Beyond Prosthetics: the First Steps Towards Identifying Key Regulators of Limb Regeneration

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ABSTRACT

Despite the advances in prosthetic and robotic alternatives to limb loss, little progress has been made in human limb regenerative therapy. While mammals have the ability to heal after an amputation, most are unable to form a blastema. Currently, the genes needed for blastema formation are not known, but by examining the genes used during Tribolium castaneum beetle regeneration, these potential factors can begin to be identified. Knocking down the expression of enhancer of zeste (ezh2) and polycomb (pc) led to prolonged maintenance of blastema after Tribolium larval legs were ablated, indicating that these genes may play major roles in larval limb re-patterning. RNA-seq showed that POU domain protein (pdm) and metabolism genes may be important players in limb regeneration. Once the genes necessary for forming and maintaining a blastema are identified, it may one day be possible to turn on these blastema-inducer genes in humans and promote human limb regeneration.
INTRODUCTION

Overview of regeneration

Regeneration is a reparative process that replaces lost body parts and organs. This ability is observed in a host of invertebrates, including arthropods, flatworms and echinoderms, and many vertebrates, such as amphibians, teleost fishes and even some tissues and organs of humans. However, humans are unable to regenerate their limbs. While there has been research done in the realm of limb regeneration in invertebrates and vertebrates, we still have not been able to find a way to make human limb regenerative therapy possible. There have been advances in prosthetic and robotic alternatives to limb loss, but there currently is no regenerative therapy for amputees. The lack of progress in this field can be attributed to the fact that mammals have low ability to regenerate limbs. Most model systems, including mice and frogs, have been unable to contribute to this field of study because of their lack of ability to regenerate limbs as adults. These factors pose a major obstacle in making strides in the field of regenerative medicine.

Regeneration can be divided into two categories: epimorphosis and morphallaxis. Epimorphosis is characterized by growing back the missing parts without a drastic rearrangement of remaining tissues. During morphallaxis, the remaining part of the body is radically remodeled to regenerate all parts of the body. Limb regeneration in vertebrates is characterized by epimorphosis, and it is this mode of regeneration that we focus on in this study.

While animals have the ability to heal after an amputation, mammals are unable to grow a new arm or leg. However, there are certain animal species that maintain the ability to regenerate and may shed light on how epimorphic limb regeneration might be restored in mammals. Specifically, insects and salamanders have the ability to form blastemas, which are clusters of
de-differentiated cells that act as stem-like cells to create the new limb (Tanaka, 2003). In mammals, blastema formation does not occur and, instead, a scar tissue is formed (Rinkevich et al., 2011). It is still unclear why this is the case, but understanding how a blastema forms is critical for regenerative therapy. In order to be able to form a blastema, blastema-specific genes need to be activated. Currently, these genes are not known, but by examining the genes used during limb regeneration, we can begin to identify these potential factors.

**Regeneration in invertebrates**

Regeneration is widespread among invertebrates. Two particular invertebrates have received the most attention in terms of regenerative abilities: diploblastic *Hydra vulgaris* and triploblastic freshwater planarians, *Schmidtea mediterranea* and *Dugesia japonica* (Alavarado and Tsonis, 2006). While the loss of essential body parts, such as the head, usually leads to death for most animals, the hydra continues to due to regeneration, which usually continues without detectable cell proliferation (Holstein et al., 1991). Unlike hydra, planarians regenerate missing body parts by forming a blastema (Reddien and Sanchez Alvarado, 2004).

In addition, members of the Ecdysozoa, such as insects, arachnids and crustaceans, have the ability to regenerate limbs (Alvarado and Tsonia, 2006; Cooper, 1998). Polychaete and oligochaete worms can regenerate their heads after decapitation (Alvarado and Tsonis, 2006). Echinoderms, such as the sea cucumber, distract their predators by disposing of their digestive system and, soon after, regenerate it completely (Garcia-Arraras et al., 1998).
**Limb regeneration in vertebrates**

While limb regeneration is common in various non-vertebrate organisms, there are only a few adult vertebrates with the ability to regenerate limbs. Limb regeneration occurs in urodele amphibians, including news and salamanders (Bryant et al., 2002). Newts are considered to have the most versatile regenerative abilities among vertebrates. As an adult, it can regenerate its limbs, tail, lens and retina, hair cells, brain and spinal cord, jaws and heart (Tsonis, 2000).

Studies in urodele amphibians have shown that the process of epimorphic limb regeneration is comprised of three phases: wound healing, de-differentiation and re-patterning (Kragl et al., 2009; Endo et al., 2004). In these animals, limb regeneration is characterized by wound healing and formation of a blastema. In addition, fin regeneration in zebrafish is also governed by the formation of a blastema (Alavarado and Tsonia, 2006). While humans have the ability to regenerate many structures, they are unable to regenerate limbs. Mammalian epidermis heals at the site of limb amputation, but a scar tissue forms instead of a blastema, preventing the limb from regenerating (Gardiner et al., 1999). Mammals have differentiated cells that hardly de-differentiate and/or change fates under normal conditions, and removal of mammalian limbs usually leads to irregular wound healing with scar formation (Neufeld, 1985; Neufeld, 1989). However, neonatal mice as well as embryos can regenerate their digit tips (Reginelli et al., 1995; Han et al., 2003). This form of regeneration has also been observed occasionally in humans, where fingertip regeneration has been observed in children (Illingworth, 1974; Han et al., 2005).

