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Transcriptional correlates of proximal-distal identify and regeneration timing in axolotl limbs

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ABSTRACT

Cells within salamander limbs retain memories that inform the correct replacement of amputated tissues at different positions along the length of the arm, with proximal and distal amputations completing regeneration at similar times. We investigated the possibility that positional memory is associated with variation in transcript abundances along the proximal-distal limb axis. Transcripts were deeply sampled from *Ambystoma mexicanum* limbs at the time they were administered fore arm vs upper arm amputations, and at 19 post-amputation time points. After amputation and prior to regenerative outgrowth, genes typically expressed by differentiated muscle cells declined more rapidly in upper arms while cell cycle transcripts were expressed more highly. These and other expression patterns suggest upper arms undergo more robust tissue remodeling and cell proliferation responses after amputation, and thus provide an explanation for why the overall time to complete regeneration is similar for proximal and distal amputations. Additionally, we identified candidate positional memory genes that were expressed differently between fore and upper arms that encode a surprising number of epithelial proteins and a variety of cell surface, cell adhesion, and extracellular matrix molecules. Also, genes were discovered that exhibited different, bivariate patterns of gene expression between fore and upper arms, implicating dynamic transcriptional regulation for the first time in limb regeneration. Finally, 43 genes expressed differently between fore and upper arm samples showed similar transcriptional patterns during retinoic acid-induced reprogramming of fore arm blastema cells into upper arm cells. Our study provides new insights about the basis of positional information in regenerating axolotl limbs.

1. Introduction

Salamander limb regeneration provides an excellent model to identify endogenous mechanisms of tissue repair that might one day be translated to humans. A fundamental question in the limb regeneration field concerns the basis of positional information in cells along the proximal distal axis (McCusker and Gardiner, 2014; Bryant and Gardiner, 2016). How do limb cells that survive an amputation injury orchestrate a reparative response that reforms the appropriate distal structures? Seemingly, progenitor cells have position-specific information prior to amputation or gain this information during regeneration. The basis of this information maybe a quantitative property of cells or components of the nearby extracellular environments that cells create and maintain. For example, retinoic acid treatment of a distal limb stump reprograms blastema cells to a proximal positional identity (Maden, 1982), likely by altering gene expression (Nguyen et al., 2017). Also, when distal blastemal cells are grafted into proximal blastemal sites, their cellular movements suggest positional information is communicated via cell surface proteins (da Silva et al., 2002; Echeverri and Tanaka, 2005; Kumar et al., 2007). Additionally, positional information may correlate with other cell properties including cell adhesion, composition of extracellular environments, and bioelectricity, which likely regulate cell fate decisions during proximal-distal limb regeneration (Levin, 2014; McCusker et al., 2015; Phan et al., 2015).

Somewhat associated with the question of positional memory concerns the rate at which regeneration proceeds along the proximal-distal axis. The time from amputation to the completion of regeneration is similar regardless of where an amputation is performed along the limb axis (Iten and Bryant, 1973; Stocum, 1980). Surprisingly, limbs that are amputated at different anatomical positions pass through stages of regeneration at the same time, but more overall growth occurs in proximal amputations to replace the greater amount of missing tissue. In

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other words, it takes a similar amount of time for a salamander to regenerate an elbow, fore arm, and hand after an upper arm amputation as it does to regenerate a hand after an amputation through the wrist. Why the time to complete regeneration evolved to be similar along the limb axis is curious enough, but equally curious is the nature of the mechanisms that alter regeneration to achieve a similar offset timing.

Here we investigate the possibility that positional memory and regeneration timing are properties of transcriptional control. We reasoned that cells sampled from different anatomical positions at the time of amputation and during regeneration would express different transcripts associated with positional information and regeneration timing. Further, we reasoned that by filtering these genes against an existing list of genes that showed differential expression in response to retinoic-acid treatment (Nguyen et al., 2017), we would identify candidates for positional information. Within these contexts, we report differently expressed genes, highlighting candidates that are most likely to provide new insight about the basis of positional information and regeneration timing in axolotl limbs.

