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Epigenetic Regulation of Limb Regeneration and Radial Excision Repair in the Axolotl
(*Ambystoma mexicanum*)

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Cristian Aguilar

Dissertation Committee:
Professor David M. Gardiner, Chair
Assistant Professor Ali Mortazavi
Professor Xing Dai

2014

DEDICATION

To

all my friends and family who have supported me

but especially to my son, Andon

to whom I dedicate all my life's work and success

Andon, you are my happiness and inspiration
Simply by being, you have brought joy
and fulfillment to my life,
and so I strive to do the same for you.

You don't raise heroes, you raise sons.
And if you treat them like sons,
they'll turn out to be heroes,
even if it's just in your own eyes.
– Walter Schirra Sr

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ACKNOWLEDGMENTS

I would like to thank primarily my advisor, Dr. David M. Gardiner, who has been a source of unwavering support and guidance during this journey to my degree. More alike than we might want to admit, our time and conversations together have given me a model for success in work and in life.

I would like to thank my committee members, Drs. Ali Mortazavi and Xing Dai, as well as Dr. Susan V. Bryant and the entire Bryant/Gardiner lab for stimulating discussion and the necessary assistance to design and implement my experimental work.

In addition, I thank Dr. Barbara A. Burke of the Cal Poly Pomona SEES program for pushing me onto the path I am now travelling. Without her insistence and source of opportunities, I might not find myself where I am today. I also thank Drs. Marlene de la Cruz and Luis Mota-Bravo of the University of California, Irvine Minority Sciences Program for their support. Furthermore, I thank Dr. Nancy E. Buckley for serving as my first research advisor. Her guidance and mentorship have allowed me to develop into the scientist I am now.

CURRICULUM VITAE

Cristian Aguilar

Professional Preparation

2008 – 2014 Ph.D. Biological Sciences	**University of California** Dept. of Developmental and Cell Biology Laboratory of Dr. David Gardiner	**Irvine, CA**
2004-2008 B.S. in Biotechnology. Microbiology & Pathology Concentration, *Cum Laude*	**California State Polytechnic University** Biotechnology Undergraduate in the Laboratory of Dr. Nancy E. Buckley	**Pomona, CA**
2007 Research Internship	**University of California** Amgen Scholars Program Laboratory of Dr. Kunxin Luo	**Berkeley, CA**
2005 – 2006 Research Internship	**California State Polytechnic University** McNair Scholars Program Laboratory of Dr. Nancy E. Buckley	**Pomona, CA**

Fellowships

2013 – 2014	Pedagogical Fellows Program, University of California Irvine
2013 (Fall)	HHMI Graduate Teaching Fellows Program
2013 (Jan) - **2014 (Aug)**	Graduate Assistance in the Areas of National Need (GAANN), US Dept of Education
2010 – 2011	Graduate STEM Fellows in K-12 Education, National Science Foundation

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Teaching Experience

• All TA positions included the following duties: Monitoring an online forum for student discussion, holding a 1hr weekly office hour, composing test questions for lecture, grading exams.

2013 (Fall) **TA, DNA to Organisms**
Duties included: Designing problem based learning worksheets to supplement lecture material meant to encourage collaborative learning amongst the students. Implementing worksheets during three 50min discussion sections weekly. Creating and administering weekly quizzes.

2013 (Winter) **TA, Developmental Biology**

2012 (Summer) **TA, Cell Biology – Guest Lecturer, Stem Cell Biology**
Duties included: Designing problem based learning worksheets to supplement lecture material meant to encourage collaborative learning

amongst the students. Implementing worksheets during a 50min discussion section semiweekly. One guest lecture on the topic of stem cell biology.

2012 (Winter)   **TA, Cell Biology**
Duties included: Designing problem based learning worksheets to supplement lecture material meant to encourage collaborative learning amongst the students. Implementing worksheets during a 50min discussion section twice weekly.

2010 – 2011   **Resident Scientist, Costa Mesa Middle School, Costa Mesa, CA**
During the 2010-2011 academic year, I visited the campus for 15hrs/wk interacting with socioeconomically disadvantaged students with support from the NSF GK-12 fellowship. I worked directly with two 7th grade life sciences teachers to design innovative and interactive lessons within the established curriculum. These lessons exposed the students to college level concepts taught for 7th grade understanding. The lessons also incorporated an instructional lab component to provide the students with practical laboratory experience in addition to a higher level of engagement. As a member of an ethnic minority myself, I was able to show young minority students they too can become scientists.

2010 (Winter)   **TA, Developmental Biology**

Service

2011 – 2014 (Summers)   **Journal Club Leader – Minority Sciences Program – University of California, Irvine**
Led a journal club designed to increase undergraduate students' abilities to process and analyze scientific research. Participants were members of the undergraduate minority biomedical research support program, as well as MARC scholars.

2009 – 2014   **Judge - Orange County Science and Engineering Fair (Judge Captain 2012-2014; Sweepstakes Judge 2013-2014)**

2009 – 2014   **Judge - Intel International Science and Engineering Fair**
In addition, attending science fair project development workshops to assist students in project design/trouble shooting.

2005 – 2008   **Facilitator - Academic Excellence Workshops - California State Polytechnic University, Pomona**
Duties included: Designing problem based learning worksheets to supplement lecture material. Conducting mock midterm and final exams. Facilitating collaborative learning amongst undergraduate students. Attending weekly facilitator development meetings.

2005 – 2008   **Mentor - Science Educational Enhancement Services Undergraduate Mentoring Program - California State Polytechnic University, Pomona**
As a mentor in this program, I met weekly with 2-4 freshman students for 1hr each. I provided guidance to help minority students successfully

navigate college life, including academics and social aspects, in order to achieve their stated goals.

Awards & Honors

2014	Amgen Scholars Alumni Travel Award
2013 – 2014	Graduate Fellow Award, HHMI-UCI Teaching Fellows Program
2011	Honorable Mention (2nd place) – AAAS Annual Meeting Student Poster Competition
2009	Robert Warner Memorial Award for Outstanding Achievement in Nucleic Acid Biochemistry – University of California, Irvine
2007	Paul C. Hiemens Scholarship Award - Science Educational Enhancement Services, California State Polytechnic University, Pomona
2005	Boeing Scholarship - Science Educational Enhancement Services, California State Polytechnic University, Pomona
2004 – 2008	Dean's List – California State Polytechnic University, Pomona

Research Presentations/Meetings

2014-July   Society of Developmental Biology Annual Meeting – Poster Presentation
DNA methylation dynamics regulate the formation of a regenerative wound epithelium during Axolotl limb regeneration.

2014-June   Conference of the Association for Biology Laboratory Education – Poster Presentation
Assessment of the Impacts of BIO SCI D140: How to Read a Science Paper on Student Attitudes towards Biological Research

2014-Feb   Southern California Project Kaleidoscope Regional Network Meeting

2012-Oct   Compact for Faculty Diversity on Teaching and Mentoring

2011-Feb   American Association for the Advancement of Science Annual Meeting – Poster Presentation
Epigenetic Modifications during Dedifferentiation in the Regenerating Axolotl Limb

2007-Aug   Amgen Scholars Program Symposium – Oral and Poster Presentation
The Role of SnoN in the Induction of Cellular Senescence, a Tumor Suppression Mechanism

2007-Jan   California State University Program for Education and Research in Biotechnology – Poster Presentation
In vivo Effects of an Echinacea purpurea Ethanol Extract on Splenocyte Proliferation and Secretion of Cytokines IFN-γ, IL-2, and IL-4

2006-Aug   McNair Scholars Research Symposium – Oral Plenary Presentation
                      In vivo Effects of an Echinacea purpurea Ethanol Extract on
                      Splenocyte Proliferation and Secretion of Cytokines IFN-γ, IL-2,
                      and IL-4

Memberships

- Alliance for Graduate Education and the Professoriate
- Diverse Educational Community and Doctoral Experience (DECADE)
- Golden Key International Honor Society
- Society for the Advancement of Chicanos and Native Americans in Science

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Publications

Aguilar C*, Lee J*, Gardiner DM. (2013) Gain-of-function assays in the axolotl (*Ambystoma mexicanum*) to identify signaling pathways that induce and regulate limb regeneration. In R.G. Gourdie, & T.A. Myers (Eds.), *Wound regeneration and repair*: Humana Press

Aguilar C, Gardiner DM. DNA methylation dynamics regulate the formation of a regenerative wound epithelium during Axolotl limb regeneration. (In preparation)

ABSTRACT OF THE DISSERTATION

Epigenetic Regulation of Limb Regeneration and Radial Excision Repair in the Axolotl
(*Ambystoma mexicanum*)

By

Cristian Aguilar

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2014

Professor David M. Gardiner, Chair

The formation of a blastema during regeneration of an axolotl limb involves important changes in the behavior and function of cells at the site of injury. One of the earliest events is the formation of the wound epithelium and subsequently the apical epidermal cap, which involves *in vivo* dedifferentiation that is controlled by signaling from the nerve. I have investigated the role of epigenetic modifications to the genome as a possible mechanism for regulating changes in gene expression patterns of keratinocytes of the wound and blastema epithelium that are involved in regeneration. I report a modulation of the expression *DNMT3a*, a *de novo* DNA methyltransferase, within the first 72 hours post injury that is dependent on nerve signaling. Treatment of skin wounds on the upper forelimb with decitabine, a DNA methyltransferase inhibitor, induced changes in gene expression and cellular behavior associated with a regenerative response. Furthermore, decitabine-treated wounds were able to participate in regeneration while untreated wounds inhibited a regenerative response. Elucidation of the specific epigenetic modifications that mediate cellular dedifferentiation likely will lead to insights for initiating a regenerative response in organisms that lack this ability. To this end, a novel RNA-seq experiment has been

designed to compare the transcription profiles of tissues that have undergone

dedifferentiation to uninjured tissues and tissues that participate in simple wound healing.

In addition, tissues treated with a DNA methyltransferase inhibitor will be evaluated for

changes in gene expression that are a result of DNA methylation dynamics specifically. As

the mechanisms behind *in vivo* cellular dedifferentiation are elucidated and applied, it will

be important to use an appropriate assay in order to determine the success and extent of

dedifferentiation. I include here a developed protocol designed to assay for cellular

dedifferentiation called the Excisional Repair Model (ERM) model. Briefly, treated cells or

tissues are grafted into a cavity in the lower forearm created by the excision of a segment of

the radius. The ability of the treated cells or tissue to differentiate and replace the missing

bone fragment, and incorporate into the existing bone reveals their regenerative capacity.

CHAPTER 1 - Introduction

This project was designed to provide an understanding of changes in epigenetic patterns that result from the induction of a regenerative response and how these changes contribute to the successful formation of a wound epithelium and blastema. Epigenetic modifications can greatly influence the expression state of associated genes, which in turn dictate the state and identity of cells in an organism. During the development of an organism, cells must transition from a pluripotent state into specific cell types with designated functions. Much effort is being placed into reversing this process by transitioning adult somatic cells into induced pluripotent stem cells. It has become apparent that the pattern of epigenetic modifications within these cells significantly affects the process of inducing pluripotency. The regenerating limb of the axolotl provides a unique circumstance in which adult somatic cells dedifferentiate to a more plastic state *in vivo*. By understanding the epigenetic modifications underlying this dedifferentiation, progress will be made towards inducing a similar response in humans.

Many model systems are available in which to study the control that epigenetics exerts on states of cellular differentiation. Of note are the fields of developmental biology, stem cell biology, and cancer biology. Each of these systems provides a unique view of a common mechanism, and provides useful insights into the details of epigenetic control. For example, histone modification is one such epigenetic mechanism through which gene expression is controlled. The N-terminal tails of histones are susceptible to various forms of modification such as methylation, acetylation, ubiquitination, and phosphorylation [1]. Each of these modifications occurs at specific residues at certain locations within the N-terminal tail. By analyzing the presence of these modifications in the context of gene

expression, a correlation can be ascertained that links the modifications to a particular function. For example, genes that are being expressed may show an enrichment of acetylation on H3K9 or methylation at H3K4. Conversely, transcriptionally repressed genes exhibit an enrichment of methylation at H3K9 and/or H3K27 [2,3]. Studies investigating the chromatin state of hES cells revealed the presence of H3K4 methylation at a large number of genes, yet most of these genes did not produce an active transcript. Closer examination discovered the presence of the repressive H3K27me3 mark at the same regions. The presence of the two marks at a single locus is termed bivalency and results in the associated gene being silenced, but 'primed' for expression [4]. Many key developmental genes exhibit this bivalent chromatin mark in ES cells, but are resolved to either active or repressed states during differentiation depending on the cell fate [2]. These results show the importance of not only expressing pluripotency factors, but also silencing genes that play a role in cellular differentiation.

Within the context of stem cell biology and cellular reprogramming, the state of chromatin in embryonic stem cells (ESCs) has been determined to be hyperdynamic and more 'open' compared to the chromatin of further differentiated daughter cells. [5,6]. Experimental manipulation of chromatin modifying complexes has resulted in large-scale changes in cell behavior, such as in the mouse embryo where members of the Polycomb group complex (involved in establishing histone 3 lysine 27 methylation, a repressive mark of gene transcription) were inactivated leading to defects in cellular differentiation and the eventual death of the embryo [7]. When analyzing the histone modifications of genes associated with cellular differentiation within the developing embryo more closely, it was observed that the promoters often displayed conflicting marks of repression as well as

activation [8,9]. For instance, genes displaying the previously mentioned histone 3 lysine

27 methylation mark of repression might also display methylation of histone 3 lysine 4,

which is a mark of active transcription deposited by members of the Trithorax group of

histone methyltransferases. Often, the genes were silenced, but considered poised for

activation. By removing the repressive mark, the activating mark could be read leading to

the rapid activation of gene transcription, further underscoring the dynamic nature of

chromatin in these cells. Within stem cells, specific factors have been determined to be

important to maintaining the capacity for self-renewal, such as Nanog. Mouse ESC self-

renewal capacity is in part governed by Oct4, which controls the expression of the histone

3 lysine 9 demthylases Jmjd1a and Jmjd2c [10]. Subsequently, the histone demethylases

then regulate the expression of Nanog, highlighting the importance of histone

modifications in stem cell self-renewal abilities. This dynamic interplay must be replicated

during SCR in order to provide increased developmental potential.

Beyond embryonic stem cells, the state of chromatin has been found to be critical to

the success of reprogramming somatic cells to induced pluripotent cells. For several years,

the ability to reprogram cells using a limited number of factors, such as Oct4, Sox2, and

Nanog, has led to large advances in stem cell therapy [11]. However, the efficiency of

reprogramming is often low. In addition, the question arises as to how these few factors

are able to illicit such large changes in cellular behavior. To study this question, the Oct4

interactome was analyzed and several chromatin modifying factors were identified, such as

subunits of the NuRD complex and members of the SWI/SNF group of lysine demethylases

[12,13]. These associations suggest a high level of cooperativity between reprogramming

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transcription factors and chromatin modifying machinery during the process of cellular reprogramming.

In addition to histone modifications, DNA methylation provides another layer of epigenetic regulation of gene expression. DNA methylation occurs predominantly upon the cytosine base within CpG dinucleotides and results in a more compact chromatin structure. The enzymes responsible for the transfer of methyl groups from S-adenosyl-L-methionine to cytosine bases are collectively termed DNA methyltransferases (DNMTs). The two major classes of DNA methyltransferases are implicated in maintenance methylation and *de novo* methylation. DNMT1 is the major maintenance methyltransferase and follows the replication machinery during DNA synthesis. Through a binding preference for hemimethylated DNA [14], DNMT1 attempts to faithfully copy the methylation pattern of parent strands and replicate them on the resultant daughter strands [15]. This process is critical to maintaining patterns of gene expression throughout a population of cells, especially in the context of a tissue or organ. The DNMT3 class of methyltranferases is implicated in the addition of methyl groups to cytosines that were not previously methylated. This process of *de novo* methylation is carried out by DNMT3a and DNMT3b. These enzymes are particularly important during early embryonic development where they play a role in the control of cellular differentiation states [16]. A third *de novo* DNA methyltransferase, DNMT3L, has been identified through conservation of several domains common to DNMTs, but lacks critical methyltransferase motifs and therefore is not able to methylate DNA directly [17]. However, recent evidence suggests that DNMT3L can mediate the activity of DNMT3a and DNMT3b [18], and may aid in the targeting of DNMT3a to specific bases in the genome [19]. Throughout the genome, methylation of cytosine bases

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is often found among the promoter regions of genes in what are termed CpG islands. Methylated cytosines in the promoter region of a gene can interfere with transcription factor binding resulting in silencing of gene expression [20,21], although recent evidence of intergenic methylation suggests a role for DNA methylation in gene expression [22]. Interestingly, genome-wide analysis of gene expression in mouse ES cells lacking all DNA methyltransferases showed increased expression of tissue specific transcription factors and signaling molecules [23]. This suggests that DNMTs play a critical role in controlling the expression of genes associated with various states of differentiation. Furthermore, the addition of DNA methyltransferase inhibitors such as 5-azacytidine, or decitabine, to the media of cells undergoing reprogramming allows for a more complete reprogramming of cells stalled at an intermediate stage of dedifferentiation [24]. Clearly, the process of dedifferentiation or reprogramming is a delicate balance of changes in gene expression mediated by chromatin modifications and dynamics that must be carefully tuned in order to obtain the desired results. When these mechanisms are not regulated appropriately, aberrant cell behaviors may be observed.

The biology of cancer formation and progression provides a window through which we can observe the misregulation of many cellular processes, including chromatin modifications. While the incidence of cancer has long been attributed to the accumulation of mutations leading to the silencing of tumor suppressor genes or the activation of protooncogenes, it is now recognized that tumor suppressor genes are often hypermethylated leading to their inactivation [25,26,27]. While the DNA regions surrounding tumor suppressors are hypermethylated, the levels of 5-methylcytosine within the entire genome of cancer cells are often lower than in non-cancerous tissue [28].

This observation suggests a possible mechanism through which cancerous cells acquire new behaviors and fates, such as the re-initiation of the cell cycle and the ability to differentiate into tissues outside of the regional source of the tumor. Along with the observed changes in DNA methylation levels, the expression of DNA methyltransferases themselves is also modulated in cancer. Often, DNMT expression in increased in various forms of cancer including prostate, ovarian, and breast cancers [29,30]. The changes in DNMT expression must be reconciled with their perceived change in activity or targets in order to determine possible therapies designed to treat these epigenetic abberations.

While the methylation of DNA plays a role in large-scale changes in gene expression, it is still not well understood how these chromatin marks are deposited in a site-specific manner. As DNA methyltransferases themselves are not able to recognize specific DNA sequences, they must rely on the interaction with various other cofactors in order to successfully methylate appropriate bases. Piwi-interacting small RNAs (piRNAs) have been implicated in directing DNA methylation, at least in the context of male gametogenesis [31]. More well studied is the correlation between sites of DNA methylation and post-translational histone modifications. As previously discussed, methylation of histone 3 at lysine 4 is an indicator of active expression, yet the absence of this mark is strongly correlated with DNA methylation [32]. This suggests that DNA to be methylated must first be devoid of active expression marks on histone tails. DNMT3L, in complex with DNMT3a has been found to interact with unmethylated histone tails providing a mechanism through which DNMTs may be targeted to specific regions of the DNA [33]. Furthermore, the PWWP domain of DNMT3a is able to directly interact with histone 3 tails carrying the repressive mark of methylation of lysine 36 [34]. These findings suggest an important

interplay between DNA methyltransferases and histone modifications that results in changes to the pattern of DNA methylation, or further stabilizes previously methylated regions.

Equally important to the addition of methyl groups to cytosines, resulting in likely gene repression, is the loss of methlyation that may result in gene activation. One can easily imagine a mechanism of passive DNA demethylation that could result from a decrease in DNMT1 activity. During replication, daughter DNA strands would not receive the same pattern of DNA methylation leading to a gradual loss of these modifications [35]. This in turn could lead to the reactivation of previously silenced regions of chromatin. This mechanism, while effective, lacks the sequence specificity necessary in order to activate certain subsets of genes in order to modify cellular behavior or states of differentiation. Active DNA demethylation, on the other hand, could provide a mechanism through which only specific bases or regions are targeted for changes in their methylation states. During gametogenesis, widespread demethylation is observed that cannot be attributed to passive demethylation mechanisms [36]. Likewise, demethylation of specific loci is observed in adult neurons and lymphocytes [37,38]. To date, specific DNA demethylase enzymes have yet to be identified, complicating the elucidation of the demethylating mechanism. By studying methylated cytosine residues, it has been discovered that these modified bases can be further modified by TET enzymes that are able to convert 5-methyl cytosine to 5-hydroxymethyl cytosine [39]. After conversion, 5-hydroxymethyl cytosine can then be targeted by base excision repair (BER) machinery leading to its replacement with a completely unmodified cytosine base. This mechanism may be in play in the context of maintaining the self-renewal capacity of embryonic stem cells where the Nanog promoter

is kept in a hypomethylated state by the activity of TET1 [40]. Again, the dynamics of DNA

methylation are evidenced to be involved in the control of cellular states of differentiation.

