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UNIVERSITY OF CALIFORNIA,  
IRVINE

Organotypic Slice Culture of Salamander Limbs: Identification and Investigation of  
Nerve Derived BMP2 in an Organotypic *In Vitro* Assay

DISSERTATION

Submitted in partial satisfaction of the requirements  
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Jeffrey Irving Lehrberg

Dissertation Committee:  
Professor David Gardiner  
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2014

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## **DEDICATION**

I would like to dedicate this thesis to my beautiful fiancée, Anna Dyukareva.

She has always been the light at the end of the tunnel, the hand reaching down to raise me up from the pit of self-doubt.

Without her support, none of this would have been possible.

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## LIST OF ABBREVIATIONS

Abat, 4-Aminobutyrate Aminotransferase

Ach, Acetylcholine

ADAMTS1, ADAM Metalloproteinase With Thrombospondin Type 1 Motif 1

AEC, Apical Epithelial Cap

AET, Apical Epithelial Thickening

AER, Apical Ectodermal Ridge

Agr2, Anterior Gradient 2

Akt, V-Akt Murine Thymoma Viral Oncogene Homolog 1

ALM, Accessory Limb Model

Ankrd1, Ankyrin Repeat Domain 1

Areg, Amphiregulin

Asap1, ArfGAP with SH3 Domain, Ankyrin Repeat and PH Domain 1

Batf3, Basic Leucine Zipper Transcription Factor, ATF-like 3

Bcl2l1, BCL2-like 1

BDNF, Brain-Derived Neurotrophic Factor

BMP, Bone morphogenetic protein

BrdU, Bromodeoxyuridine

C3orf54, Chromosome 3 Open Reading Frame 54

Chd3, Chromodomain Helicase DNA Binding Protein 3

Col22A1, Collagen, Type XXII, Alpha 1

Creb1, cAMP Responsive Element Binding Protein 1

Cryba2, Crystallin, Beta A2

Ctgf, Connective Tissue Growth Factor

DAPI, 4', 6-Diamidino-2-phenylindole

Dll, Distal less

Dlx-3, Distal less homeobox-3

DNA, Deoxyribonucleic acid

Dnm11, Dynamin 1-like

DRG, Dorsal root ganglion

ECM, Extra Cellular Matrix

EdU, 5-ethynyl-2'-deoxyuridine

Egf, Epidermal Growth Factor

EGFP, Enhanced Green Fluorescent Protein

EMT, Epithelial-Mesenchymal Transition

ERM, Excisional Repair Model

Esco2, Establishment of Sister Chromatid Cohesion N-acetyltransferase 2

FBS, Fetal Bovine Serum

Fgf, Fibroblast Growth Factor

Gadd45b, Growth Arrest and DNA Damage Inducible, Beta

Gadd45g, Growth Arrest and DNA Damage Inducible, Gamma

Gas6, Growth Arrest Specific 6

GDF5, Growth Differentiation Factor 5

GGF, Glial Growth Factor

GF, Growth fraction

Gfap, Glial Fibrillary Acidic Protein

GFP, Green Fluorescent Protein

Hmox1, Heme Oxygenase (Decycling) 1

Hoxa13, Homeobox A13

Idh3g, Isocitrate Dehydrogenase 3 (NAD<sup>+</sup>) Gamma

IL-1 $\beta$ , Interleukin 1, Beta

IL-8, Interleukin 8

Irf1, Interferon Regulatory Factor 1

Kazald1, Kazal-Type Serine Peptidase Inhibitor Domain 1

Kit, V-Kit Hardy Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog

Krt17, Keratin 17

Lep, Leptin

LI, Labeling Index

Mall, Mal, T-Cell Differentiation Protein-like

MAPKKK, Mitogen Activated Protein Kinase Kinase Kinase

Marcks, Myristoylated Alanine-Rich Protein Kinase C Substrate

Mbp, Myelin Basic Protein

Mdm2, MDM2 Oncogene, E3 Ubiquitin Protein Ligase

MI, Mitotic index

Mmp, Matrix Metalloproteinase

Msx2, Msh Homeobox 2

MTOR, Mechanistic Target of Rapamycin (Serine/Threonine Kinase)

NAG, Newt Anterior Gradient

Napa, N-Ethylmaleimide-Sensitive Factor Attachment Protein, Alpha

Ndc80, NDC80 Kinetichore Complex Component

Npff, Neuropeptide FF-Amide Peptide Precursor

Npy, Neuropeptide Y

Nrg1, Neuregulin 1

Ntf3, Neurotrophin 3

Nts, Neurotensin

OSC, Organotypic slice culture

P-Smad, Phospho-Smad

P38, Mitogen Activated Protein Kinase 14

Plp1, Proteolipid Protein 1

Pmp22, Peripheral Myelin Protein 22

Prrx-1, Paired Related Homeobox 1

PVPr, Posterior-Ventral-Proximal

RA, Retinoic Acid

RARE, Retinoic Acid Response Element

RhGGF2, Recombinant Human Glial Growth Factor 2

RNA, Ribonucleic Acid

RT-PCR, Reverse Transcription Polymerase Chain Reaction

Robo1, Roundabout, Axon Guidance Receptor, Homolog 1 (Drosophila)

Shh, Sonic Hedgehog

Smad1, SMAD Family Member 1

Smc2, Structural Maintenance of Chromosomes 2

SP, Substance P

Tacc3, Transforming, Acidic Coiled-Coil Containing Protein 3

TGF-  $\beta$ , Transforming Growth Factor- $\beta$

Tgfb1, Transforming Growth Factor, Beta 1

Thbs1, Thrombospondin 1

TNF  $\alpha$ , Tumor Necrosis Factor  $\alpha$

TUNEL, Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling

QPCR, Quantitative Polymerase Chain Reaction

ZPA, Zone of Polarizing Activity

Znf697, Zinc Finger Protein 697

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McCusker C, **Lehrberg J**, Gardiner D. (2014) Position-specific induction of ectopic limbs in non-regenerating blastemas on Axolotl forelimbs. *Regeneration* 1(1), p27-34.

**Lehrberg J.** “Belial, Vice-President of Marketing.” *50-Word Stories*. fiftywordstories.com. May 20<sup>th</sup>, 2014

**Lehrberg J**, Gardiner D. (2014) Regulation of axolotl limb blastema cell proliferation by nerves and BMP2 in organotypic slice culture. (in preparation).

Ludwig C, **Lehrberg J**, (2005) Bringing them in from the cold. *All Hands*. Apr, pg 15-18.

## POSTERS

McCusker C, **Lehrberg J**, and Gardiner D. Induction of limb regeneration in non-regenerative wounds on the Mexican Axolotl: a tool to map positional information in the adult. Center for Complex Biological Systems retreat, the Langham Huntington, Pasadena. March 28-30th, 2014.

## **ABSTRACT OF THE DISSERTATION**

Organotypic Slice Culture of Salamander Limbs: Identification and Investigation of  
Nerve Derived BMP2 in an Organotypic *In Vitro* Assay

By

Jeffrey Irving Lehrberg

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2014

Professor David Gardiner, Chair

Salamander limb regeneration is a nerve-dependent process. In the absence of nerves, blastema cells (i.e. cells that give rise to the new structure) will not proliferate. A major question in regeneration biology is how the nerve exerts its growth promoting effect on blastema cells. To better understand the cellular and molecular signals responsible for growth and pattern formation during regeneration, I have adapted the use organotypic slice culture for axolotl blastemas. Using microarray, I have identified BMP2 as a possible nerve derived factor responsible for growth and proliferation during regeneration. Here I will demonstrate how organotypic slice cultures are responsive to culture conditions and the candidate neurotrophic factor BMP2 in both terms of levels of proliferation and gene expression.

## **CHAPTER 1: Introduction**

Almost 200 years ago, under the shadow of Mount Vesuvius, in the Italian city of Naples, a 34-year-old retired British Naval surgeon makes an interesting discovery. The retired surgeon, a Dr. Tweedy John Todd, was performing experiments on an animal that had the peculiar ability to regenerate its limbs. While it had long been known that when salamander limbs were amputated they regrew, Dr. Tweedy John Todd was interested in “[inducing] any derangement in the process” and observe how “nature is ever prepared to adapt herself to every new circumstance and exigency (Todd, 1823)” One of the so-called “derangements” that Todd performed was to sever the sciatic nerve simultaneously with amputation. Upon the denervation of the limb, Todd made the interesting observation that regeneration failed to proceed (Todd, 1823).

Fast-forward to the present day. Two hundred years after Todd’s observation, the exact cellular and molecular mechanisms responsible for limb regeneration are still being explored. The work presented herein provides new tools and insights that will lessen the knowledge gap currently preventing limb regeneration in humans from becoming a reality.

Along with their limbs, salamanders can regenerate a number of other tissues. While a number of different species exhibit impressive regenerative abilities (e.g. planaria), salamander limb regeneration is particularly interesting because of the shared homology between salamander and human limbs. Like humans, salamanders possess bone, muscle and skin. It is because of these similarities that salamanders are the animals of choice for regeneration studies.

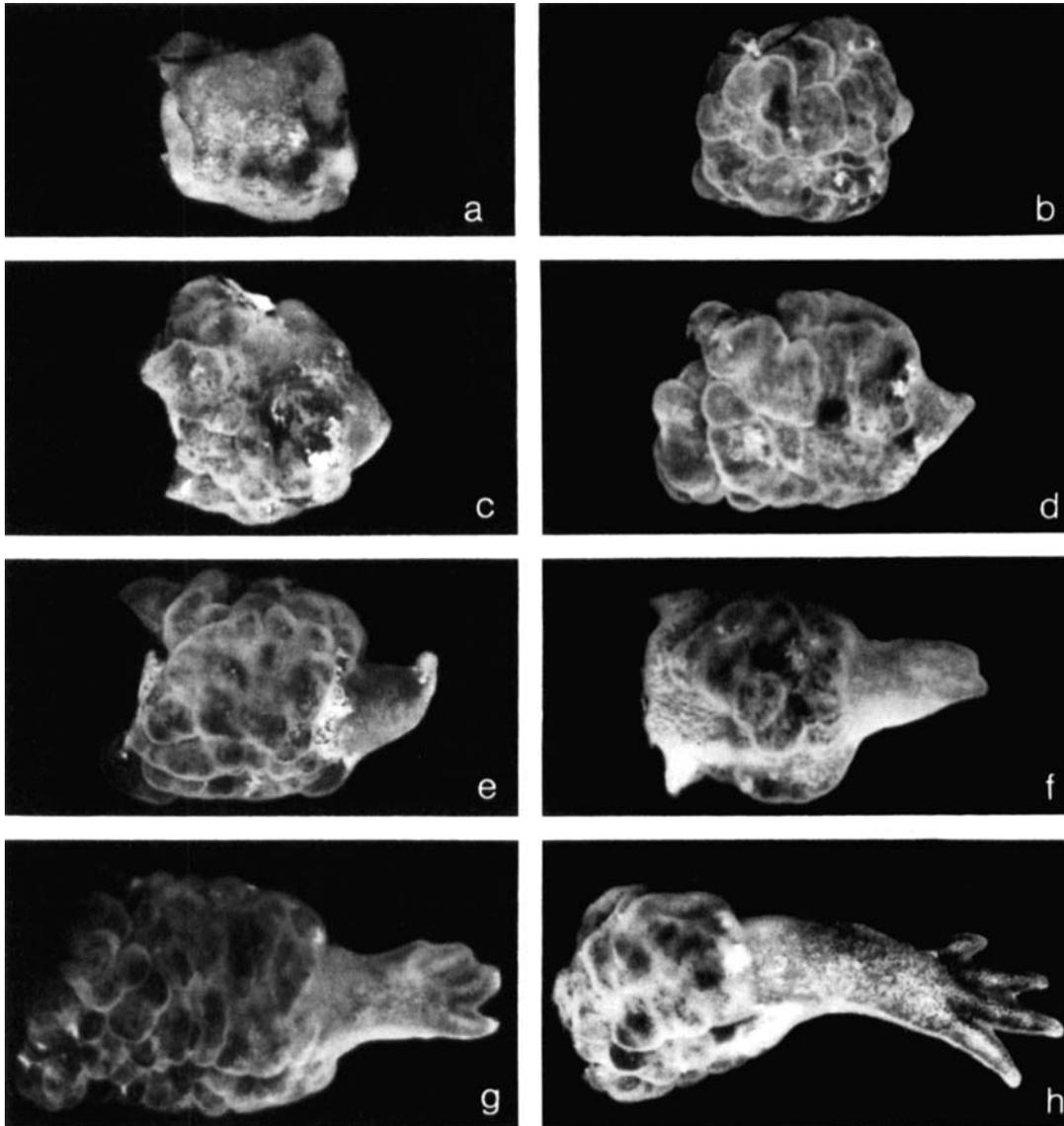
Unlike salamanders, humans cannot regenerate their limbs. At early developmental stages the extent of human limb regenerative ability can be seen in the form of small outgrowths called “nubbins” that grow in response to in utero amputation of the limb via amniotic sac (Gardiner, 2005). Losing a limb puts emotional and financial strain on the victim and also decreases their quality of life (Belisle et al., 2013; Ziegler-Graham et al., 2008). There are approximately 185,000 limb amputations a year and the costs associated with limb amputation exceed more than \$8.3 billion dollars (HCUP, 2007; Owings and Kozak, 1998). The loss of a limb affects not only the victim, but also society as a whole.

#### *Regeneration at a glance: nomenclature*

After a salamander limb is amputated, a new limb is regrown that completely and perfectly replaces the missing structures (Bryant et al., 2002; Endo et al., 2004; Wallace, 1981). Following limb amputation, a mass of highly proliferative progenitor cells form a structure called a “blastema.” (Bryant et al., 2002; Wallace, 1981). The blastema exhibits periods of growth and differentiation that are divided into different stages in order to aid in identification. It is frequently more advantageous to describe blastemas by stage rather than age as animals of different sizes can reach a given stage at different time points (e.g. larger animals usually take longer to regenerate) (Tank et al., 1976). The most commonly used nomenclature to describe these stages is based on P.W. Tank’s 1976 study, “A staging system for forelimb regeneration in the axolotl, *Ambystoma mexicanum*” (Tank et al., 1976).

The first stage in blastema nomenclature is the “wound healing stage” (Tank et al., 1976). Immediately following a limb amputation, a specialized wound epithelium rapidly covers the amputation plane (Endo et al., 2004; Repesh and Oberpriller, 1980). This early step in regeneration is important because the wound epithelium and the structure it gives rise to are both important molecular signaling centers (Bryant et al., 2002; Christensen et al., 2002; Wallace, 1981). After the wound healing stage, the next stage is the “Dedifferentiation stage” (Tank et al., 1976). In the dedifferentiation stage the wound epithelium begins to thicken into a structure called the Apical Epithelial Thickening (AET) (Bryant et al., 2002; Tank et al., 1976). The next stage is the “early bud stage” and is marked by the accumulation of blastema cells and increased osteoclastic activity at the distal end of the bone (Tank et al., 1976). It is at the early bud stage where the generation of a growing structure is first apparent to the naked eye. Following the early bud stage is the “medium bud stage” or the “mid bud stage” (Tank et al., 1976). At the mid bud stage in regeneration there is large amount of proliferation and the blastema rapidly grows in size. Most of the experiments performed in this thesis were performed on mid bud stage blastemas. The next stage in regeneration is the “late bud stage” (Tank et al., 1976). At the late bud stage, the beginnings of differentiation can begin to be observed in sections in the form of pre-cartilage condensations (Tank et al., 1976). Outward signs of differentiation can be observed in the next stage called the “palette stage” (Tank et al., 1976). At the palette stage, an epidermal invagination called a “notch” can be observed at the distal tip of the blastema. This notch separates the digit primordium that is in the process of being generated (Tank et al., 1976). The final stage of regeneration is called the digital outgrowth stage where epidermal wedges begin to

separate all the digits (Tank et al., 1976). Figure 1 shows examples of the aforementioned blastema stages.



**Figure 1: Stages of forelimb regeneration.** Reprint of a figure from Tank et al. 1976. This figure shows the stages of limb regeneration that have been defined. a) Wound healing. b) Dedifferentiation. c) Early bud. d) medium bud. e) Late bud. f) Palette. g) Digital outgrowth. h) regenerate. (Tank et al., 1976)

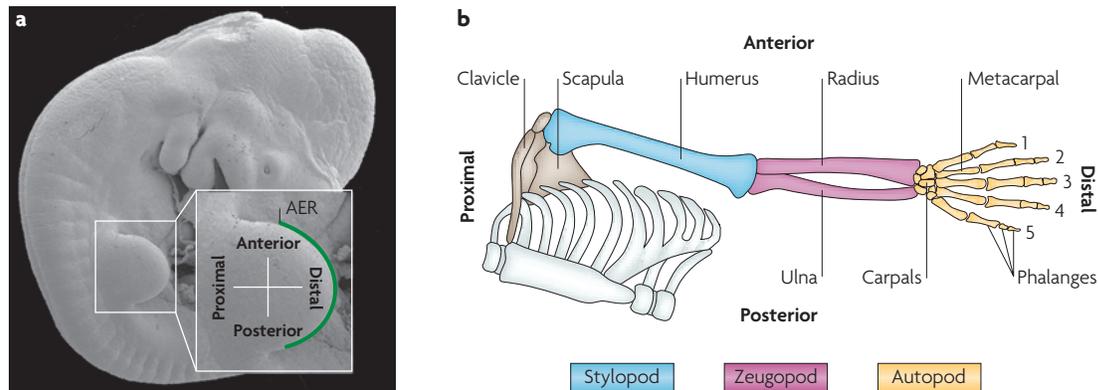
*Similarities between signaling pathways during vertebrate limb development and regeneration*

Limb regeneration and limb development share a number of similarities. Limb regeneration has been described as being comprised of three different phases. Phase I, the wound healing phase, Phase II, the dedifferentiation phase, and Phase III, the redevelopment phase (Bryant et al., 2002). Phase III, the redevelopment phase, involves increased growth and patterning and the expression of genes similar to those found in developing limbs (Bryant et al., 2002). While it is now known that the initiating steps of limb regeneration and development involve different spatial and temporal expression of genes, the function of these genes appears to be the same (Bryant et al., 2002; Gardiner et al., 1995). Along with the similar expression of genes, limb development and limb regeneration share similar morphological structures that play similar roles.

Proper limb development requires complex genetic interactions to guarantee the outgrowth and specification of a proximodistal, anteroposterior, and dorsoventral axis. The initiation of limb development occurs when regions of flank mesenchyme along the proximodistal axis are specified to become the prospective limb. It is believed that specification of anteroposterior axial patterning of the embryo is achieved through patterns of Hox genes or the restriction of Fgf8 as a result of retinoic acid (Tanaka, 2013; Zeller et al., 2009). Following the specification of the limb field, a specialized group of epithelial cells forms called the Apical Ectodermal Ridge. The AER goes on to serve as one of the two main signaling centers in limb development (Figure 2a).

Genes found in the Fibroblast Growth Factor Family (FGF) have been shown to be expressed in the AER and involved in both patterning and outgrowth during limb

(Mariani et al., 2008; Zeller et al., 2009). Fgf8 is expressed in the entire AER throughout limb bud development and Fgf4, Fgf9, and Fgf17 are expressed in posterior region of the AER (Mariani et al., 2008; Zeller et al., 2009)



**Figure 2: Anatomy of the limb.** Reprint of a figure from Zeller et al. 2009. a) A scanning electron microscopy image of a 10.5 day mouse embryo. The developing limb is outlined by the white box and the proximodistal and anteroposterior axis are labeled. The AER is outlined in green. b) The anatomy and terminology of the proximodistal regions of the limb. The most proximal element of the limb is called the stylopod contains the Humerus. The Zeugopod element contains the Radius and Ulna. The Autopod element contains the Carpals, Metacarpals, and Phalanges (Zeller et al., 2009).

Proximodistal patterning of the developing limb has had many models proposed over time. One of these models is the so-called “progress zone” model. In the progress zone model, dedifferentiated mesenchymal cells are given cell identities based on how much time is spent in the zone during outgrowth (Zeller et al., 2009). Insight gained from studies using Cre-Lox knockout mice suggests that another model called the “two signal model” might be in play (Mariani et al., 2008). The two-signal model suggests that proximal and distal identities are established first with the intercalation of corresponding identities filling in the gaps during outgrowth (Mariani et al., 2008; Zeller et al., 2009).

The proximodistal axis of the tetrapod limb is divided into three regions: the Stylopod, the Zeugopod, and the Autopod (SZA) (Figure 2b). The Stylopod element contains the human humerus the Zeugopod element contains the radius and ulna, and the autopod element contains the carpals, metacarpals, and phalanges (Figure 2b).

Knocking out certain Fgfs can result in the loss of certain SZA elements. Through the use of Cre-Lox knockout mice, Mariani et al. 2008 were able to show that double knockout Fgf4 and Fgf9-/+ mice were able to generate stylopod and autopod elements but lacked the zeugopod element (Mariani et al., 2008). This result from Mariani et al. gave support to the two-signal model of development and demonstrates the importance of Fgfs during limb development.

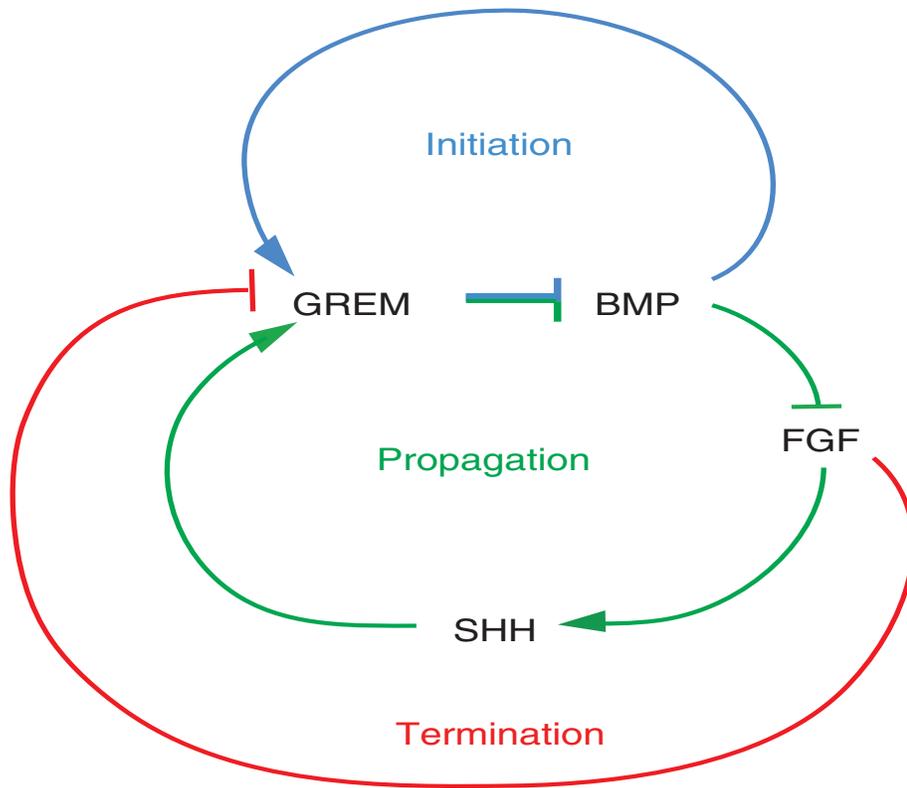
The other main signaling center during limb development is the Zone of Polarizing Activity (ZPA). The ZPA consists of a group of cells in posterior region of the developing limb bud that set up anteroposterior patterning (Benazet and Zeller, 2009). The ZPA was identified during early grafting studies where it was discovered that when posterior mesenchyme cells from the limb bud were grafted anteriorly, they were able to induce mirror image duplications of the digits (Benazet and Zeller, 2009; Zeller et al., 2009). It was later determined that that cells in the ZPA influence patterning is through the release of the morphogen Sonic Hedgehog (SHH) (Benazet and Zeller, 2009; Zeller et al., 2009)(Figure 3).

Along with the expression of genes such as those from the FGF family of genes, bone morphogenetic proteins (BMP) also play a critical role during both limb regeneration and development. BMPs are secreted proteins that are in the transforming growth factor- $\beta$  (TGF- $\beta$ ) family of proteins (Geetha-Loganathan et al., 2006). During

development, BMPs are required for growth and patterning of the limb (Geetha-Loganathan et al., 2006; Zeller et al., 2009). Bmp2, 4, 5, and 7 are expressed during limb development (Geetha-Loganathan et al., 2006; Robert, 2007; Zeller et al., 2009). Bmp2 is expressed in both the AER and the posterior mesenchyme (Geetha-Loganathan et al., 2006). BMP2 and BMP4, which are closely related proteins (i.e. similar targets and downstream affects), are also involved in early events in limb patterning (Bandyopadhyay et al., 2006). BMP4 signaling initiates the expression of its own repressor (i.e. Grem1) which then goes on to de-repress Fgf signaling in the AER (Figure 3). This Fgf signaling then goes on to form a positive feedback loop with sonic hedgehog (Shh) in the zone of polarizing activity (ZPA) (Bandyopadhyay et al., 2006; Geetha-Loganathan et al., 2006; Robert, 2007; Zeller et al., 2009) (Figure 3).

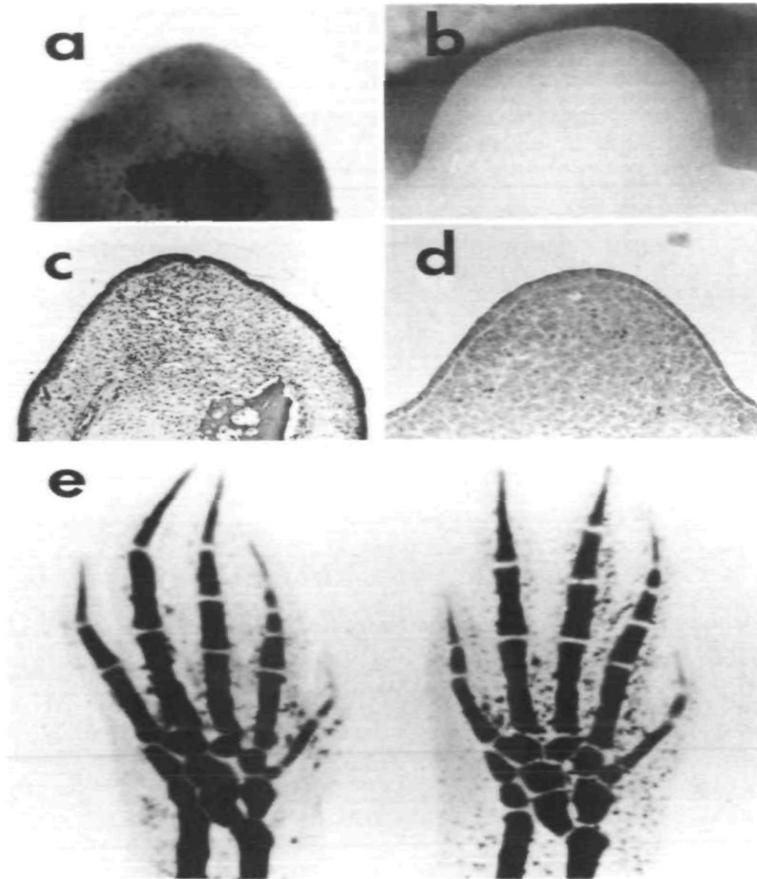
At the end of limb bud development, continued expression of Fgf from the AER goes on to inhibit Grem1 expression (Benazet and Zeller, 2009) (Figure 3). This FGF-GREM inhibitory loop results in the termination of outgrowth and patterning and signifies the end of limb development (Benazet and Zeller, 2009) (Figure 3).

