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Systemic cell cycle activation is induced following complex tissue injury in axolotl

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ABSTRACT

Activation of progenitor cells is crucial to promote tissue repair following injury in adult animals. In the context of successful limb regeneration following amputation, progenitor cells residing within the stump must re-enter the cell cycle to promote regrowth of the missing limb. We demonstrate that in axolotls, amputation is sufficient to induce cell-cycle activation in both the amputated limb and the intact, uninjured contralateral limb. Activated cells were found throughout all major tissue populations of the intact contralateral limb, with internal cellular populations (bone and soft tissue) the most affected. Further, activated cells were additionally found within the heart, liver, and spinal cord, suggesting that amputation induces a common global activation signal throughout the body. Among two other injury models, limb crush and skin excisional wound, only limb crush injuries were capable of inducing cellular responses in contralateral uninjured limbs but did not achieve activation levels seen following limb loss. We found this systemic activation response to injury is independent of formation of a wound epidermis over the amputation plane, suggesting that injury-induced signals alone can promote cellular activation. In mammals, mTOR signaling has been shown to promote activation of quiescent cells following injury, and we confirmed a subset of activated contralateral cells is positive for mTOR signaling within axolotl limbs. These findings suggest that conservation of an early systemic response to injury exists between mammals and axolotls, and propose that a distinguishing feature in species capable of full regeneration is converting this initial activation into sustained and productive growth at the site of regeneration.

1. Introduction

Differences in natural regenerative responses across the animal kingdom are profound, and underlying causes for these differences are poorly understood. Across species, an injury-inducing event prompts a systemic, global response across a myriad of tissues (reviewed in (Foex, 1999; Brochner and Toft, 2009; Cordeiro and Jacinto, 2013)). In mammals, an injury stimulus initially promotes systemic activation of immune cells and increased levels of cytokines and growth factors (reviewed in (Anthony and Couch, 2014; Hirsiger et al., 2012; Lenz et al., 2007)), followed by proliferation and remodeling to close the wound (reviewed in (Gonzalez et al., 2016)). Mammalian wounding responses can result in formation of scars, nonfunctional fibrotic tissue physically distinct from surrounding structures (reviewed in (Cohen et al., 2017; Takeo et al., 2015)). In contrast, invertebrates such as hydra and planarians, and lower vertebrates, such as salamanders and fish, are capable of perfectly regenerating complex tissues following amputation (reviewed in (Rink, 2013; Tanaka and Reddien, 2011; Sehring et al., 2016)). The wound response in planarians, for example, results in the systemic activation of resident tissue stem cells that proliferate locally at the injury surface to replace lost tissues with functional, perfectly integrated counterparts (Wenemoser et al., 2012; Wenemoser and Reddien, 2010). Whether the same pathways are active across species and how injury-induced signals are differentially incorporated between regeneration-competent and incompetent species is less clear.

As opposed to wounding models such as epidermal injury, amputation of an appendage results in the total loss of a complex structure composed of multiple tissue types. In highly regenerative tetrapods, such as axolotl salamanders, the entire limb can be regenerated regardless of amputation location (reviewed in (Tanaka, 2016)). This process is facilitated by two key structures: the wound epidermis and the blastema. The wound epidermis is a specialized skin that forms atop the cut stump soon after amputation via the migration of existing epidermal cells. The blastema is a bud-like structure that develops at the tip of the stump, beneath the wound epidermis, that houses the activated progenitor cells that will give rise to the new cells of the regenerate limb. These progenitor cells are activated either by stimu-

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lating cell-cycle re-entry of resident adult stem cells, cellular dedifferentiation, or a combination of both mechanisms (Kragl et al., 2009; Sandoval-Guzman et al., 2014).

A long-standing goal in the field has been to identify molecular mechanisms of progenitor cell activation following amputation. A common assumption is that the wound epidermis is predominantly responsible for secreting the factors that are required to promote progenitor cell activation in the internal stump tissues. This idea largely stems from the observation that when wound epidermis formation is experimentally blocked, limbs fail to grow blastemas, and they do not regenerate (Mescher, 1976; Thornton, 1957; Goss, 1956). Past studies using X-irradiation and lead shielding have demonstrated that the only cells required to divide during the process of limb regeneration are those left on the stump, but close to the amputation plane (Butler, 1931). Together, these experiments have led to a model in which progenitor cell activation occurs locally, where the limb was lost, leading to cell proliferation and tissue growth specifically where the injury occurred.

Mammals such as mice and humans are very restricted in their ability to regenerate portions of their limbs following amputation. The distal digit tip can be regenerated in mice and in young children, but more proximal amputations past the first joint fail to regenerate (Borgens, 1982; Illingworth, 1974). The extent to which mammals might employ similar post-amputation progenitor cell activation programs as other regeneration-competent species is largely unexplored. The possibility that some components of the early regeneration program are intact in mammals, but that later essential features are compromised, exists. More information is required about the very early responses to injury in highly regenerative species, such as axolotls, in order to compare these events to known post-injury or unidentified post-amputation responses in mammals. These comparisons will be critical for further understanding where in the trajectory of successful regeneration the mammalian response goes awry.