2. Materials and methods

2.1. Gene expression analysis

The experimental design and methods for collecting tissues, isolating RNA, and performing microarray analysis were previously detailed in Voss et al., 2015 (Fig. 1). That study generated comprehensive gene expression datasets for axolotl fore and upper arm regeneration, but only presented results of the fore arm regeneration dataset. Here, we use both datasets to identify genes that were expressed differently between fore and upper arm tissue samples at the time of amputation (Day 0: D0) and during specific intervals of time during the first 28 days of limb regeneration: D0–0.5, D1–D2, D3–D9, D10–D16, D16–D20, D20–24, and D24–D28. Day 0 consisted of a 1 mm thick heterogeneous cross-section of limb across the amputation plane. Post-amputation, tissue was collected within 1 mm of the distal blastemal tip. For each time interval, the average expression difference was calculated on a gene-by-gene basis between fore and upper arm replicate samples. All genes that exhibited a > 1.0 log2 average expression difference were retained for gene enrichment analysis using Panther Gene Expression tools (Mi et al., 2013), or manual curation using literature mined from Gene and PubMed databases at the National Center for Biotechnology Information (NCBI). GO terms were reported if they met a Bonferroni corrected p-value < 0.05, as implemented in the Panther statistical overrepresentation test. The fore and upper arm data are available in the Gene Expression Omnibus at NCBI.

2.2. Immunohistochemistry

Experimental procedures involving axolotls were approved by IACUC of Northeastern University under protocol number 15-1244R. Amputated limbs were collected from animals 6 cm in total length (RRID:AGSC_100J) and fixed overnight in 4% paraformaldehyde at 4 °C. Limbs were then cryoprotected in 30% sucrose and cryosectioned at 30 μm from each the center of the proximal limb segment and distal limb segment. Limbs sections were stained for myosin heavy chain (DSHB MF-20) overnight at 4 °C and muscle and bone were quantified as a fraction of the total area of the tissue section. Area was quantified using FIJI/ImageJ (Schindelin et al., 2012) to determine the relative fraction of pixels contained within each region.

3. Results and discussion

3.1. Identification of differently expressed genes

Previously, we reported on a highly-powered, transcriptional study of axolotl fore arm regeneration. In that study, tissue was collected at the time of amputation and at 19 post-amputation time points during the first 28 days of regeneration, using 10 biological replicates for each time point. To complement this body of work, we report on an equally powered dataset based on an upper arm amputation. We used fore and upper arm data to identify genes expressed differently at the time of amputation and during regeneration, as these genes might provide perspective on the molecular basis of positional information. A comprehensive description of the upper arm data will be presented elsewhere; here, we focused attention on transcriptionally and biologically significant time intervals that were discovered in the previous study of axolotl fore arm amputation. The time intervals were: 0–0.5 DPA (initial burst of transcription), 1–2 DPA (phase of decreasing transcription), 3–9 DPA (pre-bud stage), 10–16 DPA (early bud stage), 16–20 DPA (medium bud stage), 20–24 DPA (late bud stage), and 24–28 DPA (pallet stage). Quality control analyses found the 12–16 DPA upper arm samples to be outliers as all gene expression

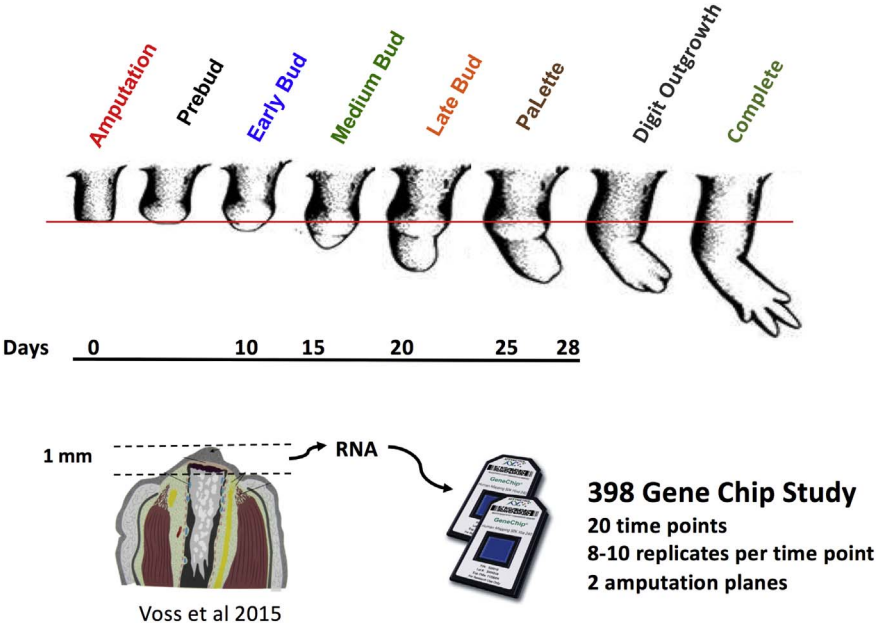

Fig. 1. Cartoon showing stages of limb regeneration relative to time after amputation, and an overview of the experimental design.