**Blastema development and regulation**

The presence (or lack thereof) of blastema formation is a crucial aspect of determining what type of regeneration has occurred. After the amputation of a limb, the wound surface is
sealed in 12 hours via rapid migration of epithelial cells. Cell migration leads to the formation of the wound epidermis, which is a transient epithelium crucial for subsequent outgrowth. Urodeles can reverse the differentiated state of cells in response to amputation or tissue removal: the postmitotic cells of the limb mesenchyme found under the wound epidermis can reenter the cell cycle and lose their differentiated character (Brockes, 1997). These blastemal cells proliferate to produce a conical mound of cells, which have been found to arise from cells located within 1-2 mm of the amputation plane (Gardiner et al., 1986). These blastemal cells then exit from the cell cycle and begin to differentiate into the cartilage, connective tissues and muscle of the new regenerated limb. Axial identity is crucial for regeneration, in that the blastema only gives rise to structures that are distal to its level of origin—a wrist blastema gives rise to a hand, and a shoulder blastema leads to the formation of an entire arm (Brockes, 1997). The blastema also has a significant amount of morphogenetic autonomy, in that if it were to be transplanted to a location different from its origin, it can give rise to a regenerate that is appropriate for its level of origin (Stocum, 1984).

The apical epidermis has been shown to influence the location at which blastema cells accumulate (Thornton and Thornton, 1965). During de-differentiation, the wound epidermis thickens and forms a structure known as the apical epidermal cap (Thornton, 1956). One of the main functions of the apical epidermal cap is the production and transport of various fibroblast growth factors (FGFs). FGFs are members of the heparin-binding growth factor family (HBGF) and are found to affect the proliferation and differentiation of cells is involved in embryonic induction, angiogenesis and the healing of damaged tissue (Basilico and Moscatelli, 1992; Pandit et al., 1998). *Fgfr1* is expressed in regenerating blastema. *Fgfr2* expressed predominantly in
blastemal mesenchyme during newt limb regeneration and is also expressed in the basal layers of wound epithelium during limb regeneration (Peters et al., 1992; Orr-Urtreger et al., 1993).

Nerve dependency is an important aspect of blastema formation in vertebrates. Denervation (the removal of axons in the limbs) concurrently with limb amputation prevents proper blastema formation and the outgrowth of blastema, thus resulting in the lack of new limb structures (Simões et al., 2014). It has been thought that neurotrophic factors produced in the neurons stimulate proliferation and allow blastemal cells to survive (Maden, 1978, Mescher and Tassava, 1975). Several genes, including FGFs and Dlx3, were shown to be downregulated by denervation (Cannata et al., 2001), suggesting that neurotrophic factors regulate molecular mechanisms that play a role in the maintenance of blastemal cells and blastema growth (Suzuki et al., 2005; Kumar et al., 2007).

**De-differentiation in blastema development**

A crucial mechanism associated with blastema formation is de-differentiation, which involves a terminally differentiated cell reverting back to an earlier stage in cell commitment. This progress enables the cells to multiply again before re-differentiating, leading to the replacement of those cells that have been lost. De-differentiation is seen to occur during limb regeneration in urodele amphibians. Soon after limb ablation, cells near the wound begin to de-differentiate resulting in the formation of a blastema. The blastema is comprised of de-differentiated cells that go on to proliferate and re-differentiate to regenerate the missing limb. The blastema has been referred to as a group of progenitor cells (Nye et al., 2003), and it has been proposed that cells adjacent to the wound de-differentiate back to pluripotent cells, where these cells have the potential to contribute to cells of any germ layer type. These cells would then
proliferate and differentiate again to create the lost limb. However, work by Echeverri and Tanaka (2002) on axolotls indicates that de-differentiated blastema cells only undergo partial de-differentiation so that the potency of the cells is limited. More recently, Kragl and colleagues (2009) using GFP-marked donor cells demonstrated that the cells in the blastema retain their cellular memory, where they retain their cell fate.

**Identification of blastema markers**

It has recently become possible to de-differentiate or reprogram fibroblasts into induced pluripotent stem (iPS) cells *in vitro* through the activation of four transcription factors (Takahashi and Yamanaka, 2006). This brings up the question of whether the *in vivo* de-differentiation seen during regeneration has similarities to the *in vitro* reprogramming of fibroblasts into iPS cells. Reprogramming factors, *myc*, *sox2* and *klf4* have been shown to be expressed during newt regeneration (Maki et al., 2009), and two reprogramming factors are expressed during *Xenopus* limb regeneration (Christen et al., 2010). It was also found that during blastema formation, some key reprogramming factors are expressed and required for regeneration to take place, suggesting a partially overlapping mechanism between the processes of iPS cell reprogramming and blastema formation (Christen et al., 2010).

