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Key Terms

- Axolotl
- Blastema
- Extracellular Matrix (ECM)
- Heparan Sulfate Proteoglycan
- Heparatinase-III
- Limb Pattern Formation
- Regeneration

The Role of the Extracellular Matrix in the Induction of Ectopic Blastemas in the *Ambystoma mexicanum*

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Abstract

As embryos, vertebrates have multipotent cells, allowing them to regenerate any body part. However, most cannot regenerate after the embryonic stage. Fully-developed salamanders, particularly the axolotl, retain the ability to regenerate any body part following an injury. Unlike most vertebrates, axolotls do not form scars; their cells have the ability to dedifferentiate and form ectopic blastemas. Axolotl cells also contain information that specifies their location in the body during development. We used nerve deviations and extracellular matrix (ECM) grafting to test for interaction between limb components of differing positional values during the axolotl wound healing process. We discovered that grafting urea-treated anterior ECM into an anterior wound site does not produce an ectopic blastema. However, urea-treated posterior ECM grafts result in asymmetric, ectopic blastema formation. Urea-treated anterior ECM grafts further treated with the enzyme heparitinase-III (HepIII) regained the ability to induce blastema development. All grafts that were treated with fibroblast growth factor-2 (FGF2) induced blastema formation in an anterior wound site. We hypothesize that heparan sulfates within the ECM play a regulatory role in growth factor activity, and that signaling from the ECM is necessary for the induction of ectopic blastemas but is not sufficient for ectopic limb development.

Faculty Mentor

Undergraduate research projects are an important opportunity for our students to experience life in the lab with its many challenges, frustrations, and successes. Rarely do such projects lead to the important insights that Tiffany and Rachele have discovered. Their discovery of how the extracellular matrix can regulate growth and pattern formation during limb regeneration has caused us to rethink how growth factor signaling is regulated. Based on their findings, we have begun a new series of experiments that likely will lead to novel therapeutic approaches to inducing a regenerative response in humans.

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Introduction

Regeneration is the ability to regrow functional limbs, digits, and other body parts. Although many vertebrates can regenerate body parts during embryonic development, the salamander, particularly the axolotl (*Ambystoma mexicanum*), has a unique innate ability to regenerate post-natally. Unlike other organisms, they do not develop scars, which are regions of fibrous tissue that replace normal skin after an injury. Axolotl skin returns to its original state and the missing structures of the limb are replaced during regeneration.

Axolotl cells have the ability to dedifferentiate and form ectopic blastemas. Blastemas are masses of dedifferentiated cells that form in a wound site following an injury, and are functionally and structurally equivalent to the limb buds of embryos during vertebrate development (Gardiner, 2005). Prior to complete limb formation, cells within each blastema and the limb bud re-develop, grow, and undergo pattern formation. These dedifferentiated cells give rise to regenerated limb structures and presumably could be directed to become any body part depending on the molecular signals available and which body part has been damaged.

Limb pattern formation involves interactions among blastema cells from different positions within the limb, which thus differ in a property called positional information. This information is encoded in the proximal-distal and the circumferential axes of the limb. In this experiment, we tested for the presence of positional information in the anterior-posterior limb axis. Anterior refers to the portion of the upper forelimb that points toward the head region of the animal (the thumb in a human), while posterior refers to the portion of the limb that is toward the caudal end of the animal (the little finger in a human).

Growth and pattern formation during regeneration is controlled by the interaction among cells with different positional information. If cells with anterior and posterior information interact with one another, the cells recognize that part of the pattern is missing and they are stimulated to proliferate. The cells that are generated fill in the missing parts of the pattern. To restore the original pattern of limb formation and development, a blastema develops in the wound site and eventually differentiates between what is anterior, posterior, and the patterning in between. The blastema thus inserts the missing pieces of the structure that correspond to the missing positional information (French et al., 1976).

In an experiment by Endo, Bryant, and Gardiner, ectopic limb formation was induced in an axolotl when a nerve deviation was performed in conjunction with grafting a piece of skin, from the side of the limb opposite to the wound, into the wound site (Endo et al., 2004). The nerve deviation involved cutting and surgically rerouting a nerve from the posterior side of the forelimb to a wound created on the anterior side of the upper arm. A posterior skin graft was then grafted to the wound site to induce formation of a limb *de novo*. In their experiment, it was not known whether the positional information within the graft was provided by the posterior cells, the posterior extracellular matrix (ECM), or both. We determined that at least some of the information is encoded by molecules in the ECM.

