

An Analysis of Cellular Growth in Developing Limbs

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ABSTRACT

This study focused on the relationship between pattern formation and the topology of localized cellular growth in the developing limbs of the Spotted Salamander, *Ambystoma maculatum*, using histology and anti-BrdU immuno-cytochemistry. Results show differences in the number of dividing cells between the regions of the developing limb that correspond to the major limb axes. These “hot spots” of cellular growth varied also with developmental stage of the limb, corresponding with “hot spots” of pattern formation and morphogenesis in these limbs. These results indicate that cellular growth is a fundamental mechanism of pattern formation in developing limbs.

INTRODUCTION

A major goal of developmental biology is to understand the cellular mechanisms underlying pattern formation and morphogenesis. Many of these studies focus on the molecular and genetic processes of limb development in amphibians because they are considered indicator organisms and some have the ability to regenerate their limbs. It is thought that for development to occur undifferentiated cells go through two processes, pattern formation and morphogenesis. Pattern formation is the set of processes by which embryonic cells establish the ordered spatial arrangement of tissue differentiation (Gilbert 2003). In other words, during pattern formation, cells are communicating with each other and making “decisions” on what structure to become relative to their neighbor, how to become that structure, and where to form that structure. How particular structures arise in particular places during pattern formation involves intercalation (Gilbert 2003). Intercalation is a process where cells interact with each other and respond by mitotic cell division to establish pattern continuity (Gilbert 2003). Intercalation can be visualized in amputation experiments where cells that are not normally next to each other, divide to relieve cellular confrontations and reestablish continuity which results in a supernumerary limb (Bryant et al. 1981).

Pattern formation is followed by morphogenesis, which is the overt differentiation of cells and tissues in their proper spatial arrangement (Gilbert 2003). This is the time when the actual building of the limb structure occurs. This is also when cells differentiate into cartilage, nerve, muscle and other tissues associated with limb structures.

The molecular components of limb development have also been studied in great detail. Selever (2004) found that Bmp4 in the mesoderm of a limb regulates the induction and maturation of the apical ectodermal ridge (AER), and implicates signaling from AER in regulation of digit number and identity. More research explains that Hedgehog (Hh) protein signals, which originate in the epithelium, targets mesenchyme cells and induces cell proliferation, promotes cellular survival, and directs cell differentiation through Ptc receptors and Gli transcription factors (Walterhouse 2003). Finally, it was found that the nested spatial patterns of the anterior portion of developing organisms were found to be controlled by Hox genes. This is because the anterior boundaries of consecutive genes are arranged anterior to posterior in the same order as their 3' to 5' positions along their chromosomes. This means that the anterior segment receives the “Hox code” which results in posteriorization of the affected segment (Irvine 2001).

While most of these studies involve extensive exploration into the molecular origins of limb development, not much work has been on the physical nature of limb development. The work that has been done on limb development has come up with three models to represent how limb development occurs: progress zone model, polarizing zone model and polar coordinate model. The progress zone model and polarizing zone model combine to give us the polar co-ordinate model. The progress zone model suggests there is a specialized region at the distal tip of the limb bud where cells are actively dividing (Muneoka 1984). The size of the progress zone is controlled by the apical ectodermal ridge and all of the cells within the progress zone are assumed to possess the same proximal-distal positional information. It was later found that positional information is carried in the mesoderm rather than the epidermis (Bryant 1987). The polarizing zone model suggests that limbs acquire positional information for the anterior-posterior axis by reading the concentration of a diffusible morphogen produced in the posterior margin of the bud (Muneoka 1984). This morphogen establishes a gradient with the high end posteriorly making the cells in the posterior margin of the bud respond to high morphogen concentrations becoming posterior limb structures, and a low end anteriorly making the anterior cells respond to low morphogen concentrations becoming anterior limb structures. The polar coordinate model suggests that cells acquire their positional information (value) in two dimensions, angular and radial components, which correspond to the proximal-distal axis of the appendage circumferentially (Bryant 1981). The most important aspect of this model is that intercalation results in patterns that show continuity where all pattern elements are adjacent to their normal nearest neighbors or to extra copies of themselves. Intercalation is the ability for cells to divide in order to eliminate positional disparities (Bryant 1987). This is why supernumerary limbs develop; the cells are dividing so as to line up their positional data in relation to one another before further progress is made forming the limb. Stock (1981) expanded this model by including digit formation stating that circumferential positional values, which were separate and nonadjacent at more proximal levels, come into contact with each other at the base of the digits. Furthermore, through surgical rotational experiments, it was found that anterior cells contribute more to the ventral, and posterior cells contribute more to the dorsal parts of the new pattern (Bryant 1992).

