and are later replaced by bone.¹² Tail NTs, themselves, are rich in neural stem cell (NSC) populations which undergo neurogenesis to yield neurons of spinal cords and dorsal root ganglia (DRG).^{13,14} Remnants of embryonic NT NSCs persist into adulthood among ependymal/radial glial cell populations lining the central canals of tail spinal cords.¹⁵ Tail regeneration begins with the growth of blastemas on amputated tail stumps.^{4,5,16} These collections of mesenchymal cells differentiate into the majority of regenerated tail tissues, including skeletons.^{5,16} Instead of NTs, ependymal tubes (ETs) sprout from original tail spinal cord ependymal populations and invade tail blastemas.^{3,5,16} Like embryonic tail NTs, regenerated tail ETs induce differentiation in surrounding cells and contain populations of NSCs.^{3,5,16} However, the extent to which adult and embryonic tail NSC populations resemble one another varies with species and appears to affect tail regenerative fidelity.³

Lizards are the only amniotes capable of tail regeneration, making them the closest relatives to mammals capable of regrowing an amputated appendage.⁴ Lizard tail regeneration also presents a unique case in which developmental and regenerative outcomes diverge greatly, particularly as they relate to skeletal tissue patterning.⁵ Tail vertebral columns that form during embryonic development are regenerated as unpatterned and unsegmented cartilage tubes. Similarly, regenerated lizard tail ETs lack the dorsoventrally patterned roof, lateral, and floor domains developed by embryonic NTs.³ We hypothesize that the lack of dorsoventral patterning in the regenerated lizard tail ETs and skeletons are directly related, and that introduction of NSC populations with the ability to dorsalize in regenerated lizard tail environments will induce patterning in both tissues. The goals of this study include comparing the differentiation potentials of embryonic and regenerated tail NSCs and the generation of the first dorsoventrally patterned regenerated lizard tails.

RESULTS

Regenerated lizard tails fail to recapitulate the dorsoventral patterning that is developed during embryogenesis and maintained into adulthood.

Embryonic lizard tails begin as tail buds, collections of mesodermal cells surrounding neural tubes (NTs) populated by Sox2⁺ neural stem cells (NSCs) (Supplementary Information Figure 1). Mesodermal cells respond to signals from embryonic NSCs to proliferate and differentiate into tail tissues. Specifically, Shh produced by tail NT NSCs induce cartilage differentiation in embryonic sclerotome from which tail skeletons are derived.¹¹ Similarly, regenerated lizard tails begin as blastemas, specialized regenerative structures made up of heterogeneous fibroblastic cell populations surrounding central ETs (Supplementary Figure 1).⁵ Like embryonic NTs, regenerated tail ETs contain populations of Sox2⁺ NSCs that act as signaling centers for regulating patterning in surrounding tissues (Supplementary Information Figure 1).³ We have previously shown that lizard tail blastema cells respond to proliferation and differentiation signals produced by ET NSCs.⁵ Specifically, Shh secreted by ET NSCs induce blastema cell cartilage differentiation, essentially recreating the signaling environment responsible for embryonic tail skeletal development. Similarly, both tail bud and blastema NSCs proliferate during tail development and regeneration, respectively, and elongate with growing tails (Supplementary Information Figure 1).

Despite these early similarities between embryonic and regenerated tails, developmental outcomes are very different, particularly as they relate to dorsoventral patterning of central nervous system (CNS) and skeletal tissues (Figure 1). Embryonic tails exhibit NTs dorsal to notochords (Figure 1A). Sox2⁺ NSCs populate NTs and undergo neurogenesis to differentiate into Tuj1⁺ neurons of early spinal cords and DRG (Figure 1A).^{13,14} Meanwhile, notochords and surrounding tissues express Col2, a marker of the early cartilaginous skeleton (Figure 1A). This pattern of dorsal CNS and ventral skeletal tissue is maintained through embryonic development and into adulthood (Figure 1B). Neural tube NSCs differentiate and add more neurons to spinal cords and DRG (Figure 1B), while notochords and surrounding sclerotome develop into the vertebrae of adult tails (Figure 1B). Sox2⁺ NSCs persist in adult spinal cords among ependymal cell populations lining central canals, the remnants of embryonic NTs (Figure 1B). Upon amputation, adult lizard tails regenerate, but dorsoventral tissue patterning is replaced by radial symmetry around central ETs (Figure 1C). ETs are derived from adult tail spinal cord ependyma populations and are enriched for Sox2⁺ NCS (Figure 1C). Cartilage tubes form from blastema cells and completely surround ETs (Figure 1C). Spinal cord neurons and DRG are not regenerated in regrown tails (Figure 1C).

The loss of regenerated tail CNS and skeletal tissue patterning is preceded by the disappearance of patterned NSC populations during NT maturation (Figure 1 D-O). Embryonic tail NTs exhibit distinct molecular differences between dorsal roof plates, ventral floor plates, and intermediate lateral domains. NT roof plates express the markers Pax7 and BMP4 (Figure 1 D, E); lateral domains express Pax6 (Figure 1 F); and floor plates express Shh and FoxA2 (Figure 1 D, F, G). Adult tail spinal cord ependyma, the direct descendants of embryonic NT cells, lack roof and lateral domain markers (Figure 1 H-J) but retain floor plate markers Shh and FoxA2 (Figure 1 H, J, K). In turn, regenerated tail ETs cells are derived from original tail ependyma cell populations and also lack roof and lateral domain identities (Figure 1L-N), being entirely made up of Shh⁺ Foxa2⁺ floor plate identities (Figure 1 L, N, O). Taken together, these results demonstrate that dorsoventral patterning of skeletal and CNS tissues developed during embryogenesis are not recapitulated during lizard tail regeneration. We hypothesize that these findings are related; regenerated lizard tail skeletal tissues lack dorsoventral patterning *because* ETs lack patterned NSC populations. Patterning of embryonic NT NSCs is lost during adult tail ependymal cell derivation, which in turn results in un-patterned regenerated tail ETs and cartilage tubes.

Interestingly, we attempted to test the regenerative potential of embryonic lizard tails, but results have been inconclusive. Embryonic tails amputated *in ovo* (7 and 14 DPO) failed to regenerate. While embryos remained viable for weeks following surgery and egg re-closure, amputated tail stumps became bound to amnion membranes, which may have interfered with tail healing. Tails of embryos removed from eggs and cultured *in vitro* also failed to regrow following amputation, but this may have been an artifact of prolonged culture conditions.

Embryonic lizard tail NSCs default toward roof plate in culture, while adult tail NSCs exhibit persistent floor plate identity.