The ECM is a meshwork of fibers, molecules, and interstitial fluid that is produced by and surrounds cells. It is involved in intercellular communication and cellular structural support. Molecules found in the ECM include polysaccharides, elastin fibers, collagen fibers, and heparan sulfate proteoglycans (HSPGs). Heparan sulfates have binding sites for signaling molecules released by cells, particularly for fibroblast growth factors (FGFs). Growth factors are naturally-produced chemical compounds that promote cellular proliferation and differentiation (Werner and Grose, 2003).

Due to their binding ability, heparan sulfates aid in cell-to-cell signaling and growth factor activity. Heparatinase III (HepIII) is an enzyme that cleaves heparan sulfates from proteogylcans, thus rendering heparan sulfates inactive. We tested our hypothesis that positional information is mediated by the ECM by the binding of growth factors to HSPG. Evidence that is consistent with our hypothesis has been published by Schaller and Muneoka. They demonstrated that supernumerary digit formation could be induced by grafting ECM derived from mouse posterior limb bud cells into the anterior portion of a chick wing bud (Schaller and Muneoka, 2001). In our study, we modified their experimental design to test the role of HSPG-mediated growth-factor signaling during axolotl limb regeneration.

We performed two surgical procedures in each of our experiments: nerve deviation and ECM grafting. Nerve deviations are done because a deviated nerve provides signals that elicit blastema formation through the dedifferentiation of fibroblasts. These are cells involved in the wound healing process, which are responsible for producing the extracellular matrix and are the progenitor cells for the early blastema. ECM grafting involved placing a sample of ECM adjacent to a deviated nerve, just beneath the newly-healed epithelium of a wound site. Such grafts are placed

into the wound site so that components within the ECM can interact with the signals released by the nerve and with the dedifferentiated fibroblasts. In addition, we performed different experiments in which we treated the grafted ECM by urea treatment, urea and HepIII treatment, and urea and FGF2 treatment, with urea used to decellularize graft samples (Schaller and Muneoka, 2001). Both anterior and posterior ECM grafts were treated with each of the treatment types. The aim of these experiments was to determine the role that the extracellular matrix plays in the formation of ectopic blastemas, as well as its role in providing positional information that is sufficient to produce a functional, ectopic limb.

Methods

Animals

Axolotls (*Ambystoma mexicanum*) were either spawned at the *Ambystoma* Genetic Stock Center at the University of Kentucky or at the Gardiner Lab at the University of California, Irvine. Animals were maintained at 20–22 °C in 40% Holtfreter's solution. The animals used ranged from 14 to 16 cm in length. For surgical and data imaging purposes, animals were anesthetized in 0.1% ethyl 3-aminobenzoate methanesulfonate salt (MS222).

ECM Preparation

ECM was isolated by incubating a 2 by 2 mm full-thickness skin sample in 2M urea for 15 minutes at room temperature (Gospodarowicz et al., 1983). The skin sample was obtained from either the anterior or posterior side of an axolotl's upper forelimb, depending on the experimental trial. For heparan sulfate inactivation, the skin sample was initially treated with 2M urea and then incubated with 0.008 IU/ml heparitinase III (Fisher) for 4 hours at 37 °C (Schaller and Muneoka, 2001). For protein incubation, the skin sample was treated with 2M urea and then incubated in 500 μg/ml FGF2 (Fisher) for 2 hours at room temperature (Schaller and Muneoka, 2001).

Surgical Procedures

Nerve Deviation. A wound window was made by removing a 2 by 3 mm piece of full-thickness skin from the anterior side of the axolotl upper forelimb. A slit, running from the elbow of the animal to its shoulder, was then made on the posterior side of the same limb. The brachial nerve was then cut, dissected from the connective tissues, threaded between the skin and the underlying muscle of the limb, and placed to one side of the anterior wound window.