The aim of this project was to study the relationship between pattern formation and hotspots of cellular growth. To visualize the development of a limb would require the observation of proliferating cells. Proliferating cells form hot spots of cellular division which would indicate the direction and morphology of growth in an organism (Bryant et al. 1981). The goal was to test the hypothesis that cellular growth is a fundamental mechanism of pattern formation. If this hypothesis is true, then we should be able to map areas of pattern formation by using patterns of cell division in a naturally growing limb.

MATERIALS AND METHODS

Ambystoma maculatum larvae were chosen at three different stages: 0-digit stage, 2-stage digit, and 3-digit stage. The larvae were immersed in 1.0% bromodeoxyuridine (BrdU) for twenty four hours then post-fixed in 3:1 ethanol: glacial acetic acid. Next, three embryos from each stage were chosen for histology and dehydrated in three changes of 100% ETOH for thirty minutes each change. Next, the embryos were soaked in two changes of 100% xylene for one hour each, then transferred to a shallow vessel containing paraffin wax and left to sit overnight. Each embryo was then oriented into a paraffin cube in such a way as to

produce either horizontal, sagittal, or cross sections of limb structures at 10um in thickness during histology. After histology, sections that contained limb structures were stained using anti-BrdU immunocytochemistry (Appendix A). Immediately after immunocytochemistry, sections were observed to confirm the presence of labeled cells and then photographed using a spot digital camera attached to a microscope and SpotAdvanced imaging software. Sections that contained the best overall image of the limb with darkly stained labeled cells were chosen. Each limb section was divided into quadrants along the major body axes, and the numbers of labeled cells were counted. Differences in numbers of labeled cells in each quadrant and between major axes (Proximal vs. Distal, Dorsal vs. Ventral, Anterior vs. Posterior) were then evaluated using ANOVA; Poisson Regression analysis which was used to compare differences in relation to developmental stage.

Cross sections of all stages under investigation were omitted from this study. Zero digit stage cross sections were omitted because these sections were destroyed during the histology process and were unstainable. Two and three digit stage cross sections were omitted from this study because the specific location of the section in relation to the limb could not be determined. The precise location of the section in relation to the limb was crucial because if we were to analyze these sections we must know if they are in the proximal or distal parts of the limb as the number of dividing cells could be influenced by this location. Omitting cross sections of all stages meant that the dorsal/ventral vs. anterior/posterior axis differences could not be investigated.

RESULTS

The results for this study are grouped by the two types of histological sections because they were the sections most useful for this study. Within these two groups are two subsections which show the difference in the number of dividing cells along the two major axes investigated. For sagittal sections, the major axes investigated were the proximal/distal axis and the dorsal/ventral axis. Individual quadrants, a third subsection, under investigation for the sagittal sections included the proximal/dorsal, proximal/ventral, distal/dorsal, and distal/ventral quadrants. For the horizontal sections, the two major axes under investigation were the proximal/distal axis and the dorsal/ventral axis. Individual quadrants under investigation for the horizontal sections included the proximal/posterior, proximal/anterior, distal/posterior and distal/anterior quadrants.

Sagittal Sections, Major Axes:

In the sagittal sections, statistically significant differences in the number of dividing cells were observed along the proximal/distal axis (ANOVA, $P < 0.05$). The majority of the dividing cells were observed in the distal portions of the limb for 0-digit, 2-digit, and 3-digit stages (Fig.1). There were also statistically significant differences in the number of dividing cells along the proximal/distal axis in relation to age with the majority of dividing cells observed in the 2-digit stage limbs and the fewest number of dividing cells found in 3-digit stage limbs (Poisson regression, $P < 0.05$).