It is clear from the examples above that limb development requires the cooperation of many different molecules and pathways and between different tissue types. An interesting aspect of the feedback loops required to generate anteroposterior, proximodistal and dorsoventral patterning is how they are linked and the communication between epithelial and mesenchymal tissue (i.e. The AER and ZPA respectively) general scheme of some of these feedback loops can be seen in Figure 3.



**Figure 3: Feedback loops involved in limb development.** Reprint of a figure from Benazet & Zeller, 2009 showing the feedback loops involved in initiation, propagation, and termination of limb development and how the processes are linked. (Benazet and Zeller, 2009).

At face value, it is easy to make comparisons between limb development and limb regeneration (Figure 4). Going beyond outward appearances, based on our current knowledge of limb development and regeneration, with the exception of the use of alternate initiating steps, the molecular pathways involved in limb development and limb regeneration are quite similar. Along with similar molecular pathways, developing limbs and regenerating limbs have similar structures that appear to play similar roles during their respective processes (Figure 4).



**Figure 4: Similarities between limb development and regeneration.** Reprint of a figure from Bryant et al. 1987 showing the similarities between development and regeneration. a) Medium bud blastema on newt. b) 11 day mouse limb bud. c) longitudinal section of newt medium bud blastema. d) longitudinal section of 10 day mouse limb bud. e) original axolotl limb and regenerated limb. (Bryant et al., 1987).

One structure that occurs in both limb development and regeneration is a group of specialized epithelial cells that serve as a signaling center. During development this structure is the AER and during regeneration it is the AEC. The AER and the AEC produce many of the same molecular signals during their respective processes. As in limb development, one of molecular signals produced during limb regeneration are members of the Fibroblast Growth Family (Christensen et al., 2002; Zeller et al., 2009). Like in the AER during development, the AEC also expresses Fgf8 during regeneration

(Christensen et al., 2002; Han et al., 2001a). During regeneration, *Fgf8* is expressed in the distal blastema at the interface where mesenchymal cells and proximal AEC cells come into contact (Christensen et al., 2002; Han et al., 2001a).

During limb development, an organizing region called the ZPA expresses *Shh* and is responsible for anteroposterior patterning. *Shh* is also expressed in the regenerating limbs of newts and axolotls and influences patterning in a similar manner to development (Imokawa and Yoshizato, 1997; Torok et al., 1999).

BMPs also play an important role in regenerating limbs (Athippozhy et al., 2014; Guimond et al., 2010; Makanae et al., 2013). When the general BMP2 antagonist *Noggin* is electroporated into a blastema, regeneration is inhibited (Guimond et al., 2010). When *GDF5* (also known as *BMP17*), is grafted into an ectopic wound site with *FGF2* and *FGF8*, ectopic blastemas can be induced (Makanae et al., 2013). Finally, in work presented in Chapter 4 of this thesis, *BMP2* gene expression was found to be increased in axolotl DRG when co-cultured with a blastema (Athippozhy et al., 2014).

Another gene that is expressed in both development and regeneration is Paired Related Homeobox 1 (*Prrx-1*). *Prrx-1* is an early transcription factor that is expressed in the mesenchyme of the limb bud during limb development (Nohno et al., 1993; Suzuki et al., 2007). *Prrx1* is expressed in the axolotl limb during limb development and regeneration and is commonly used as a blastema marker due to the fact that it is expressed in the blastema but not in uninjured stump tissues (Athippozhy et al., 2014; Makanae et al., 2013; Satoh et al., 2007; 2011). Along with being an early expressed transcription factor during development and regeneration, *Prrx-1* plays a role a number of other processes involving the differentiation of cells, some of which are especially

tantalizing due to their association with bone formation and BMP2. One process that Prrx-1 is involved in is the differentiation of osteoblasts. Osteoblasts arise from progenitor mesenchymal cells and are inhibited by Tumor Necrosis Factor  $\alpha$  (TNF $\alpha$ ) via Prrx-1 inhibition of the transcription factor Osterix (Lu et al., 2011). Along with skeletogenesis, Prrx-1 also is involved in adipogenesis. Prrx-1 expression inhibits cells from taking an adipocyte fate via the regulation of TGF- $\beta$  ligands (Du et al., 2013). Inhibition of Prrx-1 resulted in the decrease of expression of TGF- $\beta$  (Du et al., 2013) and when TGF- $\beta$  receptor is inhibited in axolotls, regeneration fails to proceed (Lévesque et al., 2007). Finally, Prrx-1 is involved with the epithelial-mesenchymal transition (EMT) and loss of Prrx-1 is required for metastasis (Ocana et al., 2012).

It is clear from the similarities observed in both the morphology and molecular signals during limb development and regeneration that the two processes are very similar. The similarities observed in limb development and regeneration lead to the popular phrase “regeneration recapitulates development” being coined (Imokawa and Yoshizato, 1997; Wang and Conboy, 2010).

#### *The requirements for limb regeneration*

In order to initiate and maintain regeneration in a salamander limb, three conditions must be met. The first condition that must be met is that a wound epithelium must cover the amputation plane (Bryant et al., 2002; Endo et al., 2004; Repesh and Oberpriller, 1980; Wallace, 1981). Following the thickening of the wound epithelium as a result of cell migration, the wound epithelium goes on to become the AEC (Bryant et al., 2002). If the

wound epithelium or AEC is interrupted by grafting full thickness skin over the amputation plane, then regeneration will not occur (Mescher, 1976).

The second condition is that there needs to be a disparity between connective tissue cells in the limb that contain positional information (Bryant et al., 2002; Endo et al., 2004; Gardiner and Bryant, 1989; McCusker et al., 2014; McCusker and Gardiner, 2013; 2014; Wallace, 1981). In the salamander, connective tissue cells contain information about their spatial organization (McCusker et al., 2014; McCusker and Gardiner, 2013). When there is a gap in positional information, it is called a positional disparity (Endo et al., 2004; Gardiner and Bryant, 1989; McCusker and Gardiner, 2013; Wallace, 1981). During regeneration, proliferation results in the generation of cells that fill in this positional disparity until the positional information is once more complete and the pattern is restored (McCusker and Gardiner, 2013; 2014).

The final condition (the condition that represents the main focus of the work presented in this thesis) is that there must be an adequate nerve supply present (Singer, 1952; Todd, 1823; Wallace, 1981). It has been proposed that the nerve releases some substance, deemed the “neurotrophic factor,” that influences blastema cells to proliferate directly or creates the conditions necessary for proliferation to occur (Singer, 1952; Wallace and Maden, 1976; Wallace, 1981).

Interruption of the nerve supply during regeneration results in two outcomes depending on the blastema stage at which denervation occurred. The first outcome is that the limb fails to regenerate and the animal will be left with a stump. This occurs during the early to mid stages of blastema growth and has been called the “nerve-dependent stage.” If the nerve supply is interrupted in the early stages of regeneration, no blastema

will form and the end result will be a stump (Singer and Craven, 1948). If an early bud or a medium bud blastema is already present when the nerve supply is interrupted, then the blastema will regress or “resorb” into the limb and the animal will also be left with a stump (Singer and Craven, 1948).

The second outcome is that the animal will have a malformed and severely hypomorphic limb. This occurs when denervation occurs in a later stage blastema (Singer and Craven, 1948). If the nerve supply is interrupted at a later stage, such as the after the medium bud stage and after, then denervation will produce a hypomorphic limb (Singer and Craven, 1948). These hypomorphic limbs are on average the same length as normal regenerated limbs but have a lower overall volume (Singer and Craven, 1948). The fact that these regenerating limbs do not resorb into a stump has led to blastema growth at these stages as erroneously being called “nerve independent.” The observation that limbs denervated at later stages form severely hypomorphic limbs indicates that in reality the nerve is required at all stages of regeneration and the terms “nerve dependent” and “nerve independent” are misleading at best.

#### *The role of the neurotrophic factor in limb regeneration*

An important question in regeneration studies pertains to how the nerve influences growth in the regenerating limb. Many clever and painstakingly detailed experiments have been performed in order to provide us with our current knowledge concerning the nerves effect on limb regeneration (Wallace, 1981). Rather than type of nerve (i.e. motor, sensory, etc.), it is the quantity of nerve that is important in regeneration (Singer, 1946b; 1946a; 1947; 1952; 1974; Singer and Craven, 1948; Singer and Egloff, 1949). In

order for regeneration to proceed, a certain threshold number of nerves must be present at the wound site (Singer, 1952). But what insight into the identity of the neurotrophic factor has the past 200 years of study revealed to us?

The identity of the so-called neurotrophic factor has been an area of scientific investigation for decades. Many potential candidates, including neuropeptides, organic molecules, cyclic nucleotides, neurohormones, growth factors, and even bioelectric signals, have been put forth with questionable results (Wallace, 1981).

Because regeneration and development share many similarities, molecules important for growth in the developing limb have been investigated as proposed neurotrophic factors. One class of molecules proposed as being the neurotrophic factor are molecules from the FGF family. Using an antibody against FGF1 resulted in a decrease in 3H-Thymidine incorporation in blastema cells in *Pleurodeles waltl* supporting the idea that Fgf1 might be involved in regeneration (Zenjari et al., 1996).

Fgf2 is expressed in nerves and maintains the expression of the homolog of *Drosophila* distalless (Dll), Dlx-3 in axolotl limbs (Mullen et al., 1996a). Dlx-3 is expressed in the AEC and its expression was found to be nerve dependent (Mullen et al., 1996a). When beads soaked in FGF2 were implanted into denervated axolotl limbs, it was found that Dlx-3 expression could be maintained (Mullen et al., 1996a).

When comparing the rates of limb regeneration in salamanders of the species *Triturus carnifax* and *Triturus vulgaris*, it has been shown that the former regenerates at a slower rate (Giampaoli et al., 2003). Upon examination of temporal expression of Fgf2, it was found that *Triturus carnifax* has a delayed expression of Fgf2, further supporting the argument that Fgf2 plays an important role in regeneration (Giampaoli et al., 2003).

Interestingly, Fgf2 seemed to be localized to the AEC rather than in the nerve (Giampaoli et al., 2003). Finally, it was found that FGF2, in combination with FGF8 and GDF5, could induce the formation of ectopic bumps in the absence of a nerve (Makanae et al., 2013).

In addition to classic growth factors known to play a role in limb development, classic nerve specific factors have also been investigated as to their role as the neurotrophic factor. One of the nerve-derived factors that has been investigated as the possible neurotrophic factor in salamander limb regeneration is the neuropeptide Substance P (SP). Substance P is a neuropeptide that is ubiquitously expressed in the human body and is responsible for numerous physiological roles ranging from emotional behavior to tumor cell proliferation (Muñoz and Coveñas, 2014). Substance P is strongly expressed in the sensory ganglia of newts (Globus et al., 1991) and furthermore, it was found that immunoreactivity to Substance P increased in the peripheral nerves of axolotls following injury (Anand et al., 1987). Substance P was then hypothesized as being a cell proliferation stimulus via initiating intracellular calcium levels through the inositol phospholipid transmembrane pathway (Smith et al., 1995). Neomycin, an inhibitor of the inositol phospholipid pathway, decreased <sup>3</sup>H-Thymidine incorporation in a dose dependent manner (Smith et al., 1995).

Glial Growth Factors (Ggf) are neuropeptide splice isoforms of the Neuregulin 1 gene (Nrg1) (Esper et al., 2006). In denervated newt limbs, treatment with GGF led to a sevenfold increase in the labeling index for blastema cells positive for the 22/18 antigen (a putative marker of blastema cells), however this response was observed for only these 22/18 positive cells, and a GGF rescue of a denervated blastema was not attempted

(Brockes and Kintner, 1986). Later experiments with recombinant human Glial Growth Factor 2 (rhGGF2) however reported that inter-peritoneal injections of rhGGF2 could rescue regeneration in denervated newt limbs (Wang et al., 2000).

Transferrins are glycoproteins that facilitate the transport of iron into cells (Kiffmeyer et al., 1991; Mescher et al., 1997). Immunohistochemistry revealed that transferrin was present in axolotl axons and Schwann cells (Kiffmeyer et al., 1991). It was found that transferrin had a similar stimulatory effect on <sup>3</sup>H-Thymidine incorporation as axolotl brain extract in axolotl blastema explants (Mescher et al., 1997).

Non-protein molecules have also been investigated as being the putative neurotrophic factor. These include cyclic nucleotides, neurotransmitters such as acetylcholine, and electrical signaling (Becker and Spadaro, 1972; Rathbone et al., 1980; Sicard, 1983; Wallace, 1981) It has also been proposed that intracellular calcium levels are the primary cell proliferation signal with the effect of calcium being mediated by cAMP and cGMP feedback loops (Globus et al., 1987; Sicard, 1983). As is the case with all other potential neurotrophic factors, the role of non-protein molecules in regeneration in regard to the neurotrophic factor is unresolved.

Acetylcholine (Ach) is an attractive candidate for the proposed neurotrophic factor responsible for cell proliferation during limb regeneration due to its presence in nerves and its well characterized effect on other physiological processes (Singer, 1959; Wallace, 1981). Acetylcholine levels were shown to increase during newt regeneration and were highest at the mid blastema stage (Singer, 1959). In experiments attempting to block the action of Ach, it was found that anticholinergic drugs and chemicals including atropine sulphate, tetraethylammonium hydroxide, procaine hydrochloride, pilocarpine

nitrate, serine salicylate, and hemicholinium-3 all had an inhibitory effect on regeneration (Hui and Smith; Singer et al., 1960). A number of experiments contradict the claim that Ach is the neurotrophic factor however, the most demonstrative example being the result that newt limbs treated with botulinum toxin continued to regenerate (Drachman and Singer, 1971), and the fact that sensory neurons contain a lower content of Ach yet induce and maintain regeneration just as readily as motor neurons (Singer, 1946a; 1952; Wallace, 1981).

Rathbone et al. hypothesized that noradrenaline was the neurotrophic candidate and investigated its effect on cyclic AMP levels on whole mount explants *in vitro* (Rathbone et al., 1980). It was found that cyclic AMP increased but did not maintain the level of mitotic index found in explants cultured with newt brain extract (Rathbone et al., 1980)

In addition to proteins and small organic molecules, it has previously been proposed that the neurotrophic factor is a bioelectric signal (Becker and Spadaro, 1972; Blackiston et al., 2009; Bodemer, 1964; Wallace, 1981). The claim that bioelectric signaling from the nerve is responsible for growth and proliferation has been investigated by numerous authors (Wallace, 1981). Many early investigators have reported voltage or current changes in the regenerating limb when compared to the limb base or dorsal midline (Becker and Spadaro, 1972; Bodemer, 1964; Wallace, 1981). The changes in voltage observed in the regenerating limb however may be attributable to the properties of the wound epithelium owing to changes in ion pump concentrations or permeability (Wallace, 1981).

Attempts to stimulate regeneration via the administration of electrical shocks have met with less than impressive results (Becker, 1972; Becker and Spadaro, 1972; Bodemer, 1964). Bodemer et al. reported partial or incipient regeneration could be induced in post metamorphic frogs via the administration of 300mV shocks to the brachial nerve (Bodemer, 1964; Wallace, 1981). The result of “partial” regeneration is not particularly shocking (no pun intended), since in some species (e.g. *Xenopus laevis*) post-metamorphic frogs partially regenerate their limbs in the absence of any intervention (i.e. the regeneration of spikes) (Sessions and Bryant, 1988). Becker et al. reported that after a silver-platinum coupler was implanted into the arms of rats, cancellous bone growth was reported as “regeneration” and occurred within 7 days (Becker, 1972; Wallace, 1981).

Inhibiting regeneration by reducing current has also been attempted by keeping regenerating newts in water containing no sodium, the idea being that sodium pumps would fail to generate currents in such a medium (Borgens et al., 1979; Wallace, 1981). While newts kept in these conditions exhibited slower regeneration, regeneration proceeded none the less (Borgens et al., 1979; Wallace, 1981).

The failure to rescue and/or inhibit regeneration through the use of electrical stimulation or inhibition does not mean however that some electrical signaling is not at play. Rather directly influencing growth, electrical signals might play a role in dedifferentiation or positional identity (Adams and Levin, 2013). Changes in membrane potential, as a result of changing intracellular ion concentrations, have been implicated as a key regulator of cell proliferation (Blackiston et al., 2009). Globus et al. also have reported that blastemas co-cultured with a DRG and the divalent calcium ionophore

A23187 have increased levels of mitosis (Globus et al., 1987). Furthermore it has been reported that the V-ATPase H<sup>+</sup> pump is required for *Xenopus* tadpole tail regeneration (Adams et al., 2007). In lieu of these results, the role of bioelectrical signaling may prove to be an important factor in limb regeneration.

Recently, newt anterior gradient protein (nAG) has been identified as the putative neurotrophic factor (Kumar et al., 2007). It was shown that nAG could rescue regeneration in partially innervated newt limbs (Kumar et al., 2007). However, nAG is not detected in neurons and cannot rescue proliferation in dissociated newt blastema cells *in vitro* (Kumar et al., 2007).

*In vivo* experimentation concerning the role that the nerve plays has yielded a wealth of information. Many previous experiments investigated the effect of inhibition of the nerves influence. Studies that aimed to rescue the influence of the nerve *in vivo* have yet to identify the neurotrophic candidate. A reason that identification of the neurotrophic factor has been difficult can be attributed to experimental design challenges.

One major challenge facing the role that the nerve plays in regeneration is the abundance of nerves and the difficulty that adequately removing them presents (Singer, 1946a; 1952). Ganglionectomy must include not only the 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> spinal ganglia that innervate the forelimb, but the adjacent ganglia as well (i.e. 2<sup>nd</sup> and 6<sup>th</sup>) to ensure adequate denervation (Singer, 1946a; 1952). To verify that any denervation is successful, absence of nerve fibers through the use of staining should be performed with immunohistochemistry, a control that is not always performed (Singer, 1946a; 1952). In order to overcome the challenges that studying the nerve presents in regard to

proliferation *in vivo*, as well as attempt to further identify the molecular pathways involved in regeneration, many authors have attempted the use of *in vitro* techniques.

Limb regeneration and development share many similar structures and signaling pathways. Limb regeneration however has certain conditions that must be met in order to proceed (i.e. wound epithelium, positional disparity, and adequate nerve supply). The role that nerve plays in regeneration has been a topic of discussion for hundreds of years. There have been numerous neurotrophic candidates proposed with questionable results. One major problem facing the study of the nerves role in regeneration is the limits presented when attempting *in vivo* studies. Overcoming the experimental design challenges when studying the nerve *in vivo* has been investigated and is the subject of the next chapter.

**CHAPTER 2: *In vitro* techniques for studying limb regeneration.**

Using cell culture to investigate how limb regeneration occurs in salamanders has a long history that reaches back to the early 20<sup>th</sup> century. The first recorded experiments concerning salamander cell culture occurred during the end of the 1920's, with Johannes Holtfreter being the first to experiment with salamander tissues *in vitro* in his paper *Ueber histologische Differenzierung von isolierten Material jungster Amphibienkeime* (translated from German as 'About histological differentiation of isolated material most young amphibians germs') (Holtfreter, 1929). At the time of Holtfreter's 1929 paper, salamander cell culture was an amalgamation of simple techniques that had been used in embryology and early explant experiments up until that point (Holtfreter, 1929; Wilde, 1950). Since these early experiments, the techniques used for salamander cell culture saw relatively little change for the next 60 years (Dalton, 1950; Globus and Vethamany-Globus, 1977; Globus et al., 1987; Grillo et al., 1968; Jabaily et al., 1982; Liversage and Globus, 1977; Twitty and Bodenstein, 1939; Wilde, 1950). It is interesting to note when examining the history of salamander cell culture that there are relatively few papers that explicitly examine growth and proliferation, and report labeling index, LI (i.e. a pulse of label that shows cells in S phase at a given time) or growth fraction, GF (i.e. the total number of cells proliferating in a population) (Globus and Liversage, 1975; Holtfreter, 1929; Jabaily et al., 1982; Kumar et al., 2007; Liversage and Globus, 1977; Mescher and Loh, 1981; Tomlinson et al., 1981; 1984; Wilde, 1950).

Early experiments with salamander tissues *in vitro* began with the use of basic salt solutions or other types of creative media (e.g. Twitty et al. used the peritoneal fluid from adult *Triturus similans* (Twitty and Bodenstein, 1939). The literature concerning the

growth or proliferation of salamander cells *in vitro* is limited, and relies on difficult and or subjective techniques (e.g. 3H-Thymidine radiography and mitotic counts respectively). One of the earliest reports addressing the growth of salamander tissues *in vitro* where actual counts were performed was by H. Clark Dalton. Examining chromatophores in the posterior trunk neural fold tissue from stage 16-21 embryonic axolotls, Dalton reported a mitotic index (MI) of 4% in black axolotls and 6.5% in white axolotls, and MI index of zero after 8 days in culture (Dalton, 1950).

Another interesting early study that showed behaviors similar to the ones presented in this thesis, were performed by C.E. Wilde in his examination into the organogenesis of the limb buds and blastema of larval *Ambystoma maculatum*. Wilde used whole limb bud explants in a hanging drop culture and examined growth and behavior over a period of 21 days (Wilde, 1950). Interestingly, Wilde reported that the blastema failed to grow in Holtfreter's solution (a basic salt solution) that did not contain added nutrients (Wilde, 1950). Wilde also remarked on wound epithelium behaviors that were remarkable similar to those described herein (See Chapter 3).

From the 1930's up until the early 1990's, the use of whole explant cultures continued to be a common method of studying regeneration *in vitro*. Beginning in the late 1940's, investigators such as Marcus Singer began to research how the nerve influenced growth. As mentioned in the previous chapter, researchers realized the inherent difficulty in studying the role the nerve plays in growth during regeneration, and have tried to circumvent these challenges using *in vitro* techniques to study the nerve. The 1950's heralded a new era of using *in vitro* techniques to study the nerves role in regeneration.

Globus et al. used explant cultures to evaluate whether blastema explants required a nerve to proliferate *in vitro* (Globus and Liversage, 1975). Prior to amputation, blastemas had sensory nerves (i.e. dorsal root ganglia, DRG) implanted so that after amputation a nerve source would still be present in the explant (Globus and Liversage, 1975). Explants were then cultured in Parker's medium for 3-6 days. It was found that proliferation of blastema cells *in vitro* was correlated with the presence of an eccentrically implanted DRG, and that this growth response was most pronounced when the DRG was implanted 2-3 days prior to amputation of the blastema (Globus and Liversage, 1975). This result is very interesting in light of the results discussed in Chapter 3 with regard to the "pre-conditioning" of DRG. Liversage and Globus continued to examine the relationship between the nerve and the blastema in terms of growth *in vitro* and produced similar results as Globus et al. (1975) in that growth of blastema explants *in vitro* required prior nerve implants (Liversage and Globus, 1977).

The benefit that using an *in vitro* system bestows on the investigator was exemplified by Globus et al. (1977) in the paper "Transfilter mitogenic effect of dorsal root ganglia on cultured regeneration blastemata, in the newt, *Notophthalmus viridescens*." Globus et al. showed that the growth promoting effect of the nerve was maintained even when the blastema explant and DRG were separated by transwell filter (Globus and Vethamany-Globus, 1977). The mitotic index of blastema explants co-cultured with a DRG was 1.124% as opposed to blastema cultured without a DRG which had a MI of 0.339% (Globus and Vethamany-Globus, 1977). The importance of this work was that it showed that the neurotrophic factor was indeed a "physical reality" and could pass through a porous filter (Globus and Vethamany-Globus, 1977). This in turn

demonstrates the benefit of using an *in vitro* system to study regeneration as the information gleaned from this study would be all but impossible to attempt *in vivo*.

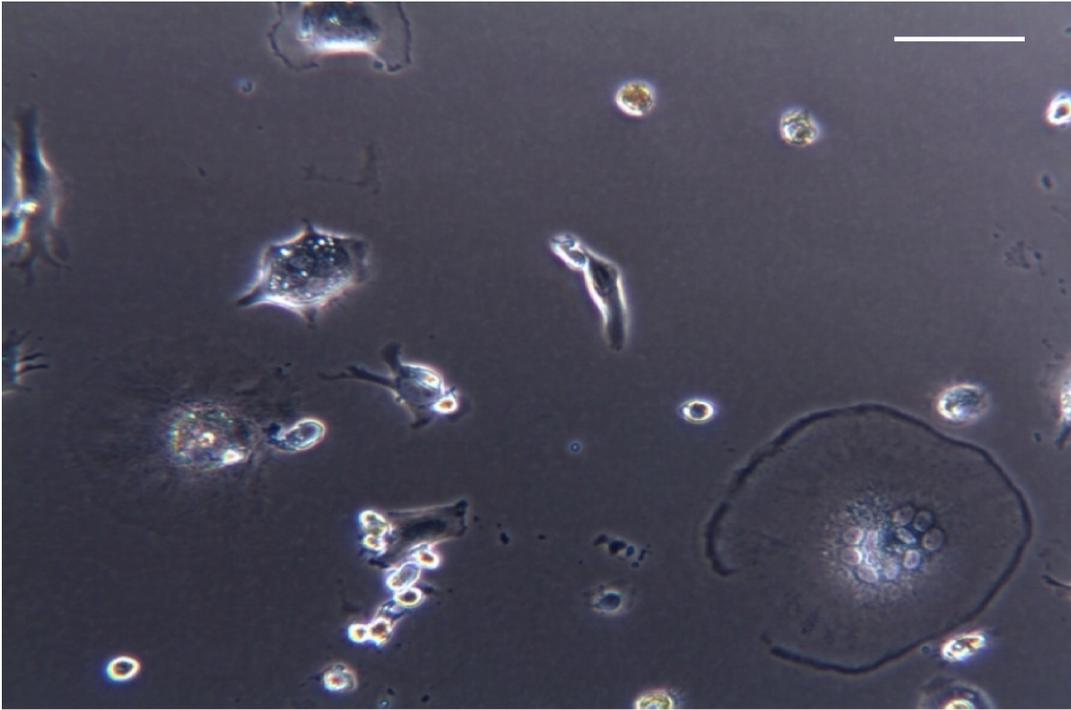
Removing a blastema and culturing it *in vitro* has a similar effect on proliferation rates to performing denervation *in vivo* (Goldhammer and Tassava, 1987; Oudkhir et al., 1986). For all intents and purposes, removal of the blastema from the animal is *de facto* denervation, and furthermore is much more efficient in regard to completely removing the nerves influence.

For all the benefits of whole explant culture, it does have its setbacks. A problem with explant cultures is that cell death can occur especially in tissues unable to exchange nutrients and gasses with the cell culture medium (Lossi et al., 2009).

An alternative approach to explant culture is the dissociation of tissues into a monolayer of cells. The technique of dissociating salamander cells began to become more widely used in regeneration research after the 1980's (Jabaily et al., 1982). The technique of dissociating cells avoids the issue of cell death like those found in explants, but also obliterates any positional connections and cytoarchitecture found in the original tissue. It is for these reasons perhaps that the LI of dissociated blastema cells is much lower than *in vivo* levels (Ferretti and Brockes, 1988; Goldhammer et al., 1992; Kumar et al., 2007; Maden, 1978; Tomlinson et al., 1984). Another issue is that since the blastema represents a heterogeneous population of cells (Kragl et al., 2009; Muneoka et al., 1986), as well as the fact that reliable cell markers are currently unavailable, dissociation of blastemas into monolayer cultures results in the inability to distinguish cells types other than via the basis of morphological characteristics. Figure 5 illustrates the wide variety of cell morphologies observed in a typical culture of dissociated blastema cells.