ECM Grafting. ECM grafting was done 18 to 24 hours after the nerve deviation. An incision was made adjacent to one side of the anterior wound window. Forceps were then inserted into the slit and slightly opened under the newly healed skin of the wound site to create a space. Using two forceps, treated ECM was inserted through the small incision and placed adjacent to, but not touching, the deviated nerve. Following each surgical procedure, the animal was placed on ice for 2 hours to ensure the healing-in of the nerve and graft.

Table 1

ECM treatments and blastema formation. Through different treatment types (urea only, urea and HepIII, and urea and FGF2) and positional information, ectopic blastemas either did or did not develop. At times, asymmetric blastemas (blastemas that did not have rounded ends) developed; however, no ectopic limbs developed from these blastemas.

Wound Type	Type of Graft + Treatment	Asymmetric Ectopic Blastema	Ectopic Blastema	No Ectopic Blastema	Total Number of Trials
Anterior	No ECM	0 (0%)	6 (75%)	2 (25%)	8
Anterior	Anterior, urea-treated ECM	0 (0%)	0 (0%)	8 (100%)	8
Anterior	Posterior, urea-treated ECM	3/7 (27.3 %)	7 (63.6%)	4 (36.4%)	11
Anterior	Anterior, HepIII-treated ECM	1/4 (25%)	4 (80%)	1 (20%)	5
Anterior	Posterior, HepIII-treated ECM	0 (0%)	2 (33.3%)	4 (66.6%)	6
Anterior	Anterior, BSA-treated	0 (0%)	0 (0%)	1 (100%)	1
Anterior	Posterior, BSA-treated	0 (0%)	0 (0%)	1 (100%)	1
Anterior	Anterior, FGF2-treated ECM	0 (0%)	2 (100%)	0 (0%)	2
Anterior	Posterior, FGF2-treated ECM	0 (0%)	2 (100%)	0 (0%)	2

Histology

Limbs with nerve deviations were collected by amputation. Portions of the limbs with wound windows were then isolated, treated with 4% paraformaldehyde (PFA) and placed on a rocker at 4 °C overnight. After the PFA treatment, the samples were treated with 10% ethylenediaminetetraacetic acid (EDTA) and placed on a rocker overnight, at room temperature, to decalcify the skeletal elements. The samples were then treated with 30% sucrose and placed on a rocker at 4 °C overnight, after which they were embedded in Optimal Cutting Temperature (OCT) media. 10 µm sections of the frozen samples were made and placed onto slides using a cryostat. To visualize skin and muscle, slides were stained with hemotoxylin and eosin (H & E). Slides were stained with acian blue to visualize cartilage, .

Data Collection

The Image Processing and Analysis by Java (Image J) program was used to quantify data. Photographic images were taken using a SPOT camera (RT Color Diagnostic Instruments, Inc.) and a dissecting microscope (Leica) in conjunction with using the SPOT Digital Imaging Software: Basic program (SPOT Imaging Solutions). Image J was used to determine the size in pixels of ectopic blastemas at 0.8x magnification.

Results

Anterior wounds with a deviated nerve formed ectopic blastemas in 75% of the cases (Table 1, Figure 2). These blastemas were symmetric in shape, began regressing 10 to 16 days after the nerve deviation, and never developed into ectopic limbs. In contrast, when urea-treated anterior ECM was grafted into an anterior wound window, ectopic blastema formation was inhibited 100% of the time (Table 1).

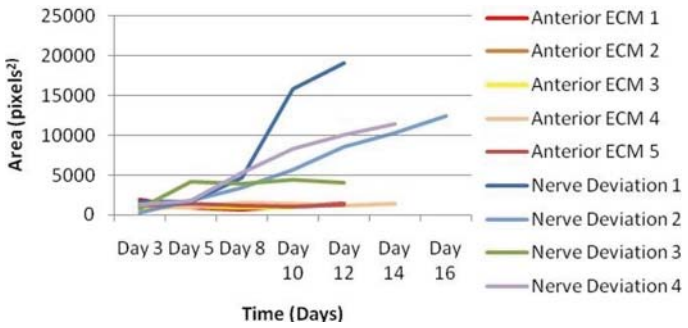

Figure 2

Comparison of blastema formation in anterior wound with just nerve deviations and those with nerve deviations plus anterior ECM grafts. Anterior ECM refers to trials in which urea-treated ECM grafts were placed into an anterior wound site. Wounds with anterior ECM grafts did not form ectopic blastemas. In trials within which only nerve deviations were done, there was evident ectopic blastema formation. These blastemas eventually regressed 10 to 16 days post-nerve deviation. Blastema sizes were quantified using Image J and expressed in pixels.

