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14. ABSTRACT This project proposes novel and unbiased genomic approaches to identify genes associated with salamander limb regeneration. The first objective will extend an ongoing study of the transcriptional basis of limb regeneration in the Mexican axolotl (<i>Ambystoma mexicanum</i>) to three additional salamander species (<i>A. tigrinum</i> , <i>A. maculatum</i> , and <i>Notophthalmus viridescens</i>). Transcripts will be sampled at four times during limb regeneration to characterize temporal changes in abundance that coincide with wound healing and blastema formation. Accomplishment of this objective will enhance an ongoing DOD Multi-Investigator Research Initiative by resolving genes and molecular					
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Report Title

Final Report: Application of Comparative Functional Genomics to Identify Regeneration-Specific Genes

ABSTRACT

This project proposes novel and unbiased genomic approaches to identify genes associated with salamander limb regeneration. The first objective will extend an ongoing study of the transcriptional basis of limb regeneration in the Mexican axolotl (*Ambystoma mexicanum*) to three additional salamander species (*A. tigrinum*, *A. maculatum*, and *Notophthalmus viridescens*). Transcripts will be sampled at four times during limb regeneration to characterize temporal changes in abundance that coincide with wound healing and blastema formation. Accomplishment of this objective will enhance an ongoing DOD Multi-Investigator Research Initiative by resolving genes and molecular pathways for translation to mammalian injury models. The second objective will use quantitative trait locus (QTL) mapping and a newly derived salamander line to identify genes that contribute to variation in limb regeneration. Genotypic and statistical approaches will be used to identify genotype-phenotype associations indicative of QTL for regeneration traits. Accomplishment of this objective will set the stage for identifying loci that explain genetic and developmental sources of variation during limb regeneration. Overall, the project is significant because novel approaches will be used to reveal fundamental regenerative processes that could be engineered in humans to dramatically improve the quality and length of life after injury.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

<u>Received</u>	<u>Paper</u>
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TOTAL:

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

<u>Received</u>	<u>Paper</u>
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TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received Paper

TOTAL:

Number of Manuscripts:

Books

Received Book

TOTAL:

Received

Book Chapter

08/22/2014 1.00 Stephen R Voss, Antony Athipposhy, Ryan M. Woodcock. Transcriptomics using axolotls, Molecular Methods: Springer, (01 2015)

TOTAL: 1

Patents Submitted

Patents Awarded

Awards

Graduate Students

<u>NAME</u>	<u>PERCENT SUPPORTED</u>	Discipline
Nour Al Haj Baddar	0.25	
Alex Palumbo	0.25	
FTE Equivalent:	0.50	
Total Number:	2	

Names of Post Doctorates

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>	National Academy Member
Stephen R Voss	0.05	
FTE Equivalent:	0.05	
Total Number:	1	

Names of Under Graduate students supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>	Discipline
Gareth Voss	0.30	
FTE Equivalent:	0.30	
Total Number:	1	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 0.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 0.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 0.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:..... 0.00

Names of Personnel receiving masters degrees

<u>NAME</u>
Total Number:

Names of personnel receiving PHDs

<u>NAME</u>
Total Number:

Names of other research staff

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
FTE Equivalent:	
Total Number:	

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

Please see attachment below.

Technology Transfer

This project had two primary objectives: (1) Perform a comparative analysis of limb regeneration transcriptomes for 4 salamander species, and (2) Perform a quantitative genetic analysis of limb regeneration using hybrid salamanders. These objectives are novel and designed to identify regeneration-associated genes.

Accomplishments: We accomplished the first objective during the final funding period. In review, we collected 54 tissue samples from regenerating limbs of *A. mexicanum*, *A. andersoni*, and *A. maculatum*. We performed RNA-seq for 36 samples (12 from each species) and then used the 18 samples from *A. mexicanum* as technical replicates for microarray analysis. In the past year, we developed an RNA-seq bioinformatic schema to re-analyze the data in a way that best leverages existing transcript resources for *A. mexicanum*. During the last 5 years, the *A. mexicanum* transcriptome has been deep sequenced. However, there are no existing transcript data for *A. andersoni*, and *A. maculatum*. Thus, we first built a reference transcriptome for *A. mexicanum*, using all available data at Sal-Site and the data generated from this experiment, and then used this as a model for annotating contigs that were generated for *A. andersoni* and *A. maculatum*. Additionally, we built a separate data partition for all 1:1 matches between *A. mexicanum* RNA-seq contigs and probe-sets on the *A. mexicanum* Affymetrix GeneChip. This later dataset will be used to specifically compare results between the two gene expression platforms. This reanalysis of the data was necessary, but it does set back slightly the completion date for manuscripts arising from this work, which should occur before the end of 2014. We did in the last year commit effort to writing a book chapter for an upcoming Regeneration Methods book, to be published by Wiley. Our chapter entitled *Transcriptomics using the axolotl* is based upon the experience that we gained under this funding mechanism and a MURI grant that comprehensively detailed gene expression during the first 28 days of limb regeneration. In our chapter, we describe best practices for performing gene expression analyses using either the Affymetrix GeneChip or RNA-seq. The information contained in this chapter will be an important deliverable to the regeneration community.