Since Sox2⁺ NSCs were detected in embryonic and adult tail tissues, we sought to characterize and compare the roof plate, lateral domain, and floor plate identities of these cells *in vivo* and *in vitro* (Figure 2). We have previously shown that adult lizard tail NSCs form neurospheres in response to FGF stimulation in culture (Fig. 2 C, D),³ and we tested

whether this held true for embryonic tail NSCs. Both embryonic tail NTs and adult tail spinal cords were isolated via microdissection, digested with papain, and cleared of myelin to yield single-cell suspensions (Fig. 2 A, C). Both embryonic NT and spinal cord NSCs formed neurospheres after 14 days in culture in the presence of FGF (Figure 2 B, D). Next, Pax7, Pax6, and Shh expression were analyzed to determine the positional identity of embryonic vs. adult tail Sox2⁺ NSCs in situ and after neurosphere formation in vitro (Figure 2 E-P). Sox2⁺ cells were detected among Pax7⁺ roof plates, Pax6⁺ lateral domain, and Shh⁺ floor plates of embryonic tail NTs (Figure 2 E-G). In contrast, adult lizard tail spinal cord ependyma and their resident Sox2⁺ NSCs expressed Shh only (Figure 2 H-J). In vitro, both embryonic NT- and adult spinal cord-derived neurospheres were enriched for Sox2⁺ NSCs (Figure 2 K-P). Embryonic neurospheres were predominantly Pax7⁺, Pax6⁻, Shh⁻ (Figure 2 K-M) (Supplementary Information Figure 2), while adult neurospheres were Pax7⁻, Pax6⁺, and Shh⁺ (Figure 2 N-P) (Supplementary Information Figure 2). These results indicated a divergence in roof plate vs floor plate identities between NT and adult spinal cord NSCs when cultured in vitro; embryonic NSC neurospheres defaulted to a roof plate identity, while adult tail neurospheres were restricted to floor plate.

Embryonic lizard tail NTs are patterned by hedgehog signaling, while adult regenerated tail ETs are unresponsive.

We next sought to compare embryonic and regenerated tail responses to hedgehog signaling. Previous studies have shown that hedgehog signaling regulates cell differentiation and patterning in both embryonic and regenerated tissues,^{3,5,16} and here we compared the responses of embryonic vs adult regenerated tails to inhibition and exogenous activation of hedgehog signaling. To treat embryonic lizard tails, freshly laid lizard eggs (n=6) were injected with either the hedgehog inhibitor cyclopamine or the hedgehog agonist SAG and allowed to develop for one week. To treat regenerated lizard tails, cyclopamine or SAG was administered to lizards (n=6) for four weeks following tail amputation. Embryonic and regenerated tail samples were analyzed for Col2 and Tuj1 expression as markers for cartilage and nerve differentiation, respectively, and for Pax7 and Shh expression as indicators of dorsoventral patterning (Figure 3). Lizard embryos treated with vehicle control developed patterned tails with Tuj1⁺ NTs and DRG in the dorsum and Col2⁺ notochords in the ventrum (Figure 3 A). Control embryonic tail NTs expressed Pax7⁺ dorsal roof plate and Shh⁺ ventral floor plate (Figure 3 B). Adult lizards treated with vehicle control regenerated tails with characteristic Col2⁺ cartilage tubes surrounding ETs exhibiting circumferential Shh⁺ expression without Pax7 expression (Figure 3 C, D). No Tuj1⁺ neurons or DRG were detected (Figure 3 C, D). Treatment with cyclopamine inhibited embryonic lizard tail Col2 expression and resulted in tails without noticeable notochords (Figure 3 E). Conversely, cyclopamine treatment increased embryonic tail Tuj1 expression and resulted in ectopic nerve differentiation throughout tail tissues (Figure 3 E). Tail NTs of embryos treated with cyclopamine exhibited abnormal shapes with ventral openings and marked enhancements in Pax7 expression and reductions in Shh signal (Figure 3 F), indicating dorsalization of NT phenotypes. Like embryonic tail cartilage, regenerated lizard tail cartilage tube formation was inhibited by cyclopamine treatment (Figure 3 G). However, unlike the observations of embryonic tails, cyclopamine did not affect regenerated lizard tail Tuj1 expression, and DRG remained absent (Figure 3 G). ETs remained positive for Shh expression, and Pax7 levels remained undetectable (Figure 3 H).

SAG treatment induced ectopic Col2⁺ cartilage formation in both embryonic and regenerated lizard tails (Figure 3 I, K). The response of regenerated lizard tails to SAG treatment was particularly strong, with extensive cartilage infiltration

into various tail regions (Figure 3 K). In embryonic tails, SAG treatment resulted in the replacement of Pax7 with Shh expression, reductions of roof plate domains, and expansions of floor plate regions, suggesting ventralization of embryonic tail NTs (Figure 3 J). Conversely, regenerated lizard tail ETs did not respond to SAG treatment; Shh expression was maintained by the entire tube, and Pax7 expression remained undetectable (Figure 3 L). Interestingly, the replacement of Pax7⁺ roof plate with Shh⁺ floor plate in tail NTs of SAG-treated embryos bore striking resemblances to regenerated lizard tail ETs, suggesting that tail environments saturated with hedgehog signaling destroyed dorsoventral patterning of NSC structures. Taken together, these results suggested patterning of both embryonic and regenerated tail cartilage was regulated by hedgehog signaling. However, only embryonic tail NTs, and not regenerated tail ETs, responded to hedgehog dorsoventral patterning signals. Furthermore, the strong response of regenerated lizard tail cartilage to both cyclopamine and SAG treatments validated the effectiveness of drug treatment methods in modulating cell differentiation responses to hedgehog signaling in regenerated lizard tails and highlighted the contrasting responses of cartilage vs ET cells to hedgehog inhibition and stimulation.

Embryonic tail NSCs ventralize in response to activation of hedgehog signaling, while floor plate identities of regenerated tail NSCs persist independent of hedgehog signaling.

We next investigated whether the differences observed between embryonic tail NT vs regenerated tail ET cell responses to modulators of hedgehog signaling extended to neurosphere cultures of these same cell populations (Figure 4). Neurospheres were generated from either embryonic or regenerated tail NSCs and treated with either cyclopamine (5 μ M), SAG (100 nM), or vehicle controls for 14 days. Neurospheres generated from embryonic tail NTs exhibited roof plate identities with high levels of Pax7 and undetectable Shh expression levels under control conditions (Figure 4 A) (Supplementary Information Figure 3) that were unaffected by cyclopamine treatment (Figure 4 B) (Supplementary Information Figure 3). In contrast, SAG treatment of embryonic NSC neurospheres induced the replacement of Pax7 with Shh expression, indicating conversion to floor plate identity (Figure 4C) (Supplementary Information Figure 3). Neurospheres derived from regenerated tail ETs were unresponsive to the same concentrations of cyclopamine and SAG (Figure 4 D-F) (Supplementary Information Figure 3), and consistently expressed Shh and not Pax7. These results supported in vivo findings, where NT NSCs ventralized in response to hedgehog activation to match the immutable floor plate identity of ET NSCs.

Embryonic lizard NSCs contribute to CNS cell lineages of the embryonic, adult, and regenerated tails.

