Editorial

Stem Cell INVESTIGATION

Getting nervous about regeneration

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Introduction

Tissue regeneration is fundamental to sustaining life, but the multi-tissue regeneration capacity of adult mammals is extremely limited. In a classic model of multi-tissue regeneration, rodents display the capacity to replace amputated distal digit tips that are comprised of many various tissues including bone, muscle, nerve, nail, and skin. The mechanisms by which these various tissues collectively generate a properly patterned replacement digit tip, however, have not been fully elucidated.

It has been previously shown that intact nerves are required for complete replacement and patterning of the mammalian digit tip (1), but the specific role of nerves or nerve-derived factors for executing regeneration has remained unclear. Adam Johnston and colleagues at the University of Toronto have recently shed light on this process by defining the requirement for neural crest-derived Schwann cells and demonstrating that their secreted cytokines can largely rescue regeneration in the absence of intact innervation (2). Here we review their specific results and discuss the broader implications for multi-tissue regeneration.

Results

Johnston *et al.* localize cells from the peripheral nervous system (PNS) in the digit tip using a GFP reporter from the *Sox2* locus. These cells are defined in this study as Schwann cell precursors (SCP) by marker analysis of *Sox2*-GFP+ cells with Schwann cell marker S100B. The SCPs were further shown to come from the neural crest developmental lineage by co-localizing S100B+ SCPs with TdTomato+ cells in a mouse harboring a *Wnt1Cre* transgenic driver and a LSL-TdTomato reporter in the *Rosa26* locus. Once establishing the presence of SCPs in the digit tip, they used an inducible CreERT2 driver inserted into the *Sox2* gene crossed to a TdTomato reporter to confer an indelible genetic TdTomato label to SCPs and their progeny during 4-week digit tip regeneration period. The TdTomato+ SCPs were found to localize to PGP9.5+ axon terminals prior to regeneration, but were found in the regenerating blastema 2-week post-amputation in the absence of PGP9.5+ axon terminals, suggesting that SCPs are the only PNS-derived cells present during early regeneration. Denervation of the limb by resection of the sciatic nerve 10 days prior to digit tip amputation resulted in the complete disappearance of axons prior to regeneration and *Sox2*+S100B+ Schwann cells within the first 2 weeks of regeneration. Consistent with previous studies (1), the denervated digit tips displayed impaired regeneration of bone and nail during compared to the contralateral control amputations.

In order to determine if the loss of SCPs contributes to impaired bone and nail regeneration, Johnston *et al.* used an inducible genetic strategy to globally delete *Sox2* using a CreERT2 driven by the *Rosa26* locus 1 week prior to amputation. The deletion of *Sox2* resulted in the loss of S100B+ Schwann cells in the blastema during early regeneration, and recapitulated the impaired bone and nail regeneration phenotype seen after denervation of the sciatic nerve, affirming that *Sox2* expression is necessary for complete digit tip regeneration. Johnston *et al.* deployed another genetic model to test the requirement for SCPs in

digit tip regeneration in which the previous *Sox2*-CreERT2 driver was used to activate a floxed TdTomato reporter and floxed diphtheria toxin A from the *Rosa26* locus, resulting in the label and cellular ablation of *Sox2*+ cells, including SCPs. Immediately after digit tip amputation, TdTomato label and diphtheria toxin-mediated cell ablation was induced with tamoxifen administration and cellular ablation monitored by the disappearance of TdTomato+ SCPs from the blastema during the first 2 weeks of regeneration. Nail regeneration was impaired early while bone regeneration was not, however, both tissues showed similar impairment by 4-week post-denervation, showing that *Sox2*+ SCPs are required for complete digit tip regeneration. In order to test whether SCPs are sufficient to rescue impaired regeneration due to denervation, Johnston *et al.* employed a similar rat model of digit tip regeneration in which isolated neonatal rat *Sox2*+S100B+ SCPs were expanded in culture and transplanted into immunocompromised rats that had undergone sciatic denervation 13 days prior to digit tip amputation. Though the regenerate supplied with exogenous SCPs displayed abnormal patterning phenotypes, the size and area of the bone and nail regenerates were significantly increased over the vehicle control groups, demonstrating that SCPs are sufficient to largely rescue denervation-impaired regeneration.