Each field of investigation, from stem cells and development to cancer cells is likely to yield

information about how epigenetic mechanisms control cellular processes, and how they

might by manipulated for practical and therapeutic applications. Yet, much of this

information is also likely to be context specific. Toward the aim of understanding

regenerative processes across species, we must investigate these conserved mechanisms

within a regenerating species in order to glean important details about how cells

participating in regeneration control changes to the epigenetic patterns and achieve

specificity. When examining the effects of manipulating epigenetic processes, an

interesting question about degrees of cellular potency arises. With each level of

differentiation, the epigenetic landscape changes. It will be important to determine the

effects of various stages of differentiation on epigenetic patterns in order to monitor this

step-wise process. To this end, a model of *in vivo* reprogramming [41] can yield

tremendous insight into therapeutically relevant changes of epigenetic modifications.

Ambystoma mexicanum, more commonly known as the axolotl, is unique among

vertebrate species in that it retains the ability to regenerate many complete body parts as

an adult. This salamander has become the focus of intense research aimed at

understanding the mechanisms of regeneration with the eventual goal of being able to

apply the information gained to improving human therapies. Severe injuries to limbs and

digits are common and can often result in loss of the appendage as a result of the initial

trauma or amputation. Thus, regeneration of limbs specifically is of paramount interest. In

addition to overall regeneration, these detailed studies in the axolotl are aimed at

understanding the developmental potential retained by axolotl cells. The process by which the axolotl regenerates a limb involves the rapid re-epithelialization of the wound site to form a specialized wound epithelium [42]. This wound epithelium responds to signals from a severed nerve that result in the dedifferentiation of basal keratinocytes as evidenced by re-expression of the developmental limb-specific transcription factor Sp9 [43]. The resultant environment instructs the dedifferentiation of cells in the limb stump to form a population of cells collectively termed a blastema. This mass of undifferentiated cells must multiply and will eventually form the missing part of the limb in its entirety [44]. Previous research has shown that blastema cells are mainly derived from connective tissue fibroblasts and therefore represent a population of cells that have the capacity to be reprogrammed into the various cell types present in a limb. The multipotency of blastema cells, as well as their ability to proliferate and maintain an undifferentiated state, lends them to direct comparison with stem cells. The axolotl represents a powerful *in vivo* model of the induction of stem-like qualities in cells that were once differentiated. A greater understanding of the induction of stem cells *in vivo* will aid in furthering the field of regenerative biology. The early dedifferentiation events are critical to the success of regeneration, yet little is know about the underlying cellular and molecular mechanisms. Additionally, investigating gene expression changes after amputation can be confounded by the induction of cellular processes not required for the regenerative response. A gain of function regenerative assay termed the Accessory Limb Model (ALM) involves minimal tissue damage allowing for the analysis of changes in gene expression that are necessary and sufficient to induce complete limb regeneration [44]. Once the earliest events leading to the initiation of regeneration are evaluated, we can look toward the completion of the

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regenerative response, which involves the correct reprogramming of dedifferentiated cells in a coordinated fashion to give rise to a functional and integrated structure. Regulation of the reprogramming process requires tight control in order to acquire the desired outcome [45]. Aberrant cell proliferation leading to expansion without a specific program or pattern is a hallmark of cancer diseases. It is imperative that cells are able to respond appropriately to signals for differentiation [46]. While reprogramming of cells *in vitro* allows for the obtainment of large numbers of cells, it can also cause an accumulation of genetic abnormalities that may compromise the proper engraftment of cells into recipient tissue [45]. The axolotl performs this feat *in vivo* and regenerates perfectly, thus serving as a model for successful dedifferentiation while maintaining the cells in a proper environment for their eventual response to differentiation signals. The chromatin state of axolotl cells during the induction of the limb regenerative response, specifically DNA methylation, has yet to be investigated. Understanding the epigenetic mechanisms controlling this process will yield insight into improved methods for induced pluripotency as well as controlling and maintaining proper differentiation in recipients of stem cell transplants. The axolotl salamander provides such a model in which changes in epigenetic factors are observed, but seemingly controlled in order to result in the proper cellular division and patterning that is a hallmark of true regeneration.

References

1. Jenuwein T, Allis CD. (2001) Translating the histone code. Science. 10;293(5532):1074-80.

2. Tomazou EM, Meissner A. (2010) Epigenetic regulation of pluripotency. Adv Exp Med Biol. 695:26-40.

3. Meissner A. (2010) Epigenetic modifications in pluripotent and differentiated cells. Nat Biotechnol. 28(10):1079-88.

4. Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K, Jaenisch R, Wagschal A, Feil R, Schreiber SL, Lander ES. (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell. 21;125(2):315-26.

5. Meshorer, E. et al. (2006) Hyperdynamic plasticity of chromatin proteins in pluripotent embryonic stem cells. Dev. Cell 10, 105–116

6. Efroni, S. et al. (2008) Global transcription in pluripotent embryonic stem cells. Cell Stem Cell 2, 437–447

7. Surface, L.E. et al. (2010) Polycomb group proteins set the stage for early lineage commitment. Cell Stem Cell 7, 288–298

8. Zhao, X. et al. (2007) Whole-genome mapping of histone H3 Lys4 and 27 trimethylations reveals distinct genomic compartments in human embryonic stem cells. Cell Stem Cell 1, 286–298

9. Bernstein, B.E. et al. (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 125, 315–326

10. Loh, Y-H. et al. (2007) Jmjd1a and Jmjd2c histone H3 Lys 9 demethylases regulate self-renewal in embryonic stem cells. Genes Dev. 21, 2545–2557

11. Takahashi K, Yamanaka S. (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 25;126(4):663-76.

12. Pardo, M. et al. (2010) An expanded Oct4 interaction network: implications for stem cell biology, development, and disease. Cell Stem Cell 6, 382–395

13. van den Berg, D.L.C. et al. (2010) An Oct4-centered protein interaction network in embryonic stem cells. Cell Stem Cell 6, 369–381

14. Pradhan S, Bacolla A, Wells RD, Roberts RJ. (1999) Recombinant human DNA (cytosine-5) methyltransferase. I. Expression, purification, and comparison of de novo and maintenance methylation. J Biol Chem. 274(46):33002-10.

15. Chen T, Li E. (2004) Structure and function of eukaryotic DNA methyltransferases. Curr Top Dev Biol. 60:55-89.

16. Okano M, Bell DW, Haber DA, Li E. (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell. 99(3):247-57.

17. Aapola U, Kawasaki K, Scott HS, Ollila J, Vihinen M, Heino M, Shintani A, Kawasaki K, Minoshima S, Krohn K, Antonarakis SE, Shimizu N, Kudoh J, Peterson P. (2000) Isolation and initial characterization of a novel zinc finger gene, DNMT3L, on 21q22.3, related to the cytosine-5-methyltransferase 3 gene family. Genomics. 65(3):293-8.

18. Chen ZX, Mann JR, Hsieh CL, Riggs AD, Chédin F. (2005) Physical and functional interactions between the human DNMT3L protein and members of the de novo methyltransferase family. J Cell Biochem. 95(5):902-17.

19. Jia D, Jurkowska RZ, Zhang X, Jeltsch A, Cheng X. (2007) Structure of Dnmt3a bound to Dnmt3L suggests a model for de novo DNA methylation. Nature. 449(7159):248-51.

20. Bird A. (2002) DNA methylation patterns and epigenetic memory. Genes Dev. 1;16(1):6-21.

21. Holliday R. (2005) DNA methylation and epigenotypes. Biochemistry (Mosc). 70(5):500-4.

22. Wu H, Coskun V, Tao J, Xie W, Ge W, Yoshikawa K, Li E, Zhang Y, Sun YE. (2010) Dnmt3a-dependent nonpromoter DNA methylation facilitates transcription of neurogenic genes. Science. 23;329(5990):444-8.

23. Tsumura A, Hayakawa T, Kumaki Y, Takebayashi S, Sakaue M, Matsuoka C, Shimotohno K, Ishikawa F, Li E, Ueda HR, Nakayama J, Okano M. (2006) Maintenance of self-renewal ability of mouse embryonic stem cells in the absence of DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b. Genes Cells. 11(7):805-14.

24. Mikkelsen, T.S. et al. (2008) Dissecting direct reprogramming through integrative genomic analysis. Nature 454, 49–55

25. Herman JG, Latif F, Weng Y, Lermann MI, Zbar B, Samid D, et al. (1994) Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. Proc Natl Acad Sci USA. 91:9700–4.

26. Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, et al. (1995) 5′ CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nat Med. 1:686–92.

27. Esteller M, Silva JM, Dominguez G, Bonilla F, Matias-Guiu X, Lerma E, et al. (2000) Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. J Natl Cancer Inst. 92:564–9.

28. Wilson AS, Power BE, Molloy PL. (2007) DNA hypomethylation and human diseases. Biochim Biophys Acta. 1775:138–62.

29. Girault I, Tozlu S, Lidereau R, Bièche I. (2003) Expression analysis of DNA methyltransferases 1, 3A, and 3B in sporadic breast carcinomas. Clin Cancer Res. 9:4415–22.

30. Jin F, Dowdy SC, Xiong Y, Eberhardt NL, Podratz KC, Jiang SW. (2005) Up-regulation of DNA methyltransferase 3B expression in endometrial cancers. Gynecol Oncol. 96:531–8.

31. Aravin AA, Sachidanandam R, Bourc'his D, Schaefer C, Pezic D, Toth KF, Bestor T, Hannon GJ. (2008) A piRNA pathway primed by individual transposons is linked to de novo DNA methylation in mice. Mol Cell. 31(6):785-99.

32. Meissner A, Mikkelsen TS, Gu H, Wernig M, Hanna J, Sivachenko A, Zhang X, Bernstein BE, Nusbaum C, Jaffe DB, Gnirke A, Jaenisch R, Lander ES. (2008) Genome-scale DNA methylation maps of pluripotent and differentiated cells. Nature. 454(7205):766-70.

33. Ooi SK, Qiu C, Bernstein E, Li K, Jia D, Yang Z, Erdjument-Bromage H, Tempst P, Lin SP, Allis CD, Cheng X, Bestor TH. (2007) DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA. Nature. 448(7154):714-7.

34. Dhayalan A, Rajavelu A, Rathert P, Tamas R, Jurkowska RZ, Ragozin S, Jeltsch A. (2010) The Dnmt3a PWWP domain reads histone 3 lysine 36 trimethylation and guides DNA methylation. J Biol Chem. 285(34):26114-20.

35. Jones PA, Taylor SM. (1980) Cellular differentiation, cytidine analogs and DNA methylation. Cell. 20(1):85-93.

36. Morgan HD, Santos F, Green K, Dean W, Reik W. (2005) Epigenetic reprogramming in mammals. Hum Mol Genet. 14 Spec No 1:R47-58.

37. Ma DK, Jang MH, Guo JU, Kitabatake Y, Chang ML, Pow-Anpongkul N, Flavell RA, Lu B, Ming GL, Song H. (2009) Neuronal activity-induced Gadd45b promotes epigenetic DNA demethylation and adult neurogenesis. Science. 323(5917):1074-7.

38. Bruniquel D, Schwartz RH. (2003) Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process. Nat Immunol. 4(3):235-40.

39. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L, Rao A. (2009) Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science. 324(5929):930-5.

40. Ito S, D'Alessio AC, Taranova OV, Hong K, Sowers LC, Zhang Y. (2010) Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. Nature. 466(7310):1129-33.

41. Odelberg SJ. (2002) Inducing cellular dedifferentiation: a potential method for enhancing endogenous regeneration in mammals. Semin Cell Dev Biol. 13(5):335-43.

42. Endo T, Bryant SV, Gardiner DM. (2004) A stepwise model system for limb regeneration. Dev Biol. 1;270(1):135-45.

43. Satoh A, Graham GM, Bryant SV, Gardiner DM. (2008) Neurotrophic regulation of epidermal dedifferentiation during wound healing and limb regeneration in the axolotl (Ambystoma mexicanum). Dev Biol. 15;319(2):321-35.

44. Satoh A, Gardiner DM, Bryant SV, Endo T. (2007) Nerve-induced ectopic limb blastemas in the Axolotl are equivalent to amputation-induced blastemas. Dev Biol. 1;312(1):231-44.

45. Forsberg M, Hovatta O. (2012) Challenges for the Therapeutic use of Pluripotent Stem Derived Cells. Front Physiol. 3:19.

46. Oviedo NJ, Beane WS. (2009) Regeneration: The origin of cancer or a possible cure? Semin Cell Dev Biol. 20(5):557-64.

CHAPTER 2 – DNA Methylation Dynamics Control Regenerative Wound Healing

The remarkable ability of stem cells to be programmed to a specific fate will eventually allow many injuries and diseases to be treated more effectively, or even cured. In an effort to generate large amounts of stem cells, researchers are focused on somatic cell reprogramming (SCR) which will lead to the generation of stem cells from patients' own somatic cells [1]. This strategy may prevent complications associated with immune rejection such as graft vs. host disease and loss of transplanted tissue. Currently, the process of SCR is highly variable and inefficient [2]. Recent evidence has suggested that the epigenetic modifications of the genome of differentiated somatic cells are a type of 'barrier' to the reprogramming process [3,4]. Understanding the signals that regulate epigenetic patterns associated with pluripotency during development (e.g. embryonic stem cells, ES cells) and dedifferentiation during regeneration will provide insights for the design of more efficient and reliable methods of SCR. Future SCR techniques likely will involve inducing specific patterns of epigenetic modifications mimicking that of the desired state of developmental potency [5].

Epigenetic regulation of gene expression encompasses a wide array of processes such as chromatin remodeling to increase/decrease gene accessibility, recruitment of activators or repressors of transcription, and methods of translational repression such as microRNAs [6]. The best characterized of these are post-translational modifications of histone tails and DNA methylation. Methylated cytosines in the promoter region of a gene can interfere with transcription factor binding resulting in silencing of gene expression [7,8]. In addition, recent evidence of intergenic methylation suggests a role for DNA

methylation in gene expression [9]. Two main categories of DNA methylation mechanisms have been identified: maintenance methylation and *de novo* methylation. DNA methyltransferase1 (DNMT1) is responsible for maintenance methylation in which the pattern of CpG methylation is faithfully transmitted from a parent cell to a daughter cell during division [10]. The DNMT3 group of methyltransferases performs *de novo* methylation in which previously unmethylated cytosines are modified, resulting in changes in gene expression. These enzymes play key roles in embryonic development [11] and cellular differentiation [12,13,14,15], being highly expressed in undifferentiated cells (e.g. ES cells) and subsequently down regulated in cells as they differentiate. ES cells deficient in these enzymes are able to retain their pluripotent state, but are unable to differentiate unless DNMT function is restored [6,16]. On the other hand, mouse ES cells lacking all DNMTs showed increased expression of tissue specific transcription factors and signaling molecules [17], indicating that epigenetic modifications are also required to repress differentiation and maintain ES cells in an undifferentiated state.

Taken together, these findings demonstrate the important function of epigenetic modifications on the regulation of cell fate and developmental potency. During embryonic development, the epigenome changes as cells become increasing differentiated and lose developmental plasticity. The success of regenerative therapies involving induced dedifferentiation and increased plasticity likely will depend on the ability to regulate these epigenetic changes. Rather than reprogramming differentiated cells to a pluripotent state (e.g. ES-like cells), it would be more efficient to reverse the epigenetic program to the point of generating a population of lineage-specific progenitor cells (e.g. undifferentiated connective tissue progenitor cells that could regenerate cartilage, bone, ligaments and

tendons). To do this, it would not be necessary to reprogram cells to a state of complete pluripotency [18], but rather to an intermediate stage of multipotency that retains the epigenetic marks that function to stabilize the developmental state of the desired progenitor cell. By changing only the necessary epigenetic marks, the process of dedifferentiation and eventual re-differentiation of reprogrammed cells could be regulated.

Urodele amphibians such as the axolotl (*Ambystoma mexicanum)* are unique among adult vertebrates in that they are able to regenerate lost body structures perfectly, restoring previous structure and function. The success of axolotl limb regeneration is dependent on the formation of a blastema, which is structurally and functionally equivalent to a limb bud in the embryo [19]. In turn, blastema formation is dependent on signaling from a nerve that recruits undifferentiated mesenchymal cells that interact with the overlying wound epithelium [20,21]. The function of this wound epithelium is dependent on signals from the regenerating nerve that induce dedifferentiation of basal keratinocytes to form the apical epithelial cap (AEC), which is functionally equivalent to the AEC of developing amphibian limb buds and the Apical Ectodermal Ridge (AER) of developing amniote embryos. Basal keratinocytes of all three structures (blastema AEC, limb bud AEC and AER) express the transcription factor *Sp9* [21] that is involved in the regulation of FGF signaling. During regeneration, signaling from the AEC is required for dedifferentiation of cells in the limb stump (e.g. connective tissue fibroblasts) and activation of stem cells (e.g. muscle satellite cells to give rise to myoprogenitor cells), leading to the formation of a blastema [19,22]. Cells of the blastema multiply and eventually differentiate to reform the missing parts of the limb [23].

In humans, injuries to limbs and digits are common and can result in loss of the appendage as a result of the initial trauma or surgical amputation [24]. The axolotl has become the focus of research aimed at understanding the mechanisms of regeneration with the goal of being able to apply that knowledge to guide translational research to develop regenerative therapies for humans [25]. Studies from animals such as the axolotl, have demonstrated that the early nerve-dependent process leading to formation of the AEC are critical to the success of regeneration, yet little is know about the underlying cellular and molecular mechanisms regulating this process. Formation of the AEC involves re-expression of embryonic genes (e.g. *Sp9*) and re-acquisition of the ability to support blastema cell proliferation (comparable to the limb bud AEC/AER). We therefore hypothesized that nerve signaling mediates epigenetic modifications of the wound epithelium resulting in dedifferentiation of the basal keratinocytes and formation of the AEC [20,26]. We report that DNMT3a expression is regulated by nerve signaling, and that experimental manipulation of DNMT3a activity can induce a regenerative response in wounds that normally would not regenerate in the axolotl. We thus have identified a source of signaling that functions to regulate epigenetic modifications associated with the initial blastema formation leading to limb regeneration.

Results

Amputation of a salamander limb has long been the model for regeneration studies. In recent years, we have developed and optimized an alternative regeneration model, the Accessory Limb Model (ALM), which allows us to discern regeneration-specific signals that are distinct from the generalized injury signals triggered by the massive trauma of

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amputation [20,23]. The ALM is based on the discovery that a full-thickness skin wound on the side of the upper forelimb can be induced to form an ectopic blastema in response to signaling from a surgically deviated nerve [20,27]. This ectopic blastema is structurally and functionally equivalent to an amputation-induced blastema, and can be induced experimentally to form a well-patterned ectopic limb [20,23]. In the present study, we have used the ALM to identify and characterize changes in DNA methylation at the early stages of regeneration that are specifically associated with the response of the early wound epithelium (WE) to signaling from the nerve.

We initially screened the *Ambystoma* EST database (http://www.ambystoma.org/genome-resources/5-gene-and-est-database) for expression of genes associated with epigenetic regulation. In addition to a number of genes encoding for histone modifying enzymes, two axolotl orthologs of human DNA methyltransferases (DNMT1 and DNMT3a) were identified. Based on an initial PCR screen for changes in the level of expression of these genes during stages of regeneration, we identified DNMT3a as a candidate gene for nerve-dependent epigenetic modifications during axolotl limb regeneration.

DNMT3a expression is induced in regenerating tissues of the axolotl limb
As an initial characterization of epigenetic modifications associated specifically with blastema formation, we quantified changes in the global level of DNA methylation in the blastema WE and mesenchyme relative to uninjured skin and muscle tissues. Ectopic blastemas (day 10 post-surgery) that were equivalent to early bud – medium bud blastemas that form on an amputated limb were collected, and global DNA methylation was

analyzed separately for blastema epithelial and mesenchymal cells (Figure 2.1A). Although the level of DNA methylation of the blastema WE was increased relative to the uninjured skin, this difference was not statistically significant. The level of global DNA methylation of the blastema mesenchymal cells was significantly increased compared to stump muscle and uninjured skin. Since approximately 50% of the early blastema mesenchymal cells are derived from dermal fibroblasts of the uninjured skin [28], the approximately 4% increase in the level of methylated cytosines associated with the transition from dermal cell to blastema cell suggests a regeneration-specific role for DNA methyltransferases in blastema formation.

Much of the increase in global DNA methylation of blastema cells appeared to be a consequence of increased levels of DNMT3a expression. Expression of both DNMT1 and DNT3a was increased in ectopic blastemas, but only DNMT3a expression appeared to be regeneration-specific (Figure 2.1B, C). The expression of DNMT3a, a *de novo* DNA methyltransferase, was significantly higher in both the WE and mesenchyme of early/medium-bud ectopic blastemas relative to the uninjured control tissues (Figure 2.1B). Furthermore, blastema mesenchyme cells expressed DNMT3a at levels there were comparable to developing limb bud cells. Although DNMT1, a maintenance methyltransferase, was expressed at a higher level relative to stump muscle tissues, its level of expression was not significantly different between ectopic blastema tissues and uninjured, full-thickness skin (Figure 2.1C). Since expression of DNMT1 is associated with cell division, an increased level of expression would be expected in the skin since it is a continuously proliferating tissue. The highest levels of DNMT1 expression were measured in developing limb bud cells that have a high rate of proliferation. Taken together, these

data suggested that the upregulation of DNMT3a expression could account for the increase in global methylation levels of cells of the blastema as compared to the progenitor cells in the uninjured skin. Thus, we hypothesized that *de novo* methylation leading to new cytosine methylation sites within the genome of cells of a regenerating limb is linked to dedifferentiation and blastema formation.