Upon my arrival to the Gardiner lab as a rotation student, I was challenged to identify the proper conditions to permit axolotl dermal fibroblasts to proliferate *in vitro*. Dermal fibroblasts play an important role in regeneration and make up 40-80% of the cells within the blastema (Muneoka et al., 1986). Anecdotal data and personal communications from within the Gardiner lab suggested that dissociated dermal fibroblasts and blastema cells present a challenge in that the cells do not proliferate *in vitro*. Corroborating this claim is the result of recent studies that explicitly examine labeling index in dissociated cells report an extremely low LI, much lower than *in vivo* levels (Kumar et al., 2007).

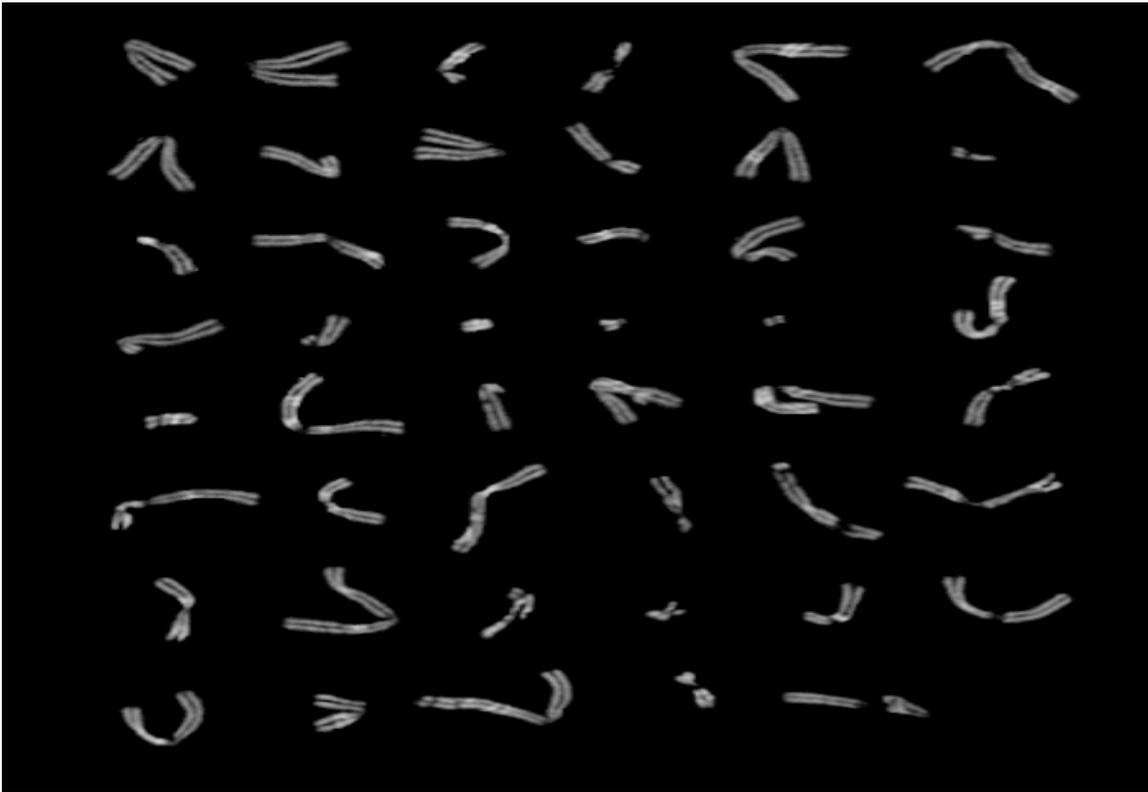
Despite many experiments examining a variety of conditions, I was unable to discover culture conditions for which dissociated axolotl dermal fibroblasts proliferated *in vitro*. The conditions examined included varying both individually and in combination the composition of media, substrate, temperature, osmolarity, and dissociation techniques. The only extant cell line derived from axolotl dermal fibroblasts was created via explant culture in the Gardiner lab more than 20 years ago. This cell line was named “AL1” cells, so called after their provenance (i.e. Axolotl Limb, AL). These cells have since been used in a number of experiments from other labs (Lévesque et al., 2007; Shaikh et al., 2011; Villiard et al., 2007; Whited et al., 2012; 2013).



**Figure 5: Cell phenotypes of dissociated blastema cells.** Phase contrast image showing the cell phenotypes that result from the dissociation of a medium bud blastema mesenchyme. Cells display a wide variety of behaviors and morphologies (e.g. multinucleate cell in the lower right resembling a fried egg). Because the blastema is a heterogeneous population of cells and due to a lack of reliable cell markers, it is difficult to distinguish the tissue origin of a given cell. Scale bar = 100 $\mu$ m

It was particularly vexing that no matter what the condition, axolotl dermal fibroblasts failed to proliferate *in vitro* when dissociated, especially with the knowledge that there was already an established cell line (i.e. AL1 cells). Two hypothesis were put forth to explain why AL1 cells might proliferate in conditions that dissociated cells might not. The first hypothesis was that AL1 cells proliferated owing to the fact that they were not obtained from dissociated blastemas but rather obtained from explant cultures. The second hypothesis was that AL1 cells proliferated as a result of a spontaneous *in vitro* transformation.

A spontaneous *in vitro* transformation is when cells spontaneously become neoplastic while *in vitro*, resulting in progressive growth (Evans et al., 1967). Spontaneous *in vitro* transformation can occur frequently, especially undifferentiated progenitor cells, for example one study reported the rate as high as 45.8% (Garcia et al., 2010).



**Figure 6: Karyotype of AL1 cells.** Mitosis was inhibited in AL1 cells through the use of colcemid and the cells were then incubated in a hypotonic medium and plated. The cells were then stained with DAPI and chromosomes were isolated and counted

A hallmark of *in vitro* transformation, and cancer cells in general, is an abnormal chromosome number (i.e. aneuploidy) (Magdolen et al., 2010; Nicholson and Duesberg, 2009). To test this hypothesis, I analyzed the karyotype of AL1 cells to determine if they

possessed a normal number of axolotl chromosomes. Upon karyotype analysis of AL1 cells I found that they contained ~ 35 chromosomes as opposed to the normal 28 (Callan, 1966), indicating that they may have undergone a spontaneous *in vitro* transformation. Perhaps, like in the instance of cancer, a spontaneous *in vitro* transformation is the reason why AL1 cells are able to proliferate *in vitro* while primary cultures of dermal fibroblasts are not. If AL1 cells are a transformed cell type then it calls into question the use of AL1 cells in certain publications (Lévesque et al., 2007; Shaikh et al., 2011; Villiard et al., 2007; Whited et al., 2012; 2013).

## **CONCLUSION**

The techniques used to study limb regeneration *in vitro* have both their advantages and disadvantages. Explant cultures maintain the cytoarchitecture of the original tissue but prevent adequate gas and nutrient exchange to tissues in the explant core. Dissociated monolayer cultures avoid the problems associated with gas and nutrient exchange however the cytoarchitecture is destroyed, as are important cell-cell and cell-ECM interactions. To address these problems, as well as address the problem of studying the nerve *in vivo* as addressed in Chapter 1, I modified the technique of organotypic slice cultures, which is the subject of the next chapter.

## **CHAPTER 3: Regulation of axolotl limb blastema cell proliferation by nerves and BMP2 in organotypic slice culture**

The following chapter describes work that shall be submitted for future publication. It describes the organotypic slice culture system that I modified and optimized in order to study the role of the nerve *in vitro*.

## **ABSTRACT**

We have modified and optimized the technique of organotypic slice culture in order to study the mechanisms regulating growth and pattern formation in regenerating axolotl limb blastemas. Blastema cells maintain many of the behaviors that are characteristic of blastemas *in vivo* when cultured as slices *in vitro*, including rates of proliferation that are comparable to what has been reported *in vivo*. Because the blastema slices can be cultured in basal medium without fetal bovine serum, it was possible to test the response of blastema cells to signaling molecules present in serum, as well as those produced by nerves. We were also able to investigate the response of blastema cells to experimentally regulated changes in BMP signaling. Blastema cells responded to all of these signals by increasing the rate of proliferation and the level of expression of the blastema marker gene, *Prrx-1*. The organotypic slice culture model provides the opportunity to identify and characterize the spatial and temporal co-regulation of pathways in order to induce and enhance a regenerative response.

## **INTRODUCTION**

Research on regenerating body parts has focused on identifying the signaling pathways involved in initiating and regulating this fascinating and biologically important process. Much of that research has focused on regenerating salamander limbs, and has involved either describing the process of regeneration, or inhibiting regeneration (e.g. by denervating the limb), and then attempting to rescue regeneration. In recent years, the gain-of-function assay for limb regeneration (the Accessory Limb Model, ALM) has identified a number of signals and pathways that are necessary and sufficient for

induction of blastema formation and subsequent regeneration of an ectopic limb (Endo et al., 2004; Satoh et al., 2008a; 2011). These experimental approaches have provided insights into the mechanisms of regeneration; however, their utility has been limited by the fact that they all involve regeneration *in vivo*. Thus it is not possible to control the spatial and temporal activation or inhibition of specific pathways without the variability associated with working on a live animal. Attempts to deal with this complexity have involved using *in vitro* culture techniques with either monolayer cultures of dissociated blastema cells, or with blastema explant cultures (Globus and Liversage, 1975; Hinterberger and Cameron, 1983; Jabaily et al., 1982; Kumar et al., 2007). These studies have been of limited utility because of the limited viability of explants, and the loss of normal blastema cell behaviors (e.g. proliferation) after enzymatic dissociation. To address this challenge, we have adapted organotypic slice culture techniques that have been used widely and successfully in the field of neurobiology (Gähwiler et al., 1997), and have investigated the response of blastema cells to nerve signals and to BMP2.

Because regeneration is a stepwise process (Endo et al., 2004; Knapp et al., 2013; Muller et al., 1999), there are critical signaling events at each step that are required for progression to the next step. Consequently, if the appropriate signaling does not occur at any one of the steps, regeneration will fail to occur (Endo et al., 2004; Muller et al., 1999). Thus each step represents a possible barrier to regeneration, and the failure to overcome at least one of these barriers likely accounts for regenerative failure in mammals (Muller et al., 1999). Identifying the steps and discovering how to provide the appropriate signals to progress beyond each step will be required in order to induce regeneration in humans. One or more of the early signals involved in the initiation of

regeneration are provided by nerves (Endo et al., 2004; Singer, 1952; Todd, 1823; Wallace, 1981), which have been the focus of research efforts for decades.

The nerve has long been recognized to be important in regeneration (Sidman and Singer, 1951; Singer, 1946b; 1947; 1952; Singer and Craven, 1948; Singer and Egloff, 1949; Todd, 1823; Wallace, 1981). Historically, most experimental work on the role of the nerve has involved *in vivo* studies designed to rescue regeneration of amputated limbs that have been denervated. Limbs that have been denervated fail to initiate regeneration, or fail to progress through the early stages of regeneration (Singer, 1952) to the point where they become independent of the nerve (Singer and Craven, 1948). If the nerves are allowed to regenerate to a point where the supply of nerves exceeds a threshold level, reinjured limbs will be able to regenerate (Kamrin and Singer, 1959; Salley-Guydon and Tassava, 2006; Singer, 1946b). Thus attempts to identify the pro-regenerative signals provided by nerves have focused on the later stages of regeneration when the regeneration blastema transitions to becoming independent of nerve signals (Mullen et al., 1996a), or on the stage when the regenerating nerve reaches the threshold for the required nerve signaling (Kumar et al., 2007; Salley-Guydon and Tassava, 2006; Tassava et al., 1987). Although denervation experiments have repeatedly demonstrated that a nerve is required (loss-of-function), they have not identified the signals that are required to initiate regeneration and allow for progression through to the late stages at which regeneration is no longer nerve-dependent. The variability associated with *in vivo* experiments (e.g. the age of the animal which affects the timing of the regenerative response, and the degree of nerve regeneration and reinnervation of limbs following

denervation) is a challenge to understanding the precise regulation of the nerve signals that are sufficient for inducing regeneration.

The Accessory Limb Model (ALM), which is a gain-of-function assay for limb regeneration, has provided additional insights into the early steps leading to blastema formation as well as the regulation of blastema cell dedifferentiation (Endo et al., 2004; Satoh et al., 2008a). In the ALM, an ectopic blastema is induced on the side of the arm by making a full-thickness skin wound and surgically deviating the brachial nerve to the wound site (Endo et al., 2004; Satoh et al., 2007). Within a few days post-surgery an ectopic blastema develops and grows; however, without further signaling, it does not form ectopic limb structures and eventually reintegrates into the limb. The induced ectopic blastema can be induced to form an ectopic limb if a piece of skin from the opposite side of the limb is grafted to the wound site (e.g. posterior skin grafted to an anterior wound with a deviated nerve). Thus the ALM has demonstrated that there are early critical steps in regeneration and that signaling from the nerve and wound epithelium regulates these steps. These signaling pathways can be activated experimentally with FGF and BMP, and an ectopic blastema can be induced by implanting beads soaked in a cocktail of FGF2, FGF8 and GDF5/BMP7 in the absence of a deviated nerve (Makanae et al., 2013). As with denervation experiments, the ALM is limited by variability associated with experiments *in vivo* in terms of understanding the precise regulation of the pro-regenerative signals (e.g. timing and dose of growth factor delivery) that are optimal for inducing regeneration.

To address the challenges associated with studies of regeneration *in vivo*, there have been repeated attempts to isolate and study blastema cells *in vitro*. As with *in vivo*

studies, these experiments have provided insights into factors that affect the behavior of blastema cells, particularly with regards to proliferation (Boilly and Albert, 1988a; Carlone and Foret, 1979; Globus and Liversage, 1975; Globus and Vethamany-Globus, 1977; Kumar et al., 2007). Although blastema tissue can be dissociated and blastema cells can be maintained in monolayer culture, the cells quickly lose properties associated with blastema cells *in vivo*. Cultured blastema cells typically have low rate of proliferation and are highly variable; e.g. growth fractions ranging from 0.2-10% (Ferretti and Brockes, 1988; Kumar and Godwin, 2010; Kumar et al., 2007) compared to proliferation *in vivo*; e.g. labeling index of 20% to 40% (Goldhammer et al., 1992; Maden, 1978; Tomlinson et al., 1984; Wallace, 1981). The difficulty in maintaining normal behavior of blastema cells *in vitro* has been attributed to their sensitivity to culture conditions such as osmolarity and temperature (Conn et al., 1979; Ferretti and Brockes, 1988; Fimian, 1959; Jabaily et al., 1982). Short-term culturing of blastema explants has been reported in studies pertaining to the effect of hormones and growth factors on protein synthesis and cell division (Choo et al., 1978; Ferretti and Brockes, 1988; Globus and Liversage, 1975; Globus and Vethamany-Globus, 1977; Mescher and Loh, 1981). The utility of this approach is limited by variability associated with the size of the explants, as well as differences in the viability of cells in the center of the explants compared to cells at the periphery. Finally, dissociated and re-aggregated blastema cells have been cultured in fibrin clots in which they maintain the property of producing high levels of matrix degrading enzymes; however, after a week they lose the ability to signal to other blastema cells in order to induce supernumerary limb patterns when grafted back into a blastema *in vivo* (Groell et al., 1993).

In order to overcome the challenges of studying the role of the nerve in regeneration *in vivo*, we initiated a systematic assessment of techniques for culturing blastema cells. As reported by others, proliferation rates of dissociated blastema cells quickly decreased when the cells were cultured on a range of substrates and culture media, and the cultures could not be maintained and passaged over an extended period of time. We therefore reasoned that the behavior of blastema cells *in vivo* was dependent on cell-cell and cell-matrix interactions established during blastema formation. In order to maintain the *in vivo* organization of blastema cells and matrix, we optimized the technique of organotypic slice culture (OSC) that is commonly used in neurobiology. The technique uses a vibrating blade (vibratome) to make serial sections of unfixed blastemas that are of uniform thickness and maintain the original tissue architecture. Multiple sections can be collected from the same blastema, and thus different culture conditions can be tested experimentally without the variability associated with comparing blastema tissues from different animals (e.g. variability in stages of blastemas). We report here that blastema cells in OSC maintain *in vivo* levels of proliferation and tissue architecture, and are responsive to signals from co-cultured dorsal root ganglia explants (DRG), and to exogenous BMP2.

## **MATERIALS AND METHODS**

### *Ethics Statement*

This study was carried out in accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. The experimental

work was conducted in accordance with procedures approved by the Institutional Animal Care and Use Committee of the University of California Irvine.

#### *Animal Procedures and Collection of DRG and Blastemas*

Axolotls (*Ambystoma mexicanum*) measuring 15-20 cm from snout to tail tip that were spawned at UC Irvine or the *Ambystoma* Genetic Stock Center at the University of Kentucky were housed on a 12-hour light/dark cycle and fed *ad libitum*. Axolotls were anesthetized using 0.1% ethyl 3-aminobenzoate methanesulfonate salt (MS222, Sigma) at pH 7.0. Limb regeneration leading to formation of medium-late bud stage blastemas was induced by amputation at the level of the mid-humerus.

Details for the culture of dorsal root ganglia (DRG) have been reported previously (Athipozhy et al., 2014). Briefly DRG were collected post-euthanasia by surgically removing the spinal nerves that innervated either the forelimb (spinal nerves 3, 4 and 5) or the hind limb (spinal nerves 15, 16 and 17). DRG were cultured individually in 12 well Nunc nunclon plates with 60% L-15, 5% FBS, 1% Insulin, Transferrin, and Selenium (ITS), and gentamicin/amphotericin B (Sigma). Each DRG was attached to the bottom of the culture well by embedding it in a small drop (about 3  $\mu$ l) of growth factor reduced matrigel (BD Biosciences). Only DRG explants that exhibited neurite outgrowth within the first 24 hours of culture were used for subsequent experiments.

#### *Organotypic Slice Culture (OSC)*

Medium-late bud stage blastemas (mesenchyme and epithelium) were removed surgically from animals, embedded in a 4% agarose gel, and sectioned at a thickness of 250 $\mu$ m

using a vibratome. Blastema slices were then cultured on transwell membrane inserts (3.0µm pore) with 6 well plates (BD Falcon) in 60% L-15 media containing 1% Gentamicin, 1% penicillin/streptomycin/amphotericin B, and 1% ITS media supplement (Sigma) in a refrigerated incubator at 19°C. Blastema slices and blastema slice/DRG co-culture were adhered to the transwell membrane with 5µL of growth factor reduced matrigel (BD). Blastema slices receiving additional treatments were cultured in media containing one of the following supplements: 5% Fetal Bovine Serum (FBS, Atlanta Biologicals); 1ng/mL, 10ng/mL, or 100ng/mL recombinant human Bone Morphogenetic Protein 2 (BMP2, Sigma); or 5nM, 50nM, 500nM of the BMP signaling inhibitor, LDN-193189 hydrochloride (Abcam). Half the volume of the culture media was changed every other day. Slices were cultured for 3, 5, or 7 days after being explanted.

#### *Analysis of Cell Proliferation and Viability*

To analyze cell proliferation we used a Click-It EdU labeling kit (Life Technologies) according to the manufacturer's protocol. The labeling index (% of cells that were in the S phase of the cell cycle) was determined by incubating slice cultures in 80µM of EdU for five hours, which corresponds to approximately 10% of the total cell cycle for blastema cells (Wallace and Maden, 1976). The growth fraction (% cells that were proliferating) was determined by incubating the slice cultures in EdU for 80 hours, which corresponds to approximately 1.5 cell cycles (1 cell cycle = 53 hours) (Wallace and Maden, 1976; Wallace, 1981). After EdU labeling, the slice cultures were washed in PBS, fixed in 4% paraformaldehyde, and sectioned at 5µm. EdU labeled cells were then visualized using the Click-It-EdU alexa-fluor 488 or alexa-fluor 594 kit (Life

Technologies), and were counted using the cell counter plug-in for ImageJ. The total number of cells was visualized by staining with DAPI.

To determine the relative number of viable and dying cells, we used a TUNEL assay kit (Roche) to identify cells that were undergoing cell death. Slides were processed as indicated above and then processed in accordance with the manufacturer's protocol.

### *Immunohistochemistry*

Blastema slices were fixed for three hours at room temperature in 4% paraformaldehyde, dehydrated in graded alcohol followed by xylene, embedded in paraplast, and sectioned at 5 $\mu$ m. For immunohistochemistry sections were de-paraffinized, rehydrated in TBST, and incubated with anti-acetylated  $\alpha$ -tubulin (Abcam, catalog # T7451, diluted 1:250) and anti-RT-97 (DSHB, diluted 1:250) overnight at 4°C. Sections were then washed with TBST, incubated with anti-mouse 594 (Abcam, diluted 1:250) for 4 hours, washed with TBST, and mounted with ProLong Gold Antifade mounting medium with DAPI. Images were obtained using a LSM780 confocal microscope.

The intra-nuclear distribution and translocation of phospho-Smad 1/5/8 was quantified by staining sections with an anti-phospho-Smad 1/5/8 antibody (Millipore, Catalog # AB3848, Lot # 2390361, diluted 1:75). Sections were de-paraffinized and rehydrated in TBST as described above. Heat-induced epitope retrieval was performed at 100°C for 1 hour in Tris/EDTA (pH 9.0). Sections were incubated with anti-phospho-Smad 1/5/8 overnight at 4°C, washed with TBST, and incubated with anti-rabbit 488 (Abcam, diluted 1:250). 12 regions containing 100-200 mesenchymal cells from 3 different OSC (N=3) from each condition tested were selected and analyzed in a double

blind manner. The corrected nuclear fluorescence was calculated in order to normalize the intensity of nuclear staining for variation in the area of the nuclei being analyzed as well as for the background fluorescence (Gavet and Pines, 2010).

### *Quantitative PCR*

Blastema slice RNA was extracted using Trizol (Invitrogen) in conjunction with the Nucleospin RNA XS kit (Macherey-Nagel). Samples were processed to generate cDNA using the Transcriptor First Strand cDNA Kit (Roche). Real-time PCR was performed using a LightCycler 480 (Roche) and the resulting data were analyzed using the Pfaffl method (Pfaffl, 2001). Sequences for the primers for qPCR (Sigma) were based on primers published previously (Sato et al., 2007; Whited et al., 2011).

GAPDH FWD:        GACGCTGGTGCAGGCATTGCC

GAPDH REV:        ACCATCAGGTCCACAACACGCTGAC

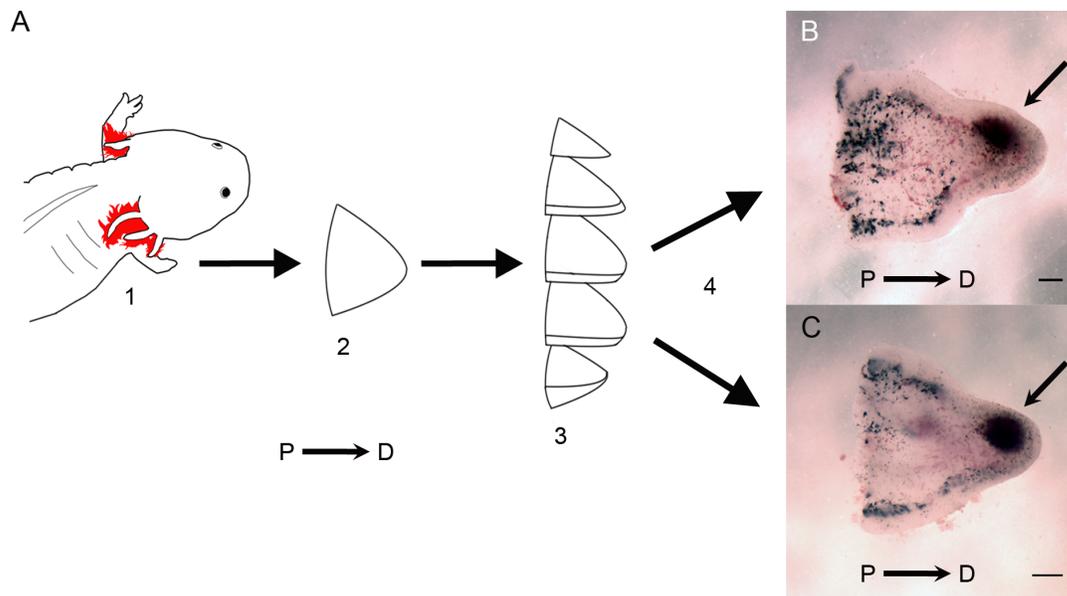
Prrx1 FWD:         GGCGAAAGTTTGCTCTTCGG

Prrx1 REV:         GGCGAAACTTTGCTCTTCGG

## **RESULTS**

Organotypic slice cultures of axolotl blastemas remain viable for several days OSC allowed for the culture of multiple sections of uniform dimensions from a single blastema (Figure 7). In response to limb amputation, a blastema (medium-late bud stage) formed in about 10-12 days (Step 1, Figure 7A). The blastema was surgically removed (Step 2), embedded in gelatin and sectioned using a vibratome that resulted in uniform 250µm thick longitudinal sections (Step 3). Depending on the size of the blastema, a

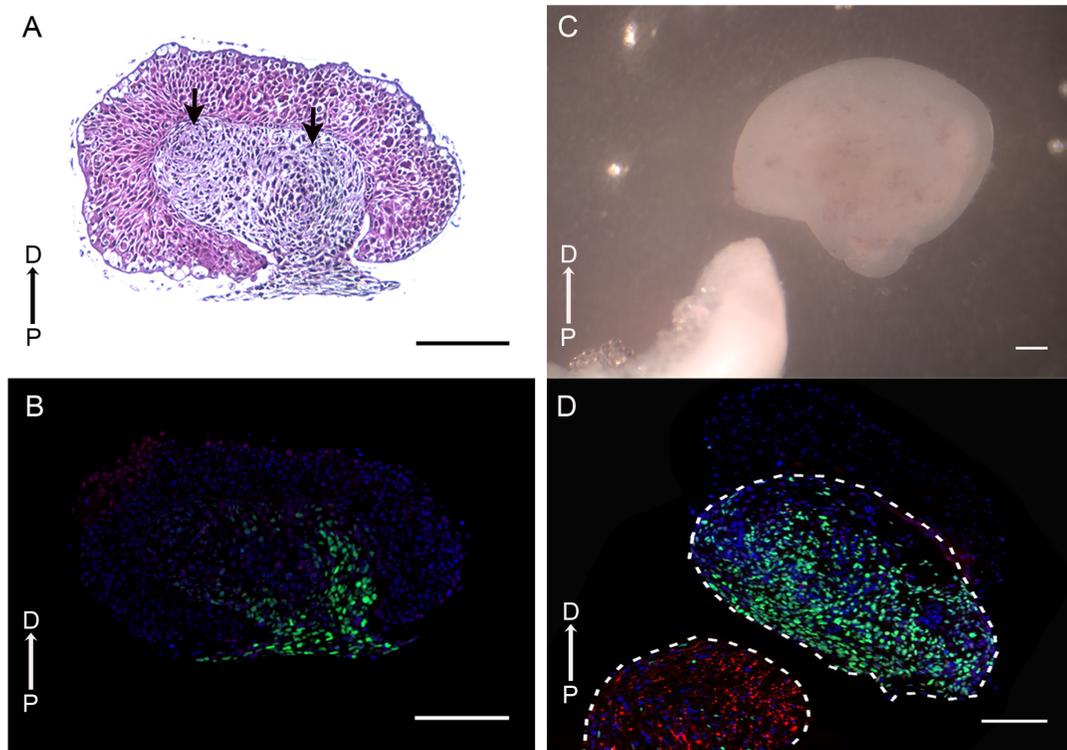
typical mid bud blastema from an adult axolotl (10-15cm) yielded 3-4 slices that were similar in shape and size. Adjacent blastema slices maintained the tissue architecture of *in vivo* blastemas (i.e. mesenchyme surrounded by a wound epithelium) (Figure 7B, C). The blastema slices were then cultured under various experimental and control conditions (Step 4).