When urea-treated posterior ECM was grafted into an anterior wound site, ectopic blastema formation occurred about 64% of the time (Table 1). Three of the posterior ECM-induced blastemas were asymmetric in shape (Figure 1). Asymmetric blastemas were collected for histological analysis when signs of regression became evident. Such signs included the widening, or bal-

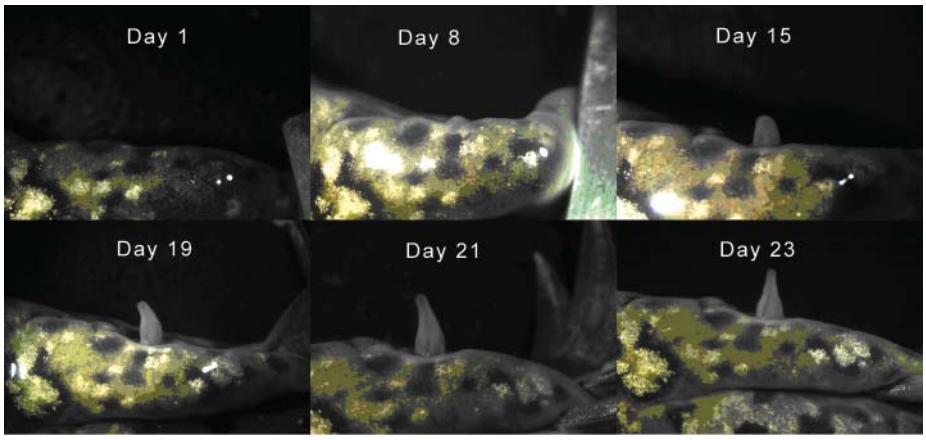

Figure 1

Progression of ectopic blastema formation due to urea-treated posterior graft into an anterior wound site. The blastema began as a rounded, symmetric 'bump.' After two weeks, however, the blastema had elongated and had an evident apical tip. It also became more asymmetric in appearance. At the end of three weeks, the blastema began to expand at the base, at which time it was collected for histological analysis.

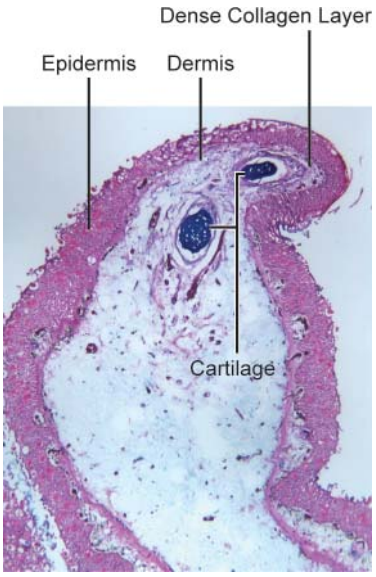

Figure 3

Cartilage formation in ectopic blastema. Using alcian blue with H & E staining, there is evident cartilage formation in the blastema and its fingerlike protrusion.

looning, of the blastema base (Figure 1, Day 23). Samples were collected two to three weeks following the nerve deviation procedure.

Heparitinase III Treatment

Removal of heparan sulfate residues prevented anterior ECM grafts from inhibiting ectopic blastema formation. Ectopic blastema formation occurred 80% of the time for urea and heparitinase III-treated anterior ECM grafts (Table 1). One of the four ectopic blastemas that formed due to such a graft was asymmetric. This asymmetric blastema had a fingerlike protrusion with a cartilaginous core that was evident after alcian blue and H & E staining (Figures 3 and 4). Blastema regression took place more than 24 days post-nerve deviation compared to around 20 days in wounds with only a deviated nerve. In wounds with posterior ECM grafts treated with heparatinase III, ectopic blastemas formed about 33% of the time (Table 1). These also regressed, but only after three to four weeks following the nerve deviation.