Expression of DNMT3a is modulated by nerve signaling

The success of regeneration is dependent on the progression through multiple steps that are dependent on signaling from nerves [20]. One of the earliest steps requires nerve signaling in order for the keratinocytes of the WE to dedifferentiate and acquire the signaling properties of the AEC this is required for blastema formation [21,26]. To determine whether changes in *de novo* DNA methylation are associated with the transition from an early WE to an AEC, we analyzed the expression of DNMT3a in keratinocytes of the WE/AEC during the first 10 days of ectopic blastema formation. Expression of DNMT3a in a WE with a deviated nerve increased significantly (about 4-fold) compared to expression in the uninjured skin (Figure 2.2). Expression was highest at 72 hours after initial wounding and declined slightly over the next 7 days. Surprisingly, DNMT3a expression increased dramatically in a WE that did not have a surgically deviated nerve (19-fold compared to uninjured skin; more than 4-fold greater than in a WE with a deviated nerve). This difference between wounds that did or did not have a deviated nerve was transient such that there was no difference in the level of DNMT3a expression at either 24 hours or 10 days post injury. Therefore wounds without a deviated nerve that regenerated the skin, but did not form ectopic blastemas, formed a WE that expressed DNMT3a transiently at high

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levels compared to wounds with a WE that was induced by nerve signaling to form an AEC and an ectopic blastema.

Wounds without a deviated nerve were created on limbs that were innervated, and consequently there was a low level of innervation of the wound even though a nerve was not surgically deviated [21]. In order to eliminate the influence of nerve signaling entirely, we surgically denervated limbs proximally at the brachial plexus, and then made skin wounds that did or did not have a surgically deviated nerve distally. For both treatments, expression of DNMT3a was not detected at either early (24 hours post-wounding) or late (5 or 10 days post-wounding) time points. There was a small, transient increase (2-fold) increase at the 72 hour time point (Figure 2.2). Since a nerve that has been denervated proximal and deviated distally does not rescue induced DNMT3a expression, we assume that the signaling that regulated DNMT3a expression was associated with viable nerves and not other cells associated with the nerve (e.g. Schwann cells). Both too high and too low a level of DNMT3a expression was associated with the failure to form a blastema, which is consistent with an hypothesis that AEC function is specifically regulated by quantitative nerve signaling, perhaps mediated by specific levels of DNMT3a activity.

Inhibition of DNMT activity induces *Sp9* expression

Increased nerve signaling from a deviated nerve is required for AEC formation [21,26] and is associated with downregulation of DNMT3a expression (this study). We therefore tested whether nerve-independent downregulation of DNMT3a activity would be sufficient to induce AEC formation. To do this, we used 5-aza-2'-deoxycytidine, also known as decitabine, to inhibit DNMT activity through its inability to receive the addition of a methyl

group to the carbon at the 5 position of cytidine when incorporated into DNA during the S phase of the cell cycle. Sol-gel beads (1mm in diameter) that contained either decitabine or 2'-deoxycytidine were synthesized and grafted under the wound epithelium of lateral wounds 24 hours after the initial surgery to create the full-thickness skin wounds.

To assay for a regenerative response associated with the inhibition of DNMT activity, we quantified the expression of *Sp9*, a marker gene for the basal keratinocytes of the AEC [21,26]. *Sp9* is a zinc finger transcription factor that is expressed in the AER of developing limb buds and is re-expressed in the AEC during limb regeneration. In response to signaling from a surgically deviated nerve, *Sp9* expression is induced throughout the WE within 24 hours after wounding, and becomes localized to the basal keratinocytes of the AEC 72 hours after the initial injury. In contrast, *Sp9* expression is not detected in wounds without a deviated nerve at 72 hours after injury [21]. We therefore grafted beads either with or without decitabine into wounds that did not have a surgically deviated nerve. Wounds that received a grafted decitabine bead re-expressed *Sp9* in the absence of a deviated nerve (Figure 2.3) at a statistically higher level than wounds that received a control bead (2'-deocycytidine). Although the level of *Sp9* expression was lower than for nerve-deviated wounds, the disparity was not statistically different, and presumably was a consequence of the need to have decitabine incorporated into the replicating DNA of the target cells of the WE in order to inhibit DNA methylation. As reported previously, *Sp9* expression was not detected in wounds without either a grafted bead or a deviated nerve; however, there was a low level increase in *Sp9* expression in control wounds receiving 2'-deoxycytidine beads. This level of expression was significantly lower than both the nerve-deviated and the decitabine-treated wounds, and may have been a consequence of

reinjuring the wound at 24 hours when the bead was grafted. Although decitabine treatment induced expression of a gene associated with formation of WE/AEC, it did not induce formation of an ectopic blastema. Therefore it appears that downregulation of DNMT activity is not sufficient to induce blastema formation, and that additional signaling pathways are involved in the early stages of regeneration.

Inhibition of DNMT activity delays reformation of the basal lamina of the WE

Skin wounds without a deviated nerve, as well as the amputation wounds of denervated limbs, reform a basal lamina within a few days of injury. In contrast, the basal lamina underlying the AEC of both nerve-induced ectopic blastemas and amputation-induced blastemas does not reform until the end of regeneration [20,21,29]. Taken together, these observations have led to the hypothesis that the presence of a basal lamina inhibits signaling between the AEC and blastema mesenchyme, and therefore blastema formation and outgrowth only can occur if reformation of the basal lamina is inhibited [29]. This regulation of basal lamina regeneration is hypothesized to be mediated by interactions between the nerve and the basal keratinocytes of the AEC [26].

To assay for a regenerative response associated with the inhibition of DNMT activity, we examined wounds that had been treated with decitabine for the presence of a basal lamina. We used trichrome staining (blue) to visualize the thin collagen layer beneath the basal keratinocytes of the WE as it reformed the basal lamina. Limbs with wounds were collected 6 days after the initial surgery to create a wound (5 days after bead grafting), sectioned, and stained for the presence of the basal lamina. The presence or absence of the basal lamina underlying the WE is best visualized at the border of the wounds where

comparison can be made with the presence of the basal lamina and dense collagen fibers of the dermis beneath the uninjured skin. As reported previously [20], wounds without a deviated nerve or a grafted bead reformed a basal lamina (Figure 2.4 A, B); whereas, nerve-deviated wounds did not reform the basal lamina underneath the WE/AEC (Figure 2.4 E, F). Control wounds that received 2'-deoxycytidine bead grafts reformed the basal lamina (Figure 2.4 C, D), and appeared similar to lateral wounds without a deviated nerve (Figure 2.4 A, B). In contrast, wounds that received decitabine beads did not reform the basal lamina (Figure 2.4 G, H), and appeared similar to nerve-deviated wounds (Figure 2.4 E, F). Although reformation of the basal lamina was delayed, an ectopic blastema did not form, indicating that downregulation of DNMT activity is associated with but not sufficient for blastema formation. At this point we cannot determine whether the observed delay in basal lamina reformation is a consequence of the downregulation of DNMT activity in the WE/AEC, the underlying mesenchymal cells of the stump, or both.

Decitabine treated wound epithelia participate in blastema formation

Re-expression of *Sp9* and the delay of basal lamina formation are both hallmarks of the early stages of regeneration, and both appear to be mediated by interactions between the nerve and the WE. In order to test the hypothesis that the regulation of DNMT activity by the nerve in turn regulates the pro-regenerative activity of the WE, we assayed for the ability of a grafted WE/AEC to participate in blastema formation. Full-thickness skin wounds were created on the anterior side of the upper arm, and either 2'-deoxycytidine beads or decitabine beads were grafted 24 hours later (Figure 2.5A). Six days after initial injury (five days after bead grafting) the wounds were collected such that there was a

border of previously uninjured skin surrounding the origin wound, and were grafted onto a new host wound along with a surgically deviated nerve. The graft was positioned such that the WE/AEC that covered the original wound (6 days earlier) was localized above the deviated nerve. In the absence of a graft, the new host wound would heal and form a functional WE/AEC and an ectopic blastema. The grafted WE/AEC with uninjured skin prevented the host wound from forming a new WE/AEC, and we therefore tested whether or not the grafted WE/AEC could respond to the deviated nerve and function in formation of an ectopic blastema.

Although control and decitabine-treated wounds appeared similar six days after the initial wounding, most of the decitabine-treated wounds participated in blastema formation when grafted to a nerve-deviated host wound (4 of 6); whereas, control wounds did not (Figure 2.5, Table 2.1). In some host wounds, a blastema formed in a gap between the peripheral margin of the grafted WE/AEC and host (a "secondary blastema'). In one limb, there was a tear in the grafted WE/AEC of a control wound that re-healed and formed a new WE/AEC that participated in blastema formation (Table 2.1). The ectopic blastemas formed with decitabine treated WE/AEC developed to the medium bud stage before regressing, which is what occurs with nerve-induced blastemas [20]. The overall time-course of blastema formation and regression was similar to that of ectopic blastemas as reported previously [20]. Thus down regulation of DNMT activity is not sufficient to induce ectopic blastema formation, but is sufficient to maintain the WE/AEC in a regeneration-competent state (consistent with the induced expression of *Sp9*) so as to be able to participate in ectopic blastema formation when grafted. At this point we cannot determine whether the keratinocytes of the WE/AEC are the direct targets of DNMT activity;

nevertheless, the experimental downregulation of DNMT activity allows the WE/AEC to respond to the signals that control the early stages of regeneration leading to blastema formation.

Discussion

In this paper we provide evidence that *de novo* DNA methylation is regulated in part by nerve signals during the early stages of limb regeneration. Specifically, expression of DNMT3a in keratinocytes of the WE was down regulated in response to increased nerve signaling (the presence of a surgically deviated nerve), and was associated with the transition of the WE to the AEC of the early blastema. When DNMT activity was inhibited experimentally by implanting beads that release decitabine, re-expression of *Sp9* was induced, and reformation of the basal lamina was delayed. Both of these phenomena are hallmarks of the early stages of regeneration, and both appear to be mediated by interactions between the nerve and the WE/AEC [21,26]. Our discovery that decitabine-treated WE/AEC can participate in early blastema formation is consistent with the hypothesis that nerve signals regulate *de novo* DNA methylation in keratinocytes of the WE leading to formation of the functional AEC required for blastema formation.

The formation of a functional WE/AEC during the early stages of regeneration is required for progression to the subsequent steps of the regeneration cascade [20,30]. The lack of this specialized structure or the surgical replacement with full thickness skin prevents the regenerative response, even in the presence of nerve signaling [31]. The WE/AEC is a relatively simple structure composed of a few layers of keratinocytes along with interspersed Leydig cells. Of these cells, the basal layer of keratinocytes that are

adjacent to the underlying mesenchymal cells appear to function in promoting blastema formation and outgrowth [21,26]. We therefore hypothesize that nerve signals target the basal keratinocytes so as to regulate *de novo* DNA methylation associated with dedifferentiation and blastema formation. Given the ability of this population of cells to respond to nerve signals and to serve subsequently as a signal center for proliferation of the underlying blastema mesenchymal cells identifies them as important for further research.

The variable response of DNMT3a expression to different amounts of nerve signaling could be related functionally to the well-documented phenomenon of a neurotrophic threshold for regeneration [32]. Regeneration is dependent on a threshold number of nerves being present in the stump, and regeneration fails to occur below that threshold [32,33]. The ALM demonstrates this phenomenon in that denervated limbs fail to regenerate (no nerves), wounds without a deviated nerve do not make a blastema (below the threshold), and wounds with a deviated nerve make an ectopic blastema (above the threshold). The variable levels of DNMT3a expression mirrored this regenerative response to variation in the level of innervation. In the absence of nerves, DNMT3a was not expressed and regeneration failed to occur. In a normal skin wound, there was innervation by sensory nerves [21], DNMT3a was expressed at high levels, and no blastema formed. In response to a deviated nerve (increased levels of nerve signaling), DNMT3a expression increased to moderate levels and a blastema formed. Thus DNMT3a expression appeared to be required for blastema formation, but the increased level of expressed must be modulated either by endogenous signals from nerves. Because the experimental inhibition of DNA methylation by decitabine can induce some of the responses induced by nerve

signals, this modulation could be quantitative (just enough and not too much *de novo*

methylation). Since decitabine treatment did not induce nerve-independent ectopic

blastema formation, there must be additional signals from the nerve that are required for

blastema formation. Therefore DNMT3a modulation also could be qualitative such that

nerve signals target DNMT3a to specific regions of DNA by controlling the expression of

cofactors that convey specificity to DNMT3a activity.

The role of DNA methylation in the regulation of limb regeneration has not been

investigated previously. DNA methylation has long been recognized as a key regulatory

mechanism of gene expression during development, cancer progression, and the control of

differentiation states in stem cells [12,13,14,15,34,35]. Therefore, it is not surprising that

DNA methylation also functions to control cell behavior and fate decisions during

regeneration as indicated by the results from the present study. The ALM has allowed us to

manipulate levels of nerve signaling experimentally in order to identify DNMT3a

expression and DNA methylation as early critical events in the regeneration cascade. The

cells of the WE/AEC respond to nerve signaling between 24 hours and 72 hours, and this

well-defined window of time provides an experimental model to identify upstream

regulation of functionally important epigenetic modifications. Future identification of the

subset of genes that are directly affected by changes in their methylation states during

blastema formation will provide insights into future therapies aimed at enhancing the

human body's inherent, although limited, regenerative responses.

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.

Animals

Experiments were performed on white and wild-type axolotls (*Ambystoma mexicanum*) measuring 12-15 cm snout to tail tip that were spawn at the University of California Irvine or at the Ambystoma Genetic Stock Center at the University of Kentucky. The animals were maintained in 40% Holtfreter's solution and were anesthetized prior to all procedures in a 0.1% solution of MS222 (Ethyl 3-aminobenzoate methanesulfonate salt, Sigma), pH 7.4.

Surgical Procedures

The technique for inducing a regenerative response from wounds on the side of the limb has been described in detail previously [20,23]. Briefly, full-thickness skin wounds on the anterior side of the limb were created by surgically removing a square of skin (3-4 mm on a side) from the anterior side of the stylopod (region of the humerus/femur), making sure that the underlying muscle was not damaged. The brachial nerve then was deviated surgically beneath the skin to bring the cut end of the nerve to the center of the skin wound. Microcarrier beads were implanted into the wound site 24 hours after the initial surgery by making a small incision through the uninjured skin proximal to the wound site. Forceps were used to create a tunnel under the wound epithelium, and a bead was inserted into the center of the wound site. Tissue samples for analysis of *Sp9* transcription were

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collected 72 hours after the initial surgery to create the wound (48 hours after bead implantation). Samples for histological analysis were collected 6 days post wounding. For the experiment testing whether or not the wound epithelium was permissive for regeneration, wounds were created and beads were implanted into wounds 24 hours after the initial surgery. These wounds were allowed to heal for 6 days, after which the graft was collected by surgically removing a piece of full-thickness skin that contained the original would in the center. The graft was placed into a host wound with a deviated nerve such that the severed end of the nerve was localized beneath the wound epithelium from the original wound. The full-thickness skin surrounding the original wound epithelium healed into place adjacent to the skin of the host wound site.

Bead Synthesis

Sol-gel beads were synthesized as described in [36]. Briefly, a solution of tetramethyl orthosilicate (TMOS) and methanol was mixed with either Decitabine or 2'-deoxycytidine solutions to yield a final concentration of 1μM. This solution was then pipetted in 1 μL volume drops onto a sheet of parafilm. The drops flattened when first pipetted such that they formed a hemispherical bead with a diameter of 1 mm. The beads were allowed to harden for 3 days at room temperature in a fume hood, and dried at 37°C overnight prior to grafting.

Histology

Samples were collected 6 days after the initial surgery to create the wound, fixed in 4% PFA, and embedded in OCT for cryosectioning. Sections were stained using Mallory's triple stain for collagen and counterstained with Hematoxylin.

Quantification of global DNA methylation

DNA was isolated from tissues using the Invitrogen Trizol® reagent. Methylation levels were assayed using the Epigentek Methylamp™ Global DNA Methylation Quantification Ultra Kit following the manufacturer's protocol.

Analysis of gene expression by qPCR

Blastema tissue samples (wound epithelium and mesenchyme) were collected from ectopic blastemas that developed on the anterior side of the arm 10 days after creating wounds with deviated nerves (surgical details described above). Samples were collected by making surgical incisions through the full thickness skin surrounding the ectopic blastema. The excised skin, including the wound epithelium was lifted from the blastema, and the border of mature skin was trimmed away from the wound epithelium. The mesenchymal portion of the blastema was then collected by surgically excising it from the side of the arm. Samples were placed in Trizol® reagent and homogenized using a 20-gauge needle and syringe. RNA was isolated after chloroform induced phase separation, and purified using the Machery-Nagel NucleoSpin® RNA XS kit. cDNA synthesis was performed with the Roche Transcriptor First Strand cDNA Synthesis kit. Roche SYBR green reagent was used for PCR quantification.

References

1. Takahashi K, Yamanaka S. (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 126(4):663-76.
2. Forsberg M, Hovatta O. (2012) Challenges for the Therapeutic use of Pluripotent Stem Derived Cells. Front Physiol. 3:19.
3. Bao S, Tang F, Li X, Hayashi K, Gillich A, Lao K, Surani MA. (2009) Epigenetic reversion of post-implantation epiblast to pluripotent embryonic stem cells. Nature. 461(7268):1292-5.
4. Ang YS, Gaspar-Maia A, Lemischka IR, Bernstein E. (2011) Stem cells and reprogramming: breaking the epigenetic barrier? Trends Pharmacol Sci. 32(7):394-401.
5. Artyomov MN, Meissner A, Chakraborty AK. (2010) A model for genetic and epigenetic regulatory networks identifies rare pathways for transcription factor induced pluripotency. PLoS Comput Biol. 6(5):e1000785.
6. Tomazou EM, Meissner A. (2010) Epigenetic regulation of pluripotency. Adv Exp Med Biol. 695:26-40.
7. Bird A. (2002) DNA methylation patterns and epigenetic memory. Genes Dev. 16(1):6-21.
8. Holliday R. (2005) DNA methylation and epigenotypes. Biochemistry (Mosc). 70(5):500-4.
9. Wu H, Coskun V, Tao J, Xie W, Ge W, Yoshikawa K, Li E, Zhang Y, Sun YE. (2010) Dnmt3a-dependent nonpromoter DNA methylation facilitates transcription of neurogenic genes. Science. 329(5990):444-8.
10. Sen GL, Reuter JA, Webster DE, Zhu L, Khavari PA. (2010) DNMT1 maintains progenitor function in self-renewing somatic tissue. Nature. 463(7280):563-7.
11. Meissner A. (2010) Epigenetic modifications in pluripotent and differentiated cells. Nat Biotechnol. 28(10):1079-88.
12. Maatouk DM, Kellam LD, Mann MR, Lei H, Li E, Bartolomei MS, Resnick JL. (2006) DNA methylation is a primary mechanism for silencing postmigratory primordial germ cell genes in both germ cell and somatic cell lineages. Development. 133(17):3411-8.
13. Epsztejn-Litman S, Feldman N, Abu-Remaileh M, Shufaro Y, Gerson A, Ueda J, Deplus R, Fuks F, Shinkai Y, Cedar H, Bergman Y. (2008) De novo DNA methylation promoted by G9a prevents reprogramming of embryonically silenced genes. Nat Struct Mol Biol. 15(11):1176-83.
14. Hu B, Gharaee-Kermani M, Wu Z, Phan SH. (2010) Epigenetic regulation of myofibroblast differentiation by DNA methylation. Am J Pathol. 177(1):21-8.
15. Challen GA, Sun D, Jeong M, Luo M, Jelinek J, Berg JS, Bock C, Vasanthakumar A, Gu H, Xi Y, Liang S, Lu Y, Darlington GJ, Meissner A, Issa JP, Godley LA, Li W, Goodell MA. (2011) Dnmt3a is essential for hematopoietic stem cell differentiation. Nat Genet. 44(1):23-31.

16. Jackson M, Krassowska A, Gilbert N, Chevassut T, Forrester L, Ansell J, Ramsahoye B. (2004) Severe global DNA hypomethylation blocks differentiation and induces histone hyperacetylation in embryonic stem cells. Mol Cell Biol. 24(20):8862-71.

17. Tsumura A, Hayakawa T, Kumaki Y, Takebayashi S, Sakaue M, Matsuoka C, Shimotohno K, Ishikawa F, Li E, Ueda HR, Nakayama J, Okano M. (2006) Maintenance of self-renewal ability of mouse embryonic stem cells in the absence of DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b. Genes Cells. 11(7):805-14.

18. Mikkelsen TS, Hanna J, Zhang X, Ku M, Wernig M, Schorderet P, Bernstein BE, Jaenisch R, Lander ES, Meissner A. (2008) Dissecting direct reprogramming through integrative genomic analysis. Nature. 454(7200):49-55.