**Figure 7: Organotypic slice culture model for axolotl blastemas.** (A1) The limb of an axolotl (*Ambystoma mexicanum*) was amputated and allowed to regenerate a medium-late bud stage. (A2) The blastema was surgically removed from the animal, (A3) was sectioned using a vibratome (A3) and cultured (A4, B, C). Depending on the size of the blastema, a typical blastema yielded 3-4 slices that appeared similar when cultured (B and C). A region of pooled blood cells in the apical region of adjacent slices is indicated by the arrows in (B and C). The proximal (P) to distal (D) orientation of the sections is indicated. Scale bars = 500 $\mu$ m.

Blastema slices appeared healthy and the cells continued to proliferate *in vitro* over an extended period of time (Figure 8). After 10 days in culture, the slices

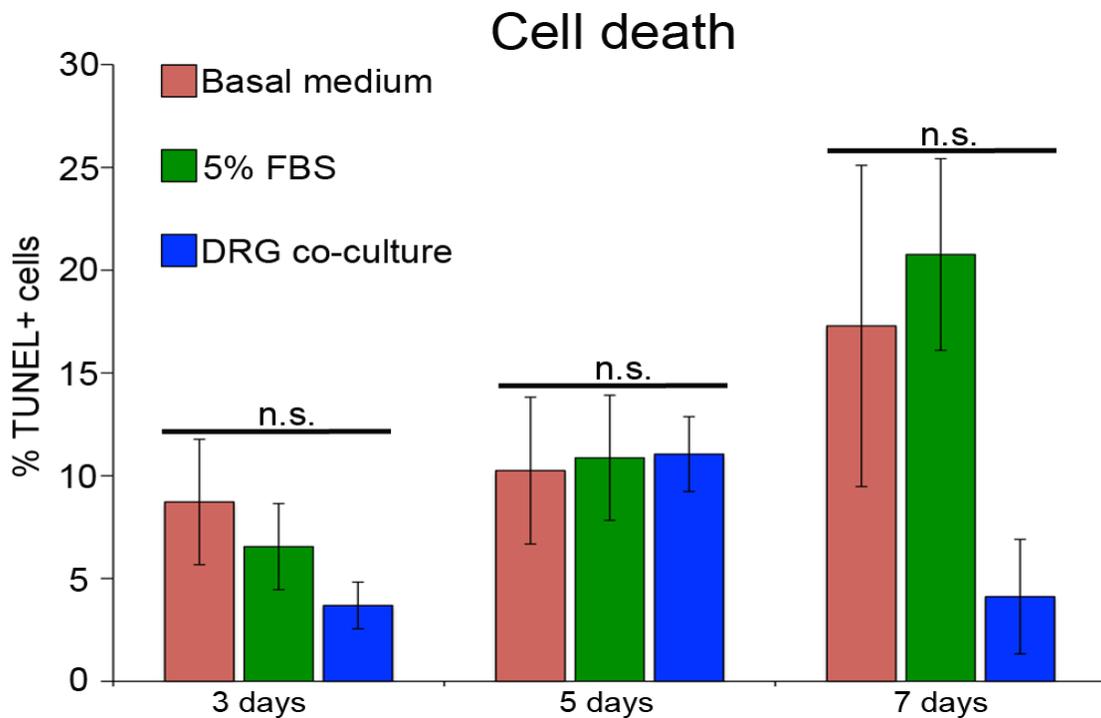
maintained a normal morphology with a thickened epithelium surrounding the blastema mesenchyme (Figure 8A). The cells within the slice appeared healthy and regions of high cell density corresponding to pre-chondrogenic condensations were evident in some sections (Figure 8A, arrows). At the proximal boundary of the slice, the epidermis began to migrate over the free surface that was created when the blastema was surgically removed from the animal. This phenomenon referred to as “epiboly”, has been described previously in whole-mount explants of amphibian blastemas and limb buds (Holtfreter, 1943; Wilde, 1950). Many of the cells of the blastema mesenchyme incorporated EdU after 10 days of culture, although cells in the regions of high cell density (visualized by DAPI staining of the nuclei) did not (Figure 8B, D). The keratinocytes of the apical epithelium (Apical Epithelial Cap, AEC) in this experiment (cultured with 5% FBS) did not incorporate EdU (Figure 8B), which was consistent with the observation that AEC cells withdraw from the cell cycle *in vivo* (Hay and Fischman, 1961; Satoh et al., 2012). As discussed below, the incorporation of EdU by keratinocytes of the AEC was variable, and determining how the proliferative response of AEC cells is regulated in organotypic slice cultures is a goal of future experiments.



**Figure 8: Organotypic blastema slices survive and proliferate *in vitro*.** (A) Hematoxylin and eosin stained section of a blastema slice that had been cultured for 10 days in the presence of 5% FBS. Regions of pre-cartilage condensations are evident (arrows). The blastema epithelium has begun to migrate over the proximal cut end. (B) EdU labeling of a blastema slice after 10 days *in vitro*. EdU positive nuclei (green) are present in the mesenchyme but absent from the epithelium. (C) A bright field image of a blastema slice (upper right) and DRG (lower left) after 10 days of co-culture. (D) Immunofluorescence of the blastema slice/DRG co-culture illustrated in (C). Proliferating cells in the slices (green) are restricted to the mesenchyme, nuclei are stained with DAPI (blue), and neurofilaments are labeled with RT97 (red). The proximal (P) to distal (D) orientation of the sections is indicated. Scale bars = 500 $\mu$ m.

As reported previously, DRG can be isolated and co-cultured with blastema explants in order to study the interaction between nerves and blastema cells (Athippozhy et al., 2014). To investigate the response of OSC blastema cells to signaling from nerves,

blastema slices were co-cultured with DRG (Figure 8C, D). Both OSC blastema slices and DRG explants appeared healthy after an extended period of time (10 day cultures illustrated in Figure 8C, D). As with blastema cultures without DRG explants, many of the mesenchymal cells not associated with regions of high cell density incorporated EdU (Figure 8D, green). As with blastema slices cultured without DRG, the keratinocytes of the AEC did not incorporate EdU. As reported previously (Athippozhy et al., 2014), the cultured DRG contained large numbers of regenerating neurons with RT97-positive neurofilaments (Figure 8D, red).

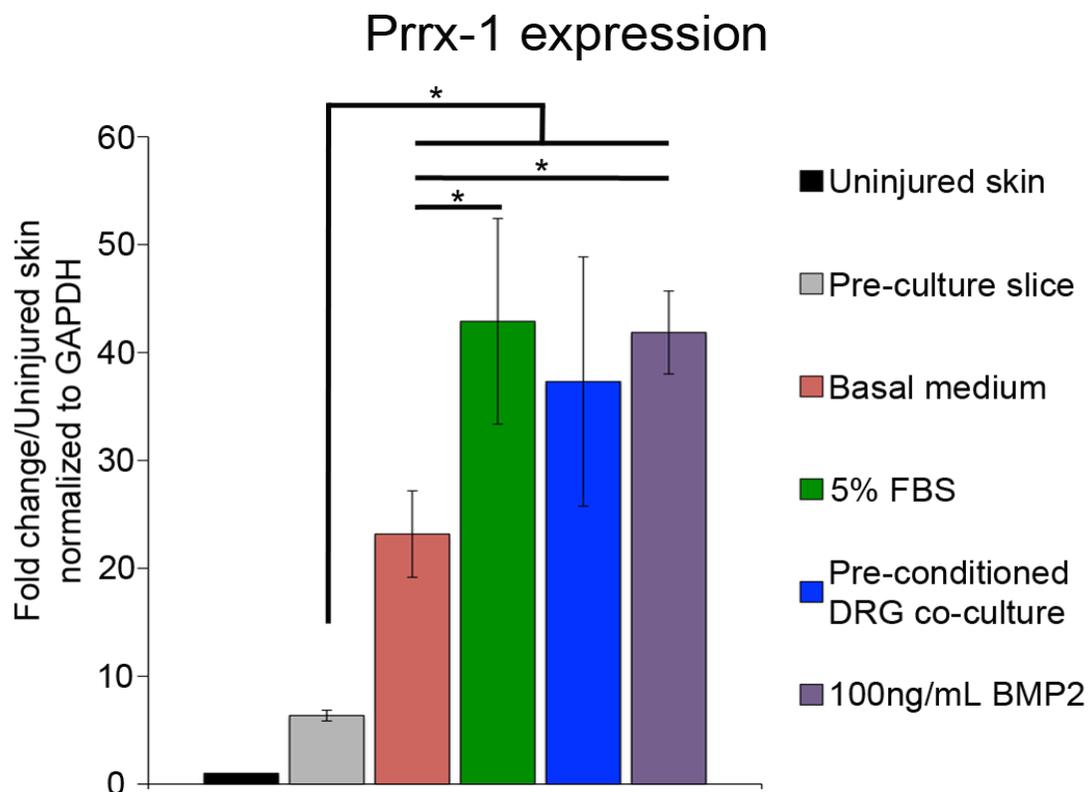


**Figure 9: Cell death in blastema slices after culture for 3, 5, and 7 days.** The percentage of TUNEL positive cells was not significantly different between treatments at any of the time points, or between the three time points. Error bars represent S.E.M, and P-values were determined by t-test with 2 tails assuming unequal variances. Sample sizes for 3, 5, and 7 days are as follows: Basal medium: 5, 5, and 3, 5%FBS: 4, 4, and 5, DRG co-culture: 3, 3, and 3.

As noted above, blastema slice cultures appeared healthy for at least 10 days in culture. During this period, some pyknotic nuclei were observed in cells along the proximal edge of the explant that was created when the blastema was surgically removed from the animal. TUNEL staining was relatively low, and was not significantly different at a given time point for slices cultured with or without FBS or co-cultured DRG (Figure 9). As with the distribution of pyknotic nuclei, most of the TUNEL-positive nuclei were restricted to the region immediately adjacent to the proximal boundary of the explant. After five days *in vitro*, TUNEL staining remained about the same as observed at three days, and did not differ depending on the culture conditions. After seven days *in vitro*, TUNEL staining was more variable and although the percentage of TUNEL-positive nuclei was higher in slices cultured in basal medium and 5%FBS, but this increase was not significant. Based on these data, we did not culture slices for longer than seven days *in vitro* so as to minimize variability associated with cell death.

Expression of Prrx-1 (Paired-related Homeobox 1) in OSC blastema mesenchymal cells was maintained and increased *in vitro* (Figure 10). Prrx-1 is a transcription factor that is expressed at high levels in developing and regenerating axolotl limb mesenchymal cells (Satoh et al., 2007), but is expressed at low levels that are not detected by *in situ* hybridization in uninjured skin. Expression of Prrx-1 in the blastema is regulated by interactions between the wound epithelium and the nerve (Satoh et al., 2007), and thus it is a marker for regenerating blastema cells (Satoh et al., 2007; 2011; Yokoyama et al., 2011). As reported previously (Makanae et al., 2013), Prrx-1 expression was detected at low levels by qPCR in uninjured skin (Figure 10). Expression

in blastema sections prior to being culture was significantly upregulated an average of 6-fold, which is comparable to the increased level of expression *in vivo* when comparing blastemas to uninjured skin (Sato et al., 2011). Blastema slices cultured in basal medium for 7 days expressed Prrx-1 at a 23-fold higher level relative to uninjured skin (about a 4-fold increase during the culture period. Since Prrx-1 expression is restricted to the distal tip of blastemas *in vivo*, this increase in expression *in vitro* could be a result of increased numbers of cells in the slices being induced to express this gene. Taken together, these data indicate that blastema slices cultured in basal media are viable and maintain expression of a gene that is characteristic of regenerating blastema cells.



**Figure 10: Prrx-1 expression in blastema slices.** Fold change in Prrx-1 levels after seven days of culture under different culture conditions. The value for “Uninjured Skin” was determined for samples of skin that

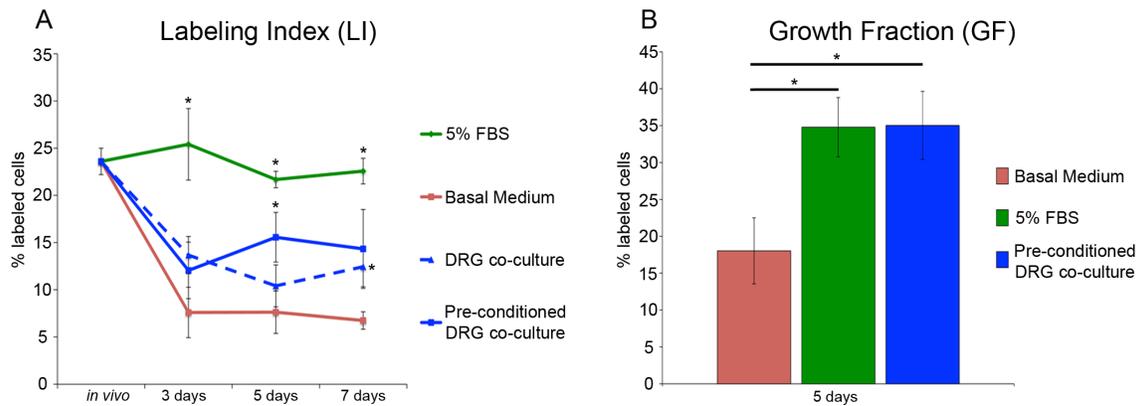
had not been cultured. The value for “Baseline” was determined for blastema slices that had not been cultured. Error bars represent S.E.M., and P-values were determined by t-test with 2 tails assuming unequal variances. Sample sizes for biological replicates for the conditions tested are as follows: Pre-culture slice: N= 5, Basal medium: N = 7, 5%FBS: N = 5, Pre-conditioned DRG: N = 5, 100ng/mL BMP: N = 3. Each biological replicate consisted of four technical replicates. Asterisk (\*) = P < 0.05.

#### *Organotypic slice cultured blastema cells responded to FBS and co-culture with DRG*

Because blastema cells remained viable when cultured in a minimal basal medium, it was possible to assay for their response to changes in the culture environment. As reported above, there was no significant change in the percentage of TUNEL-positive cells in basal medium when compared to cultures with added FBS or with co-cultured DRG. Expression of Prrx-1 was maintained, and increased over time, in slices cultured in basal medium (Figure 10). Addition of 5% FBS to the basal medium induced a significant increase in the level of Prrx-1 expression after seven days in culture (43-fold relative to uninjured skin; 7-fold relative to an uncultured blastema; nearly 2-fold relative to blastema slices cultured in basal medium). While there was an increase in Prrx-1 expression in slices co-cultured with a DRG compared to slices cultured in a basal medium (1.6-fold), the difference was not statistically significant.

In contrast to Prrx-1 expression that increased during culture in basal medium, proliferation of the blastema mesenchymal cells in basal medium decreased during the initial three days in culture and remained at a relatively low level over the next four days (Figure 11A). The labeling index (LI) of the mesenchymal cells in blastema slices prior to amputation (equivalent to *in vivo*) was about 23%, which was comparable to values reported previously for blastema cells *in vivo* (Goldhammer et al., 1992; Maden, 1978;

Tomlinson et al., 1984; Wallace, 1981). In basal medium the LI had decreased to about 7% after three days *in vitro*, and remained constant at this level until the end of the experiment (seven days). This decrease in proliferation was comparable to the rate of proliferation observed *in vivo* in denervated newt limbs (Goldhammer and Tassava, 1987) and regressing axolotl ectopic blastema (Endo et al., 2004).



**Figure 11: Labeling index and growth fraction in blastema slices.** A) Labeling index of blastema mesenchymal cells in blastema slices over a period of seven days in culture under different culture conditions as indicated. Labeling period was for five hours. The value for “*in vivo*” was determined by injecting animals with BrdU two hours prior to collecting the blastema for sectioning and counting of labeled cells without culture. Error bars represent S.E.M., and P-values were determined by T-test with 2 tails assuming unequal variances. Sample size for *in vivo*: N=22. Sample sizes for the following conditions on 3,5, and 7 days are as follows: Basal medium: N = 6, 8, and 9. 5%FBS: N = 7, 7, and 7. DRG co-culture: N= 3, 5, and 6. Pre-conditioned DRG: N = 5, 5, and 4. Asterisk (\*) = P < 0.05 when compared to the LI in basal medium at equivalent timepoint. B) Growth Fraction of blastema mesenchymal cells in blastema slices after five days of culture under different culture conditions as indicated. Labeling period was for 53 hours. Error bars represent S.E.M., and P-values were determined by t-test with 2 tails assuming unequal variances. N = 4. Asterisk (\*) = P < 0.04.

The rate of proliferation was maintained at *in vivo* levels in cultures that were supplemented with 5% FBS (Figure 11A). The proliferation rate in slices that were co-cultured with a DRG removed from animals the same day as initial culturing of OSC was higher (not statistically significant) than the rate for slices cultured in basal medium during the first five days of culture. After seven days of culture, the LI in blastema/DRG co-cultures was about 2-fold higher compared to the basal medium cultures ( $p = 0.015$ ).

Gene expression data suggest that DRG might undergo a refractory period or injury response that changes the expression of genes involved with growth and proliferation of blastema cells (Athippozhy et al., 2014). It has also been shown that the growth response to DRG was greatest when DRG were surgically grafted 2-3 days prior to whole explant culture of blastemas rather than concurrently, supporting the idea that DRG might require a period of recovery before the growth promoting effecting of the nerve is restored (Globus and Liversage, 1975). In light of these observations, we hypothesized that DRG might require a recovery period prior to co-culture with DRG in order to restore the growth promoting effect of the nerve. Because previous work has shown that there is a large change in gene expression when comparing DRG *in vitro* for 5 days to day 0 DRG (Athippozhy et al., 2014), we decided to pre-condition DRG for 5 days prior to co-culture with OSC.

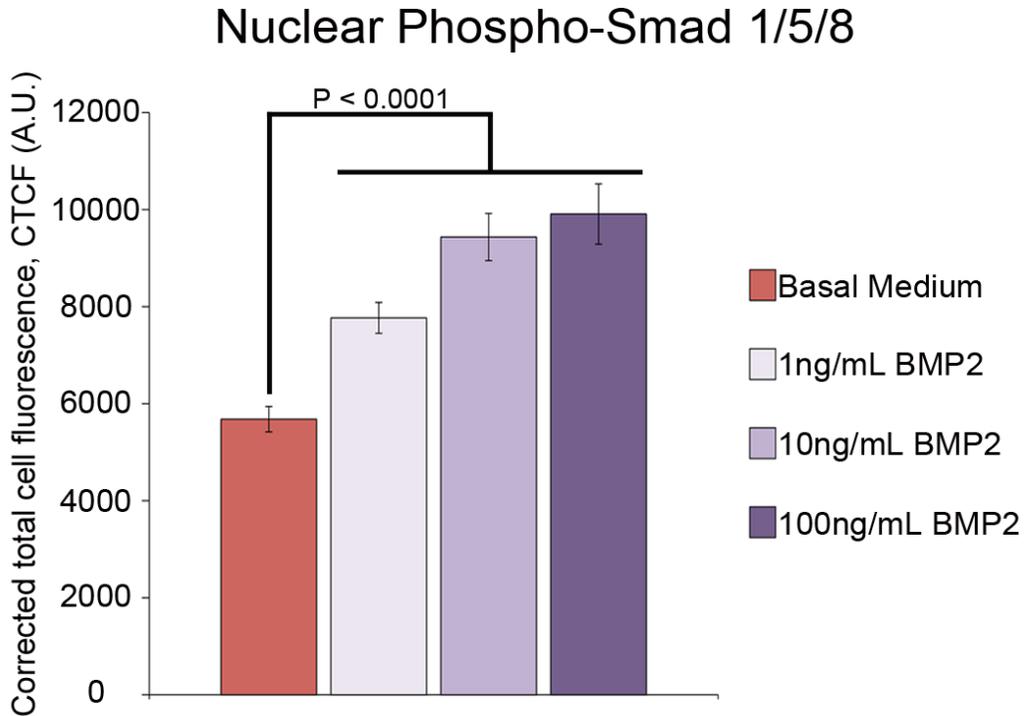
Pre-conditioning DRG led to a higher proliferation rate in OSC at day 3, 5, and 7 when compared to OSC cultured in basal medium (Figure 11A). While the proliferation rate was higher at all three time points, it was at its highest and most significant at day five, where the LI was ~15% (Figure 11A).

The decrease in LI in basal medium cultures likely was not a consequence of cell death since the percentage of TUNEL-positive nuclei was not significantly different between the different culture conditions or between the various time points of the experiment (Figure 9). However, it appeared that a significant number of cells withdrew from the cell cycle when cultured in basal medium (Figure 11A), which could account in part for the decrease in the LI. The growth fraction (1.5 cell cycles or 80 hours of continuous EdU labeling) of blastema cells cultured in basal medium was significantly lower (about 50% decrease) than that of cells cultured in medium with 5% FBS or co-cultured with pre-conditioned DRG in basal medium (both about 35%) (Figure 11B). Although the growth fraction (about 18%) decreased in the basal medium cultures without FBS or co-cultured with DRG, it was considerably higher and more consistent than what has been reported for monolayer cultures of blastema cells (growth fraction of 0.1% - 9% after 96 hours of labeling (Kumar et al., 2007).

#### *Organotypic slice cultured blastema cells respond to human BMP2*

A major advantage to OSC is that the cells remain viable when cultured in minimal basal medium, and it therefore is possible to test the effects of activating specific signaling pathways. Our previous analyses of the transcriptional response of cultured DRG identified BMP2 as a signaling molecule whose expression was specifically upregulated in response to co-culture with a blastema explant (Athippozhy et al., 2014). We therefore hypothesized that a regenerating nerve produces BMP2 in response to interacting with blastema mesenchymal cells, and that BMP2 in turn regulates the behavior of the blastema cells.

To test this hypothesis we added recombinant human BMP2 to the basal medium of OSC blastema cells at concentrations that span our best estimate for the *in vivo* concentration of BMP2 (Herrera and Inman, 2009; Honda et al., 2013; Mayer et al., 1996; Wang et al., 2011; Wong et al., 2003). We first determined that the axolotl blastema cells responded to the addition of non-axolotl BMP2 by quantifying the changes in the level of phospho-Smad 1/5/8 (p-Smad 1/5/8) immunofluorescence within the nucleus of OSC blastema cells (Figure 12). Treatment with BMP2 at all three concentrations tested (1-100 ng/ml) induced a significant increase (nearly double at 100 ng/ml) in the amount of nuclear-localized p-Smad 1/5/8 relative to basal medium, indicating that human BMP2 activated the canonical BMP signaling pathway in axolotl blastema cells.

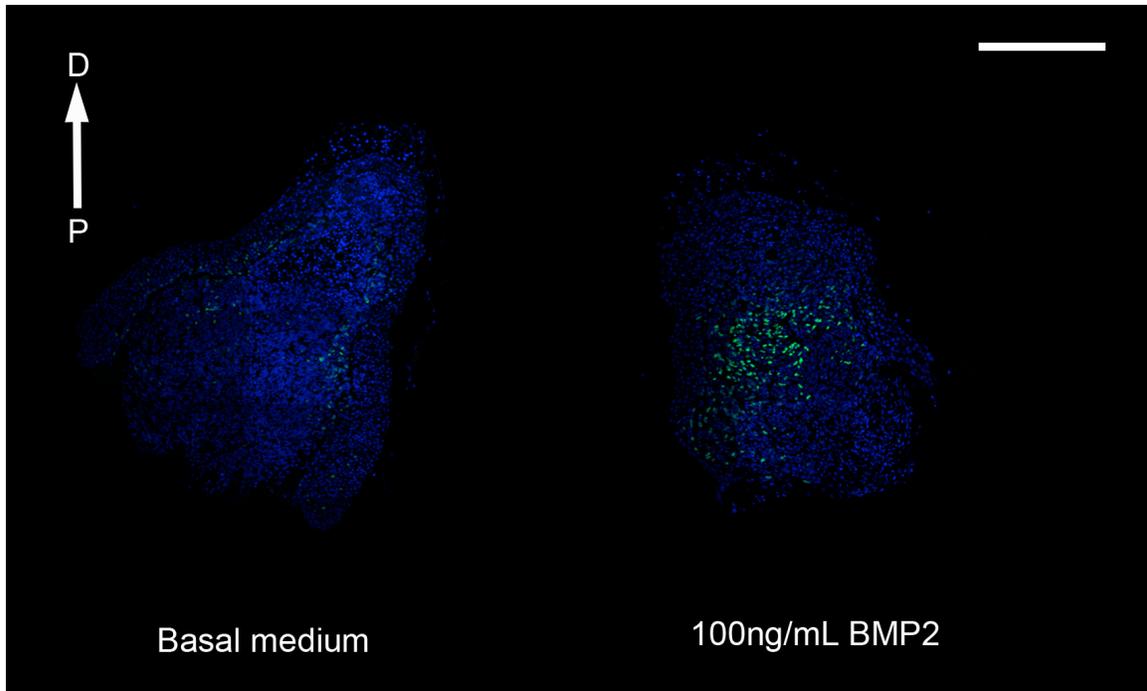


**Figure 12: Nuclear P-Smad 1/5/8 fluorescence in response to BMP2.** Nuclear Phospho-Smad 1/5/8 staining in blastema mesenchymal cells in blastema slices cultured in the presence or absence of exogenous human BMP2 in amounts as indicated. The corrected nuclear fluorescence was calculated in order to normalize the intensity of nuclear staining for variation in the area of the nuclei being analyzed as well as for the background fluorescence (Gavet and Pines, 2010). Error bars represent S.E.M., and P-values were determined by t-test with 2 tails assuming unequal variances.

OSC blastema mesenchymal cells responded to increased BMP2 signaling by increasing expression of Prrx-1 to the level observed in response to 5% FBS (Figure 10).

In addition, the rate of proliferation increased significantly (double that for cultures in basal medium) at all three concentrations of BMP2 that were tested (Figure 13, 14A). The average labeling indices for all three concentrations were comparable to each other and to *in vivo* rates of proliferation (Goldhammer et al., 1992; Maden, 1978;

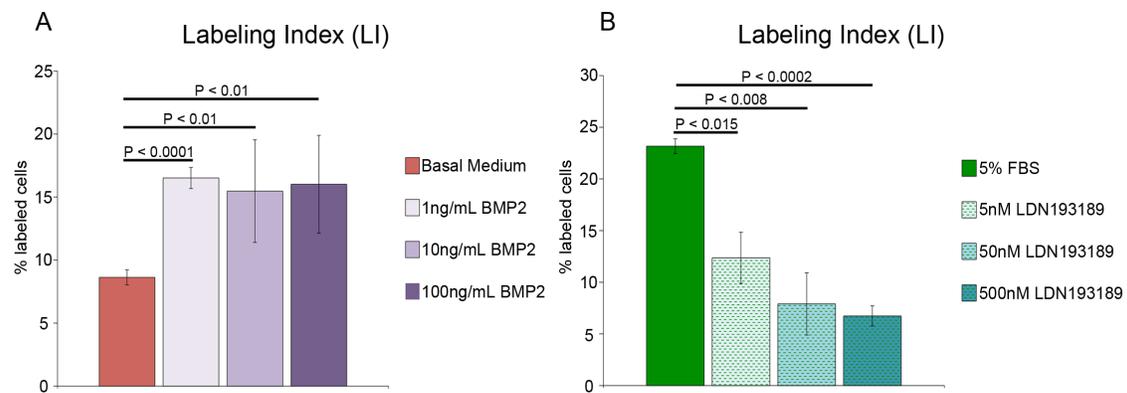
Tomlinson et al., 1984; Wallace, 1981). Since the mean LI did not change in response to increasing dose, and the variability (standard error) was considerable smaller at the lowest dose tested, we concluded that 1 ng/ml BMP2 was an appropriate dose for experiments with OSC axolotl blastema cells.