We next investigated the differentiation potential of embryonic lizard tail NSCs in vivo by tracing the cell fates and lineage contributions of Sox2⁺ NT cells through tail development and into adult tail regeneration. We used a combination of lentiviral and adeno-associated virus (AAV) vectors to deliver CreStoplight and Sox2 promoter-driven Cre recombinase (Sox2-Cre) constructs, respectively, to embryonic lizard tails. Lentiviral Sox2-Cre constructs consisting of Cre genes downstream of 1 kb inserts of the lizard (*Lepidodactylus lugubris*) Sox2 promoter region were packaged into AAV6 viral particles (See Supplementary Information Table 1 for *L. lugubris* Sox2 promoter insert sequence). CreStoplight constructs consisted of adjacent green fluorescent protein (GFP) and transcription terminator (Stop)

1 sequences under transcriptional regulation of constitutively active CAG promoters. GFP and Stop elements were flanked
2 by loxP and loxH sites and followed by red fluorescent protein (RFP) inserts.¹⁷ CreStoplight constructs were packaged
3 into VSV-G pseudotyped lentiviral envelopes. Under this Sox2-Cre/CreStoplight system, transduced cells were labeled
4 with GFP in the absence of Cre but were irreversibly converted to RFP labeling upon the co-expression of Cre in Sox2⁺
5 embryonic NSCs. Two different viral vectors were used to modulate the persistence of each packaged insert in lineages
6 derived from transduced cells. Lentivirus was chosen to deliver CreStoplight constructs because it would provide
7 permanent genomic integration of GFP/RFP genes, allowing the fates of embryonic Sox2⁺ cell progenies to be traced
8 through development and regeneration. Conversely, AAV6 was chosen to deliver Sox2-Cre constructs as episomes that
9 would be diluted through repeated cell divisions, allowing for targeted Cre-labeling of embryonic and not adult Sox2⁺
10 NSC populations. The abilities of this dual vector system to provide transient Cre expression and prolonged GFP/RFP
11 labeling were simultaneously validated via Western blots (Supplementary Information Figure 4).

12 To perform lineage tracing, lentiviral vectors were injected into the amniotic sacs of 40 freshly laid lizard eggs (0 DPO)
13 using a microinjection system (Supplementary Information Figure 5). 10 embryos were collected at 7 and 14 DPO each
14 and analyzed for RFP, GFP, Sox2, and TUJ1 expression (Figure 5B, C) as well as Pax7, Pax6, and Shh expression
15 (Supplementary Information Figure 6). At 7 DPO, GFP was expressed by various embryonic tissues (Supplementary
16 Information Figure 7), but RFP signal was restricted to cells associated with NTs (Figure 5B). The majority of RFP signal
17 was detected in Sox2⁺ cells, validating effective and specific labeling by the Sox2-Cre/CreStoplight lentiviral system
18 (Figure 5B). RFP co-localized with Pax7⁺, Pax6⁺, and Shh⁺ regions of NTs, indicating labeling of roof, lateral, and floor
19 domains, respectively (Supplementary Information Figure 6A). RFP signal was also detected in Tuj1⁺ Sox2⁻ neurons
20 immediately adjacent to NTs (Figure 5B), indicating early neurogenesis in Sox2⁺ NSCs between the time of egg injection
21 and sample collection (7 DPO). At 14 DPO, RFP signal was detected in Sox2⁺ NSCs and Tuj1⁺ neurons of NTs (Figure
22 5C). Within NTs, RFP signal continued to be distributed among Pax7⁺ roof pates, Pax6⁺ lateral domains, and Shh⁺ floor
23 plates (Supplementary Information Figure 6B). DRG had also developed by 14 DPO, and RFP signal co-localized with
24 Tuj1⁺ DRG regions (Figure 5C), indicating derivation of DRG neurons from Sox2⁺ NT NSCs.

25 20 embryos transduced with the Sox2-Cre/CreStoplight hatched and matured for 28 days post hatching (DPH) before
26 their tails were collected by amputation. The resultant regenerated tails were then re-collected 28 days post-amputation
27 (28 DPA). Samples of both original and regenerated tails were analyzed for GFP, RFP, Sox2, Tuj1, glial fibrillary acidic
28 protein (GFAP), Pax7, Pax6, and Shh expression. Original tail samples exhibited RFP signal exclusively in spinal cord
29 and DRG tissues of the CNS (Figure 5D). RFP signal co-localized with Tuj1⁺ neurons, GFAP⁺ glial cells, and Sox2⁺
30 ependymal NSC populations of the spinal cord, as well as Tuj1⁺ neurons of DRG (Figure 5D, E). Sox2⁺ ependymal cells
31 co-expressed Sox2 and GFP, validating the glial/ependymal identity of original tail spinal cord NSCs (Figure 5E). RFP
32 signal co-localized with Shh⁺ ependyma regions, but as described above, original tail spinal cord ependyma did not
33 exhibit the Pax7⁺ roof plate or Pax6⁺ lateral domains of embryonic NTs (Supplementary Information Figure 6C). In
34 regenerated tails, RFP signal was confined to ETs in glial/ependymal cells that co-expressed Sox2 and GFAP (Figure 5F).
35 As in ependymal cells of the original tail, RFP⁺ regenerated tail ependyma cells expressed Shh but not Pax7 or Pax6
36 (Supplementary Information Figure 6D).

Embryonic lizard tail NSCs undergo hedgehog-influenced neurogenesis, while adult NSCs do not.

The above experiments suggested that embryonic tail NSCs differentiated into neurons of the CNS. However, we have previously shown that adult lizard tail NSCs are unable to differentiate into neurons *in vivo* and *in vitro*.³ Here we directly compared the differentiation potential of embryonic NT and adult spinal cord NSCs *in vitro*. We also tested the effects of hedgehog inhibition and activation on the neural differentiation of these cells to gain insight into the effects of the regenerated tail hedgehog signaling environment on NSC differentiation. Embryonic NT cells and adult spinal cord ependymal cells were cultured as neurospheres and subsequently exposed to differentiation conditions for 14 days in the presence of cyclopamine, SAG, or vehicle control (Figure 6). Prior to differentiation, both embryonic and adult NSCs expressed Sox2 and GFAP in neurosphere culture, and neither expressed neuron marker Tuj1 (Figure 10A, B) (Supplementary Information Figure 8). After differentiation, only embryonic NSCs expressed Tuj1, indicating neurogenesis in embryonic, but not adult, NSCs (Figure 6C, F) (Supplementary Information Figure 8). Embryonic NSC neurogenesis was unaffected by cyclopamine treatment but was inhibited by SAG (Figure 6E, G) (Supplementary Information Figure 8). Adult NSCs did not express Tuj1 under any condition tested (Figure 6 D-H) (Supplementary Information Figure 8). These results suggested that embryonic, but not adult, lizard NSCs were capable of neural differentiation under hedgehog-free conditions.

Exogenous embryonic NSCs transplanted into adult amputated tails reconstitute ETs during tail regeneration, but differentiation into roof plate and neural identities is inhibited by endogenous hedgehog signaling.