19. Bryant, S.V., Endo, T., Gardiner, D.M. (2002) Vertebrate limb regeneration and the origin of limb stem cells. Int. J. Dev. Biol. 46, 887–896.

20. Endo T, Bryant SV, Gardiner DM. (2004) A stepwise model system for limb regeneration. Dev Biol. 270(1):135-45.

21. Satoh A, Graham GM, Bryant SV, Gardiner DM. (2008) Neurotrophic regulation of epidermal dedifferentiation during wound healing and limb regeneration in the axolotl (Ambystoma mexicanum). Dev Biol. 319(2):321-35.

22. Sandoval-Guzmán T, Wang H, Khattak S, Schuez M, Roensch K, Nacu E, Tazaki A, Joven A, Tanaka EM, Simon A. (2014) Fundamental differences in dedifferentiation and stem cell recruitment during skeletal muscle regeneration in two salamander species. Cell Stem Cell. 14(2):174-87

23. Satoh A, Gardiner DM, Bryant SV, Endo T. (2007) Nerve-induced ectopic limb blastemas in the Axolotl are equivalent to amputation-induced blastemas. Dev Biol. 312(1):231-44.

24. Zyluk A, Janowski P. (2011) Results of the treatment of major, complex hand injuries. Pol Przegl Chir. 83(2):87-94.

25. Odelberg SJ. (2002) Inducing cellular dedifferentiation: a potential method for enhancing endogenous regeneration in mammals. Semin Cell Dev Biol. 13(5):335-43.

26. Satoh A, Bryant SV, Gardiner DM. (2012) Nerve signaling regulates basal keratinocyte proliferation in the blastema apical epithelial cap in the axolotl (Ambystoma mexicanum). Dev Biol. 366(2):374-81.

27. Stephens N, Holder N, Maden M. (1985) Motorneuron pools innervating muscles in vitamin A-induced proximal-distal duplicate limbs in the axolotl. Proc R Soc Lond B Biol Sci. 224(1236):341-54.

28. Muneoka K, Fox WF, Bryant SV. (1986) Cellular contribution from dermis and cartilage to the regenerating limb blastema in axolotls. Dev Biol. 116(1):256-60.

29. Neufeld DA1, Day FA, Settles HE. (1996) Stabilizing role of the basement membrane and dermal fibers during newt limb regeneration. Anat Rec. 245(1):122-7.

30. Muller TL, Ngo-Muller V, Reginelli A, Taylor G, Anderson R, Muneoka K. (1999) Regeneration in higher vertebrates: limb buds and digit tips. Semin Cell Dev Biol. 10(4):405-13.

31. Wallace H. (1981) Vertebrate Limb Regeneration. New Jersey: John Wiley & Sons 320 p.

32. Singer M. (1974) Trophic functions of the neuron. VI. Other trophic systems. Neurotrophic control of limb regeneration in the newt. Ann N Y Acad Sci. 228(0):308-22.

33. Salley-Guydon JD1, Tassava RA. (2006) Timing the commitment to a wound-healing response of denervated limb stumps in the adult newt, Notophthalmus viridescens. Wound Repair Regen. 14(4):479-83.

34. Flotho C, Claus R, Batz C, Schneider M, Sandrock I, Ihde S, Plass C, Niemeyer CM, Lübbert M. (2009) The DNA methyltransferase inhibitors azacitidine, decitabine and zebularine exert differential effects on cancer gene expression in acute myeloid leukemia cells. Leukemia. 23(6):1019-28.

35. He S, Wang F, Yang L, Guo C, Wan R, Ke A, Xu L, Hu G, Xu X, Shen J, Wang X. (2011) Expression of DNMT1 and DNMT3a are regulated by GLI1 in human pancreatic cancer. PLoS One. 6(11):e27684.

36. Santos EM1, Radin S, Ducheyne P. (1999) Sol-gel derived carrier for the controlled release of proteins. Biomaterials. 20(18):1695-700.

Figures

Figure 2.1. DNA methylation and methyltransferase expression in ectopic limb blastemas.
(A) Total DNA methylation levels as determined through ELISA format assay. Ectopic blastema tissue samples were collected at 10 days post nerve deviation. (B, C) qPCR analysis of DNMT1 and DNMT3a expression in uninjured tissues (skin and muscle), regenerating tissues (wound epithelium, ectopic blastema mesenchyme), and developing limb buds. Ectopic blastema tissue samples were collected at 10 days post nerve deviation. (* = $p < 0.05$; ** = $p < 0.005$)
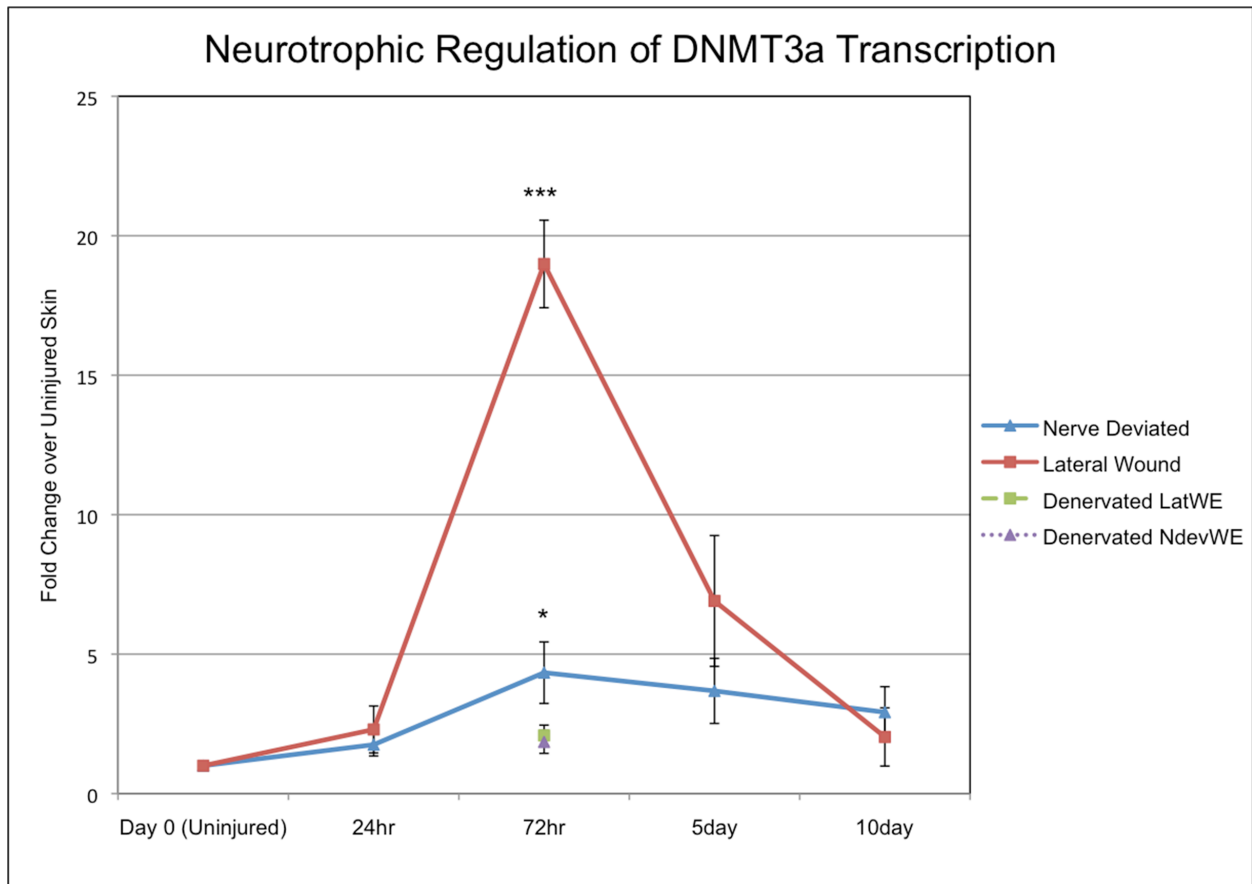
Figure 2.1
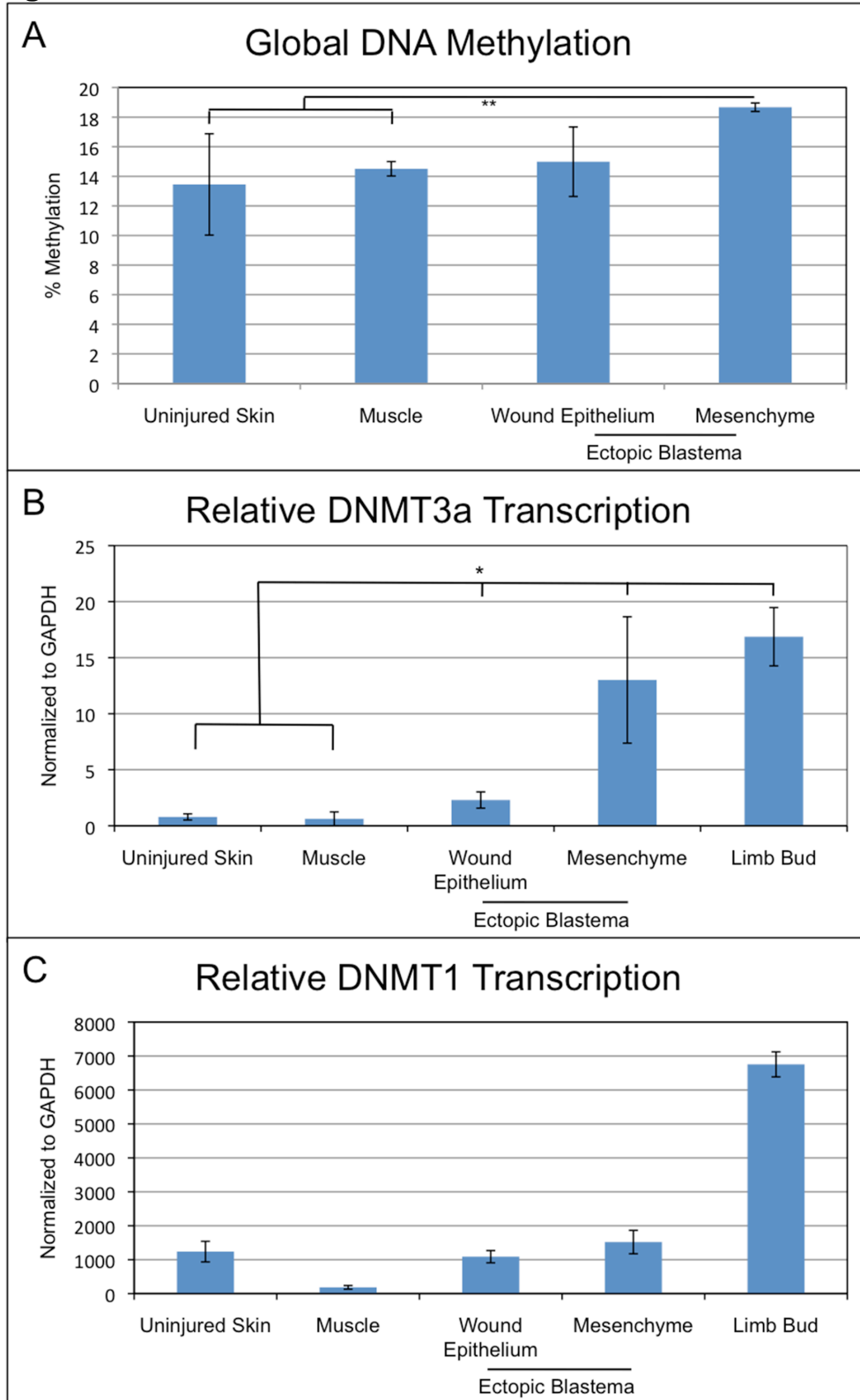
Figure 2.2. DNMT3a expression is modulated by signaling from nerves.

DNMT3a expression (qPCR) in the epithelium of wounds created on the arm of axolotls that either healed without forming a blastema (lateral wound) or received a surgically deviated nerve to form an ectopic blastema (nerve deviated). In addition the nerve supply to the limb was severed proximally (denervated) prior to making wounds either with (NdevWE) or without (LatWE) surgically deviating a nerve distally. (* = $p < 0.05$; *** = $p < 0.0005$)
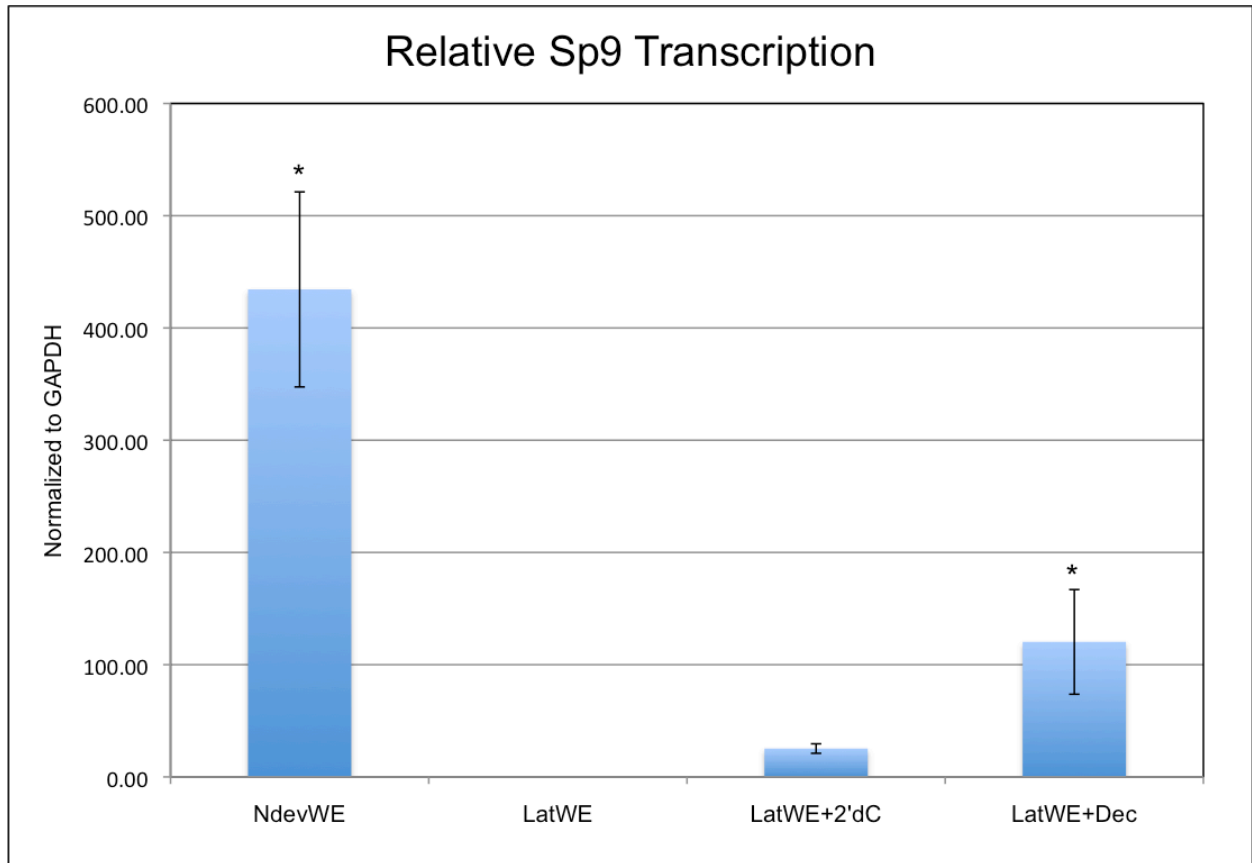
Figure 2.3. Inhibition of DNMT activity induces expression of the WE/AEC marker gene, *Sp9*.

Sp9 expression (qPCR) in the epithelium of wounds created on the arm of axolotls that healed without forming a blastema (lateral wound epithelium, LatWE); received a surgically deviated nerve to induce formation of an ectopic blastema (nerve deviated wound epithelium, NdevWE); received an implanted bead containing 2'deoxycytidine without a deviated nerve (LatWE + 2'dC); or received an implanted bead containing decitabine without a deviated nerve (LatWE + decitabine). (* = $p < 0.05$)

Figure 2.4. Inhibition of DNMT activity inhibits reformation of the basal lamina.

Trichrome staining of wounds six day post-surgery. Wounds were either untreated (A, B; mock); received an implanted bead containing 2'deoxycytidine without a deviated nerve (C, D; 2'dC); received a surgically deviated nerve to induce formation of an ectopic blastema (E, F; NDev); or received an implanted bead containing decitabine without a deviated nerve (G, H; Dec). Images in (B, D, F and H are higher magnifications of the boxed areas in (A, D, E, and G correspondingly). Dotted lines indicate the transition between the uninjured skin (left) and the wound (right). Arrows indicate the region beneath the epidermis/WE where reformation of the basal lamina has occurred or has been inhibited. Scale bars = 200 microns.
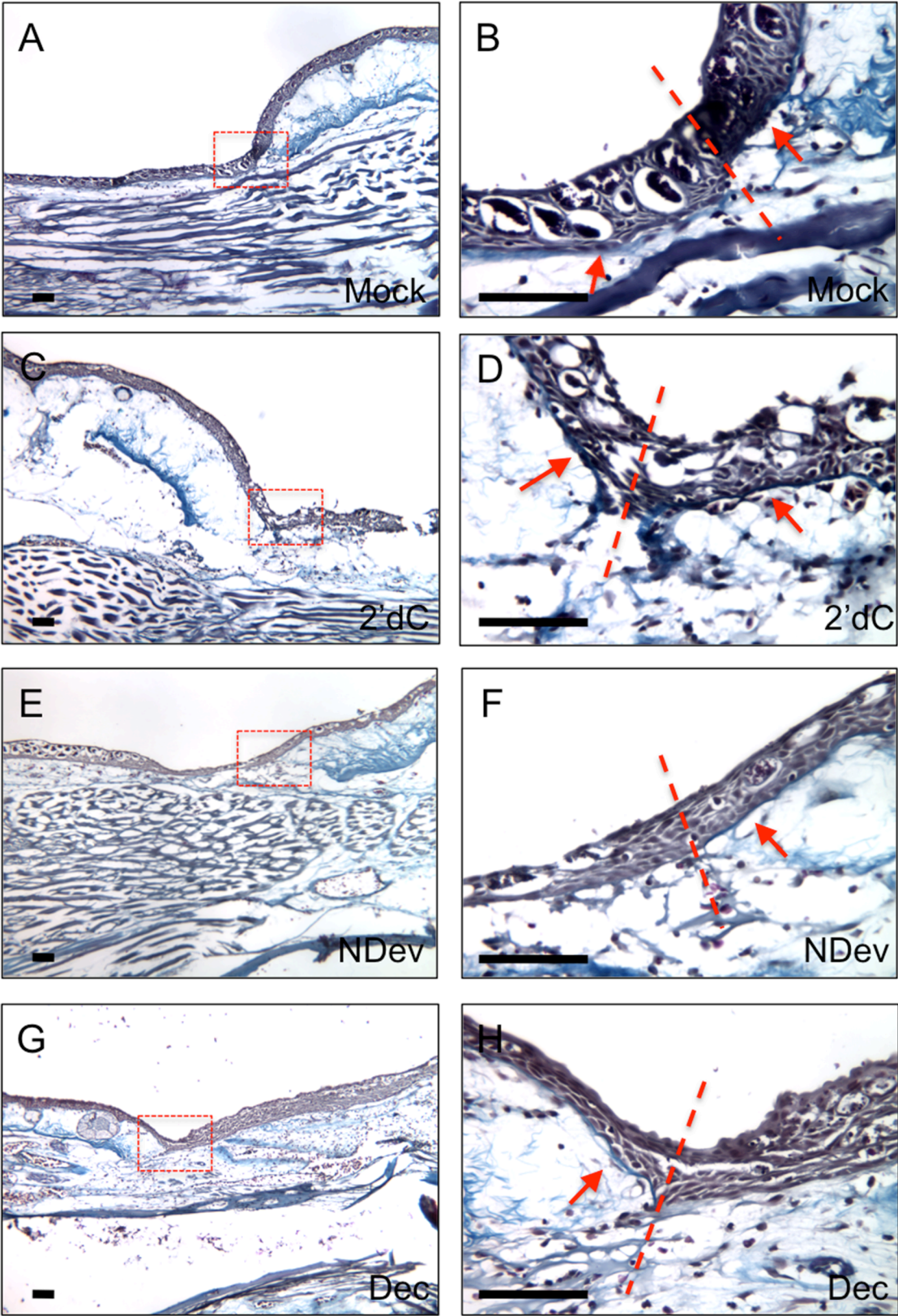
Figure 2.4

Figure 2.5. Decitabine treated wound epithelia can participate in blastema formation.
(A) Cartoon illustrating the sequence of surgical procedures for making the wound, implanting beads, and grafting the treated and control wound epithelia to a new wound with a deviated nerve. (B – E) Participation of a decitabine-treated WE in blastema formation as observed at day 3 (B), day 7 (C), day 11 (D) and day 16 (E) after grafting. (F – G) Lack of formation of an ectopic blastema when a 2'deoxycytidine-treated WE is grafted as observed at day 3 (F), day 7 (G), day 11 (H) and day 16 (I) after grafting. Arrows indicate the position of the deviated nerve (B, F) and the formation of an ectopic blastema (D, E). Scale bars = 1mm.