**Figure 13: Proliferation in response to BMP2.** Immunofluorescence showing EdU labeling of blastema slices originating from the same blastema and cultured in either basal medium or 100ng/mL BMP2. EdU positive proliferating cells are green, nuclei are stained with DAPI and are blue. The proximal (P) to distal (D) orientation of the sections is indicated. Scale bars = 1mm.

To determine if the BMP2 pathway was indeed involved with proliferation in OSC, we used the BMP2 pathway inhibitor LDN193189 in order to abrogate the effect that 5%FBS has on the proliferation rates of OSC. LDN193189 is a small molecule derivative of Dorsomorphin that prevents the BMP induced Smad1/5/8 phosphorylation

by binding to the ATP binding site in the kinase domain of Type I BMP receptors (Boergemann et al., 2010; Wrighton et al., 2009; Yu et al., 2008). We found that incubation of OSC in medium containing 5% FBS along with LDN193189 at doses of 5, 50 and 500nM all had a significant effect on the rate of proliferation in slices (Figure 14B). At LDN193189 doses of 50nM and 500nM, the LI was reduced to levels found in OSC cultured in basal medium (Figure 14B).



**Figure 14 Labeling index in response to exogenous BMP2 and LDN193189.** A) Labeling index of blastema mesenchymal cells in blastema slices over a period of five days in culture in the presence or absence of exogenous human BMP2 in amounts as indicated. N = 4. B) Labeling index of blastema mesenchymal cells in blastema slices over a period of 5 days cultured in 5%FBS and in the presence or absence of LDN193189 in amounts as indicated. N = 3. Error bars in both A and B represent S.E.M. and P-values were determined by t-test with 2 tails assuming unequal variances.

## DISCUSSION

*Maintenance of in vivo blastema cell behavior is dependent on maintenance of cell-cell and cell-matrix interactions*

Amphibian cells, including those derived from salamanders, have been used for *in vitro* experimentation since the advent of modern cell culture (Holtfreter, 1929). Early experiments using salamander tissues typically employed the technique of whole explant cultures, either using the developing limb bud or regenerating limb blastema (Carlone and Foret, 1979; Conn et al., 1979; Dalton, 1950; Globus and Liversage, 1975; Mescher et al., 1997; Oudkhir et al., 1986; Wilde, 1950). While these experiments paved the way for the study of regeneration *in vitro*, the use of explant cultures has its drawbacks; namely, increased cell death, lack of reproducibility owing to the variability of sample source and preparation, and lack of access to the interior mesenchymal tissues for experimental manipulation when blastemas are cultured with the epithelium present (Lossi et al., 2009). In addition, normal rates of proliferation are not maintained. For example, medium bud blastemas from newts were reported to have a mitotic index of 1.2% (number of cells in the M phase of the cell cycle relative to the total number of cells) that decreased to 0.2% when the blastemas were placed in explant culture (Carlone and Foret, 1979).

The technique of dissociation, culture and passage of individual cells is an alternative approach to *in vitro* studies that avoids the drawbacks of explant cultures. Although blastemas can be dissociated and blastema cells can be cultured individually, the cells quickly lose the behavior of blastema cells *in vivo*, most notably they have a dramatically lower rate of proliferation. Data for the labeling index (number of cells in the S phase of the cell cycle relative to the total number of cells) of blastema cells *in vitro* are comparable to uninjured skin (2%) in contrast to that of blastema cells *in vivo* (20%-40%) (Ferretti and Brockes, 1988; Goldhammer et al., 1992; Kumar and Godwin, 2010;

Kumar et al., 2007; Maden, 1978; Tomlinson et al., 1984; Wallace, 1981). It appears that this decrease in proliferation is in part a consequence of cultured blastema cells exiting the cell cycle when cultured. For example, the percentage of blastema cells that incorporated BrdU after 96 hours of continuous labeling (growth fraction) was highly variable (0.1% to 9%, with a median of 1%) (Kumar et al., 2007) in contrast to a growth fraction of 40% to 80% (3H-thymidine injection every 12 hours for two to four days) for blastema cells *in vivo* (Goldhammer and Tassava, 1987). With OSC (this study), the growth fraction (80 hours labeling which corresponds to approximately 1.5 cell cycles) was 35% after five days in culture (Figure 11B) and as high as 63% after 10 days in culture (N = 3 samples). Consistent with maintaining a growth fraction comparable to blastema cells *in vivo*, the labeling index of OSC blastema cells cultured with FBS also was comparable to what has been reported *in vivo* (Figure 11A).

We hypothesize that normal blastema cell behavior is dependent on maintaining normal cell-cell and cell-matrix interactions. OSC allows for cell-cell and cell-matrix interactions to occur, as well as providing conditions for all of the cells to be uniformly exposed to the cell culture media and experimental supplements (Gähwiler et al., 1997; Lossi et al., 2009). Consistent with this hypothesis are the findings that blastema cells remain viable with only a low amount of cell death, that they continue to express a blastema marker (Prrx-1), that they continue to proliferate at *in vivo* levels, that their rate of proliferation is responsive to the presence of nerve signals provided by DRG, and that they can respond to changes in the culture environment (addition of FBS or BMP2).

There are several likely reasons why OSC is an appropriate model for studying the biology of blastema cells. First, a regenerating limb blastema is comprised of a

heterogeneous population of progenitor cells (Kragl et al., 2009; Muneoka et al., 1986), which has been noted previously as to why culture techniques requiring dissociation are unadvisable (Conn et al., 1979). In addition, blastemas are not only heterogeneous in terms of cell types of different lineages, but also in terms of the state of developmental plasticity of blastema cells. The cells in the apical region of a medium-late bud blastema are undifferentiated and developmentally plastic, in contrast to those at more basal regions that are beginning to redifferentiate and have stabilized their positional information (McCusker et al., 2014; McCusker and Gardiner, 2013). The interaction of blastema cells with different positional information controls growth and pattern formation during regeneration (Bryant and Iten, 1976; Endo et al., 2004; French et al., 1976), and thus the spatial relationship of blastema cells would be expected to be important in terms of regulating their behaviors. Dissociation of a blastema into single cells disrupts the spatial organization of the blastema; whereas, OSC maintains the normal positional interactions. The stability of positional information is maintained for about a week in posterior blastema cells cultured as a micromass in a fibrin clot and grafted into the anterior of a host blastema *in vivo* (Groell et al., 1993). Testing the hypothesis that the spatial organization of positional information also is maintained in OSC is a goal of future experiments.

*Blastema cell gene expression and proliferation are responsive to nerve signals and to BMP2*

A distinct advantage to organotypic slice cultures is that cultured blastema cells remain viable without the addition of FBS. Amphibian tissues are well suited for organ culture

(Monnickendam and Balls, 1973; Monnickendam et al., 1970), and early experiments utilizing salamander tissues reported proliferation and the long-term survival of cells in basic salt solutions (Conn et al., 1979; Dalton, 1950; Wilde, 1950). OSC blastema cells also survived and proliferated when cultured in basal medium with FBS. The mitogenic activity of FBS led to its use in early studies to identify mitogenic factors, and subsequently to general use in cell culture in order to expand cell populations for passage. It has been noted that FBS evokes a specific physiological response in cultured cells that is reminiscent of wound healing (Iyer et al., 1999). Cells such as fibroblasts, which are the progenitors of many of the cells found in the blastema (Kragl et al., 2009; Muneoka et al., 1986), would encounter the factors found in FBS in the early wound environment created by tissue injury. Thus OSC blastema cells respond to FBS and proliferate at *in vivo* levels (Figure 11A), even when a nerve is not present. In spite of the response of OSC cultured blastema cells to added FBS, the utility of OSC for studying the behavior of blastema cells is that addition of FBS is not necessary; therefore, it is possible to study the mechanisms by which specific signaling pathways are involved in the regenerative response.

One pathway that likely is involved in the regulation of blastema cell behavior is BMP signaling, which previously has been implicated in controlling regeneration in a number of model systems, including mammals (Athippozhy et al., 2014; Guimond et al., 2010; Makanae et al., 2013; Reddien et al., 2007; Yu et al., 2010). The OSC model allowed us to determine that axolotl blastema cells responded to a specific, optimal dose of human BMP2 (1 ng/ml) by increasing the expression of a blastema marker gene (*Prrx-1*) and increasing the rate of proliferation. Furthermore, incubation with the BMP

pathway inhibitor, LDN193189, decreased the rate of proliferation similar to one seen in OSC cultured in basal medium. In addition, we have identified a similar response (changes in gene expression and proliferation) to signaling from co-cultured nerves, which has been associated previously with FGF as well as BMP signaling (Athipposhy et al., 2014; Makanae et al., 2013; Mullen et al., 1996a). The report that an ectopic blastema can be induced by implanting beads soaked in a cocktail of FGF2, FGF8 and GDF5/BMP7 in the absence of a deviated nerve provides additional evidence that blastema formation can be induced by the activation of well characterized and highly conserved signaling pathways (e.g. FGF and BMP)(Makanae et al., 2013). The OSC model provides the opportunity to discover the mechanism for the spatial and temporal co-regulation of these pathways in order to induce and enhance a regenerative response.

## **FINANCIAL DISCLOSURE**

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**CHAPTER 4: Characterization of *in vitro* transcriptional responses of dorsal root ganglia cultured in the presence and absence of blastema cells from regenerating salamander limbs**

This chapter is a reprint of the paper “Characterization of *in vitro* transcriptional responses of dorsal root ganglia cultured in the presence and absence of blastema cells from regenerating salamander limb,” published July 14<sup>th</sup>, 2014 in the journal REGENERATION. This paper was co-authored by Antony Athipposhy, James R. Monaghan, David Gardiner, and S. Randal Voss and is reproduced with permission from John Wiley & Sons. My contribution to this work was the designing of the experiment and performing animal surgery, cell culture, immunohistochemistry and RNA isolation. Chapter 4 shall discuss work I performed while creating the OSC system discussed in Chapter 3. While there have been a multitude of neurotrophic candidates put forth, I decided to perform microarray experiment to identify factors that would be free of historical bias. The remainder of this chapter details this work.

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## **ABSTRACT**

During salamander limb regeneration, nerves provide signals that induce the formation of a mass of proliferative cells called the blastema. To better understand these signals, we developed a blastema–dorsal root ganglia (DRG) co-culture model system to test the hypothesis that nerves differentially express genes in response to cues provided by the blastema. DRG with proximal and distal nerve trunks were isolated from axolotls (*Ambystoma mexicanum*), cultured for 5 days, and subjected to microarray analysis. Relative to freshly isolated DRG, 1541 Affymetrix probe sets were identified as differentially expressed and many of the predicted genes are known to function in injury and neurodevelopmental responses observed for mammalian DRG. We then cultured 5-day DRG explants for an additional 5 days with or without co-cultured blastema cells. On day 10, we identified 27 genes whose expression in cultured DRG was significantly affected by the presence or absence of blastema cells. Overall, our study established a DRG–blastema *in vitro* culture system and identified candidate genes for future investigations of axon regrowth, nerve–blastema signaling, and neural regulation of limb regeneration.

## **INTRODUCTION**

Salamanders and humans have structurally homologous tetrapod limbs, but only in salamanders is this structure capable of regeneration. When salamanders lose parts of their limbs from injuries, cells adjacent to the injury site are recruited to form a mass of proliferative mesenchymal cells called the blastema. The blastema grows and eventually becomes a self-organizing structure that provides progenitor cells for regrowth and

patterning of the missing limb (McCusker and Gardiner, 2013). One of the earliest discoveries of an essential regulator of the process of blastema formation and subsequent limb regeneration was that severing the nerve supply either “retarded or entirely prevented” limb regeneration (Todd, 1823). More than a century and a half later, researchers are still looking for the elusive factor(s) that the nerve provides in order to exert its effect on regeneration. Over the years many putative factors have been investigated, and now with the availability of new techniques and genomic resources it is possible to identify specific gene regulatory networks associated with this phenomenon.

Shortly after limb amputation, nerve fibers regenerate and grow distally into the injured limb tissues where they interact with the newly healed wound epithelium (Satoh et al., 2008b; Singer, 1949; Thornton and Thornton, 1970). Singer (1978) proposed that these regenerating nerves supply trophic factors (referred to in the regeneration literature as “neurotrophic factor(s)”) independent of electrical transmission that support blastema cell proliferation during the early and mid phases of regeneration. Several molecules have been proposed as the putative nerve-derived trophic factors, including fibroblast growth factors (FGF) (Mullen et al., 1996a; Satoh et al., 2008b), substance P (Globus et al., 1991), neuregulin (Wang et al., 2000), and transferrin (Mescher et al., 1997). Nerve-derived signals in theory could stimulate blastema cell proliferation either directly or indirectly by signaling to non-neuronal cells of the nerve sheath to release trophic factors. For example, anterior gradient protein 2 (*agr2*) is expressed in Schwann cells but not in neurons, and can rescue regeneration of partially innervated newt limbs (Kumar et al., 2007). Similarly, the early wound epithelium (WE) and later apical epithelial cap (AEC) appear to be direct targets of nerve signaling (Satoh et al., 2008a; 2012) and they could

signal secondarily to the underlying mesenchymal cells; for example, FGF8 produced in the WE/AEC would stimulate blastema cell proliferation as it does in developing limb buds (Han et al., 2001b).

Several properties of the neurotrophic factor(s) have been identified over many decades of research. In the case of sensory neurons, the factor is thought to be produced in the cell bodies located in the dorsal root ganglia (DRG), transported distally along nerve fibers and released distally at sites where the sensory fibers interact with basal keratinocytes of the WE (Kiffmeyer et al., 1991; Satoh et al., 2008b; 2012; Scadding, 1988; Wallace, 1972). The factor(s) is produced by sympathetic, motor, and sensory nerves innervating the limb (Singer, 1952; 1974), as well as by the spinal cord (Boilly and Albert, 1988b), brain (Singer et al., 1976), and eye (Pietsch and Webber, 1965). Finally, a critical threshold of nerve fibers is necessary for the limb to regenerate, such that if the number of nerve fibers innervating the limb is greater than this threshold, the limb will regenerate. Conversely, if the number of fibers is experimentally reduced below this threshold, the limb fails to regenerate (Singer, 1952).

The properties above suggest that the neural signaling factor is transcribed by sensory neurons whose cell bodies are located in DRG (e.g., Satoh et al. 2008). How these cells respond to injury resulting from amputation as well as their subsequent interactions with cells of the regenerating limb blastema is complex. Initially, a nerve responds to and recovers from axotomy, a process that occurs during the first few days following amputation (Singer, 1952). During this phase of regeneration, it is likely that genes encoding proteins necessary for cell survival, apoptosis, and neural development are transcribed. As axons regrow and re-innervate tissues, it is also likely that the

regenerating nerves respond to, as well as signal to, the target tissues in the blastema. Consistent with this model of bi-directional signaling between nerves and blastema cells (both mesenchymal cells and keratinocytes of the WE/AEC) is the observation that, when DRG or spinal cord explants are co-cultured with blastemas, there is an enhanced outgrowth of axons that orient towards the blastema (Bauduin et al., 2000; Dmetrichuk et al., 2005; Tonge and Leclere, 2000). This response to the presence of a blastema *in vitro* suggests that a similar signaling mechanism operates *in vivo* to modulate transcription and translation of proteins that enhance and direct outgrowth of the regenerating nerve fibers. Unraveling the complexity of reciprocal nerve–blastema signaling is key to identifying the neurotrophic requirement for blastema formation and growth. To achieve this goal, we are developing experimental *in vitro* models that will allow us to identify the temporal sequence of regeneration-specific transcriptional responses from both nerves and blastema cells (See Chapter 3).

In this study we focused on the response of regenerating DRG neurons and associated cells to signaling from the blastema. To do this, we modified the *in vitro* DRG–blastema co-culture model of Tonge and LeClere (2000) to test the hypothesis that signaling from blastema cells regulates gene transcription by cells within the regenerating nerve. We isolated DRG along with their proximal and distal nerve trunks from axolotls (*Ambystoma mexicanum*), and cultured them in the presence or absence of explanted medium bud blastemas. Since the nerve is composed of many different cell types including Schwann cells, fibroblasts, and endothelial cells in addition to the neurons, we have identified changes in gene expression in the nerve as a whole. This reflects the situation *in vivo* in which all these cell types could potentially interact with and respond

to signals originating from the blastema. We discovered that more than 1500 genes change expression in DRG and the nerve trunk during the first 5 days of culture, during which time the explanted DRG heal and begin to regenerate. After an additional 5 days of culture, a much smaller group of 27 genes were expressed differentially by DRG in response to the presence of co-cultured blastema cells.

## **MATERIAL AND METHODS**

### *Animal care and collection of DRG and blastema*

The handling and surgical manipulation of axolotls was carried out according to University of California, Irvine (UCI), Animal Care and Use guidelines. Axolotls (*Ambystoma mexicanum*) measuring 15–20 cm from snout to tail tip were spawned at UCI or the Ambystoma Genetic Stock Center at the University of Kentucky. They were housed on a 12h light/dark cycle and fed *ad libitum*. DRG were collected post-euthanasia by surgically removing the spinal nerves that innervated either the forelimb (spinal nerves 3, 4, and 5) or the hind limb (spinal nerves 15, 16, and 17). The nerves were severed where the dorsal and ventral nerve roots exit the spinal cord, and again 3 mm distal to the spinal ganglion.

### *DRG and limb blastema co-culture*

DRG were cultured individually in 12-well Nunc nunclon plates with 60% L-15, 5% fetal bovine serum, 1% Insulin-Transferrin-Selenium, and gentamicin/amphotericin B (Sigma). Each DRG was attached to the bottom of the culture well by embedding it in a small drop (about 3  $\mu$ L) of growth-factor-reduced BD matrigel (BD Biosciences). Only

DRG explants that exhibited neurite outgrowth within the first 24 h of culture were used for subsequent experiments. DRG explants were cultured for 5 days and either collected for RNA extraction or assigned to two experimental treatments: (1) nerve–blastema co-culture in which a medium bud blastema was placed directly on top of the regenerating region of the nerve, or (2) nerve culture without a co-cultured blastema.

For the samples in which the DRG were co-cultured with blastemas, we collected blastemas at the medium bud stage of regeneration from limbs that had been amputated several days earlier. The apical epithelium (AEC) was not removed prior to placing the blastemas adjacent to the cultured DRG such that the proximal cut end of the blastema covered newly sprouted neurites that were regenerating from the distal cut end of the DRG (Fig. 9C and D). The DRG and blastema were secured together with a small drop of growth-factor-reduced matrigel. For the DRG cultures without a blastema, procedures were the same as for the cultures with an added blastema, including removal of the medium, touching the surrounding matrigel with forceps, adding a drop of matrigel as described above, and then refilling the well with culture medium. Explants were cultured for an additional 5 days resulting in a total of 10 days *in vitro* after excision of the DRG from the donor animal. At 10 days of culture, the co-cultured DRG were separated from the associated blastemas under a dissecting microscope to prevent blastema cell contamination and were collected for RNA extraction. A total of 15 DRG were pooled for each biological replicate and three replicates were analyzed at both the day 5 and day 10 time points. Three replicate control samples (day 0) were prepared by pooling non-cultured DRG (12 per replicate) collected directly from euthanized salamanders.

### *RNA isolation and microarray analysis*

DRG were pooled within biological replicates prior to RNA isolation. RNA was isolated using Trizol (Invitrogen) in conjunction with the Nucleospin RNA XS Kit (Macherey-Nagel) following the manufacturer's protocol. RNA quality was assessed using an ND-1000 spectrophotometer (Nanodrop; Wilmington, DE) and a Bioanalyzer 2100 (Agilent; Santa Clara, CA). The 12 RNA samples that were obtained from the day 0, day 5, and day 10 treatments were analyzed using the Amby\_002 GeneChip (Huggins et al. 2012). The University of Kentucky Microarray Core Facility generated biotin labeled cRNA targets for all samples and hybridized each to independent GeneChips. The GeneChips were scanned and processed using the RMA algorithm in Affymetrix's Expression Console software (Affymetrix, Santa Clara, CA).

### *Statistical analysis of gene expression*

Prior to statistical analyses, probe sets with low and variable expression values were filtered. Probe sets were filtered if they registered expression values below the maximum bottom quartile value (4.27 across all arrays) for one or more replicate arrays within a treatment. To identify differentially expressed genes, data were analyzed using one-way ANOVA as implemented in JMP Genomics version 5.0 (SAS Institute, Cary, NC) and a significance cutoff of FDR = 0.05 (Benjamini and Hochberg, 1995). Follow up t-tests were used to detect significantly different changes in gene expression after the initial 5-day period of DRG culture (day 0 vs. day 5) and between DRG cultured with and without blastemas at day 10, using a P-value cutoff of 0.0125.

We used Panther (Mi and Thomas, 2009; Thomas et al., 2003) to identify gene ontology terms that were statistically enriched in our lists of differentially expressed genes. All of the genes on the *Ambystoma* GeneChips with established orthologies to human protein coding sequences were used to generate expected values. We retained all over-represented terms that were supported by >2 counts at a Bonferroni-adjusted  $\alpha$  of 0.05.

### *Immunohistochemistry*

Tissues were fixed for 3 h at room temperature in 4% paraformaldehyde and were then dehydrated in graded alcohol followed by xylene, embedded in paraplast, and sectioned at 5  $\mu$ m. To perform immunohistochemistry sections were de-paraffinized and rehydrated in Tris Buffered Saline with Tween (TBST). Sections were then incubated with anti-acetylated  $\alpha$ -tubulin (Abcam, diluted 1:250) and anti-RT97 (DSHB, diluted 1:250) overnight at 4°C. Sections were washed with TBST and incubated with anti-mouse 594 (Abcam, diluted 1:250). Following secondary antibody treatment sections were washed with TBST and mounted with ProLong Gold Antifade with DAPI. To label proliferating cells, 80  $\mu$ mol/L of 5-ethynyl-2'-deoxyuridine (EdU) was added to culture media for 5 h. Following EdU labeling, tissues were processed as described above. Proliferating cells were then visualized using the Click-It-EdU Alexa-Fluor 488 kit (Life Technologies).

## **RESULTS**

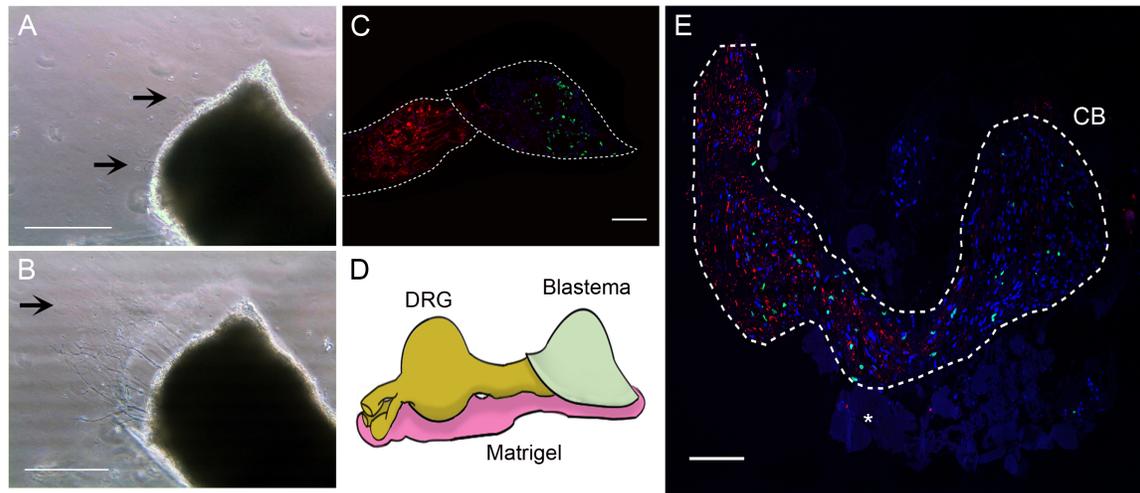
Most DRG explants survived after surgical excision and began to regenerate axons after about 24 h in culture (Figure 15A). Initially, a few growing neurites were observed to be

extending from the distal cut end of the nerve, and over the next several days of culture, increasing numbers of neurites were observed (Figure 15B). Explants that did not exhibit initial neurite outgrowth at 24 h or exhibited continued outgrowth at 5 days were discarded and not used in the experiments.

In addition to regenerating neurites, the morphology of DRG (that were used experiments) appeared normal in histological sections (Figure 15C and E). 4',6-Diamidino-2-phenylindole (DAPI) stained nuclei within both the DRG and the distal nerve trunk appeared normal with little evidence of pyknosis (Figure 15E). Cells within both the DRG and the nerve trunk were proliferating as evidenced by the presence of 5-ethynyl-2'-deoxyuridine (EdU) positive nuclei (green). Proliferation of cells within both the DRG and the nerve trunk in response to peripheral nerve injury *in vivo* has been reported previously (Clemence et al., 1989; Zochodne, 2012). Neurites were present in the nerve trunk in DRG explants that were cultured either alone (Figure 15E) or in association with blastema explants (Figure 15C) as evidenced by the presence of phosphorylated neurofilaments (stained with the RT97 antibody, red). The appearance of the RT97-positive neurofilaments within cultured axolotl DRG was similar to what has been reported previously for injured peripheral nerves *in vivo* (Bergman et al., 1999; Lawson et al., 1984). Thus, axolotl DRG appear to be viable and morphologically comparable to DRG *in vivo*, as has been reported previously (Tonge and Leclere, 2000).

After 5 days of culture *in vitro*, DRG that appeared healthy with extensive neurite outgrowth were selected for co-culture with explanted medium bud stage blastemas (Figure 15D). As with the explanted and cultured DRG, the blastema cells appeared healthy. Most nuclei appeared normal when observed in DAPI stained tissue sections,

and blastema cells continued to proliferate as evidenced by the incorporation of EdU (Figure 15C). Studies to further characterize the response of explanted blastema cells to culture conditions and to signals from DRG are in progress (See Chapter 3).



**Figure 15: Axolotl dorsal root ganglia (DRG) *in vitro*.** (A) A bright field image of a DRG *in vitro* 24 h after being explanted. Regenerating neural projections (arrows) are observed at the transected end of the nerve trunk. (B) Image of the same DRG after 4 days of culture *in vitro*. Neurite outgrowth (arrow) from the cut end of the nerve trunk was robust. (C) Fluorescent image of a sagittal section of a DRG–blastema co-culture. The blastema was placed on the distal cut end of the DRG with the proximal region of the blastema coming into contact with the regenerating neurites. Nerve fibers were visualized by immunostaining of acetylated  $\alpha$ -tubulin (red), and cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue). Proliferating cells were detected with 5-ethynyl-2'-deoxyuridine (EdU) (green). (D) Diagram of DRG–blastema co-culture set-up. The DRG and blastema were adhered to the bottom of the cell culture insert with a drop of growth factor reduced matrigel. (E) Immunofluorescence image of a longitudinal section of a DRG *in vitro*. The DRG was adhered to the cell culture with matrigel. Phosphorylated neurofilaments were visualized by immunostaining with RT97 (red). Cell nuclei were stained with DAPI (blue). Proliferating cells were detected with EdU (green). Scale bars 0.5 mm.