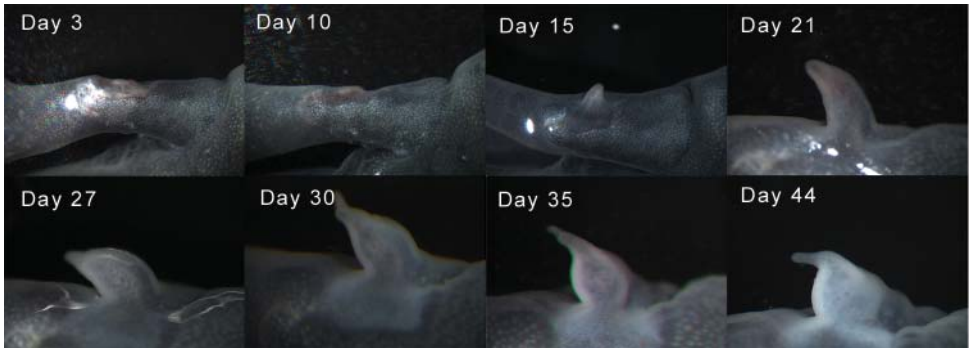

Figure 4

Progression of blastema formation in response to anterior ECM grafts treated with HepIII. Over the course of five weeks, an asymmetric blastema developed. Ectopic blastema formation became evident around two weeks after the nerve deviation was done. By Day 27 , the beginnings of fingerlike protrusion became evident. This asymmetric protrusion was most notable by Day 44.

Figure 5

Progression of blastema formation due to FGF2 treatment of ECM graft. Over the course of two weeks, a 'double bump' blastema developed. Emergence of the ectopic blastema took place earlier than ectopic blastema formation with other ECM treatments.

FGF2 Treatment

FGF2-treated ECM grafts induced ectopic blastemas. Control grafts of both anterior and posterior ECM were treated with bovine serum albumin (BSA) because BSA is non-specific and binds to all growth factor binding sites within the ECM. Neither induced ectopic blastemas; however, the sample size was very small for both (Table 1). Ectopic blastema formation was induced when grafting urea and FGF2-treated anterior and posterior ECM (Table 1; Figure 6). For both graft types, blastema formation was

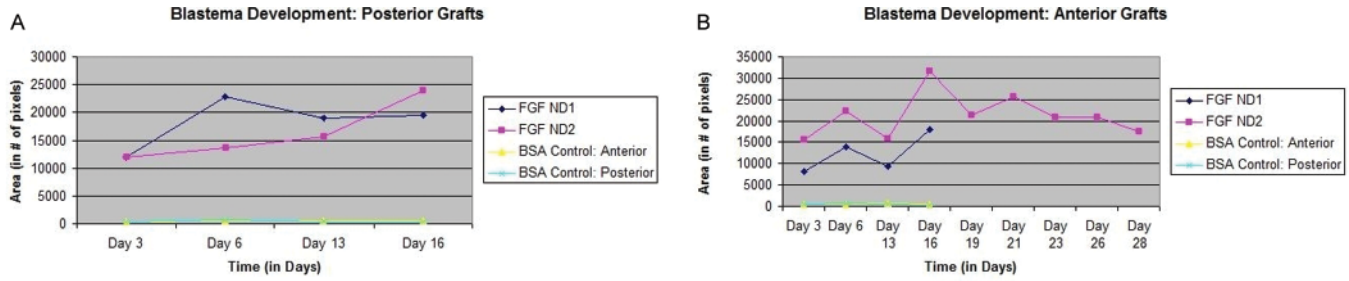

Figure 6

Blastema development due to FGF2-treated ECM graft. As a control, anterior and posterior grafts were each treated with BSA. FGF ND1= First nerve deviation done with an FGF-treated graft. FGF ND2= Second nerve deviation done with an FGF-treated graft. For both controls, (A) and (B), no blastema formation occurred with the BSA treatment. (A) Blastema induction occurred with posterior grafts treated with FGF2. Evident ectopic outgrowth occurred as early as Day 6. Peak blastema growth was noticeable about two weeks following the nerve deviation (Area$\geq$20,000 pixels). (B) Blastema induction also occurred with anterior grafts treated with FGF2. Peak blastema growth was also notable around two weeks after the nerve deviation procedure was done with areas ranging from >15,000 pixels to >30,000 pixels. Following this two week period, blastemas began to regress. The sample for FGF ND1 was collected at the onset of regression. The outgrowth for FGF ND2 was maintained for further observation. About four weeks post-nerve deviation, the sample was also collected for histological purposes.