Figure 2.5

A

Day 0
Lateral wound

Day 1
Bead graft
(2'-deoxycytidine or Decitabine)

Day 6
Nerve deviations/
Pre-treated WE graft
(LFL -> LFL, RFL -> RFL)

Day 9…
Begin imaging

B Dec

F 2'dC

C Dec

G 2'dC

D Dec

H 2'dC

E Dec

I 2'dC
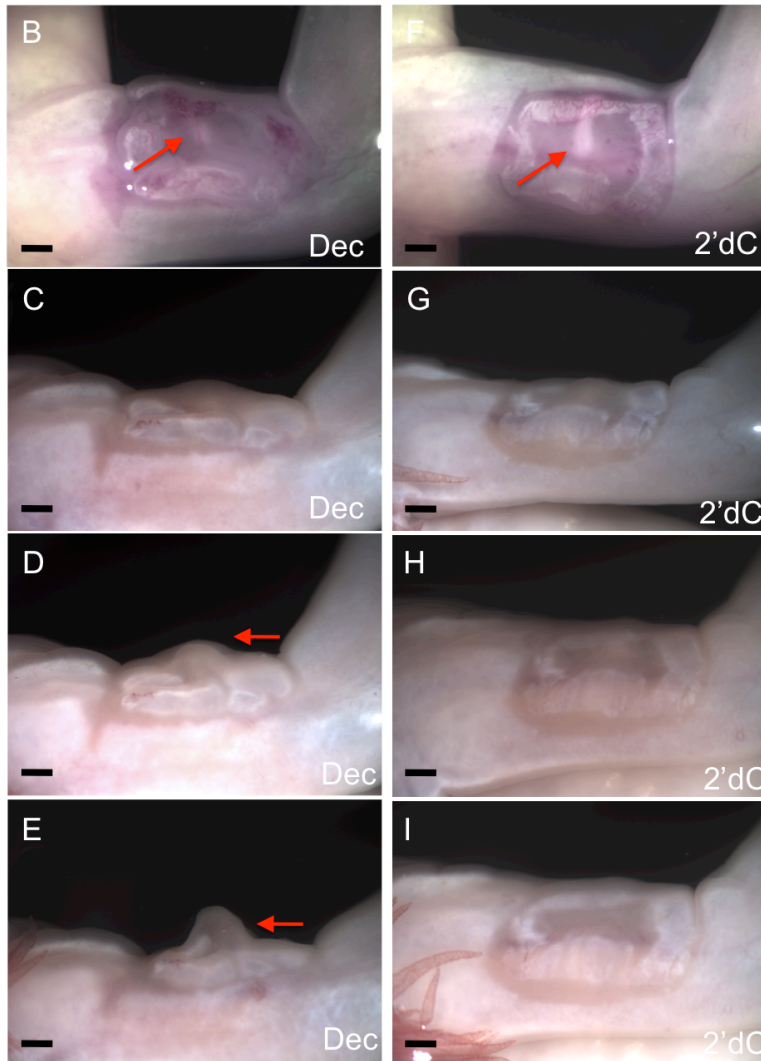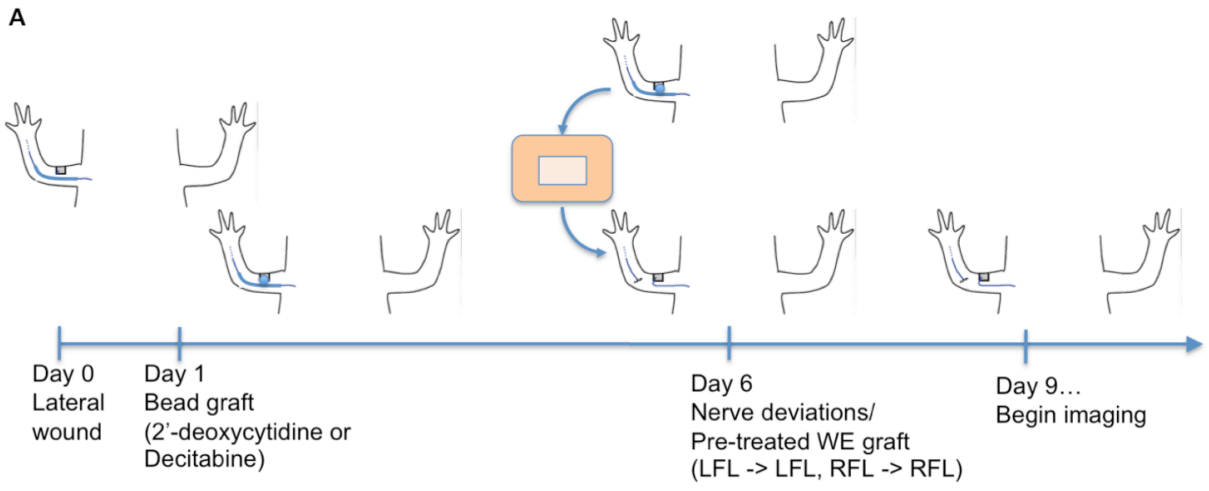
Tables

Table 2.1. Ability of decitabine-treated wound epithelia to participate in blastema formation.

Treatment	Total	1º Blastema	No Blastema	2º Blastema
Decitabine	6	4 (67%)	2 (33%)*	0 (0 %)
'2-deocycytidine (control)	8	1** (12%)	5 (63%)	2 (25%)

* The presence of a decitabine bead could not be confirmed 24 hour after grafting in two additional wounds that did not form an ectopic blastema (a total of 8 limbs were grafted), and those data were not included
** A tear in the WE/AEC of this wound that re-healed and formed a new WE/AEC was observed 24 hours after grafting

CHAPTER 3 – Accessory Limb and Excisional Repair Models in the Axolotl

Although regenerative abilities are widespread and essential to the maintenance of tissue homeostasis, they are largely limited to individual cell types within complex tissues. Many of the cell types within the human arm can regenerate (e.g. muscle, nerves, bone and blood vessels); however, the limb itself cannot regenerate when amputated. In contrast, the urodele amphibians (salamanders and newts) have the remarkable ability to regenerate complex tissues, and thus have historically been the model organisms of choice for regeneration studies.

In spite of many decades of effort studying regeneration in salamanders, we are only now beginning to take advantage of modern techniques in molecular genetics to discover the critical gene regulatory networks that regulate regeneration. Most studies have been limited in terms of describing the anatomy and patterns of gene expression associated with the regeneration process. Attempts to understand the function of candidate genes are limited by the lack of gain-of-function models for regeneration. It is paradoxical that it is experimentally challenging to test function in an animal that can regenerate perfectly. Although it is possible to test for the inhibition of regeneration (loss-of-function), it is hard to think of ways to induce a regenerative response in an animal that already regenerates. To address this issue of a need for gain-of-function assays, we began several years ago to develop and validate experimental models using the axolotl (*Ambystoma mexicanum*) to develop assays for the signaling pathways that control limb regeneration.

Initially, we focused on how to identify the signals that induce blastema formation and subsequent reformation of an entire limb. We took advantage of previous reports in

the literature indicating that ectopic limbs could be induced to form from wounds on the side of the limb (*1, 2*). Building on the insight that this response is dependent on signals from a nerve combined with interactions between dermal fibroblasts, we were able to optimize and validate the Accessory Limb Model (ALM) as an assay for the events that are induced by amputation and lead to regeneration of the entire limb (*1, 2*; Fig. 3.1A, B). We subsequently developed the Excisional Regeneration Model (ERM) in the axolotl to identify the steps and signals leading to the regeneration of skeletal defects along the proximal-distal limb axis, which are common injuries that can lead to amputation given our limited ability to repair them (*3*); Fig. 3.1 C-E). In both these models, the strategy is to start with the axolotl that we know can regenerate all the limb structures perfectly when the limb is amputated. We then create wounds that in the absence of additional signals do not regenerate. We then can deliver and test candidate factors to these wounds so as to induce a regenerative response as a gain-of-function assay.

The ALM is based on the ability to induce an entire, ectopic limb to form from a wound on the side of the arm of an axolotl (Fig. 3.1A, B). From the earlier literature, it was evident that if all the necessary signals are provided, a limb is formed *de novo*; however, if one or more of these signals is not provided the regenerative response is impaired (*see 1*).If a skin wound is made but no additional signals are provided (Fig. 3.3C), the wound becomes re-epithelialized and the dermis regenerates without forming a scar. If however, the brachial nerve is surgically deviated to the site of the skin wound, dermal cells adjacent to the wound are induced to migrate to the site of the deviated nerve, where they dedifferentiate to form an ectopic blastema (*1*; Fig. 3.1A; Fig. 3.3E). The induced ectopic blastema is equivalent the blastema formed in response to amputation (*2*), and thus the

ALM is a model for studying the induction of dedifferentiation and blastema formation. The important advantage of the ALM, in contrast to amputation, is that there is very little damage to the stump tissues, and thus extraneous signaling events associated with the trauma of amputation are not induced.

Although nerve-associated signals are necessary and sufficient to induce blastema formation, additional signals are required to induce a limb *de novo*. As has been appreciated for decades, the genesis of an entire new limb requires the interaction of cells from opposite sides of the limb (*4-6*). These cells are provided in the ALM by the grafting of skin from the side of the limb that is opposite the side on which the wound is created (e.g. a graft of posterior skin to an anterior wound; Fig. 3.3A, B, F). In response to signaling from both a deviated nerve and from connective tissue cells grafted from the opposite side of the limb, a new and perfectly patterned limb can be induced to form at a high frequency (*1*; Fig. 3.1B).

Results from ALM experiments have provided a number of insights into the mechanisms of limb regeneration. The fundamental contribution of the ALM is the demonstration that regeneration is a stepwise process, (e.g. wounding, nerve signaling, and cell-cell interactions). Experimentally it is important that the ALM allows us to study each step individually, unlike the situation with an amputation in which all the steps are activated. Secondly, the ALM demonstrates the critical role of dermal fibroblasts in controlling growth and pattern formation during regeneration. This function had been established previously from studies based on the limb amputation model (*4, 7*). With the ALM, the only tissues that are damaged are the severed nerve and the wounded/grafted dermal connective tissue. The interactions between the graft and host dermal fibroblasts in

response to nerve signals subsequently generate the signals that activate and recruit the ingrowth and patterns of the other limb tissues (e.g. blood vessels, the nerve, and myoprogenitor cells derived from satellite cells). Finally, the ALM can be used as a assay to test the function of candidate molecules and factors that can be delivered by a number of techniques (e.g. electroporation, microinjection of viral vectors, and implantation of microcarrier beads that have been soaked in growth factors). Given the ability to manipulate the steps in the limb regeneration cascade, it is possible to utilize the ALM to test for function in the control of cell migration, proliferation, and dedifferentiation leading to blastema and limb formation.

Although many injuries result in limb amputation, most involve the loss of tissues at an intermediate level along the proximal-distal limb axis. In such cases, the surgical challenge is to repair the damage and salvage as much of the remaining limb as possible so as to avoid amputation of the remaining limb tissues. Thus the ability to enhance a regenerative response would be an important advancement in treating such injuries. Although limb amputations in salamanders have been studies for decades, little is known about the regenerative response to injuries that result in structural defects in the limb. What is known is that typically such defects are not regenerated unless the entire limb is amputated (*3, 8*). As with the ALM, this lack of a regenerative response in an animal that can regenerate perfectly provides the opportunity to develop a gain-of-function assay for regeneration.

The Excisional Repair Model (ERM) involves the surgical removal of a central segment of the radius in the zeugopod. This mid-diaphyseal deletion fails to regenerate beyond a critical size limit (critical-size defect, CSD) despite the immense regenerative

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ability of the axolotl (*3, 9*; Fig. 3.1C). It is important to note that this response to injury in the axolotl is equivalent to that in the human. Mesenchymal progenitors are activated and form a soft callus structure made of fibrocartilage around the cut ends of the skeletal element, and without treatment or intervention, the gap created by the surgery will persist. However, in the axolotl the addition of multipotent blastema cells generated by dedifferentiation after limb amputation provides cells with the ability to be reprogrammed, leading to the regeneration of the excised skeletal element (*3*). Taking these results together, the ERM creates an injury environment that is permissive and instructive for a regenerative response, but does not induce dedifferentiation so as to provide a source of regeneration-competent cells.

By creating an assay in which regeneration is not the endogenous response, we can begin to identify the molecular factors for inducing regeneration in a non-regenerating wound. As mentioned previously, grafting of dissociated blastema cells into the wound site results in a regenerated skeletal element that is integrated into the existing, host bone (Fig. 3.1E). Other cell types can be grafted into the injury with various results. For example, dermal fibroblasts, the progenitors of the blastema, form dense connective tissue when grafted but do not repair the defect. However, the presence of a nerve deviated to the wound site promotes dedifferentiation of surrounding cells, as well as the grafted dermal cells, leading to regeneration of the skeletal element. Therapeutically introducing factors that induce regeneration *in situ* could also induce dedifferentiation, and the efficacy of candidate factors can be assayed based on the extent of CSD regeneration that occurs. One method of introducing factors involves the formation of micro-carrier beads encapsulating growth factors or small molecules for delivery to the wound site (Fig. 3.1D, Fig. 3.4A, D).

Alternatively, cells can be isolated and treated in culture. The extent of dedifferentiation induced during *ex vivo* culture of the donor cells (e.g. autologous dermal fibroblasts) can then be assayed by the ERM and quantified by the degree of regeneration that results.

Both the ALM and ERM provide experimental models to test the function of specific signaling pathways involved in the regulation of limb regeneration. The key element of both is that they are gain-of-function assays that identify the progressive steps and the signals that allow for progression to the next step. Combined with the power of computational biology to identify the regulatory networks that are activated in response to these injuries in the axolotl, it will be possible to discover how each step of successful regeneration progresses from one to the next. While the ability of these animals to regenerate entire organs seems extraordinary, the mechanisms of regeneration involve many basic biological processes regulated by conserved signaling pathways (*4*). Thus, we can expect to stimulate regeneration in a human by the same mechanisms used by the axolotl, and thus to enhance our regenerative responses to both acute injury and the accumulation of chronic damage associated with aging.

Materials

Methods for both surgical procedures are largely overlapping, and the following materials are often used for both (*see* Fig. 3.2).

Induction and Maintenance of anesthesia

1.   Stock Holtfreter's salt solution: To make 2 liters of stock Holtfreter's salt solution, add 320 g of NaCl, 28 g of CaCl2, 2 tablespoons of MgSO4-7H2O, and 1 teaspoon of KCl.

2.   Holtfreter's salt solution, 40%: Add 250 ml of stock Holtfreter's salt solution to 5 gallons of deionized water.

3.   Stock MS222 solution, 20X (*see* Note 1): Add 20g of MS222 (ethyl 3-aminobenzoate methanesulfonate salt, Sigma) to 1 liter of deionized water. Store the solution at 4°C and avoid light.

4.   Working MS222 solution, 1X: Add 50 ml of 20X MS222 stock solution, 1 ml of Tris-HCl (pH 7.4), and 0.5 ml of 0.5% phenol red solution (Sigma) to 900 ml of 40% Holtfreter's salt solution. Adjust the pH to 7.4 with NaOH. Adjust the volume to 1 liter with 40% Holtfreter's salt solution. Store the solution at 4°C.

5.   Plastic containers large enough to immerse an animal in the MS222 anesthetic solution (Fig. 3.2B)

Preparation of microcarrier beads for delivery of experimental agents

Sol-Gel Beads

1.   Tetramethyl orthosilicate

2.   Methanol, 100%

3.   HCl, 0.1N

4.   Glacial acetic acid, 0.1N

5.   Growth factor/small molecule of interest

Gelatin microspheres

1.   Type B gelatin

2.   Olive oil

3.   Acetone, 100%

4.   2-Propanol, 100%

5.   Glutaraldehyde solution: Combine 100mL H2O, 100μL Tween 20, and 36μL of

glutaraldehyde. Stir on stir plate for 2 min or until solution is homogenous.

6.   10mM glycine

7.   Growth factor of interest

Preparation of blastema cell grafts

Collagen clot delivery

1.   Type I collagen (rat tail), 3.6mg/mL

2.   L-15 culture media, 600%

3.   Fetal bovine serum

4.   Sodium bicarbonate, 7.5% w/v

Fibrin clot delivery

1.   Fibrinogen, 25mg/mL in 60% DMEM

2.   Thrombin, 200U/mL in 60% DMEM

Surgical procedures

1.   Lab Tissues [e.g. Kimwipes™ (Kimtech)]

2.   Holtfreter's solution, 40% in squeeze bottle

3.   Two microforceps, Dumont #5 stainless steel (0.10mm x 0.06mm tips) – World

Precision Instruments

4.   Microscissors, World Precision Instruments Noyes scissors (15mm blades), WPI

Vannas scissors (5mm blades, 0.1mm tips)

5.   Ethanol, 70%

6.   Plastic plate or tray (slightly larger than the animal) to place the animal on that will

keep liquids contained during the surgery (Fig. 3.2D)

7.   Ice and ice bucket

Sample collection and processing for histology/Immunohistochemistry/In situ

hybridization

Whole-mount staining

1.   Paraformaldehyde, 4%

2.   Alcian blue, 7.5mg/mL in acid alcohol (3:1 EtOH:Glacial acetic acid)

3.   EtOH, 100%

4.   KOH, 0.5%

5.   KOH, 4%

6.   Alizarin red (1:4 0.1% w/v Alizarin red in EtOH:0.5% KOH)

7.   Glycerol

Embedding for cryosectioning

1.   Paraformaldehyde, 4%

2.   EDTA, 10%

3.   Sucrose, 30%

4.   Tissue Tek® cryomold® intermediate, disposable vinyl specimen molds

5.   Tissue Tek®, O.C.T. compound, embedding medium for frozen tissue specimens

6.   Forceps

7.   Liquid Nitrogen

Histological staining

1.   Alcian blue, 7.5mg/mL in acid alcohol (3:1 EtOH:Glacial acetic acid)

2.   Ehrlich's Hematoxylin

3.   Eosin, 0.25% w/v in 70% EtOH + 0.4% v/v Glacial acetic acid

4.   EtOH, 70%

5.   EtOH, 95%

6.   EtOH, 100%

7.   Cytoseal™ 60 mounting media

Methods

   Preparation of surgical area

1.   Organize a surgical station that is equipped with a dissecting microscope and fiber-optics illumination.

2.   Disinfect the surgical station with 70% ethanol.

3.   Sterilize the surgical instruments (*see* Note 2) with a glass bead sterilizer (e.g. Steri 250, Inotech).

   Induction and Maintenance of anesthesia

1.   Anesthetize axolotl by immersion in 1X MS222 solution (*see* Note 1) until it is no longer responsive to a toe pinch (about 20-30 min for larger animals; less time for smaller animals).

2.   Prepare a surgery plate that has a moist (saturated with 40% Holtfreter's solution) Kimwipes™ on it.

3.   After the axolotl has been anesthetized, put the animal on the surgery plate.

4.   Cover the animal with moist Kimwipes™ to prevent dehydration of skin. Tease away the paper to expose the surgery area while keeping the rest of the animal covered (*see* Note 3).

Accessory Limb Model (ALM)

<u>Wounding</u>

1.   Expose the upper arm region by teasing back the moist Kimwipes™.

2.   Make a full-thickness skin wound on the mid-anterior region with a rectangular incision (1.0-1.5 X 2.0-3.0 mm) using microscissors (Fig. 3.3C).

3.   Remove the excised skin using fine forceps.

<u>Nerve deviation</u>

1.   To obtain a nerve fiber for surgical deviation to the wound site, lift the arm to expose the posterior-ventral side, and make an incision extending from the shoulder to the elbow (Fig. 3.3D).

2.   Locate the brachial nerve, and transect it with microscissors at the level of the elbow.

3.   Dissect the transected brachial nerve from the surrounding connective tissue being careful to not damage the nerve fiber.

4.   To reroute the transected brachial nerve to the skin wound at the anterior site, use microforceps to grab the tip of the transected nerve and guide it beneath the skin to the skin wound (Fig. 3.3E).

5.   After rerouting the nerve to the anterior skin wound, close the posterior-ventral incision using microforceps and allow the edges to heal into place (*see* Note 4).

Skin grafting

1.   Carefully turn the animal over to access the posterior side of contralateral arm (avoid having the deviated nerve retracting away from the anterior wound site).

2.   Excise a square piece of skin (~1 mm on each side) from the posterior side of the arm using the technique for making the anterior skin wound (*see* Note 5).

3.   Turn the animal over so as to access the nerve-deviated anterior wound.

4.   Trim the end of the deviated brachial nerve using microscissors and position the cut end so that it lies within the anterior wound (*see* Note 6; Fig. 3.3E). After trimming, the nerve tip should be positioned vertically in the middle and horizontally one-third on either side.

5.   Place the posterior skin within the anterior skin wound, next to the end of the deviated nerve (Fig. 3.3F). It is important that the tip of the nerve does not touch the edge of either the grafted skin or the wound (*see* Note 7).