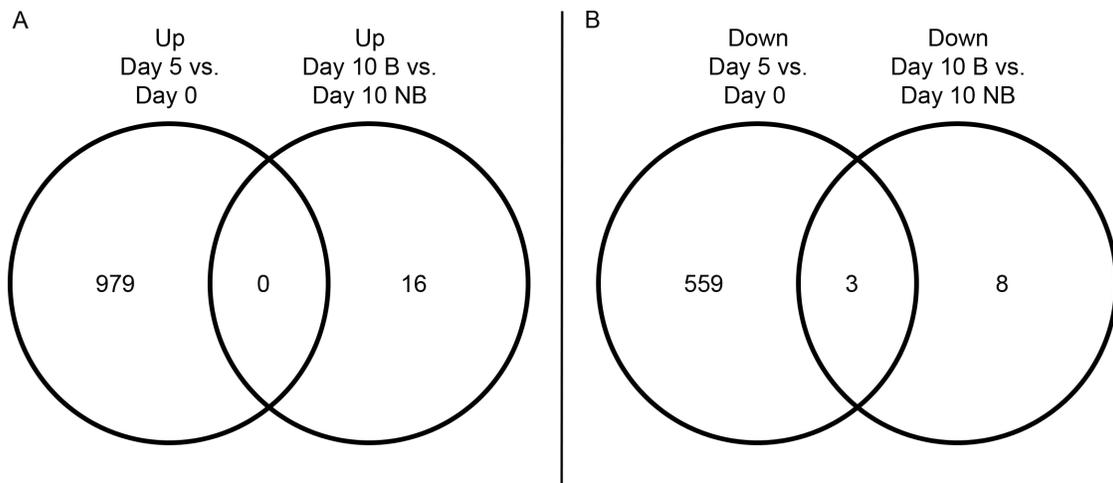
### *Differentially expressed genes identified from in vitro cultured DRG*

A total of 1541 probe sets were identified as differentially expressed between DRG at the time of removal from the donor animal (day 0) and DRG that had been cultured *in vitro* for 5 days (Figure 16A-B. A supplementary table listing 1541 probe sets that were identified as differentially expressed between dorsal root ganglia sampled on day 0 and day 5, can be found by clicking [here](#) (the full URL can be found in the appendix).

Columns indicate if a gene was significantly upregulated or downregulated for a given statistical contrast. A “1” indicates that the probe set was differentially expressed and the corresponding change was detected, while a “0” indicates there was no significant change.) Of this total, 1498 probe sets showed significant sequence identity to human RefSeq proteins ( $E \leq 1 \times 10^{-7}$ ) and we considered these to be salamander–human orthologous genes in the enrichment analyses described below. We note that >60 of these genes were identified by two to four independent probe sets. Thus, overall, statistical significance was validated for approximately 5% of the differentially expressed genes.

The majority of these probe sets (N = 979) presented higher transcript abundances at day 5 than day 0, a pattern indicating that expression of the corresponding genes was upregulated in response to injury (Figure 16A and [supplementary table](#)). For many of the genes associated with these probe sets, the magnitude of the change was dramatic, with 135 showing more than a 5-fold increase in expression ([supplementary table](#)). Of these upregulated genes, many are known to function in the regulation of inflammatory and innate immune responses (e.g., *Lep*, *IL-8*, *IL-1b*, *Tgfb1*, *Irf1*, *Thbs1*, *Mdm2*, *Hmox1*). Similarly, genes associated with cellular growth and developmental regulation

(e.g., *Bdnf*, *Ctgfntf3*, *Gadd45b*, *Gadd45g*) and axon development and regrowth (e.g., *Bmp2*, *Smad1*, *Creb1*, *Bcl2l1*, *Ankrd1*, *Npy*, *Robo1*) were upregulated. A number of over-represented biological process and Panther protein class ontology terms were identified based on genes that presented higher transcript abundances at day 5 than day 0 (Table 1). These included immune system processes, RNA and nucleic acid metabolic processes, as well as the MAPKKK signaling cascade that were identified as significantly enriched biological processes. Also, several RNA protein class gene ontologies were identified as significant, including mRNA processing factor and RNA/ribonucleoprotein binding protein (Table 1).



**Figure 16: Venn diagrams showing upregulated and down-regulated genes.** A) Venn diagram showing the number of upregulated, differentially expressed genes identified between time and treatment contrasts. Substantially more genes were identified as differentially expressed between day 0 and day 5 cultured dorsal root ganglia (DRG) than between day 10 DRG with blastema (10 B) and day 10 DRG without blastema (10 NB). B) Venn diagram showing the number of down-regulated, differentially expressed genes identified between time and treatment contrasts. Substantially more genes were identified as

differentially expressed between day 0 and day 5 cultured dorsal root ganglia (DRG) than between day 10 DRG with blastema (10 B) and day 10 DRG without blastema (10 NB).

In terms of genes that were down-regulated (N = 562), only muscle contraction was identified as a statistically enriched biological process (Table 1). Down-regulated genes associated with cytoskeletal and extracellular matrix protein classes were also identified as statistically enriched. As was observed for upregulated genes, some of the down-regulated genes are well-established regulators of cellular growth and development (Gas6 and Kit). In addition, Schwann cell biomarkers (Pmp22, Mbp, Plp1, Gfap) showed lower transcript abundances at day 5 relative to day 0. The results indicate that, after DRG are excised and cultured for 5 days, transcript abundances change for >1500 genes. The predicted functions for these differentially expressed genes are consistent with the conclusion that cultured DRG mount robust injury and neurodevelopmental responses, including changes in cytoskeletal structure, the extracellular matrix, and RNA processes associated with transcriptional and post-transcriptional regulation.

**Table 1: List of biological process and ontology terms identified as differentially expressed.** List of statistically significant ( $P < 0.05$ ) over-represented biological process (BP) and Panther protein class (PC) ontology terms for genes identified as differentially expressed in axolotl dorsal root ganglia between day 0 and day 5 of culture *in vitro*

<b>Upregulated at day 5</b>	<b>Observed</b>	<b>Expected</b>
Nucleobase, nucleoside, nucleotide and nucleic acid	193	118
metabolic process: BP		
RNA metabolic process: BP	61	22
mRNA processing	42	14

Nuclear mRNA splicing, via spliceosome: BP	34	10
Metabolic process: BP	343	283
Primary metabolic process: BP	326	268
MAPKKK cascade: BP	24	9
Response to stimulus: BP	52	30
Immune system process: BP	74	48
RNA binding protein: PC	85	40
Nucleic acid binding: PC	151	92
Ribonucleoprotein: PC	16	4
mRNA processing: PC	21	8
<b>Downregulated at day 5</b>		
Muscle contraction: BP	16	5
Cytoskeletal protein: PC	36	15
Extracellular matrix structural protein: PC	8	1
Actin family cytoskeletal protein: PC	20	8

### *Effect of blastema cell co-culture on DRG transcription*

We compared transcript abundance estimates between the two day 10 treatments (DRG co-cultured with blastema cells or DRG cultured alone from day 5 to day 10). A total of 27 genes were identified as differentially expressed in DRG in response to the presence of a co-cultured blastema (Table 2). Of these differentially expressed genes, 16 probe sets registered significantly higher transcript abundances in DRG–blastema co-cultures. Eleven of these have predicted gene names: Areg, Krt15, Krt17, Mall, Cryba2, c3orf54, Col22A1, Marcks, Chd3, Kazald1, and Tacc3. A total of 11 probe sets registered lower transcript abundances in DRG that were co-cultured with a blastema—Abat, Smc2,

Znf697, Gas6, Napa, Idh3g, Dnm11, Ndc80, Asap1, Esco2—and one unannotated probe (probe set ID axo25121). Of these 27 probe sets, the greatest expression difference was observed for Krt17 and axo31729-f, which were expressed 7.8- and 4.2-fold higher respectively in response to the presence of a co-cultured blastema. These results show that the presence of a blastema affected transcription of relatively few but potentially important genes that are expressed by cultured DRG. We did not observe differences in the level of expression of the blastema marker genes Prrx-1, Msx2, and Hoxa13 between DRG samples with or without co-cultured blastemas, indicating that blastema cells did not contaminate the DRG samples.

**Table 2: Probe sets registering significant fold changes in transcript abundances for dorsal root ganglia (DRG)–blastema co-cultures.** Fold change is measured as the ratio of day 10 DRG cultured with a blastema compared with day 10 DRG cultured without a blastema. Probe sets that are unannotated are displayed as the probe set ID with the prefix “axo”

<b>Upregulated at day 10</b>	<b>Fold change</b>	<b>P-value</b>
Keratin 17	7.82	2.44E-05
axo31729-f	4.21	1.25E-04
Kazal-type serine peptidase inhibitor domain 1 precursor	3.14	4.47E-03
axo31698-f	2.68	3.90E-05
Mal, T-cell differentiation protein-like	2.52	1.02E-03
Amphiregulin preproprotein	2.19	1.15E-03
Keratin 15	2.11	2.64E-03
axo31329-f	1.74	8.87E-05
Myristoylated alanine-rich protein kinase C substrate	1.44	9.77E-03
Crystallin, beta A2	1.42	1.76E-03
axo28274-f	1.39	1.85E-03
Similar to collagen, type XXII, alpha 1	1.39	8.02E-03
CPEB-associated factor Maskin	1.37	4.38E-03
Chromodomain helicase DNA binding protein 3 isoform 1	1.36	1.01E-02
axo30442-f	1.28	4.22E-03
Hypothetical protein LOC389119	1.28	1.18E-02
<b>Downregulated at day 10</b>		
N-ethylmaleimide-sensitive factor attachment protein, alpha	1.79	5.72E-03
Establishment of cohesion 1 homolog 2	1.69	1.24E-02
Kinetochore associated 2	1.64	7.51E-03

Structural maintenance of chromosomes 2	1.63	1.81E-04
Development and differentiation enhancing factor 1	1.57	3.26E-03
4-Aminobutyrate aminotransferase precursor	1.57	2.15E-03
Isocitrate dehydrogenase 3 (NAD+) gamma isoform a precursor	1.53	6.86E-03
Zinc finger protein 697	1.52	1.11E-02
axo25121-f	1.48	3.30E-03
Dynamin 1-like isoform 3	1.42	2.21E-03
Growth arrest-specific 6 isoform 1	1.31	1.17E-02

## DISCUSSION

An unresolved question in regeneration biology concerns the nature of the functional relationship between nerves and blastema cells. It is well documented that nerves are required for limb regeneration in salamanders, and consequently most regeneration studies have focused more on the signals that nerves may be providing (referred to in the regeneration literature as neurotrophic factor(s)) rather than the signals that the blastema provides to induce and guide the regeneration of the nerve (Globus et al., 1991; Mescher et al., 1997; Mullen et al., 1996b; Satoh et al., 2008a; Wang et al., 2000). In order to identify the genes involved in this reciprocal interaction, we have been working to develop a nerve (DRG) and blastema co-culture model (see Chapter 3). In this study we have used this model to test the hypothesis that blastema cells provide signals that regenerating nerves respond to. By culturing DRG we analyzed the transcriptional response not only of the neurons, but also the Schwann cells, fibroblasts, and other cells that are found in the nerve trunk that may be functionally important for the interaction between the nerve and the blastema *in vivo*. We observed that dissection and culture of DRG initiates dramatic changes in transcription after 5 days of *in vitro* culture that are consistent with the conclusion that nerves are recovering from injury and beginning to regenerate. At that point, the regenerating nerves respond to the presence of co-cultured

blastema cells and differentially express a relatively small number of genes that are targets for future functional studies. Below, we highlight several genes that code for proteins that are likely to be associated with cell growth and development, axon regrowth, and limb regeneration.

*Transcriptional analysis of limb regeneration processes using cultured salamander DRG*

Salamanders are unique because they provide an opportunity to understand how tissues can be regenerated endogenously. If a salamander's limb or tail is amputated, this regeneration program is activated and the missing structures are regenerated. However, salamander regeneration is complex from a transcriptional perspective (e.g., (Monaghan et al., 2009; 2007), involving thousands of gene expression changes. Accordingly, there is need to develop *in vitro* models that reduce the complexity of tissue regeneration and allow for an understanding of how the various tissues and cells respond to injury and pro-regenerative signaling. The nerve (DRG) and blastema co-culture model described here was utilized to test the hypothesis that there is reciprocal signaling between blastema cells and cells in the peripheral nerve that results in the differential transcription of genes required for limb regeneration.

Although there were specific changes in gene expression associated with interactions between DRG and blastemas, it is not known whether they occurred as a consequence of axonal retrograde transport of molecules from the blastema to DRG neuron cell bodies. It is possible that blastemas affected DRG transcription indirectly, for example via release of diffusible molecules such as retinoic acid (Prince and Carlone, 2003; Scadding and Maden, 1994) and not via axon transport. Related to this question is

the fact that the DRG consist of several different cell types (e.g., neurons, Schwann cells, fibroblasts, immune cells, and cells associated with the vasculature), and we do not have data indicating which specific cell types are involved in the observed transcriptional changes (e.g. *in situ* hybridization). The diversity of the gene expression responses that we observed is consistent with the conclusion that multiple cell types are involved in the response. For example, we observed a significant decrease in expression of Schwann-cell-associated transcripts (Pmp22, Mbp, Plp1) after 5 days of *in vitro* DRG culture, suggesting either transcriptional repression of myelin-associated proteins or progressive Schwann cell death. Similarly, cultured salamander DRG recapitulate transcriptional changes observed after mammalian sciatic nerve injury (Boeshore et al., 2004; Bosse et al., 2006; Kubo et al., 2002; Newton et al., 2000; Nilsson et al., 2005; Stam et al., 2007; Tanabe et al., 2003; Xiao et al., 2002), indicative of responses from immune cells (genes associated with inflammation) and from neurons (genes associated with nerve development and axonal regrowth).

#### *Candidate genes associated with axon regrowth and blastema cell proliferation*

The DRG–blastema co-culture model (*in vitro*) was designed to correspond to the time during limb regeneration when nerves have recovered from the injury of amputation and begun to function in the recruitment of proliferating blastema cells (*in vivo*). To model this phase of regeneration, blastemas were placed in contact with neurites that had sprouted from the peripheral branch after 5 days of culture. Historically, nerve–blastema signaling has been modeled as a process where axons release molecules that directly stimulate blastema cell proliferation. However, nerve signaling may be indirect, with

axons signaling wound epithelial cells, epithelial glands, or Schwann cells to secrete molecules to support blastema cell proliferation (Kumar et al., 2007; 2010; Satoh et al., 2012). We note that whether signaling occurs directly or indirectly, axon regrowth is a necessary first step in the genesis of the trophic effect required for blastema maturation, and axon regrowth after nerve injury requires transcription (Smith and Skene, 1997).

In our study, DRG culture elicited a diverse transcriptional response involving many of the same gene expression changes observed in previous studies of cultured mammalian DRG as well as in DRG with axotomized peripheral nerves (Boeshore et al., 2004; Nilsson et al., 2005; Szpara et al., 2007). The vast majority of genes that were differentially expressed in cultured salamander DRG (compared with day 0) were expressed similarly in the presence or absence of a blastema. Thus, blastema co-culture had a relatively minor effect on the overall DRG injury response program. Of the genes that were differentially expressed as a function of blastema co-culture, several are predicted to regulate cellular growth and axon regrowth. The differentially expressed gene that exhibited the largest change in expression (up-related in response to co-cultured blastemas) was Keratin 17 (Krt17), which is rapidly induced in response to injury via the Akt/mTOR signaling pathway in mammals (Kim et al., 2006). Activation of the mTOR pathway after peripheral injury of murine DRG neurons is associated with axon regrowth and target innervation (Abe et al., 2010). Since Krt17 generally is associated with epithelial cells and appendages in mammals, and not DRG, further analysis of its regulation during axolotl regeneration is needed to determine its possible function in nerve–blastema interactions.

It is generally thought that the nerve requirement for limb regeneration is a consequence of one or a few trophic factors that are synthesized and secreted by nerves as they innervate the blastema niche (blastema cells and the overlying apical epithelium). Studies over the years have identified several characteristics of these presumptive trophic factors: they are secreted peptides or proteins; they are produced by sensory, motor, and autonomic nerves; they increase in level/activity in regenerating axons and after priming/conditional lesions; and they stimulate mitosis (Boilly and Bauduin, 1988; Carlone and Foret, 1979; Choo et al., 1978; Deck, 1971; Globus and Liversage, 1975; Globus and Vethamany-Globus, 1977; Kamrin and Singer, 1959; Lebowitz and Singer, 1970; Singer, 1952; Singer et al., 1976). We observed that expression of several trophic factors and neuropeptides increased significantly in cultured DRG whether or not they were co-cultured with a blastema (Ntf3, Batf3, Npy, Bdnf, Npff, Nts, Ctgf). Kumar et al. (2010) reported that ectopic expression of Agr2 rescues limb regeneration in partially innervated newt limbs, and expression is associated with Schwann and Leydig cells in the epidermis. We did not observe significant changes in Agr2 expression; however, a potential downstream target of Agr2, Areg (Dong et al., 2011), was upregulated modestly, but not significantly (~1.5-fold,  $P = 0.03$ ), between day 0 and day 5 of culture, and then was expressed at a significantly higher level in DRG–blastema cultures compared with DRG cultured without a blastema. In mouse DRG, Areg is one of the most highly upregulated genes in response to sciatic nerve transection and application of AREG to DRG cultures induces neurite outgrowth (Nilsson et al., 2005). As a member of the epidermal growth factor family, AREG is a mitogen for Schwann cells and fibroblasts; the latter cell type gives rise to the majority of cells in the early axolotl

blastema (Muneoka et al., 1986). Finally, Areg is secreted by neurons and Schwann cells (Kimura et al., 1990) and thus could be produced locally at the wound site or in neural cell bodies and transported to blastema cells via regenerating nerves. Thus AREG is a strong candidate for functioning as a neurotrophic factor given its pleiotropic potential to stimulate axon regrowth as well as blastema cell proliferation.

Our study identified Kazald1 as a second gene with properties that would be expected of a trophic factor that could stimulate proliferation of blastema cells. Kazald1 expression was increased 3-fold in day 10 DRG–blastema co-cultures compared with day 10 DRG without blastemas. Kazald1 is a secreted member of the insulin growth factor binding protein family that is transcriptionally upregulated during the early phase of bone formation and regeneration, and it is associated with proliferation of osteoblasts (Shibata et al., 2004).

Expression of a third signaling factor, Bmp2, was upregulated 1.5-fold in response to co-culture with a blastema (with a P-value of 0.015 which was greater than the cutoff of 0.0125 for genes listed in Table 2). Bmp2 was significantly upregulated (nearly 3-fold) during the initial period in culture (days 0–5) and its expression continued to increase when co-cultured with a blastema. Members of the BMP family of growth factors are involved in the regulation of growth and pattern formation during both limb development and regeneration (see Chapter 1). Recently it has been demonstrated that ectopic limb blastemas can be induced in axolotls by treating wounds with a cocktail of BMP and FGF *in vivo* as a substitute for signaling from a nerve (Makanae et al., 2013). Further studies on the role of BMP2 signaling in the regulation of blastema cell proliferation are in progress (see Chapter 3). Previous studies demonstrated that

expression of keratinocyte growth factor (Fgf7) is induced in DRG in response to limb amputation (Sato et al., 2008b). Since a probe set corresponding to the Fgf7 gene is not present on the Amby\_002 GeneChip, we could not document changes in Fgf7 expression. We did not observe significant changes in the expression of other members of the FGF family, including Fgf1, Fgf2, Fgf8 and Fgf10.

## **CONCLUSION**

Our results show that a robust transcriptional response is activated in cultured axolotl DRG that is comparable to the responses observed in mammalian DRG explanted into culture or after transection of peripheral nerves. Co-culture with a blastema resulted in a low number of potentially important changes in gene expression in axolotl DRG. At least three of the differentially expressed genes that we identified encode secreted, mitogenic proteins, supporting classical ideas that nerves may provide one or a few factors that function in blastema formation and cell proliferation.

## **CHAPTER 5: Conclusion**

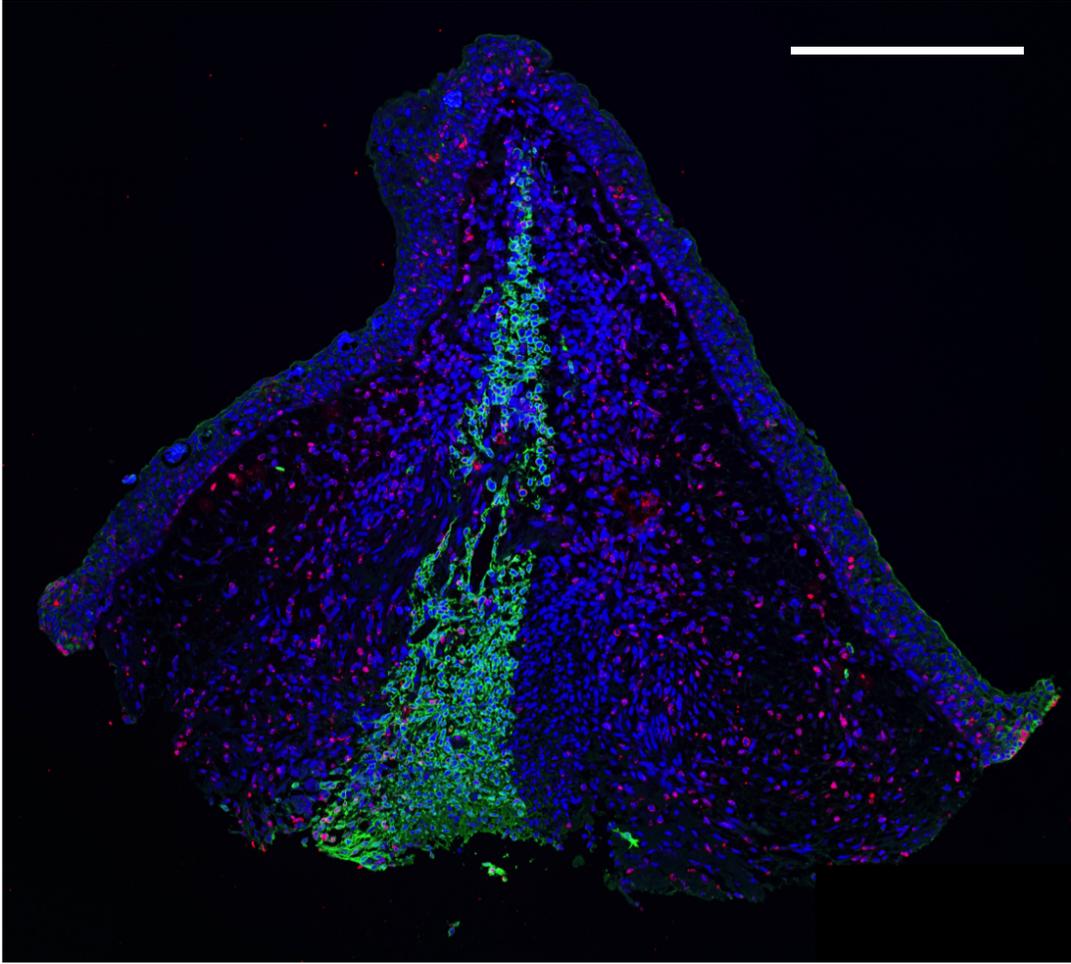
I have adapted organotypic slice culture (OSC) for use on the regenerating axolotl limb (Chapter 3). Using microarray analysis, I have identified Bmp2 as a possible nerve derived factor responsible for growth and proliferation (Chapter 4). Using the OSC system, I have tested the effect of exogenous BMP2 on OSC proliferation and gene expression (Chapter 3). I have shown that OSC are a useful tool for studying regeneration due to the fact that they are responsive to culture conditions in terms of both proliferation rates and gene expression.

The OSC system provides the opportunity to investigate the cellular and molecular mechanisms of limb regeneration in a way that has until now been unavailable. An example of how the OSC system can be of benefit to the investigator is seen in the titration experiments performed in Chapter 3 concerning the BMP2 titration experiments and the response of p-Smad 1/5/8 nuclear localization to BMP2. A similar titration experiment could not have been performed in a salamander in the *in vivo* setting. The advantages afforded by the OSC system opens the door to a number of exciting possibilities and potential experiments.

A future question that remains to be answered and that presents a future avenue of research is the determination of which cells in the blastema slice are being affected by FBS and/or BMP2. It is interesting that LI of slices cultured in basal medium with BMP2 remained at approximately 15%, as opposed to the ~23% LI seen in 5%FBS or *in vivo* (Figure 11A). One explanation for why OSC cultured with BMP2 have a LI of ~15% instead of ~23% is that nerve derived BMP2 is a sufficient signal for proliferation in only certain cell types in the blastema (e.g. fibroblasts and/or osteochondral progenitors), but

not others (e.g. Muscle cells) (Figure 18(2)). These “other” cells (i.e. cells that require some other factor besides BMP2 to proliferate) are unaffected by BMP2 in terms of proliferation resulting in a lower LI observed when compared to LI in slices cultured with FBS (Figure 11A, 18). This might explain why levels of proliferation in slices co-cultured with pre-conditioned DRG or treated with 1ng/mL BMP2 are roughly the same as both conditions lack these unknown factors (See Chapter 3, Figures 11A, 14A). Rather than being seen as a detractor, I believe this demonstrates the power of this system as it allows the opportunity for additional growth factors to be applied in a combinatorial manner.

Due to the axolotl’s ability to readily accept allografts, the identity of which cells are responding to BMP2 or FBS in the OSC system could be identified. To use cartilage as an example tissue type, GFP cartilage could be removed from a GFP donor and grafted onto a wild type host. The GFP/wild type graft could be amputated and allowed to regenerate. The dedifferentiated cartilage progenitors in the blastema that develops following amputation will then be able to be identified due to the fact they are GFP positive. The GFP/wild type blastema could then be cultured in the OSC system and the proliferation of presumed cartilage progenitors could be observed based on co-localization of proliferation markers such as EdU and GFP. Grafting experiments such as these are common and an example of one can be seen in Figure 17 (Kragl et al., 2009).



**Figure 17: GFP/Wild type grafting strategy to identify BMP2 target cells.** Immunofluorescence image of a medium bud blastema that had a GFP cartilage graft prior to amputation. GFP limb cartilage was grafted into the limb of a wild type host and then the limb was amputated through the graft. The blastema that developed contains GFP cartilage progenitor cells. Proliferation in response to BMP2 can then be analyzed via the co-localization of GFP and EdU. Cell nuclei are stained with DAPI (blue), GFP cells are stained with anti-GFP (green), and proliferating cells are stained with EdU (red). The proximal (P) to distal (D) orientation of the sections is indicated. Scale bars = 500 $\mu$ m.