Next we compared the differentiation capacities of embryonic vs adult NSCs during lizard tail regeneration *in vivo*. We have previously shown that exogenous NSCs implanted into tail spinal cord ependyma populations contribute to regenerated ETs and maintain spatial arrangements reflecting their initial positions at injection.³ For example, labeled cells injected into dorsal regions of original tail spinal cord ependyma proliferated and contributed to dorsal, but not ventral, regions of regenerated tail ETs. Furthermore, the differentiation of exogenous NSC populations into neural lineages within regenerated tails was shown to be influenced by native hedgehog signaling environments.³ We adapted this system for use with embryonic and adult tail NSCs combined with systemic cyclopamine treatment to directly test the effects of hedgehog signaling on NSC differentiation *in vivo*. NSCs were isolated from embryonic NTs and adult tail spinal cords, cultured and proliferated as neurospheres, dissociated into single cells, and labeled with the fluorescent dye DiI (Figure 7A). DiI-labeled NSCs were implanted into dorsal spinal cord ependymal regions of amputated tails (Figure 7A). Following 28 days of tail regrowth and treatment with vehicle control or cyclopamine, regenerated tails were collected and analyzed for DiI co-localizations with neural differentiation markers Sox2, Tuj1, and GFAP and dorsoventral patterning markers Pax7 and Shh (Figure 7). Exogenous embryonic and adult NSC populations contributed to regenerated tail ETs and maintained positional localization to dorsal regions following implantation into adult tail ependyma (Supplementary Information Figure 9). Similarly, both embryonic and adult NSCs retained their Sox2⁺ GFAP⁺ glial/ependymal cell identity within regenerated tail ETs and did not differentiate into Tuj1⁺ neurons under control conditions (Figure 7B, C). Furthermore, ET regions derived from exogenous embryonic NSCs did not express Pax7 as

1 observed in neurosphere culture (Figure 7B). Instead, embryonic NSC-derived cell populations expressed Shh and were
 2 indistinguishable from endogenous regenerated tail ET cells under control conditions, suggesting the loss of roof plate and
 3 ventralization of embryonic NSC identities (Figure 7B). Reconstitution of regenerated tail ETs by exogenous embryonic
 4 and adult NSCs was not affected by cyclopamine (Figure 7B, C). However, cyclopamine treatment did affect the abilities
 5 of ET regions derived from exogenous embryonic NSCs to retain roof plate identity and differentiate into neurons (Figure
 6 7B). Tails regenerated by lizards implanted with DiI-labeled exogenous embryonic NSCs and treated with cyclopamine
 7 exhibited characteristic “figure 8” double ETs consisting of dorsal DiI-labeled exogenous tubes and ventral unlabeled
 8 endogenous tubes (Figure 7B). Only DiI-labeled ET regions expressed Pax7 and Tuj1 and not Shh (Figure 7B). Instead,
 9 Shh expression was limited to endogenous DiI⁻ ET regions, resulting in the first patterned lizard ETs exhibiting dorsal
 10 Pax7⁺ Shh⁻ roof plates and ventral Pax7⁻ Shh⁺ floor plates (Figure 7B). Patterned ETs were only observed in conditions
 11 concerning both embryonic NSCs and cyclopamine treatment (Figure 7B). Otherwise, all other exogenous cell progenies
 12 fully incorporated into the endogenous ET cell populations. In particular, exogenous adult NSCs contributed to ET floor
 13 plate identities only and were unresponsive to native hedgehog signaling environments (Figure 7C). Taken together, these
 14 experiments created the first regenerated lizard tails with dorsoventrally patterned ETs and newly differentiated neurons.
 15 Relief from endogenous hedgehog signaling allowed embryonic NSC populations to recreate NT-like regions within the
 16 regenerated lizard tail ET.

17
 18 **Gene-edited embryonic NSCs resistant to hedgehog signaling introduce roof plate-associated structures and**
 19 **dorsoventral patterning of skeletal tissues in regenerated lizard tails.**

20 The above experiments suggested that embryonic NSCs implanted into adult tails incorporated into regenerated tail ETs,
 21 where they introduced roof plate structures and dorsoventral patterning, but only if hedgehog signaling was inhibited.
 22 Otherwise, progeny derived from exogenous embryonic NSCs were converted to floor plate, became undistinguishable
 23 from endogenous ependyma, and formed neither roof plate nor neurons. The goal of this study was to induce dorsoventral
 24 patterning in both CNS and skeletal tissues, but since hedgehog signaling was also responsible for induction of blastema
 25 cell cartilage differentiation, systemic cyclopamine treatments used in above experiments were unsatisfactory because
 26 they also inhibited skeletogenesis. We hypothesized that a more targeted approach was needed; embryonic NSCs
 27 genetically altered to be unresponsive to hedgehog signaling would retain roof plate identity and resist ventralization
 28 following incorporation into the Shh-saturated regenerated lizard tail environment. The simultaneous repression of Shh
 29 signaling and enhancement of NCS roof plate differentiation capacity would induce dorsoventral patterning in the lizard
 30 ET and, hence, regenerated cartilage.

31 Intracellular hedgehog signaling passes through the essential membrane protein smoothened (Smo), often referred to as
 32 the “switch” of hedgehog signaling.¹⁸ Indeed, affecting Smo activity is the most efficient means of modulating hedgehog
 33 signaling, and both cyclopamine and SAG target Smo to induce inhibition and activation, respectively.^{19,20} Without Smo,
 34 cells become unresponsive to hedgehog signaling even when stimulated by high levels of hedgehog ligands. For these
 35 reasons, we chose to create embryonic NSCs that are unresponsive to hedgehog signaling by knocking down Smo. Here
 36 we used CRISPR/Cas9 gene editing to replace Smo with GFP genes in embryonic NSCs. First, the *L. lugubris* Smo gene

(LISmo) was sequenced and used to design gRNA to exon 2 (LISmo-gRNA) (See Supplementary Information Table 1 for sequences). LISmo gRNAs were tested for their efficacy in knocking out LISmo and rendering embryonic NSCs unresponsive to hedgehog stimulation *in vitro* and *in vivo*. Homology directed repair (HDR) donor template plasmids contained appropriate left and right homology arm sequences specific for each gRNA-directed double strand breaks and included GFP gene inserts. Promoter-free GFP HDR inserts were used to limit background fluorescence produced by unintegrated HDR plasmids, thereby eliminating the need for episomal dilution through repeated passaging prior to sorting. Single cell suspensions of embryonic lizard tail NSCs were transfected with Cas9/LISmo-gRNA/HDR complexes, and FACS was used to isolate populations of GFP+ cells representing successfully edited Smo-CRISPR NSCs (Figure 8A). This method yielded an editing efficiency of 90% (Figure 8B). Control-CRISPR NSCs consisted of identically treated cell populations that were transfected with nonbinding “scramble” gRNA rather than LISmo-gRNA and served as controls for Smo-CRISPR NSCs. Smo-CRISPR cells derived from individual GFP+ neurospheres (Figure 8C) were expanded and passaged to yield 50 “near-clonal” lineages (See Supplemental Information Materials and Methods for description of expansion process). Smo-CRISPR clonal populations were individually tested for Smo RNA expression by real-time RT-PCR (Supplementary Information Figure 10) using primers to LISmo (See Supplementary Information Table 1 for sequences). All 50 Smo-CRISPR clonal lineages exhibited significantly lower SMO mRNA levels than those expressed by Control-CRISPR NSCs (Supplementary Information Figure 10). Smo-CRISPR NSC populations exhibited a range of Smo mRNA amounts, and some lineages expressed undetectable levels (Supplementary Information Figure 10). Smo- and Control-CRISPR clonal populations were also stimulated with SAG and analyzed for Gli1 mRNA expression via real time RT-PCR (Supplementary Information Figure 11) and Gli1 promoter activity via luciferase-based Gli1/hedgehog pathway reporter assays (Supplementary Information Figure 12). SAG treatment induced significant upregulation in Gli1 mRNA expression and Gli1 promoter activity in Control-CRISPR cells (Supplementary Information Figures 11 and 12). Among Smo-CRISPR cells, several populations exhibited unresponsiveness to SAG stimulation (Supplementary Information Figure 11 and 12). These hedgehog-resistant clonal populations were the same ones that exhibited the lowest Smo mRNA expression levels (Supplementary Information Figure 10). Taken together, these *in vitro* assays validated and identified Smo-CRISPR embryonic NSC populations in which Smo genes were replaced by GFP and hedgehog signaling was interrupted.