In the context of regenerative medicine, since humans are unable to generate a blastema, it is crucial to pinpoint blastema markers in order for us to begin understanding the molecular underpinnings of this process. There are two approaches for identifying possible blastema markers: one is to look for candidate genes that might be associated with blastema formation. We might expect that the overall levels of pluripotency associated factors would increase to an expression level comparable to that of a pluripotent reference cells (Christen et al., 2010). Thus,
examining the expression of pluripotency genes might allow us to identify blastema-specific markers. The second method is to perform de novo sequencing to identify differentially expressed genes. This is an unbiased way to identify genes uniquely expressed in the blastema. In this study, we sought to identify blastema markers using both of these strategies.

Blastemas form from cells in de-differentiated states that are often associated with changes in the state of epigenetic regulation. When embryonic gene expression is activated, it has been found to parallel with epigenetic reprogramming (Bouniol et al., 1995). We hypothesize that epigenetic factors may be important for blastema formation.

Epigenetics and the role of polycomb group (PcG) proteins

Epigenetics is defined as a heritable change in phenotype that does not include a change in the underlying DNA sequence. Histone modifications are considered epigenetic regulators of chromatin. The histone code determines higher-order chromatin structure by influencing contacts between different histones and between histones and DNA (Kouzarides, 2007). It has been shown that silent chromatin can be converted into an active state through loss of specific histone modifications, occurring at various genes during regeneration (Stewart et al., 2009). These histone modifications are created by the collaborative action of Polycomb and Trithorax groups.

Polycomb group (PcG) and Trithorax group (TrxG) proteins are conserved regulatory factors that play an important role in modifying chromatin of target genes by changing the accessibility of DNA to factors that are necessary for gene transcription. PcG genes are involved with silencing chromatin-based genes through regulating the methylation as well as removing acetyl groups, while TrxG genes are responsible for counteracting the silencing effects of chromatin in order to maintain gene activity (Orlando, 2003); describe how TrxG proteins
function to counteract silencing. PcG proteins were initially thought to maintain gene silencing by keeping active genes in a heterochromatin-like environment that prevents transcriptional activators from entering and is considered incompatible with RNA synthesis (Orlando, 2003). In this manner, the silent chromatin would irreversibly program differentiated cells to not leave their fate. However, recent studies indicate that PcG-mediated silencing is the result of an equilibrium between opposing but coexisting transcriptional forces. These forces include both activators and repressors that not only maintain terminally determined states but also allow for the switching of states (Orlando, 2003).

There have been approximately fifteen PcG proteins identified that participate in two separate multiprotein complexes: PRC1 (Polycomb repressive complex 1), containing Polycomb (PC), Polyhomeotic (PH) and Posterior sex combs (PSC) (Shao et al., 1999); and PRC2 (Polycomb repressive complex 2) composed of EZH2, SUZ12 (Suppressor of zeste 12) and EED (Kuzmichev et al., 2004; Kuzmichev et al., 2005). EZH2 is a H3K27 methyltransferase, and SUZ12 is required for this activity (Cao and Zhang, 2004). ES cell lines are unable to be established from EZH2-deficient blastocysts (O’Carroll et al., 2001), which suggests that PRC2 plays a role in regulating pluripotency and self-renewal. In Drosophila, the ESC-E(Z) (extra sex combs-Enhancer-of-zeste) complex is the equivalent to PRC2. It acts during the early stages of embryogenesis and is thought to set the stage for long-term memory PRC1 complex (Orlando, 2003). Both PRC1 and ESC-E(Z) complexes are conserved between flies and humans (Levine et al. 2002).

The path by which these complexes find their way onto chromatin and convey epigenetic inheritance is still unclear. In Drosophila, both PcG and trxG complexes exert their epigenetic function by binding to specialized, switchable modular DNA elements, known as Polycomb
response elements (PREs) or cell memory modules (CMMs) (Lyko and Paro, 1999). PREs, along
with promoters, allow for heritable silenced transcription patterns in an epigenetic manner. When
triggered by a transiently expressed activator, the same element is able to maintain the active
state indefinitely, including through female germline transmission (Cavalli and Paro, 1998). It
has been determined that the E(Z) SET domain methylates H3 at K9 and K27 (Cao et al., 2002)
and PC has a strong affinity for H3 methylated at K27 (Muller et al., 2002).

As noted previously, ESC-E(Z) is active in the early embryo in combination with the cell
fate determination system. Lack of maternal esc and E(z) gene products results in severe
homeotic transformation that can be only partially rescued by paternally derived zygotic product
(Jones and Gelbart, 1990). PcG loss-of-function experiences in imaginal discs show that the
initial imprint is stable enough to allow silencing even after transient reactivation of Bithorax
complex (BX-C) genes (Bechle et al., 2001). This indicates that transcription is not sufficient in
removing the PcG silencing epigenetic tag.