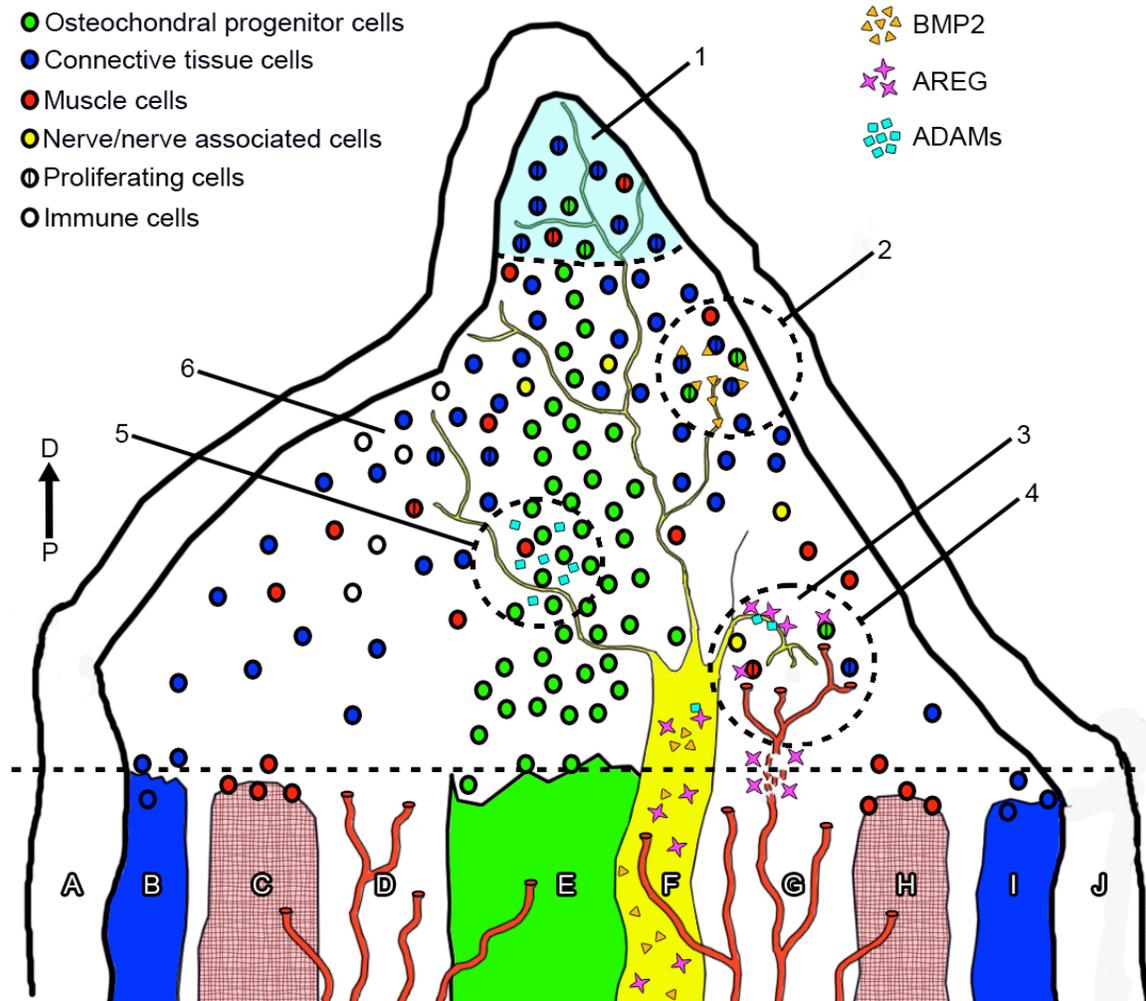
There are a number of possible targets identified in the microarray (Chapter 4) that could be investigated further (e.g. *Areg*, *Kazald1*, *Krt17*) using the OSC system. Perhaps instead of directly influencing the proliferation of cells, one of these candidate

genes identified in the microarray (along with or independent of BMP2) might be responsible for creating the microenvironmental conditions necessary for the blastema to reach its normal *in vivo* level of proliferation (Figure 18(1)). When culturing an OSC with a DRG, there are number of *in vivo* factors and processes that are absent (e.g. blood supply, immune system) (Figure 18D, G). One possible reason for the lower LI seen in OSC cultured with BMP2 when compared to *in vivo* is that in the *in vitro* system there are is no immune system. It has been shown that the systemic ablation of macrophages results in the inhibition of regeneration and increased wound fibrosis in axolotls (Godwin et al., 2013). Perhaps immune cells such as macrophages either directly or indirectly influence proliferation in the blastema and due to their absence in the culture system, a lower level of proliferation is observed (Figure 18(6)).

As mentioned above, the OSC system provides the chance for the combinatorial addition of such factors as those that might be absent *in vitro*. One process that would not be observed in the current OSC system would be the response of the blastema to signals regulating angiogenesis. A factor found in the microarray that had significantly higher expression in DRG co-cultured with a blastema is Amphiregulin (Areg), which is involved with a wide variety of developmental processes including angiogenesis.

Amphiregulin or Areg is a growth factor that is a member of the Epidermal Growth Factor family (EGF) (Ma et al., 1999; Zhou et al., 2012). Areg has been implicated in playing a role in tumor angiogenesis, cell proliferation, differentiation and liver regeneration (Bles et al., 2010; Dong et al., 2011; Liu et al., 2012; Ma et al., 1999; Wang et al., 2005; Zhou et al., 2012). AREG is also activated by the binding of CXCL12 to CXCR4 (Kasina et al., 2009). Cxcr4 was identified in microarray and had the same

pattern of expression as Bmp2 (i.e. an increase of expression from freshly dissected DRG to 5 day DRG, and then a continued increase in expression in response to blastema, see Chapter 4, Appendix).



**Figure 18: How the nerve affects proliferation: A model.** This illustration shows the potential molecular mechanisms responsible for nerve induced cell proliferation discussed in this thesis. The proximal (P) to distal (D) orientation of the sections is indicated. The horizontal dotted line near the bottom of the figure indicates the site of amputation. Stump tissues below the amputation plane are A, J) Epithelium. B, I) Dermis. C, H) Muscle. D, G) Blood vessels. E) Bone. F) Nerve. Dedifferentiated progenitor cells migrate and proliferate and form the blastema. Cell types and behavior are indicated in the upper left corner and are osteochondral progenitors (green), connective tissue cells (e.g. fibroblasts)(blue), muscle cells (red),

nerve/nerve-associated cells (e.g. Schwann cells)(yellow), proliferating cells (vertical line through cell), immune cells (e.g. macrophages)(white). Nerve derived proteins are indicated in the top right corner and are BMP2 (orange triangles), AREG (purple stars), and ADAMs (cyan squares). Proliferation and continued growth the blastema depends on an adequate nerve supply. How the nerve might be influencing proliferation: 1) The nerve could create a microenvironment conducive to blastema cell proliferation. The microenvironment is indicated (light blue) and is shown at the distal most region of the blastema. 2) BMP2 released from the nerve directly influences the proliferation of osteochondral progenitors and connective tissue cells only and not other cells. 3) ADAMs activate AREG which then go on to promote proliferation and/or angiogenesis. 4) AREG released from the nerve directly influences the proliferation of cells or indirectly promotes proliferation by stimulating angiogenesis, which in turn brings nutrients and growth factors to the blastema. 5) ADAMs degrade the ECM and allow more cells to accumulate. 6) Immune cells (e.g. macrophages) directly or indirectly influence proliferation of blastema cells.

Areg is also involved in mammary gland development, a dynamic process that like limb development and limb regeneration, includes the communication between epithelial and mesenchymal tissues, outgrowth and proliferation, and differentiation (Hens and Wysolmerski, 2005; LaMarca and Rosen, 2007; McBryan et al., 2008). Areg works in a paracrine manner to regulate mammary gland duct epithelial proliferation and morphogenesis through its binding of the EGF receptor (LaMarca and Rosen, 2007; McBryan et al., 2008). During mammary gland development, AREG is activated by genes belonging to the ADAM family (LaMarca and Rosen, 2007).

Genes belonging to the ADAM family possess the potential for cell adhesion and protease activities (Primakoff and Myles, 2000). ADAM Metalloproteinase With Thrombospondin Type 1 Motif 1 (ADAMTS1) has been shown to degrade ECM and promote bone metastasis (Lu et al., 2009). There was a significant increase of ~4 fold in

ADAMTS1 expression in DRG cultured for 5 days when compared to freshly dissected DRG (see Chapter 4, Appendix). There was also a significant ~3.2 fold increase in ADAMTS1 in DRG at 10 days and co-cultured with a blastema when compared to freshly dissected DRG (Chapter 4, Appendix).

ADAM Metallopeptidase With Thrombospondin Type 1 Motif 12 (ADAMTS12) has been shown to confer anti-tumorigenic properties via its modulation of the ERK signaling pathway (Llamazares et al., 2007). ADAMTS12 showed a significant decrease of approximately ~2.2 and ~2.5 fold in DRG at 10 days and co-cultured with blastema at 10 days respectively when compared to freshly dissected DRG.

ADAMs could potentially play a role in degrading ECM and/or activation of AREG (Figure 18(3, 4)). ECM degradation could permit the migration of dedifferentiated progenitor cells to the blastema (Figure 18(5)). One of the earliest events during limb regeneration in both newts and axolotls is the remodeling of the ECM via matrix degrading proteins (Vinarsky et al., 2005; Yang et al., 1999). Matrix Metalloproteinase 9 (Mmp9) is expressed during limb regeneration and is sensitive to denervation (Yang et al., 1999). ADAMs like MMPs might assist in ECM remodeling and promote migration in the early stages of regeneration. ADAMS might also or in concert with their ECM degrading ability, serve to activate AREG which then might go on to induce vascularization of the blastema resulting in the proliferation of cells not receptive to BMP2 (Figure 18 (3, 4)). This might also explain why addition of BMP2 or co-culture with a pre-conditioned DRG results in a LI of ~15% and why addition of FBS mimics the *in vivo* LI level of ~23% as fibroblasts, which make up 40-60% of the

blastema, would encounter the growth factors found in FBS in the context of a wound (Iyer et al., 1999; Muneoka et al., 1986)

Neurotransmitters can affect leukocyte and tumor cell migration and tumor cell growth, invasion and metastasis (Entschladen et al., 2002; 2007; 2006; Palm and Entschladen, 2007; Voss and Entschladen, 2010). The blastema has been likened to a tumor in that it is a structure comprised of plastic cells with a high rate of proliferation (Delriotsonis and Tsonis, 1992; Oviedo and Beane, 2009). Perhaps then like a tumor, which requires vascularization for growth and metastasis (Folkman, 1992), the blastema might also require a sufficient blood supply for continued growth. The idea that the nerve emits pro-angiogenic signals is supported by the evidence that denervation results in either the total inhibition of vascularization in early blastemas or limited vascularization in later stage blastemas (Smith and Wolpert, 1975). Perhaps the pro-angiogenic quality of the nerve is facilitated through the release of AREG (Figure 18 (4)).

Another possible future experiment that could be performed using OSC would be to examine further the BMP2 regulatory pathway. It has been shown here and by others that BMP2 is expressed in the nerve during regeneration (Guimond et al., 2010), Chapter 3 and 4). BMP2 has also been shown to induce ectopic Prrx-1 expression when BMP2 coated beads are implanted in chick embryos (Ocana et al., 2012). As reported in Chapter 3, addition of BMP2 to culture medium results in the increased expression of Prrx-1. It would be interesting to see if nerve-derived BMP2 is directly regulating the expression of Prrx-1 during limb regeneration.

BMP2 signaling generally falls under the category of either canonical or non-canonical signaling. The extracellular BMP2 ligand forms a heterotetrameric signaling

complex with type I or type II transmembrane receptors (Upton et al., 2008; Wang et al., 2013). BMP2 has a higher binding affinity to type I receptors which has led to the binding of BMP2 to the heterotetrameric receptor complex to be deemed canonical signaling and the binding of BMP2 to type II receptors to be deemed non-canonical signaling (Upton et al., 2008; Wang et al., 2013). While it was previously thought that the BMP signaling pathway inhibitor LDN193189 inhibited type I receptors by interfering with kinase activity via the ATP binding domain, increasing concentrations of LDN193189 have been shown to have an inhibitory effect on type II receptors as well (Boergermann et al., 2010). In work presented here, I have shown that while not statistically significant, higher doses of LDN193189 result in a lower mean labeling index in OSC (see Chapter 3). The OSC system presents the opportunity to utilize alternate inhibitors of BMP signaling to further dissect the BMP pathway. For example, as opposed to inhibition of canonical BMP signaling through the use of LDN193189, non-canonical BMP signaling could be investigated through the use of the p38 MAPK inhibitor SB203580, which would be useful due to its lack of off target MAPK independent activities (Boergermann et al., 2010; Fu et al., 2003; Zhou et al., 2010). Any attempt at performing the above mentioned experiments *in vivo* would be challenging, especially in the salamander and especially in terms of dose response. The OSC system avoids these problems and would allow these molecular pathways to be further examined.

Another area of investigation that the OSC system opens the door to are those concerning positional information. A disparity in positional information is one of the requirements necessary for limb regeneration to proceed, yet the physical manifestation

of positional information is unknown (Bryant et al., 2002; Endo et al., 2004; Gardiner and Bryant, 1989; McCusker and Gardiner, 2013; 2014).

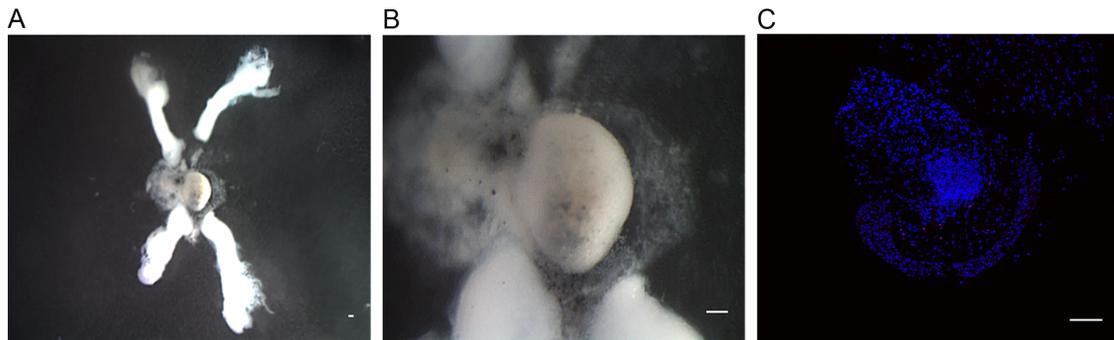
The OSC affords the opportunity to investigate the role of positional information in a number of ways. OSC allows for chemicals and growth factors to be easily added to the culture medium. Where previously an animal would require a large dose via an intraperitoneal injection, the OSC system allows for a much smaller dose and titration experiments to be performed. One such chemical that could be applied to OSC is the vitamin-A derivative, Retinoic Acid (RA). It has been shown that positional information can be manipulated through the use of the RA (Bryant and Gardiner, 1992; Maden, 1983; McCusker et al., 2014; Scadding and Maden, 1994; Stocum and Thoms, 1984; Tickle, 1991). RA changes the positional information of a blastema to one of a posterior-ventral-proximal position (PVPr) (Bryant and Gardiner, 1992; McCusker et al., 2014). Recently it has been shown that RA can substitute a contralateral skin graft in the ALM and allow ectopic limb growth (McCusker et al., 2014).

RA could be added to OSC medium followed by RNA-Seq analysis to identify how RA modulates positional information. Since many slices can be obtained from the same blastema, increasing doses of RA could be applied or at different time points increasing the statistical power of the results and reducing variability.

The OSC also allows live imaging experiments to be performed and gives the observer a look into the inner workings of the blastema that would previously only be available after fixation and sectioning. Through the use of the RARE:EGFP transgenic axolotl (Monaghan and Maden, 2012), real time imaging of internal blastema cells, visible as a result of vibratome sectioning, could be observed in response to RA.

Analysis of this sort could only previously be accomplished using multiple animals and through the use of fixation and sectioning.

The versatility of the OSC system allows for a number of creative grafting experiments to be performed. So-called “cut and paste” experiments have revealed a wealth of knowledge concerning positional information and regeneration in general (Bryant and Iten, 1976). OSC could be positioned or “sandwiched” in a number of ways to not only repeat some of the classic studies but also gain new insight. Preliminary experiments involving the sandwiching of different OSC along with multiple DRG resulted in a “blob” that was still proliferating after 15 days *in vitro* and also displayed an unusual pattern (Figure 19.)



**Figure 19: OSC positional confrontation experiment.** A) OSC were positioned in a manner where the proximal ends of each slice came into contact with each other. OSC were then co-cultured with 4 DRG for 15 days. B) B is an enhanced view of panel A. C) Section of OSC co-culture in panels A and B. Red cells show EdU positive cells. Scale bars = 500 $\mu$ m.

Future work with the OSC system that I have developed here has the potential to close the gap between basic research and any potential therapeutic treatment in a clinical setting. Before any attempt aimed at stimulating regeneration in humans can be pursued,

a thorough understanding of the molecular and cellular events that occur in salamanders during limb regeneration must be obtained. The OSC presents the opportunity to investigate these molecular and cellular events that has heretofore been difficult to pursue in the *in vivo* setting. In addition to moving potential therapies into the clinic, the OSC offers the chance to answer questions about the evolution of regeneration.

Many questions about the evolution of regeneration abound. Was regeneration an adaptation that was gained in salamanders, or was a trait seen in a common ancestor that was subsequently lost in non-regenerating lineages? Perhaps limb regeneration is nothing more a pleasant pleiotropic side effect or debatably useful inborn error of development, passed on down the line from some ancient multicellular organism. Based on phylogenetic analysis of regenerating phyla, the most parsimonious explanation is that the origin of regeneration was an epiphenomenon of development that occurred in an early ancestor, coincident with the rise of multicellularity (Bely and Nyberg, 2010).

There are generally two schools of thought when it comes to the evolution of limb regeneration in salamanders. One school of thought claims that humans may have lost the ability to regenerate but this ability has the potential to be restored due to the molecular and cellular similarities seen during limb development and limb regeneration (Bryant et al., 2002; 1987; Han et al., 2001a). In other words, humans (or any non-regenerating tetrapod) can't regenerate but that's not because they lack some salamander specific protein or process as many of the signaling pathways during limb development and limb regeneration are the same (see Chapter 1). This view is supported by the similarities seen during both development and regeneration, phylogenetic analysis, and work presented here (e.g. the ability of mammalian serum to maintain *in vivo* levels of

proliferation in the absence of the nerve, see Chapter 3)(Bely and Nyberg, 2010; Bryant et al., 2002; 1987).

The alternative school of thought regarding the evolution of salamander limbs is that salamanders have adapted or co-opted common molecular signals and/or cellular processes for regeneration that are unique to salamanders (da Silva et al., 2002; Garza-Garcia et al., 2010; Kumar et al., 2007; 2010; Sandoval-Guzman et al., 2014; Yun et al., 2014). Two examples of molecular signals touted as serving a unique or derived regeneration specific role in salamanders are the proteins newt Anterior Gradient protein (nAG) and Prod1. The first example, nAG, is the newt ortholog of the Anterior Gradient protein family and has been proposed to be the elusive neurotrophic factor (Kumar et al., 2007) (see Chapter 1). The orthologous AG proteins were first identified as being involved in *Xenopus* cement gland formation (Kumar et al., 2007; 2011). Interestingly, even though nAG has been reported to be the neurotrophic factor, it is only expressed in distal Schwann cells and not in neurons (Kumar et al., 2007).

The other example of a protein being co-opted for salamander limb regeneration is Prod1. Prod1 is a transmembrane protein that is orthologous to human CD59, a protein that regulates complement mediated cell lysis (da Silva et al., 2002; Garza-Garcia et al., 2010; 2009). Prod1 has been implicated in proximodistal identity in newt limb regeneration and has also been proposed to be the receptor for nAG (da Silva et al., 2002; Garza-Garcia et al., 2009; Kumar et al., 2007; 2010; 2011).

Prod1 and nAG are examples of two proteins that have orthologous human counterparts and yet are claimed to be involved in widely different roles in their respective species. Unlike the re-expression of developmental genes during regeneration,

the adaption of these proteins (found in both humans and salamanders) for regeneration specific purposes in salamanders suggests that they changed their function (in either humans or salamanders). The neofunctionalization of a protein resulting in major changes to its function are rare and one must make the leap (i.e. in the instance of traits arising from gene duplication) that not only did salamanders undergo duplication of the genes in question, but also incurred selection pressure on regeneration sufficient enough to preserve the duplicated genes and their new function (Beisswanger and Stephan, 2008; Force et al., 1999; Graur and Li, 2000; Xue and Fu, 2009). Furthermore, this hypothesis hangs on the assumption that regeneration increases the fitness of salamanders. Recently it has been claimed that newts and axolotls undergo muscle dedifferentiation through two divergent processes as a result of “micro-evolutionary selection pressures (Sandoval-Guzman et al., 2014).” What these so-called micro-evolutionary selection pressures might be are not explained, however one would think they must be strong enough to cause the regeneration to arise and be fixed via two different mechanisms in two different species. While some analogous behaviors or traits do evolve independently (e.g. flight or gliding in fish, bats, and birds or fins in whales and sharks), these traits, behaviors, or structures usually bestow a quantifiable fitness advantage or allow a group to fill a niche. Unlike the ability to fly or swim however, there is absolutely no evidence that regeneration confers a fitness advantage for salamanders. Limb regeneration may in fact be disadvantageous to salamanders as resources allocated for regeneration (i.e. food and energy) are taken at the expense of somatic growth and reproduction and have been shown to reduce growth and fecundity in salamanders, lizards and arthropods (Maginnis, 2006).

The OSC has shown that in contrast to the notion that salamanders possess unique or derived characteristics that allow them to regenerate, instead common molecules expressed during development (i.e. BMP2) and mammalian factors such as FBS are able to influence proliferation and gene expression (see Chapter 3). The OSC system presents the opportunity to further test molecules such as Prod1 and nAG and gain further insight into the evolution of limb regeneration.

In summary, I used microarray to identify BMP2 as the possible nerve derived factor that affects growth in the regenerating limb and then tested it in an organotypic slice culture system that I adapted for use on the axolotl blastema. The OSC system provides innumerable opportunities to investigate limb regeneration in salamanders. The OSC will hopefully be implemented into future regeneration studies and help usher in a new age of discovery in the field of regeneration. Perhaps with innovative tools like the ones presented here, we can one day take limb regeneration out of the realm of science fiction and make it a reality.

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## APPENDIX

The appendix contains work I performed during my graduate studies in addition to that found in the main body of the thesis. The appendix is a reprint of the paper “Position-specific induction of ectopic limbs in non-regenerating blastemas on axolotl forelimbs,” published February 16<sup>th</sup>, 2014 in the journal REGENERATION. This paper was co-authored by Catherine McCusker, and David Gardiner and is reproduced with permission from John Wiley & Sons.

The supplementary table URL in Chapter 4 can be found at the end of the appendix. The supplementary table is a reprint from the paper “Characterization of *in vitro* transcriptional responses of dorsal root ganglia cultured in the presence and absence of blastema cells from regenerating salamander limb,” published July 14<sup>th</sup>, 2014 in the journal REGENERATION. This paper was co-authored by Antony Athippozhy, James R. Monaghan, David Gardiner, and S. Randal Voss and is reproduced with permission from John Wiley & Sons

## **ABSTRACT**

Ectopic retinoic acid (RA) has been hypothesized to reprogram the positional identity of cells in developing and regenerating limbs to a single positional value corresponding to the posterior-ventral-proximal (PVPr) position on the limb. We tested this hypothesis by using RA to reprogram the information of blastema cells that were induced to form at different positions around the limb circumference. We observed that RA treatment of blastemas in anterior and dorsal locations, but not posterior and ventral locations, resulted in the induction of complete ectopic limbs. These position-specific differences in limb induction are probably due to differences in the positional disparity between the RA-reprogrammed blastema cells and the cells at the periphery of the wound. These observations are consistent with the hypothesis that RA treatment reprograms the information in blastema cells to the PVPr position on the limb, since anterior and dorsal positions have the largest disparity and posterior and ventral have the smallest disparity from the PVPr identity.

## **INTRODUCTION**

The goal of regenerative biology is to understand the mechanisms driving the regeneration of complicated biological structures so that they can be recapitulated to stimulate a regenerative response in humans. Urodele amphibians serve as an excellent model to study the mechanism of regeneration in a vertebrate system because they have the amazing capacity to regenerate complicated body structures including their jaws, limbs, and tails (Ferretti, 1996; Han et al., 2005; Tanaka, 2003). Our laboratory has used a gain-of-function assay (the accessory limb model, ALM) in the axolotl (*Ambystoma*

*mexicanum*) to study the role of different cell types and signaling pathways during limb regeneration. The ALM is based on the observation that ectopic limbs can grow from a wound on any region of the limb provided a severed nerve is present and that cells from a different position on the limb axis are grafted into the wound site (Endo et al., 2004). Thus, the ALM can be used to test molecules that are either involved in (1) the neurotrophic response (Singer, 1974) or (2) establishing positional diversity in the limb regenerate (Bryant et al., 1981; French, 1978).

The objective of the current study was to use the ALM to study whether the reprogramming of positional information of blastema cells could elicit a regenerative response in blastemas that normally are non-regenerative. Non-regenerative blastemas, which undergo the initial stages of blastema formation but eventually stop growing and are re-integrated into the limb without generating new structures, are induced to form by deviating a nerve to a wound on the side of the limb. Our hypothesis is that non-regenerative blastemas do not generate limb structures because they lack the diversity of positional information required to generate a complete limb field (Endo et al., 2004). To test this hypothesis we utilized the reprogramming capacity of exogenous retinoic acid (RA), which has been well documented in its ability to reprogram the positional information in developing and regenerating limbs but not in differentiated cells (Bryant and Gardiner, 1992; Eichele et al., 1985; Kim and Stocum, 1986; Larsen and Janners, 1987; Ludolph et al., 1990; Maden, 1983; Noji et al., 1991; Stocum and Thoms, 1984; Tickle, 1991; Wanek et al., 1991).

We observed that only anterior and dorsally located blastemas formed complete ectopic limbs when treated with RA. In contrast, posterior and ventrally located

blastemas had a minimal capacity to form ectopic structures when exposed to exogenous RA. These observations suggest that exogenous RA can substitute for a tissue graft in the ALM because it results in the formation of positional diversity between the cells in the wound site and the RA-reprogrammed blastema. Furthermore, these results demonstrate for the first time the chemically induced formation of ectopic limbs in the absence of a tissue graft and provide a useful tool for the future mapping of positional identities within the limb.

## **MATERIALS AND METHODS**

### *Animal husbandry*

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The experimental work was approved by the Institutional Animal Care and Use Committee of the University of California Irvine.

All of the experiments in this study were performed on wild type Mexican axolotls (*Ambystoma mexicanum*) measuring approximately 5–8 cm snout to tail tip (3–4 cm snout to vent). Experimental animals were either spawned at UC Irvine or obtained from the Ambystoma Genetic Stock Center at the University of Kentucky. Animals were anesthetized using a 0.1% MS222 solution (ethyl 3-aminobenzoate methanesulfonate salt, Sigma, St. Louis, MO, USA), pH 7.0.

### *Nerve deviation surgery*

The induction of ectopic blastemas was performed as described previously (Endo et al., 2004). In brief, the brachial nerve bundle was deviated into a square wound site (1–2 mm on a side) on the most anterior, dorsal, posterior, or ventral region of the proximal forelimb (stylopod). Given the ventral location of the brachial nerve, it was technically more challenging to deviate the end of the severed nerve to wounds created on the opposite side of the limb (dorsal), resulting in a reduced frequency of ectopic blastema induction (Table 3). Mid-bud stage blastemas typically formed 7–10 days after surgery.

#### *Retinoic acid treatment*

Ectopic blastemas were allowed to develop until mid-blastema stage (approximately 7–10 days), at which point animals were injected intraperitoneally in the flank with RA (150 µg/g of body weight as described in (Niazi et al., 1985). Animals were kept in the dark for 2 days following the injection to minimize the photo-inactivation of RA. Live images of blastemas were taken on a weekly basis starting on the day of RA injection and continuing until fully regenerated skin had formed over the regenerate or wound site, at which time the limbs were collected for further analysis.

#### *Histology staining*

Tissues were fixed in 3.7% paraformaldehyde. Tissues for histological analysis were prepared for cryosectioning. For fluorescent histology, sections were stained with phalloidin-rhodamine for F-actin and 4',6-diamidino-2-phenylindole (DAPI) for nuclei. The sections were stabilized with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Fluorescent images were obtained using a 20× objective on a Zeiss

LSM780 (two-photon) confocal microscope. Tissue sections were also stained with 0.03% Alcian blue/0.1% HCl/70% ethanol for 30 min, followed by standard hematoxylin and eosin Y staining.