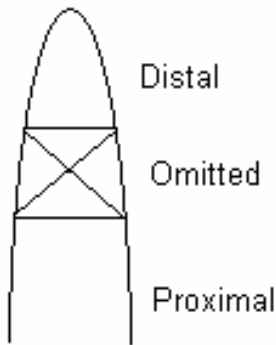
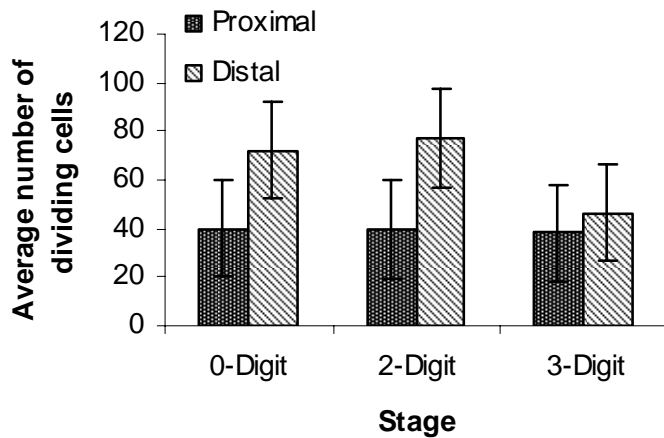


Figure 1. Diagram showing the average number of dividing cells per developmental stage for the proximal/distal axis for sagittal section (left) with limb diagram (right).

There was also a statistically significant difference in the number of dividing cells along the dorsal/ventral axis in relation to age (Poisson regression, $P < 0.05$). These differences showed that the majority of dividing cells along the dorsal/ventral axis occurred in 2-digit stage limbs and the fewest number of dividing cells were found in 3-digit stage limbs. However, there were no statistically significant differences in the number of dividing cells along the dorsal/ventral axis (ANOVA, $P > 0.05$). There was an observable shift in the region where the majority of cells were dividing at different stages. The majority of dividing cells were observed in the ventral region for 0-digit and 3-digit stage limbs whereas 2-digit stage limbs were observed to have more dividing cells in the dorsal region of the limb (Fig. 2).

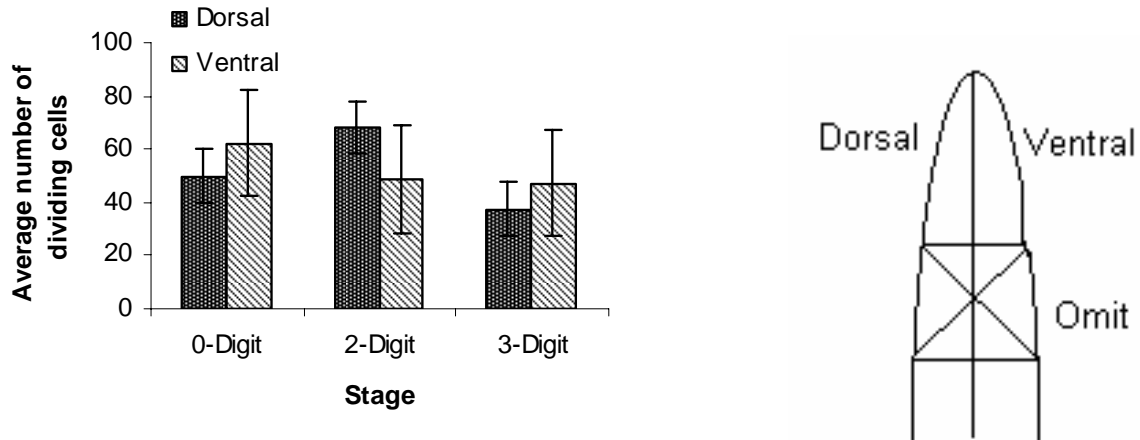


Figure 2. Diagram showing the average number of dividing cells per developmental stage for the dorsal/ventral axis for sagittal section (left) with limb diagram (right).