In this study we wish to determine the role of these epigenetic regulators during
regeneration and their potential role in maintaining a de-differentiated state. We hope to show
that epigenetic regulators play a major role in regeneration and epigenetic regulators may be
required for silencing blastema specific factors. By following this logic, we hypothesize that
silencing of PcG proteins would enable de-differentiation, leading to the growth of permanent
blastemas and detection of blastema markers.

Hypothesis and strategy

In this study, the red flour beetle, Tribolium castaneum, was used to determine blastema
markers during limb regeneration. The Tribolium polycomb (pc) and enhancer of zeste homolog
2 (ezh2) genes were silenced as key regulators of chromatin modification to determine whether their absence might disrupt the regeneration process. We hypothesized that if a permanent blastema can be formed, genes expressed in the blastema can be analyzed so that we might be able to identify blastema markers.

**Tribolium life cycle and regeneration**

The flour beetle *Tribolium castaneum* are easily cultured and have relatively short generation time, allowing us to garner a large sample size. It is also easy to see regeneration occurring in these insects, which develop limbs externally from limb buds (Beermann, *et al*., 2001; Shah *et al* 2011). The *Tribolium* genome has also been completely sequenced (*Tribolium* Genome Sequencing Consortium, 2008), allowing us to make comparisons between insects and vertebrates. RNA interference (RNAi), a tool to knock down and analyze the function of genes, has been successfully used on *Tribolium* beetles (Tomoyasu *et al*., 2008). Its ability to regenerate, short regeneration time, sequenced genome, and compatibility with RNAi make *Tribolium* an ideal model system for studying factors responsible for limb regeneration.

**RNA interference**

RNA interference (RNAi) refers to post-transcriptional silencing of gene expression that results from the introduction of double stranded RNA (dsRNA) into a cell (Fire *et al*. 1998). RNAi was first described in plants as a means of post-transcriptional gene silencing, but grew to become a useful tool for determining gene function in other organisms. In animals, this technique was first successfully implemented in *C. elegans* (Fire *et al*. 1998). When dsRNA is introduced into the cell, the cellular enzyme Dicer binds to the dsRNA and cleaves it into shorter pieces of
about 20 nucleotide base pairs, known as small interfering RNAs (siRNAs). These siRNAs then bind to the RNA-induced silencing complex (RISC), and the siRNA is reduced to single-stranded RNA. The complex finds the complementary sequence in the target mRNA, and RISC then clips and degrades the mRNA (Grishok et al., 2005). Thus, the nuclease activity of RISC leads to the inactivation of gene expression. RNAi is now widely used to knock down specific genes of interest. In our study, we injected dsRNA into Tribolium to determine the effects of specific gene silencing on function. In this species, RNAi works systemically, and RNAi is used as a targeted gene knockdown to allow for the testing of gene functionality during Tribolium larval leg regeneration (Shah et al., 2011).

**Candidate gene approach**

Because Tribolium blastema markers have yet to be identified, we decided to take the candidate gene approach and picked four genes that would potentially serve as good markers in indicating the presence of a blastema. Yamanaka factors are transcription factors that restoring pluripotency in somatic cells. Oct4, Sox2 and Myc are key transcription factors that are expressed in undifferentiated pluripotent embryonic stem (ES) cells. Myc is a regulator that plays a role in cell cycle progression and proliferation as well as pluripotency maintenance of embryonic stem cells (Sato et al., 2012). Sex determining region Y-box 2 (sox2) is a stem cell factor and is responsible for pluripotency. Ventral veins lacking (Vvl) shares the highest sequence similarity with Oct4, a well-known vertebrate pluripotency marker. In vertebrates, these factors have been found to carry the ability to reprogram somatic cells and convert them to induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006). Because of their pluripotent nature, these factors are potentially viable genes to look at. Along with Yamanaka
factors, Twist (Twi) is a mesodermal marker that serves as a blastema marker in vertebrates (Kragl et al., 2013).

The Yamanaka factors have been used as pluripotency markers across various vertebrates. Myc has been used as a pluripotency marker in zebrafish (Kinikoglu et al., 2014), *Xenopus* (Perry et al., 2013) and mice (Asadi et al., 2013). Sox2 has also been used as a pluripotency marker in *Xenopus* (Perry et al., 2013).

**RNA Sequencing**

RNA sequencing (RNA-Seq) is a method used for mapping and quantifying transcriptomes in all organisms, including mouse and human cells (Mortazavi et al., 2008). This technique takes a RNA population and converts it to cDNA fragments with adaptors attached to one or both ends. Each molecule is then sequenced, with readings typically of 30-400 bp, depending on the type of DNA-sequencing technology used (Wang et al., 2009). RNA-Seq provides several advantages, where it can determine the exact location of transcription boundaries as well as information on exon connections. These aspects of RNA-seq make this method useful for studying complex transcriptomes. Because RNA-Seq has a very low background signal, it has a large range of expression over which transcripts can be detected. In order to determine which genes are turned on or off during regeneration, we can use RNA-Seq to analyze the global gene expression changes without any bias and to identify all the genes that are differentially expressed.