Cell proliferation during digit tip regeneration quantified by nucleoside analog Edu incorporation into dividing cells 13-day post-amputation indicated significantly more proliferation in the distal mesenchyme of blastema and nail bed epithelium of digit tips with intact innervation as compared to digit tips that were denervated prior to amputation, suggesting that the loss of SCPs by denervation may impact regeneration by impairment of proliferation. This led Johnston *et al.* to hypothesize that SCPs contribute a mitogenic effect on mesenchyme via secreted growth factors. In support of this hypothesis, they demonstrate that conditioned media from cultured neonatal rat SCPs increased proliferation of rat dermal mesenchymal cells as compared to those cultured with unconditioned media. The transcriptomes and cell surface proteomes of these cell types were analyzed in an integrated discovery strategy aimed at identifying candidate pairs of secreted ligands and receptors that could enact the mitogenic effect of SCPs on mesenchymal cells. Of seventeen candidate ligand-receptor pairs, dimeric alpha-chain Platelet-Derived Growth Factor (PDGF-AA) and oncostatin M (OSM) ligands were functionally validated by their ability to expand dermal mesenchymal sphere cultures and by detection in SCP-conditioned media by ELISA. A similar transcriptome analysis was carried out directly on uninjured and regenerating digit tips, identifying 46 potential ligand-receptor pairs, including *OSM* and corresponding receptors *OSMRB* and *GP130*, and *PDGFA* and *PDGFR-a*. Consistent with their model, ligand expression was elevated specifically in regenerating digit tips relative to uninjured digit tips and expression was enriched in SCPs, with some redundant expression of *PDGFA* in other blastema cell types, while receptor expression was localized to mesenchymal blastema cells. OSM and PDGF-AA were each assayed separately for sufficiency to rescue denervation by injection of 200 ng at 3 and 14 days post-amputation. Despite heterogeneity in patterning outcomes, OSM and PDGF-AA each ultimately proved sufficient to largely rescue denervation-impaired digit tip regeneration by 4 weeks post-denervation.

Discussion

Vertebrate appendage regeneration represents a complex series of events in which multiple disparate cell types must simultaneously coordinate the generation of new cells and adopt proper patterning in order to produce functional replacement tissue. The phenomenon of appendage regeneration has been scrutinized for centuries (3), and includes studies in a variety of vertebrates including teleost fish, salamanders, rodents, and humans (4-9). Several long-standing questions have yet to be fully clarified, including: what is the extent of dedifferentiation of mature cells, the identity and contribution of progenitor cells, the mechanism of injury sensing, the coding of positional information, and the contribution of the PNS. Recent studies have begun to address several of these aspects of appendage regeneration. Studies in the salamander limb and mouse digit tip used lineage tracing techniques to demonstrate that regenerating cells retain their lineage identity, evidence that dedifferentiation of remaining cells to a pluripotent state does not occur (10,11). Wnt-agonist Lgr6 has recently been shown to be a marker of progenitor populations of nail matrix, bone, and sweat glands that contribute to digit tip regeneration in rodents (12). A FGF8-SHH circuit encoding positional information was identified in the regenerating salamander limb (13). The requirement of PNS innervation for regeneration competence of appendages has been well documented in fish and salamanders (1,9,14-17), while denervation experiments in a rodent model found that regeneration in the absence of nerves results in an abbreviated regenerate with abnormal patterning (1). Yet, as

no evidence of a specific cellular and molecular contribution of the PNS to the mammalian regenerative process had previously been reported, the experiments performed by Johnston *et al.* demonstrate the necessity of Schwann cells in multiple mammalian models of regeneration, including dermal wound healing and digit tip regeneration, and are thus of particular relevance to tissue engineering and regenerative medicine.