Sagittal Section, Quadrants:

There was no statistically significant difference in the number of labeled cells within each quadrant of the limb in relation to age (Poisson regression, $P > 0.05$). For 0-digit stage limbs, the fewest number of dividing cells were seen in the proximal/dorsal quadrant and the greatest amount of dividing cells were seen in the distal/ventral quadrant (Fig. 3). The 2-digit stage limbs had the fewest number of dividing cells observed in the proximal/ventral quadrant whereas the majority of the dividing cells were found in the distal/dorsal quadrant (Fig. 3). The 3-digit stage limbs had the fewest number of dividing cells in the proximal dorsal quadrant and the majority of dividing cells in the proximal ventral quadrant (Fig. 3).

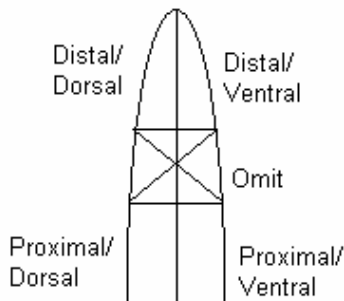
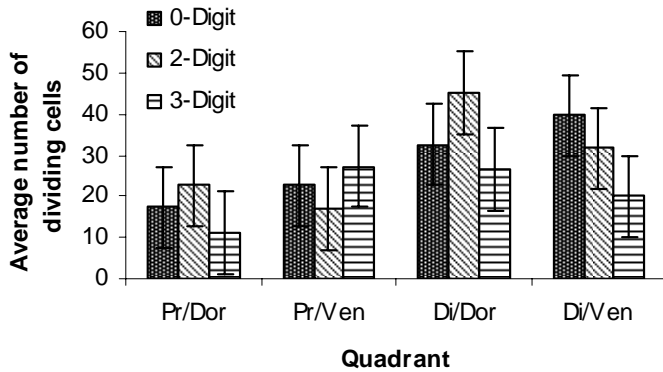


Figure 3. Diagram showing average number of dividing cells in each quadrant per developmental stage for sagittal section (left) with limb diagram (right). Note: Pr/Dor = Proximal/Dorsal; Pr/Ven= Proximal/Ventral; Di/Dor = Distal/Dorsal; Di/Ven= Distal/Ventral.

Horizontal Sections, Major Axes:

For horizontal sections, there were statistically significant differences in the number of dividing cells along the proximal/distal axis (ANOVA, $P < 0.05$). The distal region of the limb was observed to have the greatest number of dividing cells in both the 2digit and 3-digit stages (Fig. 4). There was also a statistically significant difference in the number of dividing cells along the proximal/distal axis in relation to age (Poisson regression, $P < 0.05$).

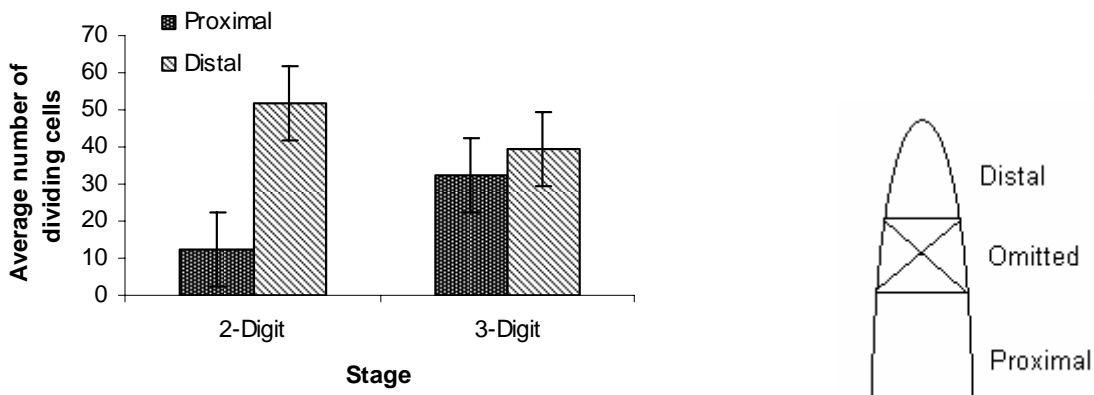


Figure 4. Diagram showing average number of dividing cells per developmental stage for the proximal/distal axis for horizontal sections (left) with limb diagram (right).