The second objective of this project was to perform a limb amputation experiment and then pursue QTL analysis to identify genes that explain variation in rate of limb regeneration. Early in this project we completed a QTL analysis using adult axolotls and a tail regeneration model. The results from this study were published in *PlosOne* and other results from this experiment were published in *Heredity*. This past year, we were unable to make a hybrid cross to perform the limb regeneration QTL analysis...it is not always straightforward to mate different salamander species using *in vitro* fertilization. Thus, we turned our attention to developing a new axolotl model for regenerative biology. As it turned out, this was an incredibly smart decision. Below, I detail some of our early results that justify a grant submission to obtain funding to further exploit this model.

One of the biggest limitations in working with highly regenerative salamander models is the time it takes to rear them to juvenile and adult stages. Developmental rate is longer for axolotls than *Xenopus* and *Danio*, which evolved in warmer

climates. The axolotl embryonic period spans 20-25 days and sexual maturity is reached after 1-1.5 years. There is need for an early stage, axolotl regeneration model that would increase the pace of experimentation, increase efficiency of animal use, and allow more robust experimental designs. While regeneration assays have been established for early stages of the *Xenopus* and *Danio*, no comparable assays have been established for the highly regenerative Mexican axolotl. Conceptually, the development of an early stage assay for the axolotl would reveal the extent to which regeneration programs are conserved among these disparate, highly regenerative models. While it seems likely that some conserved signaling pathways are similar, novel regenerative mechanisms may be unique to these evolutionarily distant, non-mammalian vertebrates.

We developed an early stage tail regeneration assay using axolotl embryos. There are several advantages of this model. First, the assay uses ~20 day old (i.e. post fertilization) embryos that have sufficient yolk reserves to fully regenerate an amputated, distal tail tip in 7 days. The approximately 1 cm embryos are efficiently reared in microtiter plates with out need for feeding. At this stage of development, axolotl embryos do not have capacity to mount an adaptive immune response and the innate immune response is immature. The immature state of the axolotl embryo immune system is hugely advantageous for interpreting gene expression patterns from heterogeneous tissue samples. Generally, the innate immune response involves activation of phagocytic and antimicrobial responses of local and infiltrating cells at the amputation site. The diversity and magnitude of immunological gene expression makes it difficult to measure the expression of key transcription factors and signaling molecules that are generally expressed at low levels. We show below that transcripts for key transcription factors and signaling molecules are robustly estimated using the axolotl tail amputation model. We used the model to screen 40 chemical inhibitors of major signaling pathway and described regenerative outcomes. We then used microarray analysis to explore one of the positive hits, a chemical inhibitor of WNT ligand secretion. Our results show that WNT signaling networks with multiple signaling pathways associated with development and regeneration. We detail our findings in the tables and figures below.

Table 1. Chemicals, targeted pathways, and experimental outcomes. Seven chemicals partially inhibited regeneration and six chemicals completely inhibited tail regeneration. All chemicals were tested at 10 uM unless otherwise noted.

Toxic

Pathway	Chemical	Pathway	Chemical
Wnt	Tideglusib	ATPase	Brefeldin A
Wnt	TWS119	ATPase	Blebbistatin
PI3K	Wortmannin	WNT/GSK3	BIO ³

Complete Regeneration

Pathway	Chemical	Pathway	Chemical
Wnt	ICG-001	Notch	Semagastat
Wnt	KY02111	Notch	MG-132
Wnt	Agonist II SKL 2001	Hedgehog/Smoothed	Vismodegib
TNFa	Lenalidomine	FGFR	Danuserib
MMP	Doxycycline	Apoptosis	NS3694
Stat1	Fludarabine	Translation	Azithromycin

Partial Regeneration

Pathway	Chemical	Pathway	Chemical
Wnt	XAV-939 ¹	EGFR	Gefitinib
Wnt	Agonist I	IL Receptor	Dexamethasone ²
Wnt	Nicotin	FGFR	SU-5402 ⁵
FGFR	BAZD4547		

No Regeneration

Pathway	Chemical	Pathway	Chemical
Wnt	C59 ^{4,5}	TGFB/activin	SB-505124
Wnt	IWR-1-endo	IL6 inhibitor	Luteolin
FGFR	BGJ398	V-ATPase H+ blocker	Concanamycin A ⁶