Notable strengths of this study include the deployment of sophisticated genetic reagents with high cellular resolution. *Sox2*+S100B+ Schwann cells are identified as PNS-derived cells that persist in the blastema region using a knock-in GFP reporter combined with marker analysis. Evidence is provided for the direct requirement of *Sox2* for digit tip regeneration using an inducible gene knockout strategy. Finally, they demonstrate that Schwann cells are necessary for complete regeneration using an inducible genetic cellular ablation of *Sox2*+ cells. Multiple strong lines of evidence leave little doubt that local Schwann cells are indeed the effectors of the PNS contribution to digit tip regeneration, although organism-wide gene perturbation and cell ablation methods leave open the possibility that *Sox2*+ cells at distant sites like the lung and gut may indirectly impact regenerative capacity. However, the possibility of a significant contribution from these sources is not likely given the demonstration of a substantial rescue of regenerative outgrowth by direct transplantation of *Sox2*+S100B+ SCPs adjacent to the blastema in a similar rat model of digit tip regeneration.

While hundreds of potential candidates' ligand/receptor pairs were identified by transcriptome analysis of neonatal SCPs, neonatal dermal mesenchymal cells, and adult blastema, the use of complementary proteomic analysis and functional assays to narrow down the candidate list of Schwann cell-derive mitogens represents a sagacious application of both technologies and the accompanying informatics. While candidates PDGF-AA and OSM both showed efficacy in promoting regeneration after denervation, these two ligands accompany other ligands which have previously been demonstrated to promote regeneration, including BMP 2, BMP4, BMP7, SDF-1a, and Flightless I (18-21). PDGF-AA was also found to be expressed in blastema cell types other than SPCs, and may be additionally supplied by platelets and macrophages recruited to the wound environment, so precise delineation of the contribution of each of these ligands and their cellular source to normal regeneration will require cell type-specific deletion of each.

Despite the significant pro-regenerative effect of SCP, OSM, or PDGF-AA treatment, the authors note that patterning defects of the regenerated bone and nail are still observed, hypothesizing that precise spatial arrangement and distribution of ligands is required for regenerate morphology. Other parameters could be considered to affect the final patterning of the regenerate which would be difficult to control using exogenous cells or recombinant sources of ligand, including precise control of ligand levels, however, the possible importance of positional memory underscores the complexity of tissue regeneration. Future studies may implement the use of ligand-conjugated beads to test the effects of positional ligand gradients on regenerate patterning, or supplement pro-regenerative ligands with ligands known to confer positional information, like FGF8, SHH, or retinoic acid.

Rigorous characterization of the cell state of SCPs prior to, during, and after amputation will be instrumental for discerning the molecular cues by which SCPs are activated to initiate their secretion of ligands after injury. Whether they themselves sense injury and initiate the regenerative response, or whether another cell is upstream in the injury response and signals to activate SCPs will be informative for elucidating the molecular control system of regenerating blastema. It is suggested here that Schwann cells are precursors, and that they undergo dedifferentiation, but evidence is not presented here to precisely characterize the various cell states of SCPs. The results of the transcriptome analysis, however, indicate that OSM and PDGF-AA are upregulated in SCPs after dermal wounding and digit tip amputation, suggesting that SCPs respond to an injury-induced stimulus in order to stimulate the expansion of blastema mesenchyme. Identification of an injury sensing mechanism will likely be a focus of future studies and may be exploited to initiate regeneration in other contexts of tissue regeneration where SCPs are present and regeneration is not normally competent, for instance, diabetic wound healing or amputations proximal to the nail bed.

The potential to induce competence for multi-tissue regeneration holds much promise for efforts aimed at tissue engineering and regenerative medicine. While the reconstitution of lost limbs for patients may represent a seemingly insurmountable challenge, recent findings in the digit tip regeneration model may prove instrumental in furthering efforts aimed at complex tissue regeneration applications. Defining the necessary cell types and factors may lead to the establishment of a comprehensive "parts list" that now includes Lgr6+ progenitors, SCPs, and

a growing list of pro-regenerative ligands. The proper deployment of the required cellular and molecular components will hinge on the clarification of how positional memory is stored and deployed, and by defining the injury-sensing mechanism that stimulates the cellular regenerative response.

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Footnote

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