The clonal Smo-CRISPR embryonic NSC population with the lowest Smo expression and weakest responses to exogenous hedgehog signals was chosen for *in vivo* experiments (Figure 8). These Smo knockout (KO) NSCs were tested for their differentiation potential and effects of tissue dorsoventral patterning during lizard tail regeneration (Figure 8A). Smo KO neurospheres were trypsonized to yield single-cell suspensions and injected into the dorsal regions of amputated tail ependyma (Figure 8A). Control tails received injections of DiI-labeled embryonic NSCs derived from Control-CRISPR neurospheres. After 4 weeks regeneration, tail samples were collected and analyzed for GFP/DiI fluorescence to trace contributions of injected cells to regenerated tissues and for expression of markers Col2, Tuj1, Sox2, Shh, and Pax7 to determine effects on regenerated tissue differentiation and patterning (Figure 8). Tails collected from lizards treated with either SMO KO or control NSCs exhibited ETs with fluorescently labeled dorsal regions (Figure 8D-X), indicating that Smo KO did not interfere with exogenous NSC engraftment and contribution to regenerated ependyma populations.

1 As observed with embryonic NSC transplants during cyclopamine treatment, Smo KO embryonic NSCs contributed to the
 2 dorsal halves of distinct figure-eight double ETs (Figure 8D, H, L-O). Interestingly, tails that received injections of Smo
 3 KO NSCs exhibited Col2+ cartilage rods dorsal to ETs (Figure 8D) rather than the cartilage tubes that completely
 4 surround ETs in control tails (Figure 8P). Quantification of dorsal and ventral Col2 immunofluorescence (IF) signals
 5 along tail lengths indicated that the presence of dorsal ET regions derived from exogenous SMO KO embryonic NSCs
 6 significantly reduced dorsal and ventral cartilage areas compared to control tails (Supplementary Information Figure 13).
 7 Dorsal regions of tails regrown by Smo KO NSC-treated lizards also exhibited Tuj1+ “DRG-like” neural structures
 8 (Figure 8D-G) that were absent from control tails (Figure 8P). Unlike cartilage regions, ectopic “DRG-like” structures co-
 9 expressed GFP, indicating their derivation from exogenous Smo KO embryonic NSC implants (Figure 8D-G). Analysis of
 10 patterning markers revealed that ETs regenerated in animals treated with Smo KO NSCs exhibited dorsoventral patterning
 11 (Figure 8H-O), while those of control tails did not (Figure 8Q-X). Dorsal regions derived from SMO KO, but not control,
 12 NSCs expressed Pax7 (Figure 8I, R). Pax7 expression co-localized with dorsal GFP+ regions (Figure 8L), while Shh
 13 expression was excluded and restricted to GFP- ventral endogenous ET regions (Figure 8M). The resultant ETs
 14 regenerated in Smo KO NSC-treated lizard bore strikingly resemblances to embryonic ETs, with GFP+ Shh- Pax7+ dorsal
 15 “roof plates” and GFP- Shh+ Pax7- ventral “floor plates” (Figure 8L-O). ETs formed in lizards treated with Di-labeled
 16 control NSCs expressed Shh throughout, including dorsal DiI+ regions, and did not form roof plate domains (Figure 8U-
 17 X). These results indicated that ET cells derived from Smo KO NSCs differentiated into roof plate structures and neurons
 18 and introduced dorsoventral patterning into regenerated CNS and skeletal tissues.

20 DISCUSSION

21 Lizards evolved more than 150 million years ago, and yet no lizard species has ever developed the ability to regenerate
 22 tails with dorsoventrally patterned tissues.²¹ Using a combination of embryonic stem cell transplantations, CRISPR/Cas9
 23 genome editing technology, and a unique parthenogenetic lizard species, we created the first regenerated lizard tails with
 24 dorsoventrally patterned CNS and skeletal tissues. In doing so, we uncovered the effects of NSC identities and
 25 differentiation capacities on tail regenerative outcomes. Regenerative programs are often presented as recapitulating
 26 developmental processes to replace lost tissues. For example, during embryonic tail development, NTs differentiate into
 27 CNS tissues and provide Shh patterning signals that induce skeletogenesis in tail bud mesoderm. Similarly, during tail
 28 regeneration, ETs reform CNS tissues and direct skeletal differentiation in blastema cells via hedgehog signaling. In some
 29 animals, like salamanders and frog tadpoles, CNS differentiation and skeletal patterning during tail regeneration faithfully
 30 recapitulates embryonic tail developmental processes. Lizard tails, on the other hand, present a unique case in which
 31 regenerative outcomes fail to recreate embryonic tail formation. This study suggests that lizard tail regeneration is unable
 32 to recapitulate the CNS differentiation and skeletal patterning achieved during embryonic tail development due to limited
 33 differentiation capacity of adult NSCs that prevents roof plate differentiation. Embryonic NSCs are able to differentiate
 34 into Pax7+ Shh- roof plate domains in tail NTs that effectively limit Shh expression and subsequent hedgehog signaling to
 35 floor plates and ventral tail regions. Roof plate NSCs undergo neurogenesis and give rise to DRGs, while Shh produced
 36 by floor plate induce chondrogenesis/skeletal development in ventral tail bud cells. In contrast, adult lizard NSCs are

unable to form roof plates, resulting in uniform floor plate identity, unregulated Shh production, and, ultimately, unpatterned cartilage formation during tail regeneration.

We hypothesized the key to introducing dorsoventral patterning in the adult regenerated lizard tail is to recreate the Shh- roof plate/Shh+ floor plate spatial expression profile characteristic of the embryonic tail NT. This was accomplished by directly transplanting embryonic NSCs derived from tail NTs into adult tail spinal cords, where they contributed to dorsal regions of regenerated tail ETs. These experiments were only possible through the use of the parthenogenetic lizard species *L. lugubris*, one of the only such lizards that is both diploid (which facilitates genome editing protocols) and capable of regenerating its tail, and one of the few examples of obligate parthenogenesis among vertebrates.²² This all-female species of gecko, which reproduces asexually to generate genetically identical clonal offspring, exhibits no detectable intra-clonal variation in MHC genes,²³ and cells and tissues can be transplanted among animals within clonal lineages without rejection.^{3,22,24} The clonal characteristics of *L. lugubris* reproduction means that embryonic NSCs and their progeny are able to be implanted into any adult animal without rejection and without the use of immunosuppressants, which have been shown to adversely affect developmental and wound healing processes, including regeneration.²⁵⁻²⁷

Embryonic NSCs and their progeny were capable of roof plate differentiation when implanted into adult tails, but unchecked hedgehog signaling effectively ventralized any NSC population introduced to the lizard ET. We hypothesized that only the simultaneous repression of Shh signaling and enhancement of NCS roof plate differentiation capacity would induce patterning in the lizard ET and, hence, regenerated cartilage. Smo KO embryonic NSCs generated via CRISPR/Cas9 gene editing were unresponsive to hedgehog stimulation and retained roof plate identity and potential for neurogenesis. When incorporated into dorsal regions of adult regenerated tail ETs, Smo KO NSCs resisted ventralization, differentiated into roof plate structures and neurons, and prevented floor plate Shh signals from inducing chondrogenesis in dorsal tail regions. These edited embryonic NSCs were sufficient for recreating developmental dorsoventral patterning in adult regenerated tails. Future work will focus on the functional ramifications of neurogenesis and skeletal patterning introduced to regenerated lizard tails.