¹ 40.0 uM; ² 25.0 uM; ³ 15.0 uM; ⁴ 5.0 uM; ⁵ 2.5 uM; ⁶ 1.0 uM

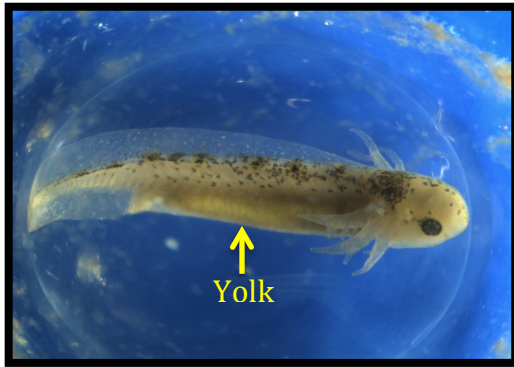


Figure 1A. Picture of a 1.0 cm axolotl embryo (Stage 39). At this stage, embryos can be manually removed from their egg membranes and reared individually in wells of microtiter plates. Thus hundreds to thousands of chemicals could be screened in a single experiment. Note the large yolk reserve in the gut that allows regeneration to be completed before the feeding stage is reached.

Figure 2. Axolotl tail regeneration assay. Axolotls embryos are hatched 1-2 days prematurely and 2 mm of the tail tip is removed with a sterile razorblade. Individuals are photographed before and after tail amputation, and then 5-7 days later. The left panel shows non-amputated, amputated, and regenerated tails. The right panel shows examples where chemical inhibitors completely blocked regeneration. For each inhibitor, no regeneration was observed 7 days post amputation (DPA).



Table 2. We performed a microarray experiment to identify genes that are differentially expressed as a result of blocking WNT ligand secretion using WNT-C59. The table below shows genes that were differently expressed at 48, 72, and 120 hrs post-amputation. The bolded genes were also significant at 168 hrs. These genes identify multiple signaling pathways (WNT, BMP, IL, NGF, MYCN, FGF, HOX, and EGF) associated with a normal regeneration response.

<u>Probeset</u>	<u>Gene ID</u>
axo01743-f_at	APCDD1
axo02472-f_at	HAPLN3
axo04593-f_at	ETV4
axo07001-f_at	BMP2
axo07256-f_at	EPAS1
axo07306-r_at	IL8
axo07473-f_at	AREG
axo07816-f_at	FGF9
axo08059-r_at	LAMB1
axo08282-f_at	NGFR
axo09045-f_at	PHLDA2
axo10046-f_at	DYNC1I1
axo11014-r_at	MYCN
axo12159-f_at	SLC2A1
axo13739-f_at	ANKRD1
axo13755-f_at	DKK2
axo18467-f_at	DUSP6
axo18474-f_at	HOXC8
axo23290-r_at	unknown
axo29791-f_at	FGFR3
axo29801-f_at	AXIN1
axo11014-r_at	MYCN
axo27228-f_at	INHBB
axo29536-f_at	SP7
axo29905-f_at	SP7
axo07974-f_at	INHBB
axo27128-f_at	WNT5A
axo28029-f_at	CYP26B1
axo01779-r_at	TMEM92
axo03170-f_at	PRICKLE2
axo07700-f_at	CTGF
axo07929-r_at	HMOX1
axo08144-f_at	MAS1
axo08178-f_at	MMP3
axo10448-r_at	<u>LHX2</u>

Figure 3. Examples of genes that were differentially expressed as a function of treatment (WNT-59 inhibitor is blue line, DMSO vehicle control is black line). The y-axis shows log₂ expression and the x-axis shows time in hours post amputation. The early disruption of FGF9 transcription at 24 hours post-amputation suggests FGF9 is an early, direct target of WNT signaling. Note that FGF9 steadily increases in expression during normal regeneration. At 48 hrs, transcription of WNT (e.g. *dkk2*) and FGF pathway members is affected, and at 72 hrs, genes associated with retinoic acid signaling (*crabp2*) are transcriptionally altered. As tail outgrowth increases in rate, transcripts for negative regulators of cell proliferation decrease in abundance (e.g. *ctnna2*). Altered expression of *mrc1* at 168 hrs suggests phagocytic responses are not resolved when tail regeneration is inhibited. Many additional key developmental genes are significantly altered in the dataset, including *bmp2*, *hoxc8*, *hoxc10*, *hoxa3*, *hoxa13*, *pparg*, *axin*, *tgfb1*, *wif1*, *spry1*, *etv4*, *dll1*, *klf10*, *smad7*, *bmp7*, *id3*, *areg*, *ctgf*, *rarg*, *mmp3*, *mmp2*, *mmp1*, *cyr61*, *wnt5a*, *pax6*, *ngfr*, *adssl1*, *lep*, *ereg*, *fos*, *egr1*, *cdkn1b*, and many others.