**MATERIALS AND METHODS**

**Beetle husbandry**
*Tribolium castaneum* GAL strain were acquired from Dr. Richard Beeman (USDA ARS Biological Research Unit, Grain Marketing & Production Research Center, Manhattan, Kansas). The beetles were raised on organic wheat flour containing 5% nutritional yeast, and were kept in a 29°C incubator with ~50% relative humidity in plastic containers.

**mRNA isolation and cDNA synthesis**

Larvae were dissected in 1X-phosphate-buffered saline (PBS; 0.02 M phosphate, 0.15 M NaCl, 0.0038 M NaH₂PO₄, 0.012 M Na₂HPO₄; pH 7.4) to take out the gut and the fat body. The remaining tissue was homogenized in Trizol (Invitrogen), and RNA was then extracted using chloroform, treated with DNase (Promega), and precipitated in isopropyl alcohol. cDNA was synthesized from 1 μg of total RNA via the cDNA synthesis kit (Fermentas) using the manufacturer’s instructions.

**Cloning and double stranded RNA synthesis**

Sequences of *pc* and *ezh2* were acquired from sequences deposited in GenBank (*pc* GenBank accession number XM_008199399; *ezh2* GenBank accession number XM_001811600). The amplified cDNA product was isolated and cloned into a TOPO TA vector (Invitrogen). After plasmid identity was confirmed by sequencing, plasmid DNA was linearized through restriction digestion.

The strands of dsRNA were synthesized with T3 and T7 MEGAscript Kits (Ambion) using the manufacturer’s instructions. Single-stranded RNAs (ssRNAs) were combined and annealed to create dsRNA (Hughes and Kaufman, 2000). The annealed product was analyzed via
gel electrophoresis to confirm annealing, then were stored at -80°C until use. The final concentrations of the dsRNA were 2 μg/μL for pc and ezh2.

Table 1. Primers used in this study

<table>
<thead>
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<th>Gene</th>
<th>Direction</th>
<th>Sequence (5’ → 3’)</th>
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<tbody>
<tr>
<td>pc</td>
<td>Forward</td>
<td>TTCTCACCGCCACAACTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTCCGTTTACCGCCACCTTT</td>
</tr>
<tr>
<td>ezh2</td>
<td>Forward</td>
<td>TGGGCGATGATTTACTGG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTCTTTCTCTCTGTCTTTGTCTT</td>
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<td>rp49</td>
<td>Forward</td>
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<tr>
<td>(qPCR)</td>
<td>Reverse</td>
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<tr>
<td>myc</td>
<td>Forward</td>
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<tr>
<td>(qPCR)</td>
<td>Reverse</td>
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<tr>
<td>sox2</td>
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<td>(qPCR)</td>
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<td>vvl</td>
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<tr>
<td>(qPCR)</td>
<td>Reverse</td>
<td>GGCATTCCCTCCATCATCTC</td>
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</tbody>
</table>

Double stranded RNA injection

Day zero sixth instar larvae (seventh or eighth instar is considered the final instar within our colony) were injected with approximately 0.5 μg (0.25 μL) of dsRNA between the first and second abdominal segments of their dorsal side with a pulled 10-μL glass capillary needle.
connected to a syringe. *ampicillin resistance (amp*) dsRNA was injected into larvae as the control. These dsRNA-treated larvae were maintained at normal conditions (29°C in whole wheat flour containing 5% yeast) until their appendages were ablated two days later. Ablation was delayed to ensure that the RNAi-mediated knockdown was fully effective prior to appendage cuts (Shah et al., 2011).

**Leg ablations**

Two days after dsRNA injections, the larval mid and hind limbs were cut close to the base of the femur on one side. The larvae were anesthetized on ice and were placed on a slide covered with double-sided tape with the ventral side up. Under a dissecting microscope, their legs were ablated using fine microscissors.

**Quantitative PCR**

Day zero sixth instar larvae were injected with *ezh2* or control *amp* dsRNA. For each treatment, ventral plates containing the mid- and hindlegs of sixteen larvae were collected in Trizol after two days after molting into the seventh instar and were then homogenized to isolate their RNA. After RNA isolation, 0.5 μg of the RNA was converted to cDNA and amplified using SYBR Green Supermix and qPCR primers of *twi, vvl, myc, Ribosomal protein 49 (rp49)* (0.5 μL cDNA, 0.5 μL forward primer, 0.5 μL reverse primer, 10 μL SYBR Green PCR Master Mix and 8.0 μL deionized water). Each sample was assayed in triplicates.