We sought to examine early cell activation events that occur globally post-amputation in axolotls, and we found that these cellular activations are not restricted to the site of amputation. Cell-cycle re-entry was observed on intact limbs contralateral to limbs that were amputated on the same animal. Activated cells were not restricted to any particular tissue lineage, and activated cells were additionally identified in several organs throughout the body. We also discovered that early cell cycle re-entry distant to the site of amputation does not rely upon the activity of the wound epidermis formed atop the regenerating limb. These results point to an early, systemic cellular activation in response to the amputation injury that is independent of local structures later required for successful regeneration of the amputated limb. Intriguingly, a fraction of distant, activated cells have engaged the mTOR signaling pathway, recently shown to be required to promote distant quiescent stem cell activation in mice following injury (Rodgers et al., 2014). We propose that global cell cycle activation post-injury is an evolutionary conserved response amongst species, and we hypothesize that axolotls are capable of converting this early systemic response into productive, localized growth at the amputation plane to reproduce a functional limb.

2. Results

2.1. Cells distant to the site of amputation are cued to re-enter the cell cycle

To identify cells that re-enter the cell cycle in response to limb amputation, we performed an EdU time course experiment to label cells actively synthesizing DNA throughout the regenerative process (Fig. 1). The baseline level of EdU-positive nuclei in limbs in which the axolotl has not undergone an amputation anywhere at all (intact, uninjured) is < 0.5% (Fig. 1**A,M; intact**). Total cell nuclei were visualized by DAPI stain. We compared the fraction of EdU+ cells in unamputated limbs contralateral to amputated limbs from the same animal at various time points up to 28 days post-amputation (dpa), until digit reformation has begun. At each time point, animals received a single pulse of EdU at 18 h before tissue harvest (Fig. 1**A′).** In all cases, both the right forelimb and the right hindlimb were amputated, while the left limbs were both left unmanipulated (Fig. 1**A′**). Left limbs therefore allowed for contralateral, distant effects to be examined.

In the amputated limbs, local cellular activation responses were observed consistent with expectations. Cell cycle re-entry skewed toward epidermis in early time points (3 dpa and 5 dpa) (Fig. 1**B, G**), while later time points (7 dpa through 14 dpa) (Fig. 1**C, D**) exhibited more proliferation within the underlying internal tissues of the stump. By 21 and 28 dpa, both epidermis and underlying tissues displayed similar percentages of proliferating cells (Fig. 1**E, F**). In all cases, we quantified total EdU+ nuclei within a millimeter of the amputation plane and under the epithelium in amputated limbs (Fig. 1**G**) to reflect the population of activated cells that would contribute to the regenerating limb.

Meanwhile, we also harvested and processed the contralateral limbs and performed the same histological analysis. We observed an upregulation of EdU+ cells in contralateral limbs at 3 dpa, and while this effect was largely restricted to the skin, significantly more nuclei in the internal tissues of the contralateral limb were EdU+ compared to intact controls (Fig. 1**H, $p < 0.05$**). This trend increased in contralateral limbs between 5 through 9 dpa, with cell cycle activation within contralateral limbs significantly higher than in limbs of intact, uninjured animals (Fig. 1**I, M**, $p < 0.001$ for all time points). By 14 dpa, the fraction of cells in S-phase began to decline (Fig. 1**J**), and it continued to do so through the end of our sampling at 28 dpa (Fig. 1**K-L** summarized in **M**). Single limb amputations were also sufficient to induce this effect (data not shown), suggesting the induction of cell cycle entry following amputation on contralateral, uninjured tissues is systemic throughout the axolotl.

To determine if cells that have been provoked to re-enter the cell cycle and synthesize DNA progress further in the cell cycle and undergo mitosis, we stained adjacent tissue sections from our above analysis with anti-phospho-histone H3 (pH3). We found that in intact limbs from animals with no prior amputations anywhere, pH3+ cells are exceedingly rare (Fig. 1**N**). However, in limbs contralateral to regenerating limbs, a detectable fraction of cells are pH3+ as early as 3 dpa, and by 15 dpa, this effect is highly significant (Fig. 1**O,** quantified in **P**). To rule out that there exists a compensatory cell death mechanism counteracting proliferation in contralateral limbs, we additionally stained for anti-activated caspase-3 and TUNEL to label for both early and late stages of cell death, respectively. With either technique, we did not detect significant cell death within control intact limbs or within limbs contralateral to regenerating limbs (Supplemental Fig. 1). Thus, we conclude that an amputation leads to cellular proliferation in the stump of the amputated limb, as expected; however, it also leads to an upregulation in cellular proliferation in the uninjured, contralateral limbs, and that these activated cells undergo at least one round of mitosis in response to the distant amputation injury.

2.2. A subset of distantly-activated cells are muscle satellite cells

We sought to determine the types of cells in contralateral limbs that became activated to enter the cell cycle in response to amputation of another limb (Fig. 2). We therefore examined the repertoire of responding cell types within limbs contralateral to amputations using EdU and DAPI (Fig. 2**A-C′**). Using cell and tissue morphology and location, we separately quantified activated cells in contralateral epidermis (Fig. 2**D**), skeletal elements (bone, cartilage; Fig. 2**E**), and other internal tissues (dermis, muscle, nerve, perichondrium, joint, tendon; Fig. 2**F**).

This analysis revealed that different tissues have distinct temporal patterns for cell cycle activation within contralateral limbs in response