Stem cell heterogeneity and regenerative competence: the enormous potential of rare cells

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Reconstitution of complex multi-tissue organs is one of the most impressive feats of biology and is observed across regeneration-competent vertebrate species, including teleost fish (e.g., zebrafish), urodeles (e.g., axolotls and newts), and some lizards. Regenerative ability within these species ranges from muscle (including cardiac), skeletal structures, to complex systems such as the brain, spinal cord and parts of the eye which are all capable of structural and functional repair following injury (Tanaka and Ferretti, 2009). In stark contrast, re-establishment of multi-tissue structures is very rarely observed following embryogenesis in regeneration-incompetent mammals. Regrowth of digit tips is the most dramatic example of mammalian regeneration, but pales in comparison to other species in the animal kingdom. Undoubtedly, a complete recapitulation of complex organs or structures in mammals will remain out of reach for a considerable time; however, an improved understanding of regenerative mechanisms would likely enhance the development of novel regenerative medicine strategies. Here we focus on the diversity and commonalities of stem cells, which could underlie complex tissue regeneration.

Endogenous stem cells for central nervous system (CNS) repair and regeneration: Endogenous stem and progenitor cells play a fundamental role in the success of repair in regenerative-competent vertebrate species. Considering the cardinal properties of stem cells including their capacity for self-renewal and their ability to generate all of the mature cells within a given organ system (multi-potency) (Reynolds and Weiss, 1992), unlocking and harnessing their potential remains a fundamental target of regenerative medicine approaches. Stem cells are located throughout the body, including within the brain and spinal cord. In mammals, the largest pool of neural stem and progenitor cells (NSPCs) are found in a well-defined region lining the ventricular system within the developing and mature nervous system. In the brain, periventricular NSPCs are neurogenic throughout life, proliferating to give rise to neuroblasts that migrate anteriorly along the rostral migratory stream to the olfactory bulb under homeostatic conditions (Doetsch et al., 1999). In contrast, NSPCs in the spinal cord are aneurogenic in adulthood (Reynolds and Weiss, 1992). Despite these differences in behavior under baseline conditions, both NSPC populations respond following injury by proliferating, migrating to the site of injury, and differentiating primarily into glial cells (astrocytes) that serve to limit the spread of injury, among other roles. Despite this response to injury, NSPC activation within the CNS does not facilitate structural or functional regeneration in mammals, including humans.

In stark contrast, NSPCs have been shown to contribute to tissue regeneration following injury to the CNS in regeneration-competent species. One of the most striking examples is in the axolotl *(Ambystoma mexicanum*), where complete structural and functional repair of the spinal cord occurs just 6 weeks following blunt injury (Thygesen et al., 2019). Despite the fact that NSPCs across regeneration-competent and -incompetent vertebrates are located in homologous anatomical regions, express the same markers (including SRY-box 2, glial fibrillary acidic protein and nestin), and are universally responsive to injury, differences in regenerative capacity across groups remains poorly understood. A key question and area of current research focuses on understanding the cellular and environmental factors that could unlock improved regenerative competence following injury in mammals. Our recent work exploring a novel population of "primitive" neural stem cells suggests that multiple stem cell subpopulations could be harnessed for endogenous repair within the CNS (Sachewsky et al., 2014, 2019). The importance of characterizing and considering stem cell heterogeneity across organ systems and in different species is a refreshed opportunity to explore exciting new questions in the field of regenerative biology.

Stem cell heterogeneity: Rapidly advancing scientific approaches, including single-cell sequencing, barcoding technologies, and complex transgenic approaches have allowed us to begin to answer fundamental questions about stem cell heterogeneity and are rapidly revealing the complexities of stem cells with an increasing level of detail (Ayyaz et al., 2019; Sachewsky et al., 2019). Historically, it was believed that all stem cells were created equal, but recent work in mammals has highlighted that this is not the case. For example, some stem cells are elite in terms of their ability to survive and change fate through reprogramming (Shakiba et al., 2019). In a recent study, it was shown that a unique subset of stem cells (derived from the neural crest) have a competitive advantage in identical reprogramming conditions, giving rise to larger, more abundant colonies of cells (Shakiba et al., 2019). This highlights heterogeneity of stem cells, which could have important implications for regenerative medicine when considering which stem cell populations to target for enhanced tissue repair. Here, we further define heterogeneity based on stem cells' unique cellular signatures, activation responses and kinetics. While heterogeneity within a stem cell population was first recognized in hematopoietic stem cells, this phenomenon has been characterized across other organs including the intestine and brain (Muller-Sieburg et al., 2004; Ayyaz et al., 2019; Sachewsky et al., 2019). It is currently unknown whether homologous stem cell subpopulations exist in regeneration-competent species, however, at least within an invertebrate model, *Drosophila*, different neural stem cell populations have been observed based on their cell-cycle arrest in either G0 or G2 (Otsuki and Brand, 2020). Moreover, understanding the role of intrinsic and extrinsic factors that regulate stem cell behavior could shed light on dramatic differences in regenerative competence across species.