This study outlines novel workflows to genetically manipulate tail NSC populations through the introduction of exogenous cells gene-edited *in vitro*. In other, more traditional model organisms, this work would typically be accomplished through the creation of stable transgenic lines. However, aspects of reptile reproductive biology present hurdles for generating stable transgenic lizard lines. Reptiles are generally slow to reach sexual maturity and/or reproduce seasonally in response to environmental triggers. Many species are also difficult to maintain in captivity and lose fecundity without revitalization from outbreeding with wild-caught stock. Finally, reptilian oviposition takes place at advanced stages of embryogenesis compared to birds, precluding the application of transgenesis protocols established for chickens to lizards. Despite these hurdles, the first transgenic lizard line was recently produced through direct injection of Cas9 ribonucleoprotein complexes into ovarian follicles of the brown anole lizard (*Anolis sagrei*),²⁸ a sexually-reproducing species. This tremendous achievement certainly provides proof-of-concept for developing transgenic lizard models for investigating a wide range of biological processes in lizards. However, the realities of investigating adult lizard biological processes, such as lizard tail regeneration, are not compatible with the current state-of-the art of lizard transgenesis. For example, gene knockdowns are necessary to investigate the specific molecular regulators of tail

1 regeneration. Lizard tail regeneration involves many of the same signaling cascades involved in embryonic tail
2 development, including hedgehog signaling. Constitutive organismal knockouts of members of these fundamental
3 pathways, such as Smo, would not survive embryonic development, limiting their usefulness in investigating adult wound
4 healing processes. Unfortunately, the inducible knockouts that are needed to avoid these complications are beyond current
5 lizard transgenesis capabilities, and the realities of lizard sexual reproduction means time commitments of multiple years
6 for the generation of each of the required lines. Thus, there was a need to directly target regenerating lizard tail cells with
7 genetic modifications and manipulations. Previous studies have applied transfection^{29,30} and transduction³¹ methods to
8 introduce exogenous sequences coding for mRNA or shRNA to the already-formed blastema. However, these techniques
9 were non-specific, making it impossible to target specific cell populations, and impermanent, suffering from episomal
10 dilution in highly proliferative cell populations. Here we overcame these shortcomings and described a platform for
11 introducing gene-edited NSCs to specific regions of regenerated tail ETs in the asexually reproducing lizard *L. lugubris*.

12 In summary, the first regenerated lizard tails with dorsoventrally patterned spinal cord and skeletal tissues have been
13 created. In reaching this milestone, this study provides insight into the principles governing the fidelity with which
14 regenerative processes recapitulate embryonic development. Specifically, the ramifications of NSC cell states at times of
15 initial tail amputations on final regenerative outcomes are highlighted. In salamanders, embryonic tail NT dorsoventral
16 patterning is maintained into adulthood, and adult salamander spinal cord NSC populations exhibit roof plate and floor
17 plate identities that carry over to ETs during tail regeneration.^{3,32} Conversely, lizard tail NT dorsoventral patterning is not
18 maintained during embryonic development, and adult lizard tail ependyma exhibit floor plate identity only. Upon adult
19 tail amputation, these lineage-restricted populations are the only NSCs available to reconstitute regenerated tail ETs,
20 resulting in the loss of dorsoventral patterning in regenerated tail tissues. This study demonstrated that regenerated lizard
21 tail patterning can be restored if endogenous adult spinal cord ependyma cell populations are supplemented with
22 exogenous, roof-plate competent NSC populations at the onset of tail regrowth. While anamniotic salamanders and frogs
23 are able to “turn back the clock” and reform embryonic structures in adult ectoderm-derived tissues,^{3,32,33} amniotic lizards
24 have lost this ability. This sacrifice in cell plasticity is one of the trade-offs between evolution and regenerative
25 potential,^{4,34} but this study highlights ways that this trend can be reversed. Future work will investigate the epigenetic
26 changes that occur in lizard tail NSC populations through development and into regeneration with the aim of identifying
27 the specific genetic regions responsible for divergent roof plate differentiation capacities.

28 **Materials and Methods**

29 All reagents/chemicals were purchased from Sigma-Aldrich unless otherwise specified. Additional materials and methods
30 can be found in Supplementary Information.

31 **Lizards**

32 All experiments were carried out with the mourning gecko *Lepidodactylus lugubris*. This parthenogenetic, all-female
33 lizard species reproduces asexually to yield clonally identical offspring.²² Cells and tissues can be transplanted among

colony members without the use of immunosuppressant and anti-rejection therapeutics,³ which have been shown to negatively affect tail regeneration. *L. lugubris* is believed to be a hybrid species, and more than twenty clonal populations are spread throughout islands of the Pacific and Indian Oceans, several of which exhibit polyploidy.²² The founding members of our research colony were collected from Hawaii, which we verified as belonging to a diploid clonal population via karyotyping (service provided by the Molecular Cytogenetics Laboratory, Department of Veterinary Integrative Biosciences, Texas A&M University) (Supplementary Figure 14). All lizard experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee at the University of Pittsburgh (protocols 15114947, 16128889, and 18011476) and the University of Southern California (protocol 20992).

Tail Sample Collection

Embryonic lizard tails were collected 7 and 14 days post-oviposition (DPO). Adult lizard tails were collected 28-112 days post hatching (DPH). Regenerated tail samples were collected 28 days post-tail amputation (DPA). Ethyl chloride spray was used to numb surgical sites, and tail amputations were performed with number 22 scalpel blades.

Drug Treatments

Embryonic and adult lizards were treated with the hedgehog inhibitor cyclopamine or the smoothened (Smo) agonist SAG in vivo. To treat lizard embryos, a microinjector system was used to deliver drug solutions in ovo (Supplementary Figure 6). Lizard eggs were injected with 3 μ l of 500 μ g/ml cyclopamine or 800 μ g/ml SAG. To treat adult lizards, cyclopamine (50 μ g/g) or SAG (40 μ g/g) were administered via intraperitoneal (i.p.) injections every 24 hours. In vitro, neurosphere cultures were treated with 10 μ M cyclopamine or 500 nM SAG.