**RNA Sequencing**
Because we observed partial regeneration in \textit{pc} dsRNA-injected larva, we decided to use \textit{ezh2} dsRNA-injected animals and \textit{amp'} dsRNA-injected animals as the control for the RNA-seq. Day zero sixth instar larvae were injected with \textit{pc}, \textit{ezh2} or control \textit{amp'} dsRNA. For each treatment, sixteen larvae were collected in Trizol two days after molting into the seventh instar and were then homogenized to isolate their RNA. Collected RNA went through DNase treatment and was sent off to SeqMatic (Fremont, CA) for RNA-seq analysis.
RESULTS

Knockdown of pc expression inhibits limb regeneration

To elucidate how pc silencing affects Tribolium regeneration, pc dsRNA was injected into day zero sixth instar larvae (n=10). Mid- and hind-legs on one side of these larvae were cut two day later, and regeneration of the ablated appendages was recorded after every molt (Table 2). Treated animals were observed during their subsequent life stages. In amp' dsRNA-injected control animals (n=7), wound healing and the formation of blastema-like structures were formed after the first larval molt. After the second larval molt, segments were reformed and both mid- and hind-leg growth were consistent with general leg morphology (Fig. 1A). amp' dsRNA-injected larvae that became pupae after one and two larval molts exhibited regenerated limbs as well (not shown).

In pc knockdown animals, all larvae showed wound healing at the leg ablation sites similar to that of amp' dsRNA-injected controls after one larval molt. These animals also exhibited blastema-like structures at the ablation sites. After the second molt, six of the surviving pc dsRNA-injected larvae showed no further regeneration (Fig. 1B), while three of the larvae exhibited partial regeneration (Fig. 1C). The one larvae that made it to the pupal stage also exhibited no regeneration (Fig. 2). These results indicate that pc is required for blastema re-differentiation and complete regeneration in Tribolium.

ezh2 is essential for the regeneration of appendages

In order to determine how ezh2 knockdown affects Tribolium regeneration, ezh2 dsRNA was injected into day zero sixth instar larvae (n=13). Mid- and hind-legs on one side of these
larvae were cut two day later, and regeneration of the ablated appendages was recorded after every molt. Treated animals were observed during their subsequent life stages. In *ezh2* knockdown animals, all larvae showed wound healing at the leg ablation sites similar to that of *amp* dsRNA-injected controls as well as *pc* dsRNA-injected animals after one larval molt. These animals also exhibited blastema-like structures at the ablation sites. After the second molt, all *ezh2* dsRNA-injected larvae showed no regeneration (n=6, Fig. 1D). The larvae that pupated after both one and two larval molts also exhibited no regeneration (not shown). These results indicate that *ezh2* is essential for blastema cell growth and complete regeneration in *Tribolium*. Because an *ezh2* knockdown was more effective in maintaining a blastema than *pc*, it may be responsible for suppressing more genes that involved in *Tribolium* limb regeneration. Since *ezh2* dsRNA-injected animals consistently showed lack of regeneration, we decided to focus our blastema gene expression study on this particular gene.

**myc, sox2, twi and vvl expressions are not uniquely expressed in *ezh2* knockdown blastemas**

To investigate whether any of the candidate genes might be expressed in the blastema, the expressions of *myc, sox2, twi and vvl* were determined in blastemas isolated from day two sixth instar *ezh2* and *amp* dsRNA-injected larvae. Since these larvae typically undergo a second molt approximately four days after the first molt, we reasoned that by day two after the first molt, the re-differentiating *amp* dsRNA-injected legs would have shut off the de-differentiation genes. A quantitative PCR analysis of candidate gene expressions showed that relative to the *amp* dsRNA-injected animals, *ezh2* dsRNA-injected animals had a 1.95-fold higher expression in *myc*, 2.35-fold higher expression in *sox2*, a 0.42-fold higher expression in *twi*, and a 1.09-fold higher expression in *vvl*. These results indicate that these candidate genes do not serve as
sufficient blastema markers because they are expressed in \textit{amp} dsRNA-injected re-
differentiating legs. Thus, we proceeded to investigate gene expression in an unbiased manner 
through RNA sequencing.

\textbf{Differential gene expression found through RNA-seq}

To determine which genes were differentially expressed in \textit{amp} and \textit{ezh2} knockdown animals, day zero sixth instar \textit{amp} and \textit{ezh2} dsRNA-injected larvae were collected two days after the first molt and homogenized for RNA isolation. The collected RNA was treated with DNase and then sent to SeqMatic (Fremont, CA) for RNA-seq analysis. Eighty genes were uniquely expressed in leg stumps of \textit{ezh2} dsRNA-injected larvae but absent in leg stumps of \textit{amp} dsRNA-injected larvae, and seven genes were uniquely expressed in \textit{amp} dsRNA leg stumps but absent in \textit{ezh2} dsRNA leg stumps (Table 3). There were two genes that were expressed in both \textit{ezh2} and \textit{amp} dsRNA leg stumps but were significantly upregulated in the \textit{ezh2} knockdown animals. There was one gene that was expressed in both \textit{ezh2} and \textit{amp} dsRNA leg stumps but was significantly upregulated in the \textit{amp} knockdown legs. Out of the uniquely expressed genes, 21 were metabolic genes, seven were neuronal genes and six were chitin modifier genes. The rest of the genes fell into the categories of cell death, growth, structure, adhesion transport as well as protein kinases and scaffolding proteins.