6.   Place the animal on the ice to let the wound heal for two hours (Fig. 3.2F).

7.   Place the animal back in a housing container with 40% Holtfreter's salt solution.

Excisional Regeneration Model (ERM)

Both types of ERM wounds (simple and deep w/nerve deviation) are host sites into which microcarrier beads and/or cells can be grafted. Beads and cells are grafted into the surgically created cavity between the cut ends of the radius. Grafting is done before closing the skin flap in the case of the simple wound, or after nerve deviation in the case of the

deep wound (there is no skin flap and the wound heals by reepithelialization from the surrounding epidermal keratinocytes).

Simple wound

1.   Anesthetize an 8-10cm axolotl in MS222 solution for 25min.

2.   Prepare the animal for surgery, laying the axolotl such that the subject limb is easily accessible (Fig 3.2D, E; *see* Note 3).

3.   Using microscissors (5mm blades, 0.1mm tips), create a skin flap on the dorsal zeugopod by making three incisions in the shape of a rectangle. The attached side of the rectangle should be the posterior side (Fig 3.4A,B).

4.   Reflect the flap back so as to expose the underlying muscle tissue. Using forceps, expose the radius by carefully reflecting the muscle fibers, taking care to not sever major blood vessels or damage the muscle itself (*see* Note 8).

5.   Excise a 2mm mid-diaphyseal segment of the radius using microscissors (15mm blades) (Fig 3.4C).

6.   Return the displaced muscle fibers to their original position.

7.   Close the skin flap, ensuring contact on all sides with the adjacent, uninjured skin. Suturing is not necessary (*see* Note 4).

8.   Place kimwipes to cover the animal completely except for the wound site created by the surgery. Soak kimwipes in 40% Holtfreter's solution.

9.   Place the animal on the ice to let the wound heal for two hours (Fig. 3.2F) before returning it to the housing container with 40% Holtfreter's salt solution.

Deep wound and nerve deviation

1.   Begin preparation as described above.

2.   Using microscissors (5mm blades, 0.1mm tips), create a skin window on the dorsal zeugopod by making four incisions in the shape of a rectangle.

3.   Surgically remove the underlying muscle tissue until the radius is exposed.

4.   Excise a 2mm mid-diaphyseal segment of the radius using microscissors (15mm blades).

5.   Continue to remove muscle tissue until the pair of nerves running between the radius and ulna is exposed (*see* Note 9).

6.   Sever the nerves distally (at the carpal level) and position the cut ends in the center of the deep wound (Fig 3.4D)

7.   Place kimwipes to cover the animal completely except for the wound site created by the surgery. Soak kimwipes in 40% Holtfreter's solution.

8.   Place the animal on the ice to let the wound heal for two hours (Fig. 3.2F) before returning it to the housing container with 40% Holtfreter's salt solution.

Preparation of Microcarrier Beads

Sol-Gel beads (*see 10*)

The following protocol describes the procedure for making 100μL total volume of sol-gel solution. For larger volumes, the amounts specified can be scaled up. All work should be carried out in the hood unless otherwise specified.

1.   In a microcentrifuge tube combine 37.6μL H2O, 31.6μL tetramethyl orthosilicate (TMOS), and 8.5μL methanol. Tetramethyl orthosilicate is extremely hazardous (*see* Note 10).

2.   Add 0.1N HCl until the pH of the solution is below 2. Approximately 2µL should be sufficient.

3.   In a separate tube, prepare a mixture of the growth factor/small molecule of interest in 0.1N acetic acid. The final volume of this mixture should be 20µL. Use the appropriate amount of growth factor/small molecule and add 0.1N acetic acid until the volume is 20µL.

4.   Add the resultant growth factor/small molecule solution to the TMOS mixture (*see* Note 11).

5.   Dispense the solution into molds of a desired shape. Beads must be small for grafting into the ERM. The simplest method of bead formation is to dispense 1µL volumes onto a sheet of parafilm.

6.   Allow the beads to harden for 3 days at room temperature in a hood.

7.   At this stage, the beads can be removed from the hood. Dry the beads at 37°C overnight.

<u>Gelatin microsphere beads</u> (see *11*)

1.   Add 5g type B gelatin to 50mL H2O. Microwave until completely dissolved.

2.   In a 500mL beaker, heat 250mL olive oil to 40°C on a hot plate/stirrer. Add a stir bar.

3.   Slowly pour the gelatin solution into the olive oil while stirring. Continue stirring for 5 minutes. The speed of stirring will affect the size of the beads produced. Faster speeds will produce smaller beads.

4.   Transfer the beaker to a cool stir plate (no heat) and continue stirring for 30 minutes at room temperature.

5.   Add 100mL acetone to the beaker and increase the stir speed to the highest possible. The acetone will fix the beads. Remove any large aggregates that form during the stirring process. Keep the beaker covered to prevent contamination.

6.   The solution will have separated into two layers. Remove the top layer and wash the beads with 75mL of 2-propanol.

7.   Transfer the mixture to two (2) 50mL conical tubes and centrifuge at 2000 x g for 5 minutes at 4°C.

8.   Decant the supernatant and add 20mL of glutaraldehyde solution. Immediately re-suspend the pellet and pour into a 200mL beaker.

9.   Stir the mixture for approximately 2 minutes or until most of the beads are separated. Remove large aggregates and incubate covered for 12 hours at 4°C.

10.   Transfer the contents of the beaker to a 50mL conical tube and centrifuge at 2000 x g for 2 minutes at 4°C.

11.   Decant the supernatant and wash the beads in 50mL H2O. Centrifuge at 2000 x g for 2 minutes at 4°C.

12.   Decant the supernatant and transfer the beads to a beaker containing 100mL of a 10mM glycine solution. Stir covered for 1 hour at 37°C.

13.   Transfer the contents of the beaker to two (2) 50mL conical tubes and centrifuge at 2000 x g for 2 minutes.

14.   Wash beads 2X: Decant supernatant and add 50mL H2O. Re-suspend and centrifuge at 2000 x g for 2 minutes.

15.   Decant the supernatant and transfer the beads to a large Petri dish. Remove any large aggregates and dry the beads at 4°C overnight.

16.   In order to add a growth factor of interest, prepare the desired concentration of a growth factor solution. Soak beads of the desired size (usually 300-500μM) in the growth factor solution overnight at 4°C.

Preparation of Blastema Cell Grafts

Collagen clot delivery - 10μL volume graft

1.   In a sterile microcentrifuge tube, combine: 27μL type I collagen, 5μL L-15 culture media (600%), 2.5μL FBS, 13.5μL autoclaved H2O, and 3μL NaHCO3 (7.5% w/v). Mix well.

2.   Transfer 10μL of the collagen solution into a tube containing a cell pellet of approximately 5 x 104 cells. Mix gently and place the mixture into a 35mm tissue culture dish to clot.

3.   Once clotted, graft the collagen clot into the gap created by excising a mid-diaphyseal segment of the radius.

Fibrin clot delivery

1.   Centrifuge approximately 7.5 x 105 cells into a loose pellet at 200 x g for 5 minutes.

2.   Decant the supernatant and re-suspend in 150μL fibrinogen solution. Centrifuge into a loose pellet at 200 x g for 5 minutes.

3.   Decant the supernatant without disturbing the pellet.

4.   Add 1.0μL of thrombin to the pellet and spin at low speed (20 x g for 1 minute).

5.   Once the clot is set, you can trim the clot to the appropriate size/shape and graft into the gap created by excising a mid-diaphyseal segment of the radius.

Sample Collection and Processing

Whole mount staining

1.   Fix collected tissue in 4% PFA overnight at room temperature.

2.   Rinse the sample twice with H2O.

3.   Stain the sample with alcian blue for 24 hours at room temperature.

4.   Wash the sample twice with acid alcohol for 5 minutes.

5.   Place the sample in 100% EtOH overnight.

6.   Transfer the sample to 0.5% KOH solution for destaining. Once the sample sinks to

the bottom of the vessel, replace the solution with fresh 0.5% KOH and leave overnight.

7.   Stain the sample with alizarin red for 24 hours.

8.   Destain in 4% KOH, replacing the solution with fresh 4% KOH periodically.

9.   Once the destaining is complete, clear the sample in 50% glycerol. After the sample

sinks, transfer to 80% glycerol. Once the sample sinks again, transfer to 100% glycerol.

This process will take several days.

Embedding for cryosectioning

1.   Fix collected tissue in 4% PFA overnight at room temperature.

2.   Replace the 4% PFA with 10% w/v EDTA and incubate overnight at room

temperature. This step is necessary for samples containing bone.

3.   Replace the 10% EDTA with 30% w/v sucrose and incubate overnight at 4°C with

gentle shaking. Proceed to the next step when the sample sinks to the bottom of the vessel.

4.   Fill a cryomold halfway with Tissue Tek®, O.C.T. compound and add the tissue

sample. The sample should be submerged and in contact with the bottom of the mold.

5.   Incubate the sample at 4°C for at least 4 hours in order to remove bubbles.

6.   Add liquid nitrogen to a Styrofoam cooler. Place the cooler on a slight incline so that the liquid nitrogen is present on one side only. Place the cryomold on the elevated side of the cooler. Avoid direct contact with the liquid nitrogen. Cover the cooler.

7.   Once frozen, store the cryomold at -20°C or colder.

<u>Histological staining</u>

1.   Rehydrate sectioned tissue in H2O for 3-5 minutes.

2.   Using a coplin jar, stain the slides with alcian blue for 30-45 minutes. The stain intensity depends upon the length of time spent in the staining solution.

3.   Rinse the slides in H2O.

4.   Stain the slides in Ehrlich's hematoxylin for 5 minutes.

5.   Place the slides under running tap water for 10 minutes. The water stream should not come into direct contact with the slides. Place the slides in a slide holder, and place into a water tight container. The slides should face away from the stream, so as to not disturb the sections.

6.   Rinse the slides in 70% EtOH.

7.   Stain the slides in Eosin for 2 minutes.

8.   Rinse the slides in H2O.

9.   Rinse the slides in 70% EtOH.

10.   Wash in 95% EtOH for 30 seconds.

11.   Wash in 100% EtOH twice for 3 minutes.

12.   Allow the slides to dry before mounting a cover slip using Cytoseal™ 60 mounting media.

Notes

1.   MS222 is acidic, and if not buffered appropriately (or if the pH of the solution changes over time during storage) the animals lose the ability to efficiently transport oxygen in their blood and will die when anesthetized. Addition of phenol red prevents the inadvertent use of MS222 that is at the wrong pH.

2.   It is important to protect the mucous layer covering the skin of the animal (e.g. do not use antiseptic solutions to wash the surface of the skin prior to making an incision). Mucous in amphibians has antimicrobial activity, and therefore the surgical incisions do not get infected so long as the mucous layer is present. Similarly, autoclaving the surgical instruments is not effective since once you make the first incision, the instrument is contaminated. Use of a hot-bead sterilizer allows you to resterilize as necessary throughout the surgical procedures.

3.   During surgery and subsequent recovery, keep the axolotl moist by periodically squirting 40% Holtfreter's solution over the length of the animal. Axolotls have both lungs and gills, and therefore tolerate being out of the water; however, their skin does not have the water barrier function necessary to prevent dehydration during extended periods out of the water.

4.   Generally it is not necessary to suture the edges of the wounds or the skin grafts. The edges of the skin adhere to the underlying connective tissues and reepithelialization is rapid in these animals, occurring within 4-6 hours post-surgery (*12*).

5.   Pay attention to the orientation of skin grafts to avoid flipping them over (dermis side up) during transferring and grafting.

6.   The brachial nerve is white, surrounded by a fibrous bundle, and can be distinguished easily from the adjacent blood vessels. It is very important not to damage either the blood vessels or the brachial nerve fiber during surgery. To avoid damage to the nerve fiber, lift only the tip of the nerve, which will then be trimmed off after being rerouted to the anterior skin wound.

7.   The protocol for deviating the nerve is based on the assumption that posterior skin graft is going to be made. For experiments that involve induction of only an ectopic blastema (wound with deviated nerve but no posterior skin graft), the end of the nerve can be placed anywhere within the wound bed. Signals from the deviated nerve attract migrating cells from the periphery such that the ectopic blastema will form symmetrically over the cut end of the nerve.

8.   Be sure to avoid severing major blood vessels or muscle tissue. In the event that a blood vessel is severed, control the bleeding before proceeding. Using a dry kimwipe, gently blot the wound until bleeding subsides. Remove any blood clots using forceps or a stream of 40% Holtfreter's solution.

9.   After removing the muscle tissue, keep the deep wound dry by regularly absorbing excess Holtfreter's solution with a dry kimwipe. This will aid the surgery by increasing visibility of the nerve.

10.   TMOS is extremely hazardous and volatile. Lab coat, safety goggles, and double nitrile gloves are required. When creating the sol-gel solution, begin with the water in the microcentrifuge tube, then add the TMOS. This will minimize the amount of TMOS exposure. Once TMOS and water are combined, the TMOS is hydrolyzed and safe.

11.   Sol-gel beads are clear and may be hard to visualize. You may add a dye in order to

increase their visibility.

Acknowledgements

References
1. Endo, T., Bryant, S. V., and Gardiner, D. M. (2004) A stepwise model system for limb regeneration, *Dev Biol 270*, 135-145.
2. Satoh, A., Gardiner, D. M., Bryant, S. V., and Endo, T. (2007) Nerve-induced ectopic limb blastemas in the Axolotl are equivalent to amputation-induced blastemas, *Dev Biol 312*, 231-244.
3. Satoh, A., Cummings, G. M., Bryant, S. V., and Gardiner, D. M. (2010) Neurotrophic regulation of fibroblast dedifferentiation during limb skeletal regeneration in the axolotl (Ambystoma mexicanum), *Dev Biol 337*, 444-457.
4. Bryant, S. V., Endo, T., and Gardiner, D. M. (2002) Vertebrate limb regeneration and the origin of limb stem cells, *Int J Dev Biol 46*, 887-896.
5. Bryant, S. V., French, V., and Bryant, P. J. (1981) Distal regeneration and symmetry, *Science 212*, 993-1002.
6. French, V., Bryant, P. J., and Bryant, S. V. (1976) Pattern regulation in epimorphic fields., *Science 193*, 969-981.
7. Muneoka, K., Fox, W., and Bryant, S. V. (1986) Cellular contribution from dermis and cartilage to the regenerating limb blastema in axolotls., *Developmental Biology 116*, 256-260.
8. Goss, R. J. (1969) *Principles of Regeneration*, Academic Press, New York.
9. Hutchison, C., Pilote, M., and Roy, S. (2007) The axolotl limb: a model for bone development, regeneration and fracture healing, *Bone 40*, 45-56.
10. Santos, E. M., Radin, S., and Ducheyne, P. (1999) Sol-gel derived carrier for the controlled release of proteins, *Biomaterials 20*, 1695-1700.
11. Tabata, Y., Hijikata, S., Muniruzzaman, M., and Ikada, Y. (1999) Neovascularization effect of biodegradable gelatin microspheres incorporating basic fibroblast growth factor, *J Biomater Sci Polym Ed 10*, 79-94.
12. Ferris, D. R., Satoh, A., Mandefro, B., Cummings, G. M., Gardiner, D. M., and Rugg, E. L. (2010) Ex vivo generation of a functional and regenerative wound epithelium from axolotl (Ambystoma mexicanum) skin, *Development, growth & differentiation 52*, 715-724.
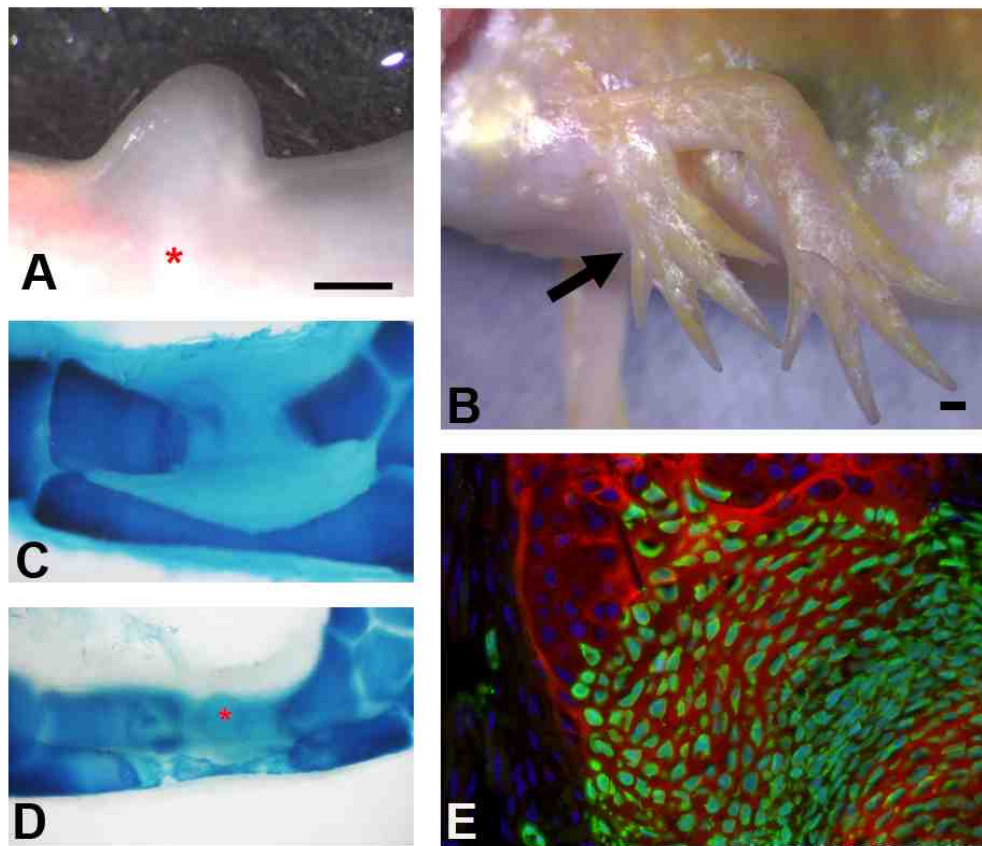
Figures