In the CNS, two distinct, lineally related populations of stem cells exist (Sachewsky et al., 2014, 2019; Xu et al., 2016). Definitive neural stem cells (dNSCs) represent the largest stem cell population along the neuraxis and are characterized by their expression of glial fibrillary acidic protein and responsiveness to epidermal and fibroblast growth factors *in vitro* (Reynolds and Weiss, 1992). A second, more rare, and mostly quiescent "primitive" neural stem cell (pNSC) population is upstream of the dNSCs (Sachewsky et al., 2014; Reeve et al., 2017). pNSCs express the pluripotency marker Oct4 and are responsive to leukemia inhibitory factor *in vitro*. Both populations are injury-responsive, as evidenced by increased clonal dNSC- and pNSC-derived colonies (termed neurospheres) following CNS injury (Sachewsky et al., 2014; Xu et al., 2016). Transplantation and lineage tracking studies reveal that pNSCs not only contribute to neurogenesis, but are the source of dNSC repopulation in models of stem cell ablation (Sachewsky et al., 2014, 2019; Reeve et al., 2017). Establishing the specific role of pNSCs during CNS neuro-regeneration, and determining whether a similar population exists along the neuraxis of regenerative-competent species are compelling questions.

Support for the idea that rare stem cell populations have a role to play in tissue regeneration comes from a recent report showing that a strikingly similar stem cell subpopulation can regenerate the intestine. Using single-cell RNA sequencing, intestinal stem cells were profiled and shown to cluster into two main groups (Ayyaz et al., 2019). One cluster identified a well-delineated intestinal stem cell population

expressing a leucine-rich repeat containing G-protein coupled receptor 5 (LGR5$^+$) known as crypt-base columnar cells, which are responsible for homeostatic turnover of the intestinal epithelium. A second, quiescent population expressed clusterin, and were termed "revival" stem cells (revSC) based on their response to injury (Ayyaz et al., 2019). revSCs are rare, quiescent, and lineally related to crypt-base columnar cells and have the capacity to generate LGR5$^+$ stem cells following intestinal ablation (Ayyaz et al., 2019). pNSCs and revSCs share several key attributes: they are a largely quiescent, exceedingly rare, and are notably responsive to injury where they serve to repopulate downstream stem cell populations that generate all of the major cell types within the respective tissues.

Stem cell heterogeneity and comparative regenerative potential: Characterizing the presence and relative abundance of these rare subpopulations of stem cells across species of varying regenerative ability could reveal the breadth of their reparative potential. When considering stem cell heterogeneity, one of the most striking phenomena is the enormous potential of exceeding rare cells. One can imagine that stem cells with extraordinary potential are only needed in small numbers as it only takes a few of them to produce all of the cells needed for regenerating lost tissue. Equally plausible, is that the rarity of the stem cells with extraordinary potential is what dictates the poor regenerative capacity of mammals. How can we best delineate these possibilities? A first place to start would be the explore stem cell heterogeneity and the relative frequency of pNSCs and revSCs (for example) in regenerative competent species. Future studies should include an analysis of stem cell population dynamics; lineage tracking to explore their respective contributions to regeneration, and utilize knock-out models to inhibit their role following injury in regeneration competent-species. These experiments would elucidate whether rare stem cell populations could be expanded in mammals and exploited to improve endogenous repair. Additionally, single cell-RNA-sequencing now permits the evaluation of how similar CNS-derived pNSCs and intestine-derived revSCs are to their developmental counterparts, and would enable comparison of these cell populations across species of varying regenerative abilities. An investigation of these novel stem cell populations including their frequency, gene profile, cell cycle kinetics and differentiation potential across species would inform a prediction about which stem cells have the most "regenerative potential". This knowledge would help to inform future efforts to target distinct stem cell populations for neurorepair.

Even with powerful techniques that enable single cell analysis, a direct comparison of stem cell populations across species will not fully address differences in regenerative potential. An additional level of complexity comes from the micro-environment or "niche" where the stem cells reside in species with different regenerative capabilities. To date, it remains unclear how the factors released into the niche via circulation, cerebrospinal fluid, or paracrine factors influence the dynamics of stem cell populations, and how this may differ between species. More detailed information on how the composition of the niche varies between regeneration-competent and -incompetent species could help us to re-model and enhance stem cell niches in mammals to improve regenerative medicine approaches.

On a translational level, we predict that stem cell heterogeneity underlies an even more important finding: that different stem cells have different regenerative capacity and play different roles in tissue repair. Understanding heterogeneity serves as a critical component of the pipeline for targeting or enriching for populations of seemingly similar cells, to essentially target those that underlie regeneration. Are different ratios of 'primitive' stem cells associated with different regenerative capacities? Or are the stem cells themselves intrinsically different in species with varying degrees of regenerative competence? Does the stem cell niche account for regenerative outcome? How did these differences between species arise? To date, it remains unclear whether regeneration was an ancestral trait (lost during evolution in non-regenerative species), or an adaptive trait (arisen during evolution in regenerative species) (Tanaka and Ferretti, 2009). Exploring the similarities between stem cell populations across species with varying regeneration potential could help to unlock the regenerative switch as well as reveal the evolutionary origins of regeneration within complex organ systems. Clearly there is much to be learned. Species capable of regeneration serve as a unique and important resource for revealing new information about factors that control the precision of injury repair throughout the body. Understanding stem cell heterogeneity could help to enhance regenerative outcomes in mammals, and ultimately have exciting implications for regenerative medicine approaches.

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