The difference in the number of dividing cells along the anterior/posterior axis was not statistically significant (ANOVA, $P>0.05$). However the posterior region of the limb, for both 2-digit and 3-digit stages, is where the majority of cells were dividing (Fig. 5).

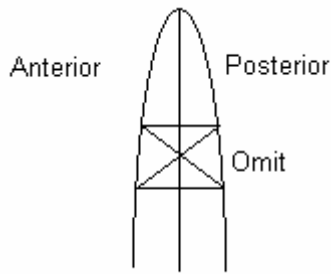
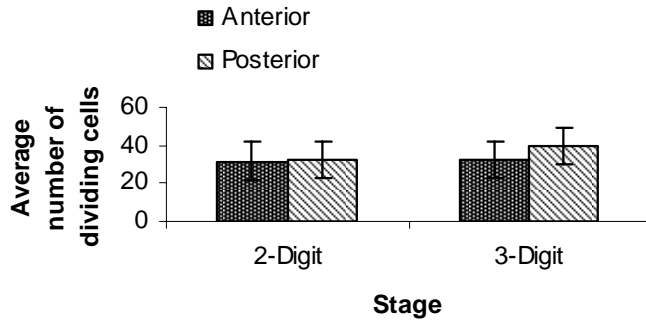


Figure 5. Diagram showing average number of dividing cells per developmental stage for the anterior/posterior axis for horizontal sections (left) with limb diagram (right). Note: posterior region of the limb lays closest to the body.

Horizontal Sections, Quadrants:

No statistical difference was found in the number of dividing cells with in each quadrant in relation to age (Poisson regression, $P>0.05$). For 2-digit stage limbs the fewest number of dividing cells were observed in the proximal/posterior region of the limb while the distal/posterior region had the greatest number of dividing cells (Fig. 6). 3-digit stage limbs had the fewest number of dividing cells in the proximal/anterior region whereas the distal/posterior region showed the greatest number of dividing cells (Fig. 6).

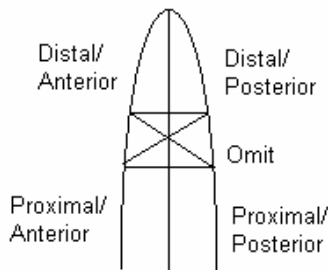
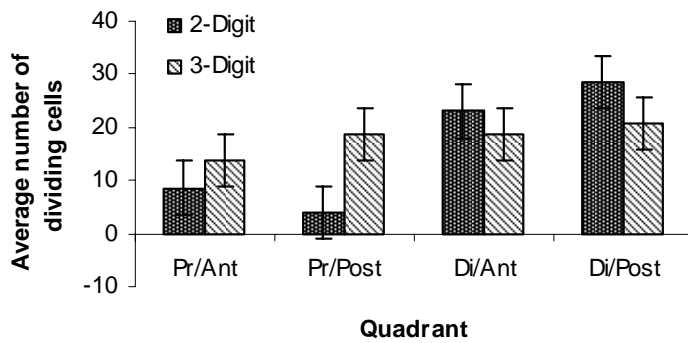


Figure 6. Diagram showing average number of dividing cells in each quadrant per developmental stage for horizontal sections (left) with limb diagram (right). Note: Pr/Ant= Proximal/Anterior; Pr/Post= Proximal/Posterior; Di/Ant= Distal/Anterior; Di/Post= Distal/Posterior. Posterior region of limb lays closest to the body.