Figure 3.1. Regeneration of an entire new limb or a radial defect in the axolotl.
(A) In response to signals from the wound epithelium and a deviated nerve (red asterisk), dedifferentiated blastema cells accumulate on the side of the arm. (B) If in addition to the wound and nerve signals, cells from the side of the limb that is opposite to the wound are grafted into the wound (*see also* Fig. 3.3A,B), a normally patterned ectopic limb is induced to form *de novo* (arrow). (C) Surgical removal of the mid-diaphyseal region of the radius results in a defect (critical size defect, CSD) that is not regenerated. (D) Regeneration of a CSD can be induced by a variety of experimental manipulations, including the implantation of gelatin microcarrier beads soaked in BMP2 (red asterisk). (E) Grafted blastema cells (grafted cells are green from a GFP donor animal) regenerate the CSD and differentiate into chondrocytes that integrate into the cut ends of the host defect (red is immunostaining for Type II Collagen).
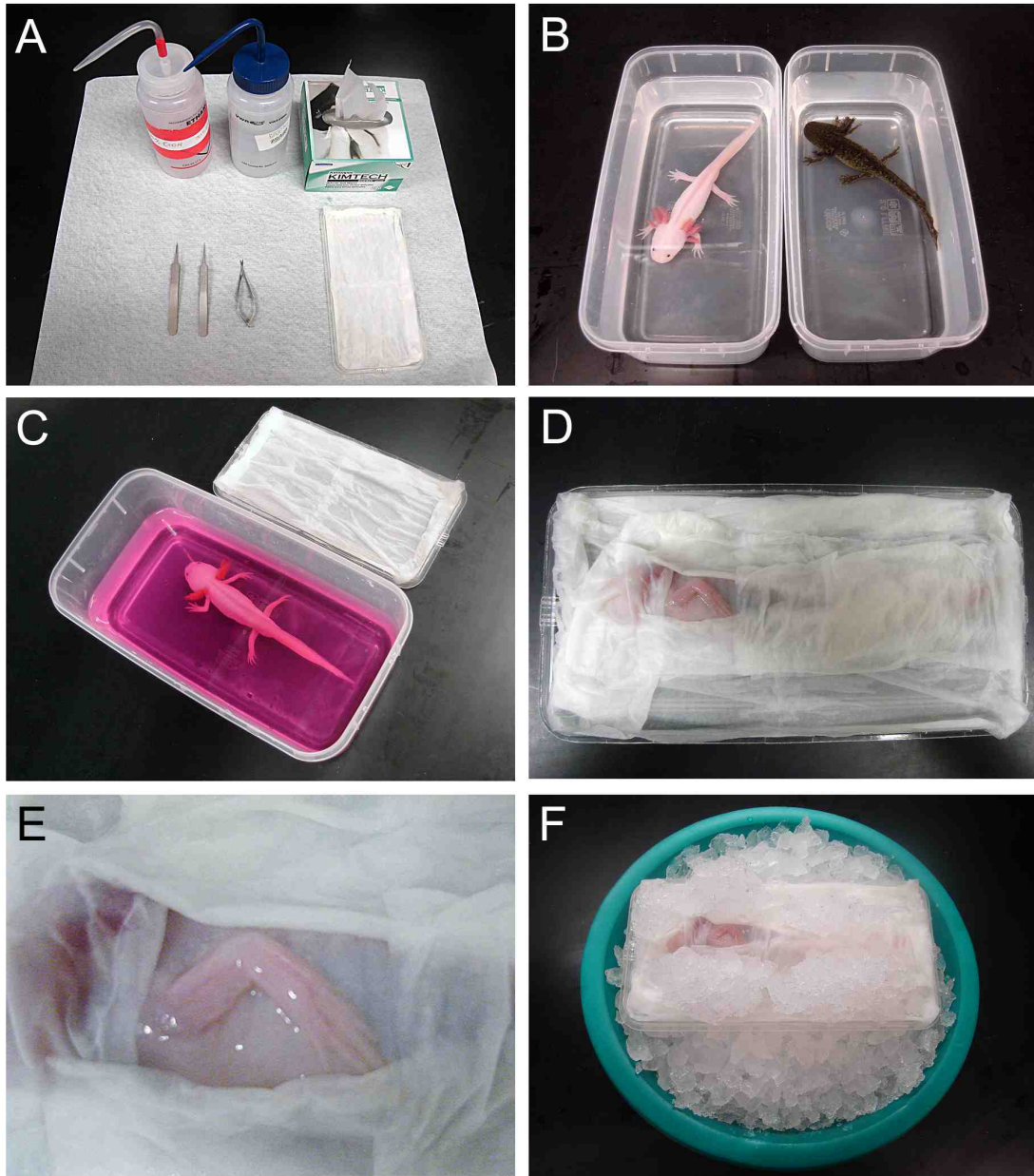
Figure 3.2. Surgical and post-surgical handling of the axolotl (*Ambystoma mexicanum*).
(A) Surgical supplies. Clockwise from upper left-hand corner: 70% ethanol, 40% Holtfreter's solution, Kimwipes™, surgery plate, microscissors, microforceps. (B) White (left) and wild type (right) axolotls in 40% Holtfreter's salt solution. (C) The white axolotl is be anesthetizing in MS222 solution. MS22 solution contains phenol red for monitoring pH change during anesthesia, so the solution is pink color (*see* Note 1). (D) The animal is placed on the surgery plate and covered with moist Kimwipes™. (E) Surgery area (upper arm) is exposed from the moist Kimwipes™. (F) For recovery, the animal on the surgery plate is placed on ice.
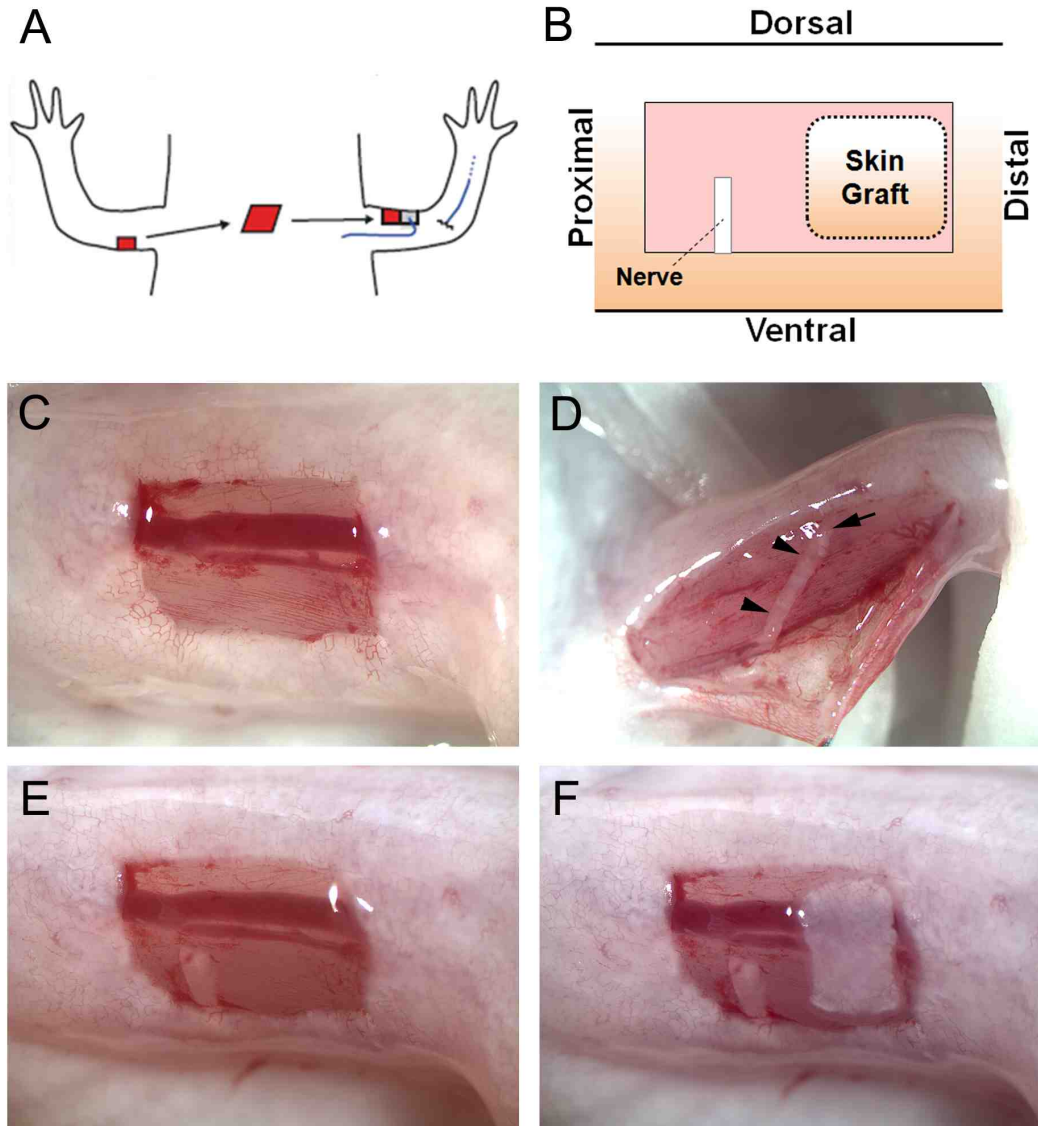
Figure 3.3. Wounding, nerve deviation and skin grafting to induce an ectopic limb (Accessory Limb Model).
(A) Summary of surgical procedure for induction of ectopic limb. (B) A diagram that shows the relative positions of the wound, deviated nerve and skin graft after a completed surgery. (C) Wounding on the anterior side of the upper arm showing the major blood vessel and nerve running from proximal (left) to distal (right) along the anterior side of the limb. (D) Incision on the posterior side of the arm, exposing the dissected brachial nerve (arrowheads) and the tip of the nerve that has been severed distally (arrow). (E) Deviated nerve on the anterior skin wound. (F) The posterior skin from the contralateral arm is grafted on the skin wound adjacent to the deviated nerve as in (B).
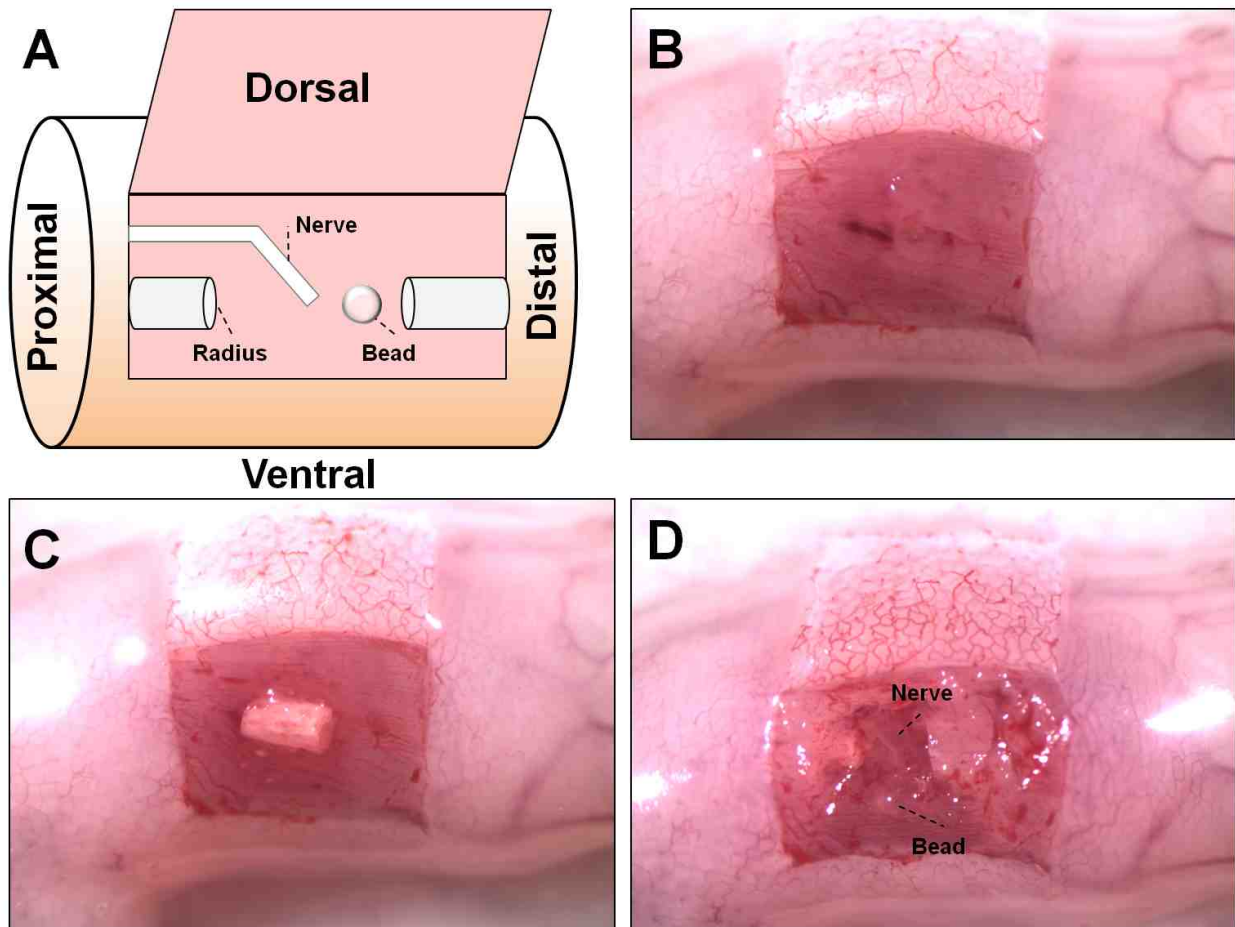
Figure 3.4. **Excision of the diaphyseal region of the radius to create a critical size defect in the Excisional Regeneration Model (ERM).**
(A) Schematic representing a completed excision with a deviated nerve and microcarrier bead graft. Placement of the bead is subject to investigator's aim and discretion. B) Skin flap window created by three incisions on the anterior side of the zeugopod. The underlying muscle has been reflected to allow access to the radius. Note the minimal damage to surrounding tissue. C) 2mm mid-diaphyseal segment of the radius after excision. D) A deep wound surgery with a deviated nerve and microcarrier bead graft. Mesenchymal tissue has been removed from the top of the wound to below the radius. The cut ends of the radius are visible with the deviated nerve placed between them.

CHAPTER 4 – Histone Modifiers during Early Regeneration Events

Beyond the previously described work detailing the expression patterns and regenerative relevance of DNA methyltransferases as epigenetic modifiers, I also characterized the expression of histone modifiers in ectopic blastemas as well as in various wound epithelia during the course of regeneration. Here I report the findings and their potential importance to the initiation of regeneration in the axolotl.

Epigenetic modifications of DNA and histones play a significant role in the control of gene expression during the differentiation process as well as during the reprogramming of somatic cells to pluripotent cells [1,2]. The axolotl EST database was searched for available sequences of epigenome modifying enzymes and the sequences of four histone modifying enzymes were found. Specifically, MLL3 and SMYD3, which are involved in histone 3 lysine 4 methylation; EZH2, which is involved in histone 3 lysine 27 methylation; and Kdm6b, which demethylates histone 3 lysine 27 [3]. These modifications are indicative of active transcription (H3K4me) or repressed transcription (H3K27me). Since large-scale changes in cell behavior are likely accompanied by large-scale changes in gene expression, these modifications are likely to be in flux during the process of dedifferentiation. Ectopic blastemas created through the accessory limb model were collected at 10 days post nerve deviation. The wound epithelium was separated from the mesenchymal portion of the blastema and treated as a separate sample. These regenerative samples were compared to uninjured mature skin and muscle tissue as non-regenerative samples as well as hind limb buds to determine the relative expression in a developing limb sample.

The histone methyltransferase MLL3 shows elevated expression within regenerating tissues when compared to non-regenerating skin and muscle (Figure 4.1A).

However, the expression observed in the 10 day blastema and wound epithelium is significantly less than in the developing limb. The histone methyltransferase SMYD3 shows elevated expression within regenerating tissues when compared to uninjured muscle, but not when compared to non-regenerating skin (Figure 4.1B). Interestingly, the levels of expression within the blastema, wound epithelium, and mature skin are comparable to that in the developing hind limb. The mark of active transcription deposited by these enzymes may be correlated with an increase in expression of developmental genes, perhaps more so in the case of SMYD3.

I next investigated the expression of the histone 3 lysine 27 methyltransferase EZH2 (Figure 4.2A), and the demethylase Kdm6b (Figure 4.2B). In both cases, the expression level within regenerating tissue was not significantly different than non-regenerating tissue, namely mature skin. In addition, the expression detected within the developing hind limb bud was significantly higher than the blastema or wound epithelium samples for both enzymes. This suggests that there is not a significant role of histone 3 lysine 27 methylation that is specific to regeneration. This is in contrast to studies conducted in the zebrafish caudal fin [4].

In order to focus on the earliest events of regeneration including dedifferentiation within the wound epithelium, I performed a time course analysis of expression of the histone modifiers (Figure 4.3). The time points collected were 24hrs, 72hrs, 5 days, and 10days post nerve deviation. Nerve deviated wounds created through the accessory limb model were compared to simple wounds that did not receive a nerve deviation, as well as to uninjured skin. I have observed that Kdm6b and SMYD3 show an increase in transcript levels at 24hrs post nerve deviation when compared to uninjured skin (Figure 4.3A).

Kdm6b and SMYD3 then begin to drop back down to the levels observed in uninjured skin. The expression of MLL3 and EZH2 remain at similar levels between nerve deviated wound epithelia and uninjured skin for the first 5 days of wound healing, but show a 2-fold increase in expression at the 10th day (Figure 4.3A). The roles of Kdm6b and SMYD3 as a remover of a repressive mark and a depositor of an active mark, respectively, is particularly interesting when analyzing the initial increases in expression at 24 hrs as this may indicate a true move of the genome towards the global activation of genes. Presumably, these could be genes associated with a less differentiated state allowing for the interaction with, and receiving of, signals from the nerve leading to the initiation of a regenerative response.

When comparing the relative expression patterns of histone modifiers between nerve deviated wounds and simple wounds (Figure 4.3B), a different trend is observed. The expression of SMYD3 remains fairly constant throughout the 10 day time course, but the other three modifiers show a decrease in expression of approximately 5-fold between the 72hr and 5 day time points before beginning to return to comparable levels at 10 days. The simultaneous decrease in expression of Kdm6b and EZH2 as modifiers of histone 3 lysine 27 methylation again suggests a minor role, if any at all, for this repressive transcription mark during the early stages of regenerative wound epithelium formation. Although there is a decrease in MLL3 expression between 3 and 5 days, the relatively stable expression of SMYD3 makes it difficult to make a strong conclusion about the role of histone 3 lysine 4 methylation in regenerative wound healing as compared to simple wound healing. Of course, the range of modifiers examined here is not an exhaustive list of histone modifers. Further analysis of histone modifications during the early stages of regeneration is

warranted in order to more completely elucidate the intricacies of this mechanism in

controlling gene expression.

References

1. Tomazou EM, Meissner A. (2010) Epigenetic regulation of pluripotency. Adv Exp Med Biol. 695:26-40.
2. Meissner A. (2010) Epigenetic modifications in pluripotent and differentiated cells. Nat Biotechnol. 28(10):1079-88.
3. Jenuwein T, Allis CD. (2001) Translating the histone code. Science. 10;293(5532):1074-80.
4. Stewart S, Tsun ZY, Izpisua Belmonte JC. (2009) A histone demethylase is necessary for regeneration in zebrafish. Proc Natl Acad Sci U S A. 106(47):19889-94.
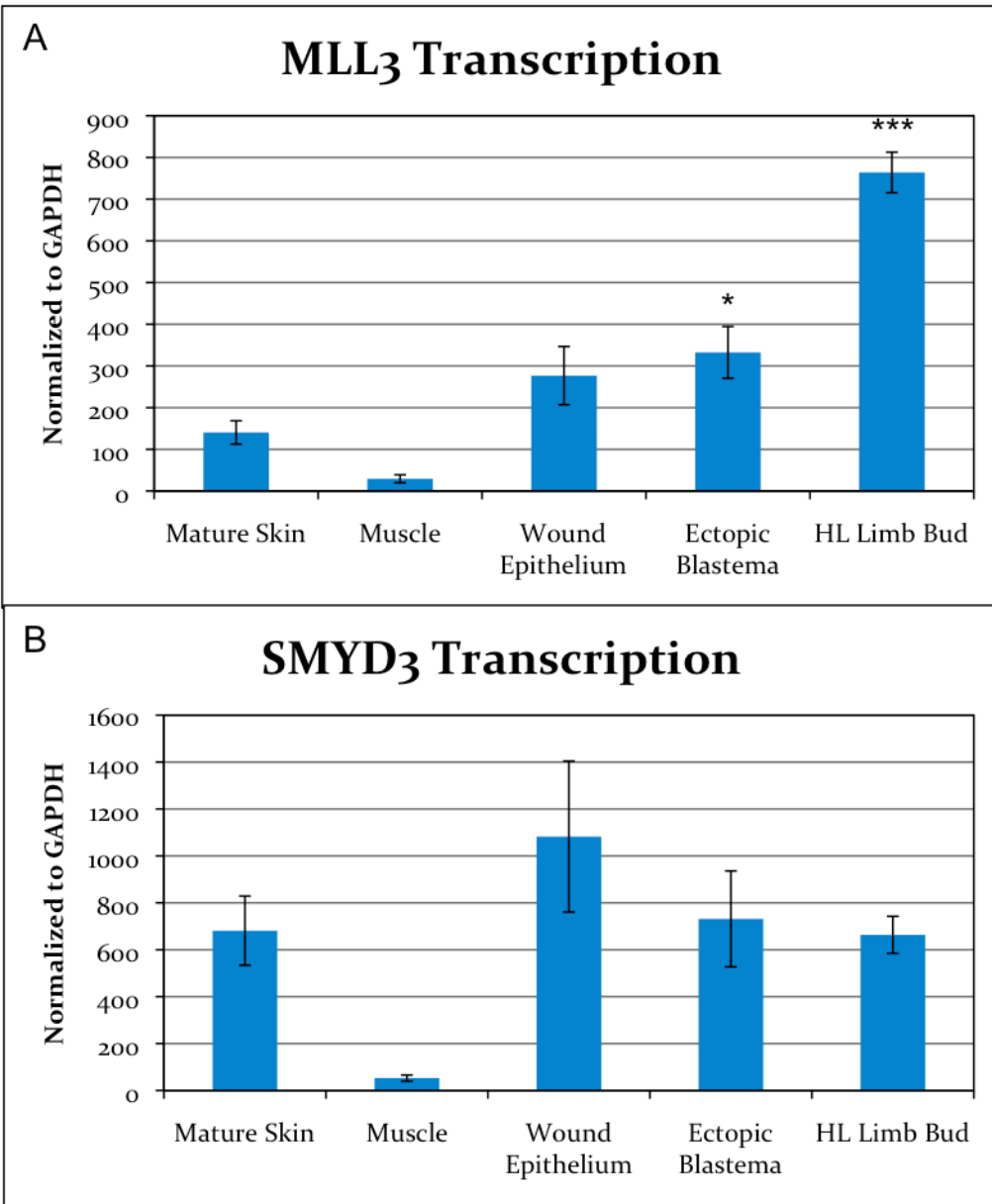
Figure 4.1. Histone 3 Lysine 4 methyltransferase expression.