asymmetric and had a "double bump" appearance (Figure 5). The two ectopic blastemas induced by FGF2-treated anterior ECM (FGF ND1 and FGF ND2), were collected for histological analysis (Figure 6B). As soon as regression began for FGF ND1, the sample was collected (Figure 6B). FGF ND2, however, was observed to determine if further development would occur (Figure 6B). This limb continued to regress, and was collected about four weeks after the nerve deviation.

Discussion

The Role of the ECM in Blastema Formation and Limb Regeneration

We have demonstrated that the extracellular matrix plays a necessary role in ectopic limb formation. Although the ECM can influence whether or not a blastema is formed, a graft consisting of ECM alone is not sufficient to induce formation of a functional, ectopic limb. When an ectopic blastema was induced, it eventually became elongated and in some cases asymmetric, but all showed signs of regression eventually. The most advanced asymmetric blastema that formed during the course of this experiment was one with a fingerlike protrusion (Figure 4). However, development did not progress further than this. No ectopic limbs formed during this experiment. Due to the formation of such asymmetric ectopic blastemas, we hypothesized that the extracellular matrix is necessary in producing a blastema but not sufficient to produce a complete ectopic limb.

We presume that the ECM graft lacks something that is necessary for ectopic limb formation. This missing component may be what interacts with the deviated nerve and eventually allows for further ectopic limb development.

The experimental basis of this project was the previous study by Schaller and Muneoka on the effects of posterior extracellular matrix in the development of the chick wing bud (Schaller and Muneoka, 2001). Although we did not observe the formation of supernumerary structures in our study, we nevertheless did observe that the ECM was bioactive, and that anterior and posterior ECM differed in their ability to induce blastema formation. We note that Schaller and Muneoka focused on the autopod, or hand, while this project focused more on the stylopod, or upper forelimb (Schaller and Muneoka, 2001).

Our experimental paradigm was based directly on the previous studies of Endo, Bryant, and Gardiner. In their experiment, a skin sample from the side of the limb that was opposite to the site of the wound was grafted into the wound site (Endo et al., 2004). This skin graft consisted of both skin cells and extracellular matrix. Considering that a functional, accessory limb developed in their experiment, and not in ours, the skin cells may provide a necessary component to was missing in our grafts. Perhaps for a future experiment, a graft consisting of just skin cells can be used in conjunction with a nerve deviation to observe what growth develops.

The Effects of Urea and HepIII Treatments

We discovered that modifications of the ECM in turn altered the ability of the ECM to induce an ectopic blastema. When considering the three treatments used, there was a difference in how posterior and anterior ECM interacted with the deviated nerve within the wound site. When grafts were treated with just urea, anterior ECM grafts inhibited the formation of ectopic blastemas, while posterior ECM grafts did not (Table 1). We infer that this observed difference is a consequence of differences in positional information between anterior and posterior ECM. Blastema formation, following the grafting of posterior ECM into the anterior wound site, may be due to the resolution of the positional incongruity between the wound site and the graft (French et al., 1976). With an anterior ECM sample, there would not be a positional difference, and thus no growth, and blastema formation would occur. The anterior sample may be recognized as having the same positional information as the wound site and is thus incorporated as if it were part of the wound window.

Mediation of ECM Positional Signaling by HSPG

The signaling ability of the anterior ECM was modified by the removal of HSPG, such that HepIII-treated anterior ECM grafts induced the formation of ectopic blastemas (Table 1; Figure 4). The results for the HepIII-treated posterior grafts were more variable. While posterior ECM grafts treated with HepIII still formed ectopic blastemas, they did so at a much reduced frequency as compared to the untreated posterior grafts (Table 1). We hypothesize that heparan sulfates may play different roles in anterior and posterior ECM samples. This role may be involved in the recognition of positional values.