DISCUSSION

My results show that differences in the numbers of dividing cells along the proximal distal axis are statistically significant. These results also reveal several shifts in the distribution of dividing cells during limb development in Spotted Salamanders. For zero-digit stage embryos the majority of the dividing cells were located in the distal and ventral portions of the limb. This is to be expected as the limb at this stage in development is a limb bud where outgrowth should be occurring at the outer most part of the limb structure. The significance of the majority of the cells being located in the ventral portion of the limb bud is debatable, but there may be higher concentrations of positional information among cells in this area that could account for the difference.

Two-digit stage limbs showed limb outgrowth to be in the distal direction as well. At this point the cells are differentiating into carpals, metacarpals and digits which would require outgrowth in the distal direction in order to form these structures. The majority of cellular growth was also found in the dorsal region and was nearly equal between the anterior and posterior portion of the limb. This corresponds well to normal limb growth since digits one and two form first in a more anterior and dorsal orientation before digits three and four develop (Oster 1988).

For three-digit stages, the ventral and posterior axes showed the greater concentrations of dividing cells. This shift appears to be linked to pattern formation as digits three and four form after digits one and two (Oster 1988). The majority of the cellular divisions occurring in the ventral axis of a three digit limb could signify that the resulting digit structures come from

this rather than from the dorsal region. This may also indicate that the ventral region is more “enriched” with positional information relative to the dorsal region.

This evidence shows that “hot spots” of cellular growth correspond to regions of pattern formation in developing salamander limbs. As observed in this study, hot spots of cellular growth appear before morphogenesis, signifying that pattern formation is occurring. These hotspots then fade as morphogenesis occurs and the structures are built in accordance with the decisions made during cellular growth and pattern formation. If no cellular growth had occurred, pattern formation would not have occurred, no “hot spots” would have been observed, cellular decisions would not have been made, and structures would have either ceased forming or developed into deformities.

Since we can observe differences in the number of labeled cells between certain regions but cannot say they are statistically significant, further work is needed in this area. Reasons for absence of differences along some axes and regions could be due to sample size. More embryos may allow for more accurate overall values to be obtained. Another explanation could be individual variation during development; individual embryos develop differently from one another and this could cause a wide range of variation that could account for certain insignificances.

While this study supports the idea that cellular growth is a fundamental mechanism of pattern formation in salamander limbs, what is lacking is a better understanding of the molecular basis of the pattern formation process itself. While we already know a few of the essential proteins involved (i.e. Sonic Hedgehog, BMPs, etc...) we do not know how they initiate or are involved in how cells make the decisions they do during pattern formation. It might have a lot to do with molecular control of cellular growth. Further research needs to be done to determine what and how cells make these decisions amongst one another.

Further research also needs to be done to determine what molecules are involved in the spatial arrangements of cells. We already know of a few molecules, namely Hox genes and Sonic Hedgehog proteins (Gilbert 2003). Certain cells migrate and become particular structures during limb development, but the component that determines whether a cell is dorsal or ventral, for example, is not known. We do know that intercalation is involved in passing along this spatial “road map” between cells, yet we do not know what makes up this “road map” and why cells follow it (Gilbert 2003).

Improvements could also be made to this study. This study only looked at three different stages of limb development. A better approach would have been to sample developing embryos at closer intervals so that a more comprehensive and progressive picture of the development of pattern formation “hot spots” could have been observed. This approach along with the addition of more embryos, therefore increasing sample size, would also improve upon this study.

The implications of this study encompass many areas of research. The presence of “hot spots” could help in predicting where cancerous tumors may develop or spread. Evolutionary development could also benefit by recognizing where patterns of cellular division occur in related lineages to determine how specific structures arose or how certain structures developed from a common ancestor, for example, development of the lobe fin of a lobe finned fish compared to a salamander limb. This may also be a new way to analyze homologies at the developmental level and be useful in studying heterochrony.

The results of this study confirm that pattern formation requires cellular growth to develop the pattern to which limb structures will form. While more understanding is needed

of the specific molecular mechanisms involved in pattern formation, this study furthers our knowledge of the complex processes of limb development. With more research and deeper investigations, these developmental mysteries will begin to be solved.

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