Although computational analysis considered these insignificantly different, we found 755 genes that were uniquely expressed in \textit{ezh2} dsRNA leg stumps but absent in \textit{amp} dsRNA leg stumps, and 511 genes that were uniquely expressed in \textit{amp} dsRNA leg stumps but absent in \textit{ezh2} dsRNA leg stumps.
Table 2. Summary of effects of patterning gene knockdowns on larval leg regeneration in Tribolium. The sixth instar larvae were injected with ezh2 (2 µg/µL), pc (2 µg/µL), and amp (2 µg/µL), and their legs were ablated.

<table>
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<tr>
<th></th>
<th>Larvae after 1 molt</th>
<th>Larvae after 2 molts</th>
<th>Pupae after 1 molt</th>
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<td>Died before pupation</td>
<td># of larvae with regenerated legs</td>
<td># of larvae with no regenerated legs</td>
<td>Total N</td>
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<td></td>
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<td>After 1 larval molt</td>
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Concen. (µg/µL): ezh2 2, pc 2, amp 2.
Figure 1: Effects of *pc* knockdown on *Tribolium* larval leg regeneration after two larval molts. Day zero sixth instar larvae were injected with 2 μg/μL *amp′, ezh2* or *pc* dsRNA, and mid- and hind-legs were cut two days after. (A) Mid- and hind-legs of *amp′* knockdown animal. (B) Mid- and hind-legs of *pc* knockdown animal. (C) Mid- and hind-legs of *pc* knockdown animal; partial regeneration seen. (D) Mid- and hind-legs of *ezh2* knockdown animal.
Figure 2: Effects of \textit{pc} knockdown on pupal \textit{Tribolium} larval leg regeneration. (Left) Whole body day zero pupa injected with 2 \(\mu\text{g}/\mu\text{L}\) \textit{pc} dsRNA at day zero sixth instar. (Right) Close-up of the mid- and hind-legs. Arrowheads indicate the base the ablated mid- and hind-legs.
Figure 3: Expression of myc, sox2, twi and vvl in regenerates of ezh2 and amp' dsRNA-injected Tribolium larvae. Day zero sixth instar larvae were injected with ezh2 and amp' dsRNA as a control. qPCR analysis was done as described in Methods and Materials. A. qPCR analysis of myc expression (A), sox2 expression (B) twi expression (C) and vvl expression (D) in ezh2 and amp' dsRNA-injected animals.
Table 3. Genes that were differentially expressed in RNA-seq

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<th>Uniquely expressed in <em>ezh2</em></th>
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<td>brachyurin</td>
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<td>carboxypeptidase B</td>
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<td>brachyurin-like</td>
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<td>cadherin-related tumor suppressor</td>
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<td><strong>Significantly expressed in amp'</strong></td>
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<td>dnaJ homolog subfamily C member 28</td>
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**DISCUSSION**

**pc and ezh2’s potential role in repressing de-differentiation genes**

In this study, the role of epigenetic regulators of chromatin, *pc* and *ezh2*, were examined during larval leg regeneration through RNA interference. When *pc* expression was silenced, limited regeneration was observed at the sites of ablated larval legs. Knockdown of *ezh2* resulted in the complete inhibition of leg regeneration (Fig. 1, 2 & 3). Along with their effects on regeneration, silencing of *ezh2* and *pc* expression resulted in permanent blastemas. Together, these results demonstrate that *ezh2* and *pc* are important for limb regeneration.

Ezh2 and Pc are part of the PcG proteins, which are a set of general transcriptional repressors that play an important role in the development of many organisms. PcG proteins regulate gene expression by keeping certain genes turned off throughout development. In our study, we knocked down both *ezh2* and *pc*, and our results suggest that these PcG proteins actively repress many genes during re-differentiation step and that the derepression of genes led to a permanent blastema. The blastema consists of de-differentiated cells that proliferate and proceed towards re-differentiation to re-pattern a new limb. Considering PcG proteins’ role as transcriptional repressors, the genes that they are responsible for repressing may include de-differentiation genes. De-differentiation genes may play a crucial role in maintaining the blastema state.

**Pluripotency genes are not upregulated in ezh2 knockdown animals**

Analyses of our four candidate genes failed to identify strong candidates for blastema formation. Christen and colleagues (2010) used *myc* and *sox2* pluripotency markers in *Xenopus* and found that there was upregulation of these two transcription factors in the blastema;
however, they could not definitively conclude that these Yamanaka factors served as good blastema markers. Together, these results indicate that cells responsible for the regenerative process in both invertebrates and vertebrates do not revert to a completely pluripotent state. Rather, they support the notion that only partial de-differentiation occurs during limb regeneration. In fact, Konstantinides and Averof (2014) have shown that during crustacean limb regeneration, cells retain their cellular memory and tissue identity. Thus, we believe Tribolium blastemas are also not fully de-differentiated structures and comprise of partially de-differentiated cells. Because our candidate gene approach did not result in strong blastema markers, we sought to identify differentially expressed genes in an unbiased manner using RNA-seq.