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AUTHOR CONTRIBUTIONS

TPL, RL, AXS, and MLH performed experiments, analyzed data, and revised and edited the paper. TPL acted as mentor to RL, AXS, and MLH.

COMPETING INTERESTS

None of the authors have competing interests to declare.

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FIGURE LEGENDS

Figure 1: Dorsoventral patterning of skeletal and CNS tissues established during embryogenesis is not recapitulated during lizard tail regeneration. Cross sections of (A) embryonic (14 days post-oviposition; DPO), (B) adult (28 days post-hatching; DPH), and (C) regenerated lizard tails (28 days post-amputation; DPA) were analyzed by Col2, Sox2, and Tuj1 IF to highlight the spatial arrangements of skeletal (cartilage), NSC, and nerve tissue, respectively, during tail development and regrowth. (D-O) Cross sections of NT and spinal cord ependymal structures were analyzed by Pax7, Pax6, Shh, and FoxA2 IF to compare the expression of roof plate, lateral domain, and floor plate identities among (D-G) embryonic, (H-K) adult, and (L-O) regenerated tails. cc, central canal; ct, cartilage tube; DPA, days post-amputation; DPO, days post-oviposition; DPH, days post-hatching; drg, dorsal root ganglion; et, ependymal tube; fp, floor plate; nt, neural tube; rp, roof plate; rsc, regenerated spinal cord; sc, spinal cord; sce, spinal cord ependyma. Bar = 50 μ m.

Figure 2: Embryonic lizard tail NTs and adult lizard spinal cord ependyma both contain NSC populations but differ in dorsoventral positional identities. (A-D) Summary schematic of embryonic tail NT and adult tail spinal cord NSC isolation and culture as neurospheres. Neurospheres derived from embryonic tails default to roof plate identity, while neurospheres derived from adult tails persist as floor plate identity. (B and D) Light microscopy images of 14-day-old neurospheres formed *in vitro* from cells isolated from (B) embryonic NTs and (D) adult spinal cords. (E-J) Cross sections of (E-G) embryonic tail NTs and (H-J) adult tail spinal cord ependyma analyzed by IF for roof plate (Pax7), lateral domain (Pax6), and floor plate (Shh) markers. Sox2 IF marks resident NSC populations. (K-P) Roof plate, lateral domain, and floor plate IF analysis of Sox2+ neurospheres derived from either (K-M) embryonic or (N-P) adult tails and cultured for 14 days *in vitro*. Note the absence of lateral and floor plate markers in embryonic neurospheres contrasted to the lack of lateral and roof plate markers in adult neurospheres. ns, neurosphere; nt, neural tube; sce, spinal cord ependyma. Bar = 50 μ m.

Figure 3: Hedgehog signaling regulates dorsoventral patterning of embryonic tail NTs but not regenerated tail ETs. Cross sections of (A-B, E-F, I-J) embryonic and (C-D, G-H, K-L) regenerated lizard tails following treatment with vehicle control, cyclopamine, or SAG were analyzed by Col2 and Tuj1 or Pax7 and Shh IF. Neural and ependymal tube regions enclosed in white boxes are shown in magnified views to the right of the corresponding image. c, cartilage; et, ependymal tube; nt, neural tube. Bar = 50 μ m.

Figure 4: Ventralization of neurospheres derived from embryonic tail NTs is regulated by hedgehog signaling, while neurospheres derived from regenerated tail ETs maintain floor plate identities regardless of hedgehog inhibition. Neurospheres derived from (A-C) embryonic tail NTs or (D-F) regenerated tail ETs were treated with vehicle control, cyclopamine (5 μ M) or SAG (100 nM) and analyzed for roof (Pax7) and floor (Shh) plate identities via IF. Bar = 50 μ m.

Figure 5: Embryonic lizard NT cells contribute to nerve, glia, and NSC populations within the embryonic, adult, and regenerated tails. (A) Experimental scheme for tracing the differentiation capacity and lineage contributions of Sox2+ NT cells *in vivo*. Freshly laid (0 DPO) lizard embryos were injected with lentiviral and AAV6 vectors containing CreStoplight and Sox2-Cre constructs, respectively. This system permanently and specifically labeled Sox2+ NT cells and their progeny with RFP fluorescence. The differentiation and contribution of RFP-labeled cells to various tail tissues were traced in embryonic lizards 7 and 14 DPO, as well as in post-hatching lizards (28 DPH) and in lizards with regenerated tails (28 DPA). Cross sections of (Row B) 7 DPO and (Row C) 14 DPO tail samples were analyzed for GFP and RFP fluorescence and by Sox2 and Tuj1 IF. Cross sections of (Row D, E) 28 DPH original tails and (Row F) 28 DPA regenerated tails were analyzed for GFP, RFP, Sox2, Tuj1, and GFAP expression. Row E presents higher magnification views of tail spinal cord regions of 28 DPH lizards analyzed in Row D. Insets of Row F depict lower magnifications of examined regenerated tail ET regions. For all rows, individual signal channels are presented to the left, and merged images of select channels are presented on the right to highlight specific co-expression patterns. White arrow heads denote cells that co-express RFP and Sox2, light grey arrow heads label RFP+Tuj1+ NT/spinal cord/ET neurons, dark grey arrow heads mark RFP+ Tuj1+ DRG neurons, and brown arrow heads point out RFP+ GFAP+ glial cells. drg, dorsal root ganglion; et, ependymal tube; nc, notochord; nt, neural tube; sc, spinal cord; sce, spinal cord ependyma. Bar = 50 μ m.

Figure 6. Comparisons of differentiation capacities of embryonic vs adult lizard tail NSCs and sensitivities to hedgehog signaling in vitro. (A,B) Representative undifferentiated neurospheres derived from (A) embryonic lizard tail NTs and (B) adult tail spinal cords analyzed by Sox2, GFAP, and Tuj1 IF. (C-H) Representative embryonic and adult neurosphere cultured under differentiation conditions for 14 days treated with (C, D) vehicle control, (E, F) cyclopamine, or (G, H) SAG analyzed for Sox2, GFAP, and Tuj1 expression. Bar = 50 μ m.

Figure 7: Embryonic lizard tail NSCs introduced into adult tails contribute to the regenerated ETs and are ventralized by the native hedgehog signaling environment. (A) Experimental scheme for introducing embryonic and adult NSCs into regenerated tail ETs. NSCs were isolated from embryo NTs and adult spinal cord ependyma, cultured as neurospheres, dissociated and labeled with DiI. DiI-labeled cells were injected into dorsal regions of adult spinal cord ependyma of tails that had been amputated. Lizards were treated with cyclopamine or vehicle control for 4 weeks while tails regenerated. Regrown tails were collected, and the differentiation and patterning of DiI-labeled exogenous cells were analyzed. (B, C) Cross sections of ETs of tails grown from stumps implanted with (B) embryonic or (C) adult NSCs. For each type of NSC, images of tails treated with vehicle control are depicted in rows on top of cyclopamine-treated tail images. For all rows, individual signal channels are presented to the left, and merged images of select channels are presented on the right. et, ependymal tube. Bar = 100 μ m.