#### *Whole mount bone and cartilage staining and phenotype scoring*

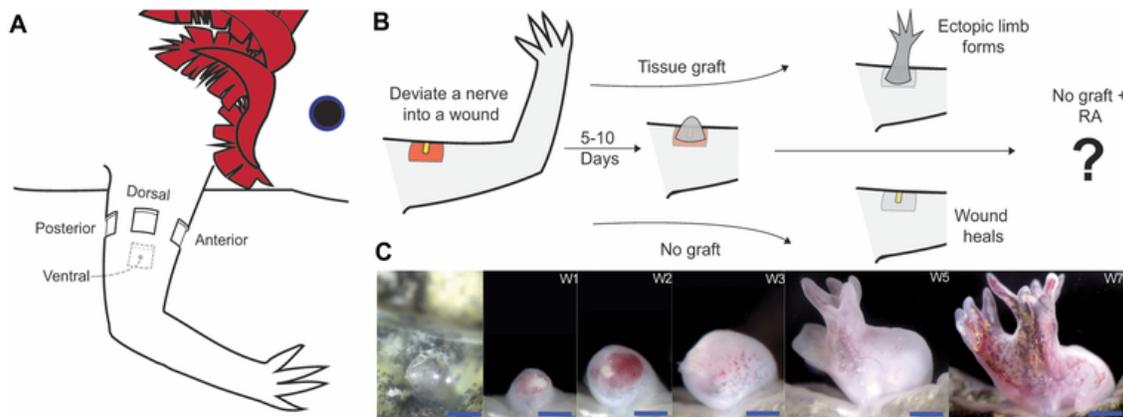
For the phenotype analysis presented in Table A. 1 and Figure A. 3(B), the presence and complexity of ectopic skeletal elements were assessed by the use of whole mount bone and cartilage staining as described in (Horton and Maden, 1995). Representative images of the skeletal staining on samples that exhibit each of the different phenotypes from blastemas treated with RA are shown in Figure A. 5.

Samples that formed two blastema-like structures that either developed into independent limbs or had skeletal fusions in the stylopod but formed independently from the elbow-joint distally were scored as “paired limbs.” The “single limb” phenotype was scored on samples that resulted in the formation of a single limb with stylopod, zeugopod, and autopod elements. One of the single limbs had extra digits, and the other was missing digits. Samples that were scored as “multiple symmetrical elements” had multiple skeletal elements that were separated by joints. The “single cartilage element” samples had one, small, spherical or ovoid shaped cartilage element that had no joints and did not display any obvious characteristics of a particular limb skeletal element. Blastemas that resulted in “no growth” after treatments with RA did not form a bulbous mass and eventually integrated into the limb.

## **RESULTS**

*Retinoic acid treatment induces the formation of ectopic limbs from “non-regenerating” anterior blastemas*

Previous studies have shown that ectopic blastemas are induced when a nerve is surgically deviated to an anterior wound site (Endo et al., 2004; Maden and Holder, 1984). These blastemas eventually will cease to grow and then integrate into the existing limb tissue without generating ectopic limb structures unless tissue with opposing positional information (posterior) is grafted into the wound site. If tissue from the posterior side of the limb is grafted into the anteriorly located wound, the positional disparity between cells of the graft and the wound site stimulates the intercalation of a complete limb field, and an ectopic limb is generated (Figure A. 1A, B) (Endo et al., 2004).



**Figure A. 1: Treatment of a “non-regenerative” anterior wound with retinoic acid (RA) induces the formation of ectopic limbs.** (A) Diagram of an axolotl forelimb indicating the positions at which wounds were made around the limb circumference. (B) Diagram illustrating the experimental procedures performed on anterior wound sites. An anterior wound site with a deviated nerve forms an ectopic blastema within 5–10 days. Without a posterior skin graft, the ectopic blastema integrates into the limb and the wound heals over without forming an ectopic limb (Endo et al. 2004). If a skin graft from the posterior side of the limb is grafted into the wound site, an ectopic limb is generated (Endo et al. 2004). Ectopic blastemas without a graft (non-regenerating) were treated with RA to determine whether RA can replicate the positional

confrontation normally induced by the posterior tissue graft. (C) Images of the same ectopic limb induced by RA treatment of an anterior ectopic blastema taken over a period of 7 weeks. The first image is the blastema on the day of RA treatment. The blastema forms an amorphous mass that eventually forms two well patterned ectopic limbs. These images are representative of what was observed in 10 of 20 ectopic structures that eventually formed paired ectopic limbs from an RA-treated anterior wound (see Table 3). Blue scale bars are 1 mm in

Ectopic RA has been hypothesized to reprogram the positional identity of cells in developing and regenerating limbs (Noji et al., 1991; Wanek et al., 1991) to a single positional value corresponding to the posterior-ventral-proximal (PVPr) position on the limb (Bryant and Gardiner, 1992). We tested this hypothesis by using RA to reprogram the identity of cells in non-regenerating anteriorly located blastemas to generate sufficient positional disparity (i.e., cells with posterior positional identity) to induce ectopic limb structures (Figure A. 1). Within 2 weeks following RA exposure, most of the anterior blastemas had increased in size (Figure A. 1C). Many of the blastemas formed a bulbous mass, as was previously observed in RA-treated frog tail blastemas (Mohanty-Hejmadi et al., 1992), which eventually formed limb structures. Most (71%) of the RA-treated anterior blastemas generated skeletal elements (Table A. 1). Of the blastemas that generated ectopic structures, 50% of them formed paired limbs.

There was some variability on where the double limbs formed from the bulbous mass. Some of the paired limbs formed from blastema-like bumps on opposite ends of the mass, while others formed from blastema-like bumps that arose from the same region of the mass (compare Figure A. 1 with Figures A. 2 and A. 3A). In a few samples, we also noticed that the double blastemas developed at slightly different rates (Figure A. 1C, week 5 image), suggesting that the paired limbs developed independently of each other.

The limb morphology of the paired limbs was usually normal, although we did notice that paired limbs that developed from blastemas that formed close together often had patterning defects, such as skeletal fusions and missing digits.

In the RA-treated blastemas that formed limbs, we observed that the skeletal elements in the basal region of the ectopic growth were not integrated with the humerus at the site of the wound. This lack of integration of the skeleton of ectopic limbs was also observed with ectopic limbs induced by a posterior skin graft (Endo et al., 2004; Satoh et al., 2007). In many cases the entire ectopic growth (both skeletal elements and associated soft tissues) eventually became connected to the host arm site only by a thin bridge of soft tissues. This phenotype is comparable to what has been reported in regenerating frog tail blastemas that were induced to form ectopic limbs when exposed to exogenous RA (Mohanty-Hejmadi and Crawford, 2003).

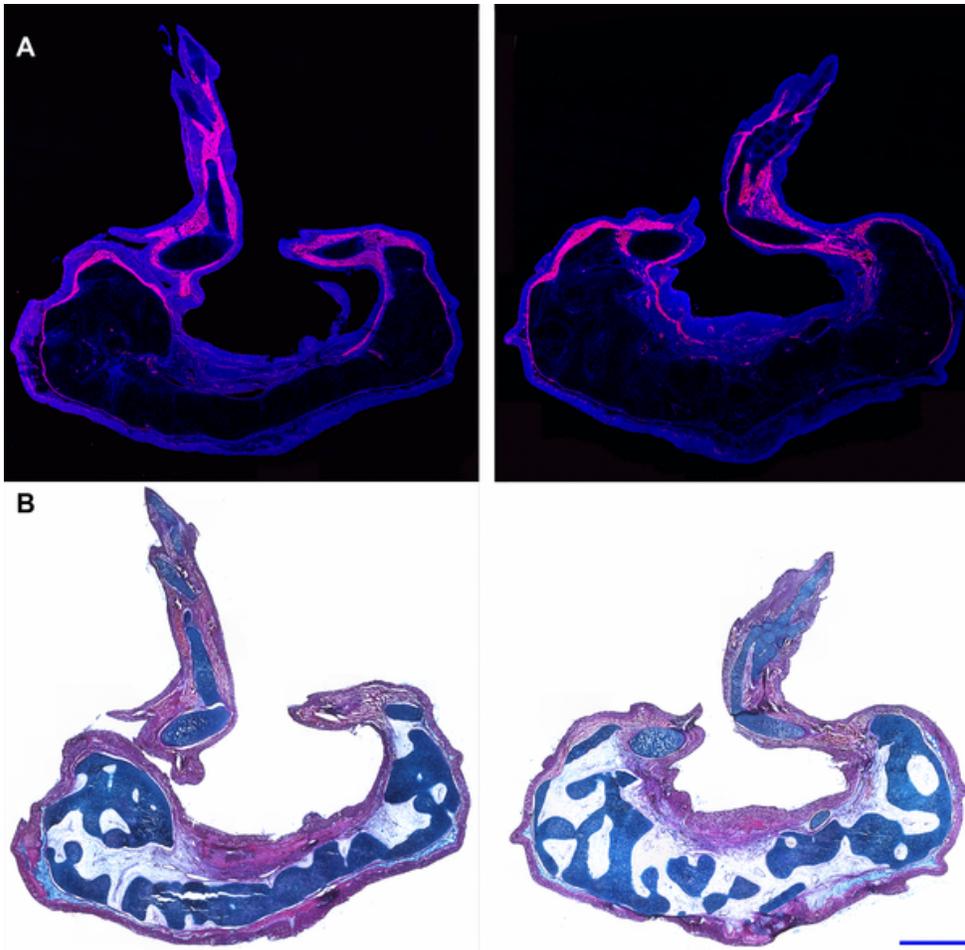
**Table A. 1: Limb phenotypes resulting from RA treatment of animals with ectopic blastemas**

Wound location	Total surgeries performed	Surgeries that developed blastemas*	Blastemas that formed ectopic structures	Single cartilage element**	Multiple symmetrical cartilage elements**	Single limb**	Paired limbs**
Anterior	30	28 (93%)	20 (71%)	4 (20%)	6 (30%)	0	10 (50%)
Dorsal	34	18 (53%)	9 (50%)	2 (22%)	1 (11%)	2 (22%)	4 (44%)
Posterior	26	26 (100%)	5 (19%)	4 (80%)	1 (2%)	0	0
Ventral	21	18 (86%)	1 (6%)	1 (100%)	0	0	0

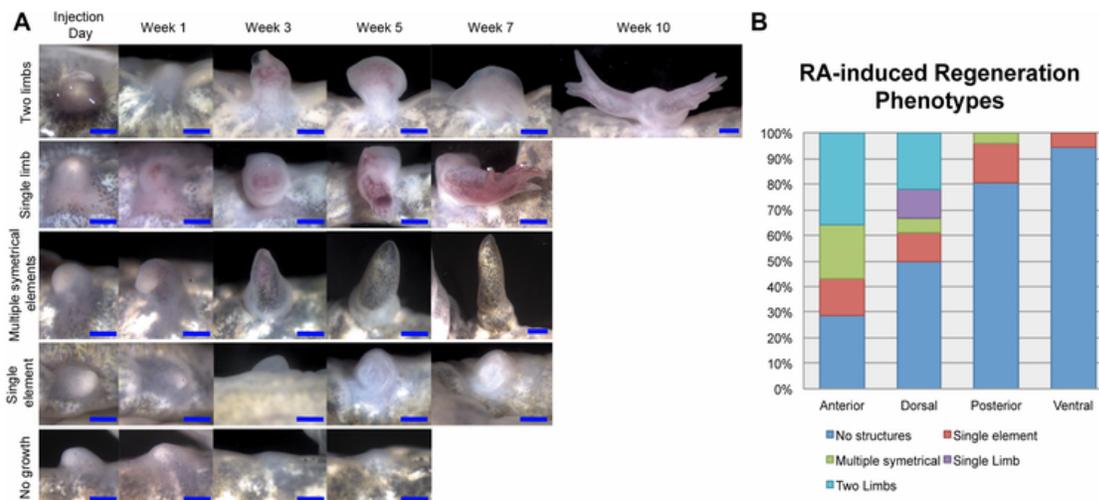
\*Percentage of surgeries that developed blastemas.  
\*\*Percentage of ectopic blastemas that formed structures.

In addition we observed that the skin of the bulbous proximal region of RA-treated ectopic blastemas had few or no pigment cells, whereas the ectopic limb structures that formed more apically were pigmented (Figure A. 1C). The presence of pigment cells is considered to be the final step in skin maturation (Seifert et al., 2012) suggesting that the bulbous mass might be composed of undifferentiated cells; however, that was not the case (Figure A. 2). The more apical limb structures exhibited skeletal patterns that were typical of normal limbs and contained muscle and cartilage. In contrast, the bulbous proximal region lacked muscle, but contained other differentiated limb tissues including cartilage and connective tissue. Although well differentiated, the basal cartilaginous elements could not be identified as corresponding to skeletal elements that are part of the normal limb pattern (Figure A. 2). Despite the absence of pigment cells, the skin of the basal bulbous masses appeared to be well differentiated as evidenced by the presence of the basement membrane and gland cells (Figure A. 3).

As with normal limbs, the pigment in the skin of the apical ectopic limbs was asymmetrically distributed (e.g., Figure A. 1C) such that the pigment was restricted to approximately half of each limb. All paired limbs exhibited a pigment pattern in which the surfaces of the limbs apposed to each other were pigmented and the opposite surfaces were not pigmented. In normal limbs, pigmentation is restricted to the dorsal half of the limb, and thus ectopic paired limbs appear to exhibit a double-ventral phenotype with their dorsal surfaces facing each other and their ventral surfaces facing outwards (Figure A. 1C).



**Figure A. 2: Histology of RA-induced paired limbs from an anterior wound site.** Histological analyses were performed on sections that transected each of the two ectopic limbs that grew from an RA-treated anterior wound site, harvested 10 weeks post-treatment. Two complete limbs, including the zeugopod, stylopod, and autopod, formed from this experimental manipulation. A large mass of tissue formed proximal to the limb structures. (A) Fluorescent images were obtained of the cryosectioned limbs stained with DAPI (blue) and phalloidin-rhodamine (red). Muscle tissue, rich in F-actin (stained with phalloidin-rhodamine), was distributed throughout the more distal limb structures but was not observed in the mass of tissue located proximally. (B) Sections were stained with eosin Y, hematoxylin, and alcian blue for histological analysis. The proximal mass of tissue predominantly differentiated into connective tissue and cartilaginous elements (dark blue) that could not be identified as corresponding to skeletal elements that are part of the normal limb pattern. Blue scale bars are 1 mm in length.



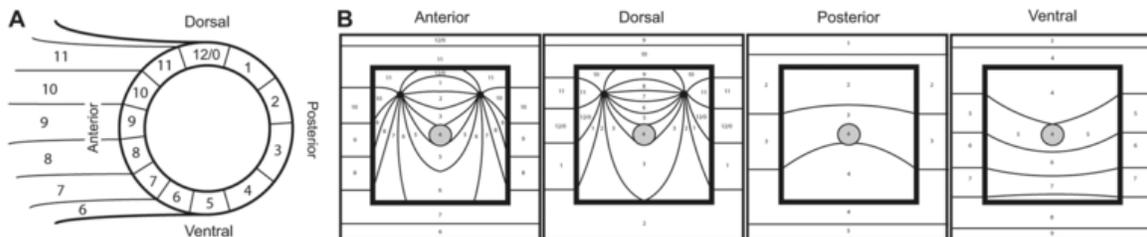
**Figure A. 3: RA-induced regeneration phenotypes are position-specific.** Quantification of the regeneration phenotypes for RA-induced blastemas. Upon the completion of differentiation, the ectopic outgrowths were collected and stained for bone and cartilage in whole mount preparations in order to analyze the presence of skeletal elements in the regenerate. From our observations of these skeletal preparations, we divided the regeneration phenotypes into five categories: (1) no ectopic structures; (2) a single skeletal element; (3) multiple (jointed) symmetrical skeletal elements; (4) a single limb; or (5) two paired limbs (also see Table 1). (A) Examples of blastemas that exhibited the five different regeneration phenotypes that were quantified in this study. Images were taken of the blastemas starting the day of RA injection and ending when the tissue in the wound site had completely differentiated (determined by the formation of mature skin). (B) The histogram represents the percentage of ectopic blastemas located on the anterior, dorsal, posterior, or ventral axis that differentiated into each phenotype. Only the wounds located on the anterior or dorsal axis generated one or two paired limbs when treated with RA (also see Table 1). Blue scale bars are 1 mm in length.

*Retinoic acid induction of ectopic limbs occurs in anterior and dorsal but not posterior and ventral located ectopic blastemas*

As noted above, exogenous RA is hypothesized to reprogram the positional information of cells in the blastema or the limb bud to a ventral as well as a posterior identity (Bryant and Gardiner, 1992). Since anterior and dorsal locations would have the largest

positional disparity relative to the PVPr positional information induced by RA, a prediction of this hypothesis is that RA treatment will induce the formation of ectopic limb structures at high frequency from dorsal blastemas (the position opposite from ventral), but at much lower frequencies from posterior or ventral ectopic blastemas (with the same positional information as the RA-reprogrammed blastema cells). By similar reasoning, blastemas at anterior/dorsal positions should form more complex, ectopic limb patterns compared with RA-treated blastemas at posterior/ventral positions.

As predicted (Table A. 1, Figures A. 3, A. 4), dorsal RA-treated blastemas also formed ectopic structures at a high frequency (anterior, 71%; dorsal, 50%). In addition to relatively simple cartilaginous structures, most of these blastemas formed either one (dorsal, 22%) or two, paired ectopic limbs (anterior, 50%; dorsal, 44%). In contrast, very few of posterior or ventrally located blastemas formed ectopic structures (posterior, 19%; ventral, 6%), and except for one blastema with multiple symmetrical elements (posterior, 2%) these ectopic structures were limited to a single cartilage element.



**Figure A. 4: Model of RA-induced supernumerary limbs from ectopic blastemas.** (A) Diagram of an amputated limb showing the distribution of positional information around the limb circumference as described in Bryant et al. (1981) and Bryant and Iten (1976). The experiment was performed on wound sites that were made on the most anterior (“9”), dorsal (“12/0”), posterior (“2/3”), or ventral (“5”) locations. (B) Models representing the positional interactions between the RA-reprogrammed blastema (gray circle with the “4” coordinate) and the cells in the surrounding wound margin. The boundary of the square wound is indicated by heavy-weighted lines. The thin lines and corresponding numbers on the outside of the

square correspond to the distribution of circumferential positional information in the host limb as illustrated in (A). The thin lines and corresponding numbers inside the square wound are the predicated positional values that would be intercalated as a result of the interactions between the reprogrammed blastema cells (“4”) and the surrounding host cells. When the RA-treated blastema is located in anterior and dorsal wound sites, the positional disparity is sufficient to induce the formation of two complete limb axes. In contrast, the interaction between the reprogrammed blastema cells with posterior or ventral host cells does not generate enough positional diversity to induce ectopic limb axes.

## **DISCUSSION**

### *Model of positional interactions induced by retinoic acid*

Whereas the region-specific induction of ectopic limbs is predicted by the hypothesis that RA converts cells to a PVPr identity (Bryant and Gardiner 1992), the complexity of the induced pattern of the ectopic limbs is predicted by the polar coordinate model (PCM) (Bryant et al., 1981; French et al., 1976). The PCM hypothesizes that, when cells with disparate positional information interact, growth is stimulated and the new cells adopt a positional identity that is intermediate between the original cells, a process referred to as “intercalation” (French et al., 1976). Thus new structure and pattern are formed (intercalated) until the normal pattern is restored. By this view, when all the information of the circumferential limb axes is present (“complete circle” = anterior + posterior + dorsal + ventral) an entire new limb will be formed (Figure A. 4A) (Bryant et al., 1981; French, 1978).

We modeled the predicted intercalary interactions between RA-reprogrammed blastema cells and host cells surrounding the wound margin to visualize interactions that would or would not induce a complete circle of positional information (Figure A. 4B). We first represented the limb circumference as a clock face with 12 positions (details in

Figure A. 4A) (Bryant et al., 1981; French et al., 1976). By this view, RA would reprogram blastema cells to position “4” (posterior/ventral). For blastemas in posterior/ventral positions, RA reprogramming of cells is predicted to result in little or no alteration in the normal pattern of distribution of positional information (Figure A. 4B). In contrast, the intercalation of cells with positional information that is intermediate between value “4” (newly reprogrammed cells) and the surrounding host cells leads to the formation of two complete circles of positional information in both anterior and dorsal RA-treated blastemas (Figure A. 4B). We presume that predicted complete circles of positional information correspond to the observed supernumerary limbs that were induced by RA treatment of anterior and dorsal blastemas (Table A. 1, Figure A. 3). In addition to predicting whether or not supernumerary limbs would be induced, the PCM also predicts that the supernumerary limbs will have mirror symmetrical patterns. Although the steps in formation of the final supernumerary limbs presumably are complex, the final pattern of the double limbs that formed from anterior wounds all appeared to have double-ventral handedness with the unpigmented ventral sides facing away from each other (Figure A. 4B).

*Potential mechanism of RA-induced positional reprogramming in blastema cells*

The effect that ectopic RA has on the positional program in blastema cells presumably is dependent on expression of the correct retinoic acid receptor (RAR) in cells that are capable of having their positional information reprogrammed. A number of RAR isoforms have been detected in the limb blastema and one of these isoforms, RAR- $\delta$ 2, appears to be responsible for positional reprogramming in the limb blastema (Pecorino et

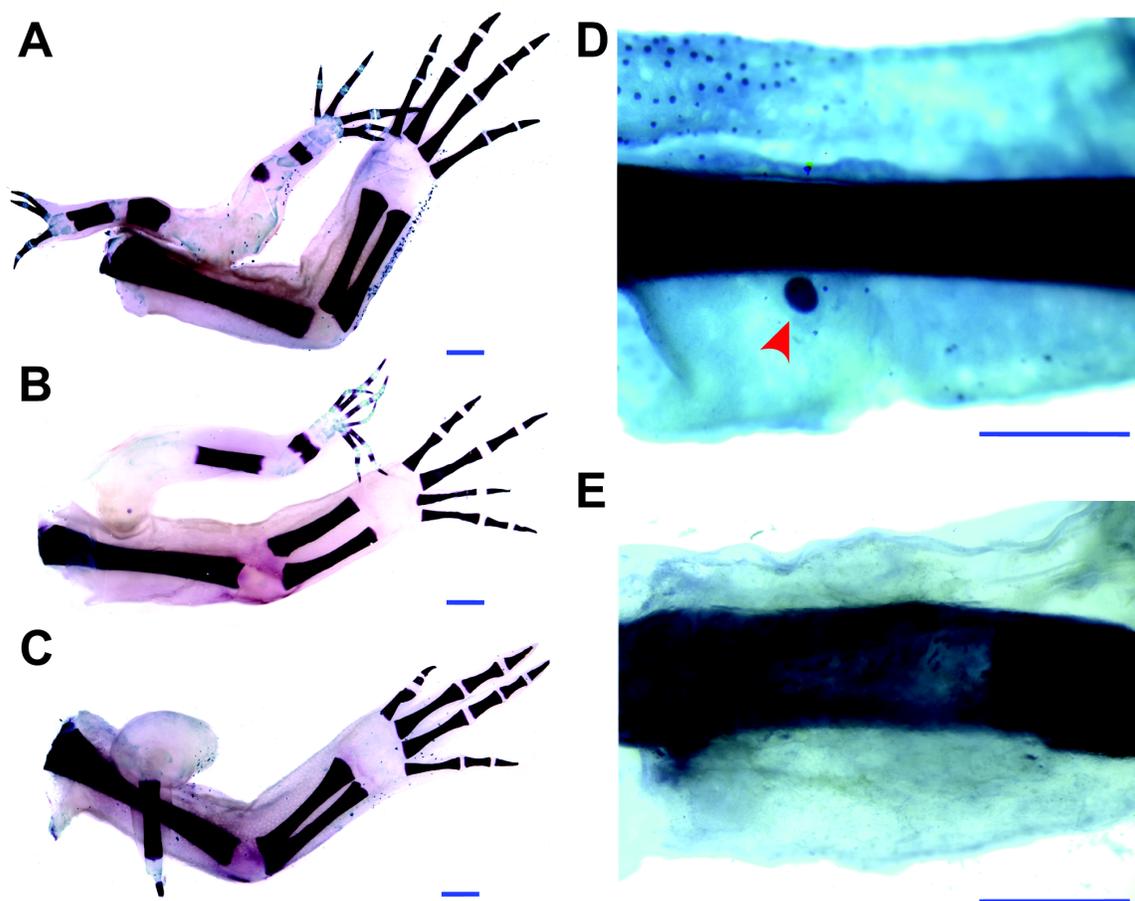
al., 1996; Ragsdale et al., 1992; 1993; 1989). However, exposing cells that express RAR- $\delta$ 2 to ectopic RA is not enough for positional respecification. Ectopic RA only appears to affect the positional information in undifferentiated blastema cells (Niazi et al., 1985), yet similar amounts of RAR- $\delta$ 2 are expressed in both the mature limb and the blastema (Ragsdale et al., 1993).

Our hypothesis is that ectopic RA reprograms the positional information in blastema cells because they are positionally plastic (i.e., are undifferentiated). Recent studies have shown that cells of the early blastema and the apical tip of the late blastema, both of which can be reprogrammed by RA, are positionally plastic and adopt the region-specific molecular fingerprint of a new host environment (McCusker and Gardiner, 2013). In contrast, mature stump tissue and the basal region of the late blastema (which is differentiating) are not positionally plastic (McCusker and Gardiner, 2013) and are refractory to the effects of RA. Since positional information is epigenetically encoded in adult cells (Rinn et al., 2006), and the expression of epigenetic modifiers is essential for regeneration (Stewart et al., 2009), it is possible that the epigenetic modifications render region-specific genes susceptible to positional reprogramming. RA signaling is upstream of a number of region-specific molecules, including multiple Hox genes, Tbx genes, *Prod1*, and *Meis1/2* (Kumar et al., 2007; Mercader et al., 2005; Mullen et al., 1996b; Simon and Tabin, 1993; Wang et al., 2006). Thus, it is possible that the “open” epigenetic state of some of these genes renders their promoters accessible to activated RAR- $\delta$ 2 in blastema cells that have been treated with RA. However, there is much to learn about the epigenetic state of positional information and how new positional information is programmed in blastema cells. Testing which epigenetic states are sensitive to positional

reprogramming by exogenous RA may bring some answers about the nature of positional plasticity.

#### *Chemical induction of regeneration*

Regeneration is a stepwise process that can be induced experimentally by signals from a deviated nerve and from grafted skin cells with differing positional information (Endo et al., 2004). Recently, a cocktail of growth factors (GDF-5, FGF2, and FGF8) has been identified that can substitute at least in part for signals from a deviated nerve leading to blastema formation (Makanae et al., 2013). In the present study we have demonstrated that RA treatment can substitute for grafting of posterior skin to an anterior wound (presumably as well as grafting of ventral skin to a dorsal wound) in order to provide the subsequent signals required for ectopic limb regeneration. Studies focused on optimizing the combinatorial delivery of growth factors and RA treatment in order to induce regeneration of an ectopic limb through specific signaling molecules are in progress.



**Figure A. 5: Representative images of whole mount skeletal preparations on ectopic skeletal elements from RA-treated blastemas**

Supplementary table URL:

<http://onlinelibrary.wiley.com/store/10.1002/reg2.14/asset/supinfo/reg214-sup-0001-TableS1.pdf?v=1&s=5c061581220a7d3eec624fd0295ad8817608be12>