**Significance of the transcription factor pdm2**

The RNA-seq results showed that there were 80 genes that were uniquely expressed in ezh2 dsRNA leg stumps but absent in amp’ dsRNA leg stumps. In contrast, there were only seven genes that were uniquely expressed in amp’ dsRNA leg stumps but absent in ezh2 dsRNA leg stumps. These results are consistent with the notion that ezh2 acts as a transcriptional repressor. In the case of ezh2, it does so by recruiting histone deacetylase or histone methyltransferase activities to chromatin (Cao et al., 2002; van der Vlag and Otte, 1999). Methylated K27 serves as an anchorage point for the recruitment of additional PcG proteins (Czermin et al., 2002), and the binding of these proteins contributes to the formation of a repressive chromatin state.

When examining all the genes that were differentially expressed, POU domain protein (pdm) was the only transcription factor that was uniquely expressed in ezh2 RNAi animals. POU domain consists of transcription factors that have a variety of functions related to the
neuroendocrine system as well as tissue morphogenesis (Assa-Munt et al., 1993). In *Drosophila*, *pdm* is referred to as *nubbin* (*nb*); it encodes a member of the POU family of transcription factors (Billin et al., 1991) and has been found to be expressed in the primordium of the adult wing prospective wing. Ng and colleagues (1995) further found that *nb* mutation led to deletion of hinge structures suggest that there is localized requirement for *nb* function in the wing hinge. It has been proposed that *nb* has been proposed to play a role in proximal-distal growth control center in the wing hinge region through acting as a downstream mediator of the wing hinge organizing center activity or specifying wing development (Ng et al.,1995).

It has also been found that *pdm2* is responsible for maintaining self-renewal capability and has been found to be transcribed in elements of the nervous system. Yang and colleagues (1993) found that *pdm2* is expressed in neuroblasts (NBs) and ganglion mother cells (GMCs); it was also noted *pdm2* is transcribed in the first progeny of neuroblast NB4-2. These observations indicate the *pdm2* specifies the cell fate of GMC-1 in NBs and potentially the cell fates of other NB lineages within the *Drosophila* CNS. POU factors seem to maintain proliferating ability and play a role in cell differentiation, with a potential link to blastemas, which are responsible for enabling cell differentiation to create a new limb.

Other POU domain proteins, such as Oct-1 and Oct-2, have been shown to exert either a direct or indirect role in regulating DNA replication (O’Neill et al. 1998; Verrijzer et al., 1990). Monuki and colleagues (1990) found that *oct-6* is expressed in proliferating but not terminally differentiated Schwann cells. He and colleagues (1989) found that mammalian POU domain proteins, including Pit-1 and Oct-2, participate in neuronal development and appear to have an earlier embryonic phase of expression in regions that contain proliferating progenitor cells. In future studies, it would be interesting to look at gene expression when *pdm2* and *ezh2* are
knocked down simultaneously. This could potentially lead to the cancelling of self-renewal capability and the differentiation of a small leg.

**Metabolism genes and their potential role in blastema formation**

The RNA-seq results also indicated that a substantial number of metabolism genes upregulated in the *ezh2* dsRNA-injected animals. Metabolism genes may play an important role in blastema formation. A recent study has shown that a heterochronic gene *lin28* is linked to the age-dependent decline in regenerative ability of mice (Shyh-Chang et al., 2013). Over-expression of *Lin-28* leads to enhanced regenerative abilities in adult mice, and this effect is accompanied by increased expression of oxidative enzymes. Through directly binding mRNAs and influencing the translation of glycolysis enzymes and mitochondrial enzymes, Lin28a increases cellular metabolism (Zhu et al., 2011; Peng et al., 2011; Shyh-Chang et al., 2013). Because the observed enhancement of oxidative phosphorylation has been shown to enhance tissue repair, Shyh-Chang and colleagues (2013) propose that by over-expressing Lin28a, the cells attain embryonic metabolic profile and regain the ability to regenerate even as adults. Thus, metabolism appears to play a critical role during regeneration, and it is interesting that the *ezh2* knockdown larvae also uniquely expressed many metabolic genes.

**Concluding remarks**

Overall, this study highlights the role that Pc and Ezh2 play in *Tribolium* limb regeneration, specifically blastema maintenance. An unbiased search for differential gene expression suggested that *ezh2* may be derepressing transcription factors and metabolism genes that may be important for limb regeneration. Further studies involving the silencing of these can
give us more insight on the role of PcG proteins in limb regeneration. If we know which genes are necessary for forming a blastema, we may one day be in the position to turn on these blastema-inducer genes in humans. Perhaps with these key pieces of information, regenerative therapy for amputees may not be so out of reach.
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