The relative expression of MLL3 (A) and SMYD3 (B) is determined by qPCR analysis. Regenerative samples (wound epithelium, ectopic blastema) are compared to non-regenerating tissues (mature skin, muscle), as well as to developing hind limb buds. (* = p < 0.05; *** = p < 0.0005)
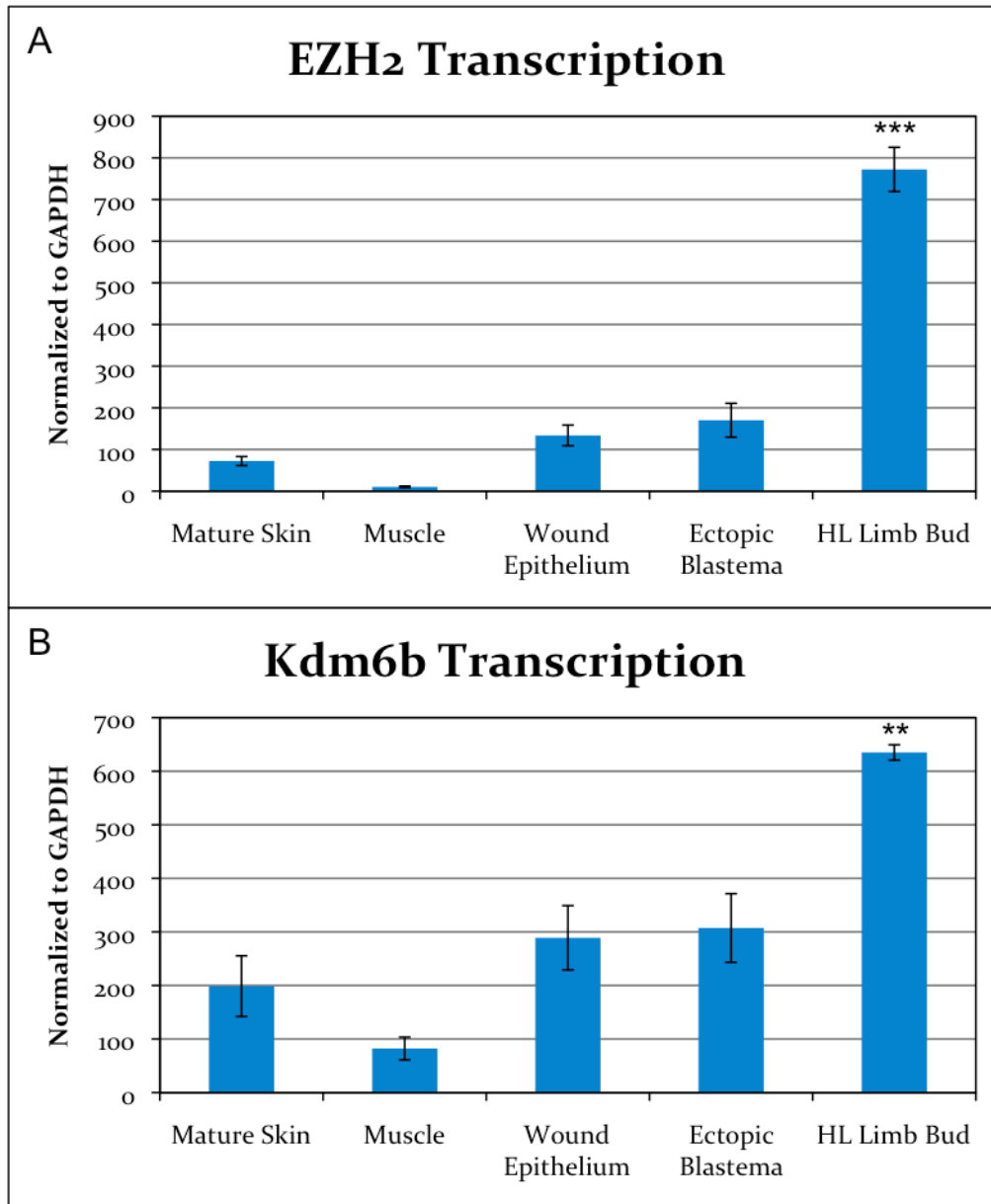
Figure 4.2. Histone 3 Lysine 27 methyltransferase and demethylase expression.
The relative expression of EZH2 (A) and Kdm6b (B) is determined by qPCR analysis. Regenerative samples (wound epithelium, ectopic blastema) are compared to non-regenerating tissues (mature skin, muscle), as well as to developing hind limb buds. (** = $p < 0.005$; *** = $p < 0.0005$)
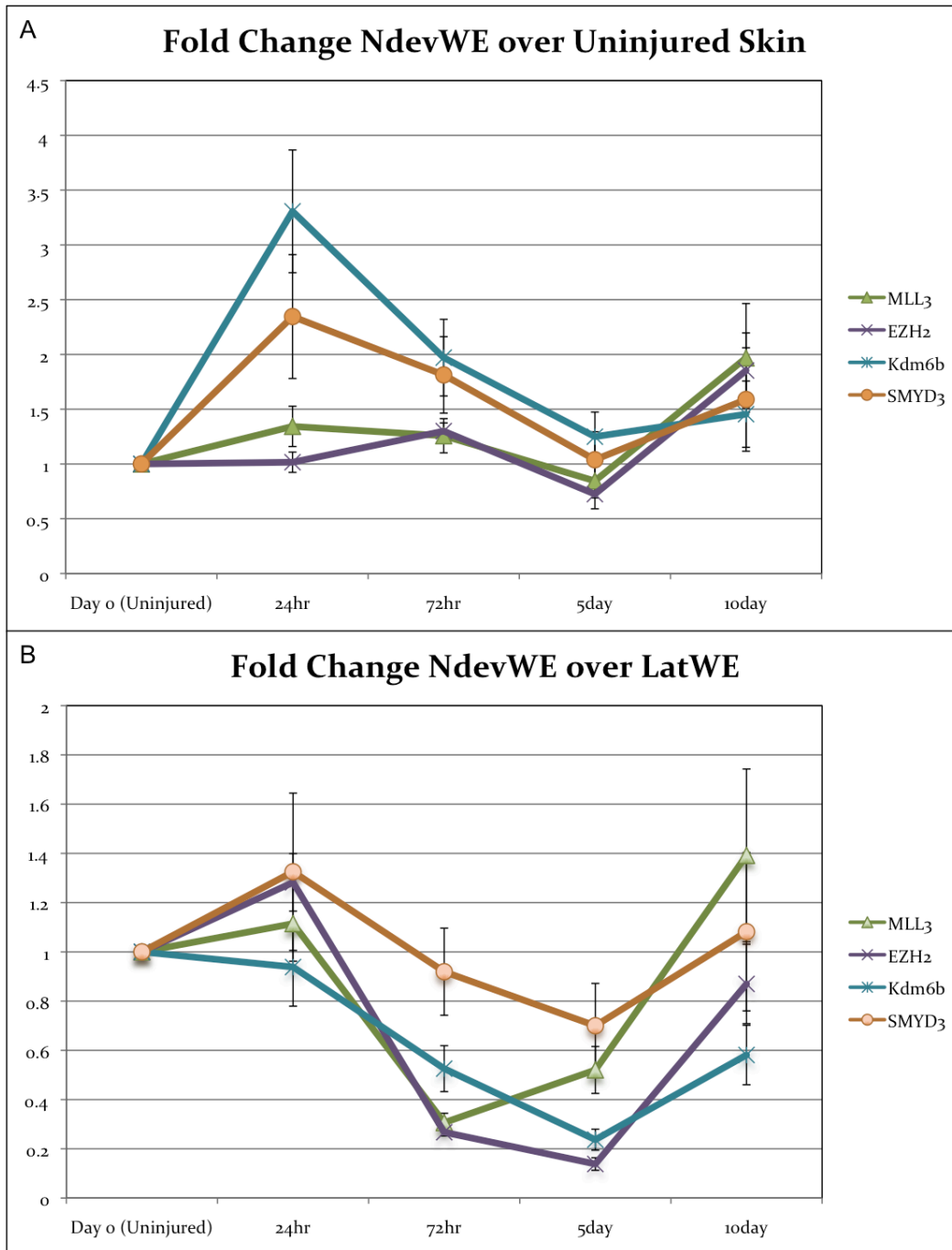
Figure 4.3. Relative expression of histone modifiers during early wound epithelium formation.
    A time course analysis of histone modifier expression was performed with wound epithelium and skin samples collected between 24 hrs and 10 days post injury. Regenerative wound epithelia formed through the use of the accessory limb model are compared to uninjured mature skin (A), and to simple wounds that did not receive a deviated nerve (B).

CHAPTER 5 – Future Directions

The analysis of expression of various epigenetic modifiers during early regeneration events in the axolotl, including dedifferentiation, has provided a first step towards understanding the vast changes in cellular behavior and states of differentiation that accompany blastema formation. The expression patterns of DNA methyltransferases specifically corroborate recent expression analyses conducted in the regenerating caudal fin of zebrafish [1]. Furthermore, the experimental manipulation of DNMT activity through the use of the cytosine analog decitabine has established that DNA methylation plays a direct role in mediating the formation of a wound epithelium that is responsive to neurotrophic signals initiating the regenerative response. In order to further this area of investigation, it would be extremely interesting to determine the subset of genes whose expression levels are affected during a regeneration event. Unfortunately, the genome of *Ambystoma mexicanum* has yet to receive the attention necessary to determine the full sequence. Complicating this endeavor is the large size of the genome as well as the high degree of repetitive sequence present [2]. Without the genome sequenced, analysis of global expression pattern changes becomes more difficult. Recently, however, the technology of RNA-seq has been successfully applied to regenerating limbs of the axolotl [2,3].

RNA-seq allows for the determination of sequence for transcribed genes. While this analysis does not include regulatory regions of DNA, it can provide the relative changes in gene expression between all genes being expressed. Briefly, RNA is isolated from tissue samples and undergoes poly adenosine selection in order to enrich the proportion of mRNA in the sample. The RNA is then fragmented in order to obtain a fairly uniform size

distribution before the RNA is reverse transcribed into cDNA. With the addition of

sequencing primers and indices, the cDNA can be sequenced and information about the

repertoire of genes expressed between samples can be obtained. The resultant

transcriptome can then be assembled from the sequencing reads produced, giving full-

length gene sequence. While this protocol provides information about all expressed genes,

it is important to remember that only genes expressed within the sample can be detected.

Therefore, a variety of tissue samples must be assayed in order to detect genes expressed

in a tissue specific manner. Likewise, the pooling of samples composed of multiple

morphologically distinct tissues may result in a decreased probability of detecting

transcripts that are lowly expressed, especially in a tissue that is not equally represented

volumetrically to other tissues in the sample. In a recent RNA-seq analysis of axolotl

regenerating limbs, researchers designed a sample collection schedule spanning the first

40 days of regeneration [3]. After limb amputation at the mid stylopod level, the

amputation-induced blastemas were collected by re-amputation. For later time points, the

samples were comprised solely of regenerated tissue owing to the large amount of

resultant structure. However, early time points in the schedule required the collection of

stump tissue proximal to the original amputation plane. Furthermore, all regenerated

tissue was collected at each time point, resulting in later time points being comprised of

more tissue mass with a lower surface area to volume ratio. Their analysis revealed a burst

in expression of oncogenes responsible for regulating cell cycle progression [3]. It is likely

that the sample collection protocol prevented the identification of genes that were lowly

expressed, especially if they are only expressed in specific cell types within the blastema.

The blastema as a whole is a very heterogeneous population of cells that are derived from

numerous lineages within the stump tissue. In addition, the blastema can be

morphologically and surgically separated into the two distinct structures of the wound

epithelium and mesenchyme. Moreover, amputation of a whole limb induces cellular

events and processes that are not integral to the regenerative process, but aid in the

prevention of infection and clearing of damaged tissue. Along with these convoluting

events comes the expression of genes necessary to carry out these processes. Again, the

expression of genes not directly involved in limb regeneration may obscure the expression

of lowly expressed genes that are necessary for regeneration.

I propose a RNA-seq experimental design (Fig.5.1) aimed at detecting changes in

gene expression that are specific to one component of the regenerating limb, in the absence

of convoluting sources of gene expression. In order to limit the activation of spurious gene

expression, I will utilize a gain-of-function assay for limb regeneration (the Accessory Limb

Model, ALM), which has allowed for the identification of a number of signals and pathways

that are necessary and sufficient for induction of blastema formation and subsequent

regeneration of an ectopic limb [4,5]. 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. The success of axolotl limb regeneration is dependent on the

formation of a blastema, which is in turn dependent on the function of a wound epithelium

(WE) (6). Within the context of the ALM, I will focus my analysis upon the wound

epithelium in order to identify genes that are relevant to the formation of a regenerative

wound epithelium and induce dedifferentiation in this relatively homogenous tissue. WE

function is regulated by signals from the regenerating nerve that induce dedifferentiation

of basal keratinocytes to a developmental state that is equivalent to the apical epithelial cap

(AEC) of the developing limb bud (7). Differentiation and acquisition of signaling function by the basal keratinocytes of the WE is associated with re-expression of the transcription factor *Sp9* that is involved in the regulation of FGF signaling (8). The loss of nerve signals (e.g. by denervation) results in the loss of *Sp9* expression, reentry into the cell cycle and regenerative failure (7). Thus the response of basal keratinocytes to nerve signaling provides an experimental opportunity to investigate the signaling and responses to signaling by a homogeneous population of target cells that play a essential functional role in blastema formation and regeneration. The early nerve-dependent signals leading to formation of the WE/AEC are critical to the success of regeneration, yet little is know about the underlying cellular and molecular mechanisms regulating this process. Nevertheless, it is intuitive that since regeneration is a stepwise process (4), the failure to progress from an early step to the next step will necessarily result in the failure of regeneration. Thus each necessary step represents a potential "barrier" to successful regeneration (9). My design will analyze the first barrier to regeneration, determining the differences between a simple wound healing response and a regenerative wound healing response (Fig.5.1). Furthermore, I will incorporate the findings of my previous work (Ch. 2), which determined a role for DNA methylation in regulating the switch between simple wound healing and regeneration. The analysis of simple wound epithelia treated with the DNA methyltransferase inhibitor, decitabine, will allow me to compare changes in gene expression to that observed in nerve deviated wound epithelia leading to the determination of the degree of regenerative similarity between the two samples. This will also shed light on genes that are directly or indirectly influenced by DNA methylation dynamics within the first 72 hours of regeneration. The regulatory pathways that are

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identified as being regulated by DNA methylation may serve as eventual targets for the induction of regenerative responses to human injury.

Beyond the analysis of regenerative wound epithelia formation as a result of DNA methylation dynamics in the axolotl limb, it will be interesting to investigate this mechanism in the context of regeneration of other structures such as the heart, lens, of the eye, or portions of the brain. The axolotl has the ability to regenerate these structures and more [10,11], and the mechanisms discovered in the limb would presumably be conserved. Determining the degree of conservation between structures may provide insight into initiating a regenerative response in a wide array of tissues, or improve the control of stem cell therapies aimed at treating diseases and injury in various parts of the body. Of course, even if DNA methylation dynamics were common to initiating a regenerative response, one would presume that each tissue and structure would be associated with its own subset of genes to be regulated. Outside of conservation between structures of the salamander, I plan to investigate the degree of conservation of DNA methylation dynamics between other species. While *Xenopus* and zebrafish do not exhibit the same degree of regenerative abilities as the salamnder [12], they are able to repair large injuries in order to regain some level of functionality. The fact that their genomes are sequenced will allow for the use of more sophisticated techniques to analyze DNA methylation mechanisms, such as bisulfite sequencing to analyze the methylation states of gene regulatory regions. Furthermore, the mouse digit has received attention from the regeneration community as a well-established mammalian regeneration model [13]. Its capacity to regenerate is normally restricted to the third phalangeal element, but recent work has resulted in the expansion of this ability to the second phalangeal element as well [14]. The analysis of DNA methylation dynamics

in the regenerating mouse digit tip, in combination with the knowledge gained from the

salamander, may provide an opportunity to quickly advance our understanding of loss of

regenerative abilities in higher organisms, and methods to return this ability to humans.

References

1. Takayama K , Shimoda N, Takanaga S, Hozumi S, Kikuchi Y. (2014) Expression patterns of dnmt3aa, dnmt3ab, and dnmt4 during development and fin regeneration in zebrafish. Gene Expr Patterns. 14(2):105-10.
2. Smith JJ, Putta S, Zhu W, Pao GM, Verma IM, Hunter T, Bryant SV, Gardiner DM, Harkins TT, Voss SR. (2009) Genic regions of a large salamander genome contain long introns and novel genes. BMC Genomics. 10:19.
3. Stewart R, Rascón CA, Tian S, Nie J, Barry C, Chu LF, Ardalani H, Wagner RJ, Probasco MD, Bolin JM, Leng N, Sengupta S, Volkmer M, Habermann B, Tanaka EM, Thomson JA, Dewey CN. (2013) Comparative RNA-seq analysis in the unsequenced axolotl: the oncogene burst highlights early gene expression in the blastema. PLoS Comput Biol. 9(3):e1002936.
4. Endo T, Bryant SV, Gardiner DM. (2004) A stepwise model system for limb regeneration. Dev Biol. 1;270(1):135-45.
5. Satoh A, Gardiner DM, Bryant SV, Endo T. (2007) Nerve-induced ectopic limb blastemas in the Axolotl are equivalent to amputation-induced blastemas. Dev Biol. 1;312(1):231-44.
6. Makanae A, Satoh A. (2012) Early regulation of axolotl limb regeneration. Anat Rec (Hoboken). 295(10):1566-74.
7. Satoh A, Bryant SV, Gardiner DM. (2012) Nerve signaling regulates basal keratinocyte proliferation in the blastema apical epithelial cap in the axolotl (Ambystoma mexicanum). Dev Biol. 366(2):374-81.
8. Kawakami Y, Esteban CR, Matsui T, Rodríguez-León J, Kato S, Izpisúa Belmonte JC. (2004) Sp8 and Sp9, two closely related buttonhead-like transcription factors, regulate Fgf8 expression and limb outgrowth in vertebrate embryos. Development. 131(19):4763-74.
9. Muller TL, Ngo-Muller V, Reginelli A, Taylor G, Anderson R, Muneoka K. (1999) Regeneration in higher vertebrates: limb buds and digit tips. Semin Cell Dev Biol. 10(4):405-13.
10. Nye, H.L., Cameron, J.A., Chernoff, E.A. and Stocum, D.L. (2003) Regeneration of the urodele limb: a review. Dev. Dyn. 226, 280–294.
11. Roy, S. and Gatien, S. (2008) Regeneration in axolotls: a model to aim for! Exp. Gerontol. 43, 968–973.
12. Katsuyama T, Paro R. (2011) Epigenetic reprogramming during tissue regeneration. FEBS Lett. 585(11):1617-24.
13. Fernando WA, Leininger E, Simkin J, Li N, Malcom CA, Sathyamoorthi S, Han M, Muneoka K. (2011) Wound healing and blastema formation in regenerating digit tips of adult mice. Dev Biol. 350(2):301-10.

14. Yu L, Han M, Yan M, Lee J, Muneoka K. (2012) BMP2 induces segment-specific skeletal regeneration from digit and limb amputations by establishing a new endochondral ossification center. Dev Biol. 372(2):263-73.
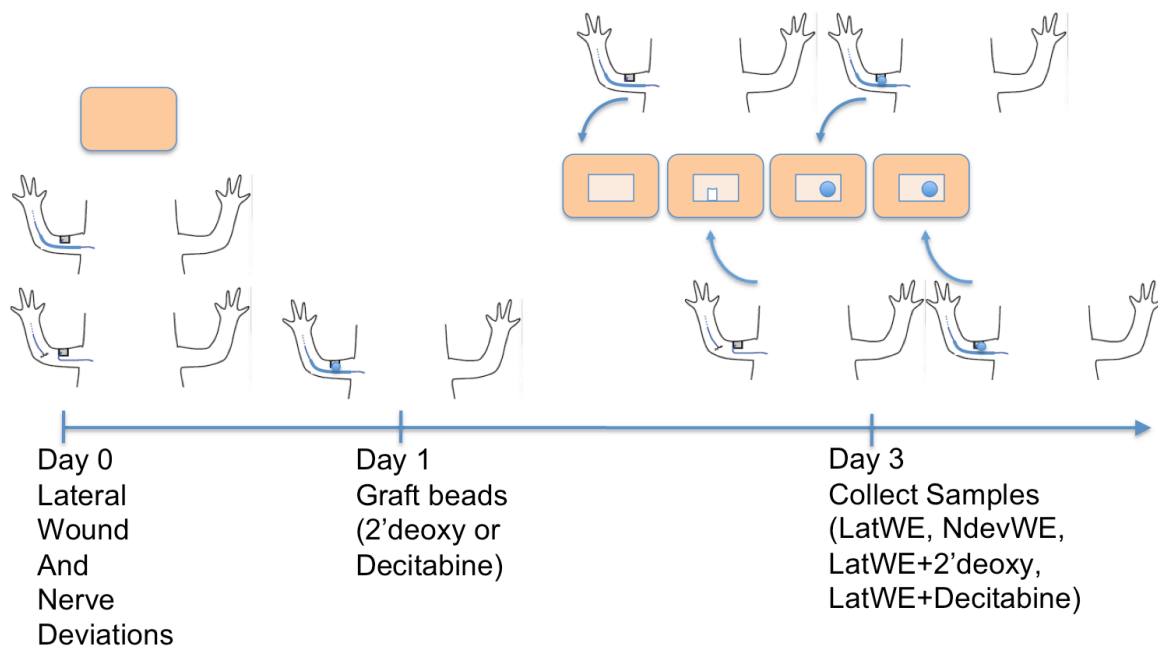
Figure 5.1. Sample creation and collection schedule for RNA-seq analysis.
  A schematic depicting the time points and specific samples to be collected for RNA-seq analysis towards the determination of differentially expressed genes. Mature skin is collected from the anterior of the stylopod. Resulting wounds are allowed to heal without initial manipulation, or receive a deviated nerve. After 24hrs of healing, non-nerve deviated wounds are treated with sol-gel beads delivering 2'-deoxycytidine or decitabine. At 72hrs after initial injury, all wound epithelia are collected without any risidual mature skin.