Figure 8: Gene-edited SMO KO neural tube NSCs recapitulate embryonic differentiation and dorsoventral patterning in adult regenerating lizard tails. (A) Experimental scheme for introducing Smo KO embryonic NSC populations into regenerating lizard tail ETs toward inducing dorsoventral patterning of regenerated tissues. NSCs were isolated from embryonic tail NTs and edited to replace Smo gene with GFP by transducing with Cas9 protein, Smo gRNA, and GFP HDR template. Smo KO NSCs were enriched by FACS, propagated as neurospheres, and injected into dorsal regions of spinal cord ependyma in amputated adult lizard tails. Smo KO NSC populations engrafted and contributed to dorsal regions of regenerated tail ETs, and regrown tails were assayed for effects on patterning of cartilage and other tissues. (B) FACS analysis of embryonic NSCs treated with Cas9, Smo-gRNA, and GFP HDR. Editing efficiency was ~90%. (C) Fluorescence micrograph of clonal Smo KO NSC neurospheres showing GFP fluorescence. (C, Inset) Brightfield micrograph of Smo KO neurospheres. (D) Representative cross section of a tail regenerated from a lizard implanted with embryonic Smo KO NSCs and analyzed for GFP, Col2, and Tuj1 expression. Note the regeneration of cartilage rods ventral to ETs. (E-G) Higher magnification view of region identified in Panel D containing ectopic neural structures analyzed by GFP and Tuj1 IF. (H-O) Higher magnification views of ET region identified in Panel D analyzed by GFP, Pax7, Shh, and Sox2 IF. (P) Representative cross section of a regenerated tail produced by a lizard implanted with control NSCs treated with “scramble” gRNA and pre-labeled with DiI. Note the normal cartilage tube completely surrounding the central ET. (Q-X) Higher magnification views of ET regions identified in Panel P analyzed by GFP, Pax7, Shh, and Sox2 IF. cr, cartilage rod; ct, cartilage tube; drg, dorsal root ganglion; et, ependymal tube. Bar = 50 μ m for Panels D and P. Bar = 10 μ m for Panels C, E-O and Q-X.

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Figure 5

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Figures

Figure 1

Dorsoventral patterning of skeletal and CNS tissues established during embryogenesis is not recapitulated during lizard tail regeneration. Cross sections of (A) embryonic (14 days post-oviposition; DPO), (B) adult (28 days post-hatching; DPH), and (C) regenerated lizard tails (28 days post-amputation; DPA) were analyzed by Col2, Sox2, and Tuj1 IF to highlight the spatial arrangements of skeletal (cartilage), NSC, and nerve tissue, respectively, during tail development and regrowth. (D-O) Cross sections of NT and spinal cord endodermal structures were analyzed by Pax7, Pax6, Shh, and FoxA2 IF to compare the expression of roof plate, lateral domain, and floor plate identities among (D-G) embryonic, (H-K) adult, and (L-O) regenerated tails. cc, central canal; ct, cartilage tube; DPA, days post- amputation; DPO, days post-oviposition; DPH, days post-hatching; drg, dorsal root ganglion; et, ependymal tube; fp, floor plate; nt, neural tube; rp, roof plate; rsc, regenerated spinal cord; sc, spinal cord; sce, spinal cord endoderm. Bar = 50 μm.

Figure 2

Embryonic lizard tail NTs and adult lizard spinal cord ependyma both contain NSC populations but differ in dorsoventral positional identities. (A–D) Summary schematic of embryonic tail NT and adult tail spinal cord NSC isolation and culture as neurospheres. Neurospheres derived from embryonic tails default to roof plate identity, while neurospheres derived from adult tails persist as floor plate identity. (B and D) Light microscopy images of 14-day-old neurospheres formed in vitro from cells isolated from (B) embryonic NTs and (D) adult spinal cords. (E–J) Cross sections of (E–G) embryonic tail NTs and (H–J) adult tail spinal cord ependyma analyzed by IF for roof plate (Pax7), lateral domain (Pax6), and floor plate (Shh) markers. Sox2 IF marks resident NSC populations. (K–P) Roof plate, lateral domain, and floor plate IF analysis of Sox2+ neurospheres derived from either (K–M) embryonic or (N–P) adult tails and cultured for 14 days in vitro. Note the absence of lateral and floor plate markers in embryonic neurospheres contrasted to the lack of lateral and roof plate markers in adult neurospheres. ns, neurosphere; nt, neural tube; sce, spinal cord ependyma. Bar = 50 μm.

Figure 3

Hedgehog signaling regulates dorsoventral patterning of embryonic tail NTs but not regenerated tail ETs. Cross sections of (A-B, E-F, I-J) embryonic and (C-D, G-H, K-L) regenerated lizard tails following treatment with vehicle control, cyclopamine, or SAG were analyzed by Col2 and Tuj1 or Pax7 and Shh IF. Neural and ependymal tube regions enclosed in white boxes are shown in magnified views to the right of the corresponding image. c, cartilage; et, ependymal tube; nt, neural tube. Bar = 50 μ m.

Figure 4

Ventralization of neurospheres derived from embryonic tail NTs is regulated by hedgehog signaling, while neurospheres derived from regenerated tail ETs maintain floor plate identities regardless of hedgehog inhibition. Neurospheres derived from (A–C) embryonic tail NTs or (D–F) regenerated tail ETs were treated with vehicle control, cyclopamine (5 μ M) or SAG (100 nM) and analyzed for roof (Pax7) and floor (Shh) plate identities via IF. Bar = 34 50 μ m.

Figure 5

Embryonic lizard NT cells contribute to nerve, glia, and NSC populations within the embryonic, adult, and regenerated tails. (A) Experimental scheme for tracing the differentiation capacity and lineage contributions of Sox2⁺ NT cells in vivo. Freshly laid (0 DPO) lizard embryos were injected with lentiviral and AAV6 vectors containing CreStoplight and Sox2-Cre constructs, respectively. This system permanently and specifically labeled Sox2⁺ NT cells and their progeny with RFP fluorescence. The differentiation and contribution of RFP-labeled cells to various tail tissues were traced in embryonic lizards 7 and 14 DPO, as well as in post-hatching lizards (28 DPH) and in lizards with regenerated tails (28 DPA).

(28 DPA). Cross sections of (Row B) 7 DPO and (Row C) 14 DPO tail samples were analyzed for GFP and RFP fluorescence and by Sox2 and Tuj1 IF. Cross sections of (Row D, E) 28 DPH original tails and (Row F) 28 DPA regenerated tails were analyzed for GFP, RFP, Sox2, Tuj1, and GFAP expression. Row E presents higher magnification views of tail spinal cord regions of 28 DPH lizards analyzed in Row D. Insets of Row F depict lower magnifications of examined regenerated tail ET regions. For all rows, individual signal channels are presented to the left, and merged images of select channels are presented on the right to highlight specific co-expression patterns. White arrow heads denote cells that co-express RFP and Sox2, light grey arrow heads label RFP+Tuj1+ NT/spinal cord/ET neurons, dark grey arrow heads mark RFP+Tuj1+ DRG neurons, and brown arrow heads point out RFP+ GFAP+ glial cells. drg, dorsal root ganglion; et, ependymal tube; nc, notochord; nt, neural tube; sc, spinal cord; sce, spinal cord ependyma. Bar = 50 μ m.

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