In addition to acting in a positive manner, it is possible that the ECM also functions to inhibit growth factor signaling. By this view, the presence of HSPG in the anterior ECM may inhibit the activity of signaling factors (e.g., growth factors) involved in pattern formation along the anterior-posterior limb axis. Therefore, HepIII removes the inhibitory activity of heparan sulfates, and thus allows blastema formation to occur.

In contrast, with the posterior ECM, HSPG may have more of an 'activator' role in that they do not bind the anterior growth factors. In addition, since posterior ECM also induced limb-like structures, it presumably also encodes for posterior positional information that is recognized by cells in the anterior wound site. When heparan sulfates in posterior grafts are inactivated by HepIII, positional incongruity may have been removed. Under this situation, there is no recognizable difference between the positional information of the wound site and that of the graft, and although ectopic blastemas developed, they did so at a lower frequency and none were asymmetric.

The Role of FGF Signaling in Blastema Formation

FGF signaling is associated with ECM-induced blastemas that have a unique form. For both anterior and posterior ECM graft samples treated with FGF2, ectopic blastemas developed with a 'double bump' appearance (Figure 5). We selected the FGF2 treatment because of previously published data demonstrating that FGFs are signals provided by nerves (Gardiner, 2005). It must be noted that this portion of the experiment had a small sample size and has yet to be explored further. Nevertheless, FGFs have been known to induce limb formation (Werner and Grose, 2003) and it is possible that exogenous FGF, provided by ECM grafts, induced position-specific growth and patterning responses.

Implications for Humans

The implication of our results is that if limb regeneration can be achieved in salamanders using grafts composed of something that surrounds their cells and that this something is also found in humans, perhaps someday humans can also have the ability to regenerate missing body parts. Anatomically, humans and axolotls may seem to have little in common. Axolotls are amphibious organisms and their skin is thin, smooth, and constantly covered in mucus. Human skin is thicker, has sweat glands, and is covered with hair. Humans and axolotls also have different injury repair systems, particularly following an amputation. Axolotls form blastemas that later develop into functional limbs. Humans do not possess the same regenerative ability. When a limb is amputated, a blastema does not form and redevelop into a new limb. Rather, wounds heal, scars form, and all that is left is a stump.

The one thing that humans and axolotls have in common, however, is that both have ECM surrounding their cells, and at the molecular and biochemical levels, the ECM is highly conserved among all vertebrates. Identification of the ECM components responsible for the regulation of axolotl limb regeneration will perhaps one day lead to the development of novel strategies to induce a comparable regenerative response in humans. We suggest that HSPG-mediated growth factor signaling by the ECM is the first place to start looking.

Author Profiles

Rachele Mariano

Rachele Mariano started conducting research in Professor Gardiner's lab in Fall 2008, where she became involved in studying the effects of the extracellular matrix in axolotl limb regeneration. Focusing on a specific aspect of skin grafts in axolotls, Rachele helped determine significant limitations on the growth of ectopic limbs, a finding that could someday help determine a method for limb regeneration in humans. Rachele hopes to continue her education, eventually earning a dental degree.

Tiffany Vu

Tiffany Vu wanted a hands-on research experience to go along with what she was learning in her classes. She found Professor Gardiner's limb regeneration research particularly interesting, working specifically on limb regeneration in axolotls. The surgeries involved in the project were an initial challenge, but Tiffany appreciated how her confidence and skill grew as the project proceeded. Tiffany hopes to pursue a career in dentistry.

Acknowledgements

We thank Dr. David M. Gardiner for his guidance, support, and encouragement. Through him, we have had an amazing opportunity to do research in such a groundbreaking and interesting field. We are also very thankful for the help, support, and understanding of our graduate student mentor, Anne Phan. Through her guidance and experience, we have learned many laboratory techniques, thinking outside the box beyond the classroom setting, and applying what we have been taught. We would like to graciously thank our fellow undergraduate peer, Cynthia Shu, for her help, collaborative efforts, and contributions to this project. Finally, we would like to thank the rest of the Bryant/Gardiner Lab for their unwavering support and constant willingness to help. They have certainly made our research experience memorable.

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