

LIMB CELL BEHAVIORS IN *MONODELPHIS DOMESTICA* GROWTH AND DEVELOPMENT: THE EFFECTS OF
FGF PATHWAY ACTIVATORS AND INHIBITORS

BY

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THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Biology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2014

Urbana, Illinois

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ABSTRACT

A fundamental question in biology is, “how is growth differentially regulated during development to produce organs of particular sizes?” Research in invertebrates has shown that answers to this question can be gained by studying how serially homologous organs of a single animal (e.g., *Drosophila* wing and haltere) become different sizes. However, the phenotypes of most traditional model vertebrates (e.g., mouse, chick) do not readily lend themselves to this approach. As a result, the mechanisms that control the growth and size of vertebrate organs remain poorly understood. This represents a fundamental gap in our knowledge, which severely limits our understanding of the many growth-related aspects of organ development, evolution and health in vertebrates.

In my thesis, I took advantage of a new model system for the study of differential organ growth, the limbs of the opossum (*Monodelphis domestica*) to investigate the cellular and molecular basis of differential organ growth in mammals. Opossum limbs are an ideal system with which to study differential growth. Opossum forelimbs grow much faster than hind limbs, resulting in newborns with large, well-developed forelimbs that are twice the size of their small, undeveloped hind limbs. Opossum fore- and hind limbs are also serially homologous structures, which ancestrally shared a common developmental program. I first leveraged the great differences in opossum fore- and hind limb growth to identify cellular processes (e.g., proliferation, death, focal adhesions), and the source of the molecular signals (i.e., internal or external to the limb) that drive them, that underlie differential limb growth (Chapter 1). I then elucidated the role of the Fgf/MAPK signaling pathway in driving these cellular processes (Chapter 2).

Results suggest that molecular signals from within the limb drive differences in cell proliferation and focal adhesion that contribute to the differential growth of the fore- and hind limbs of *M. domestica* (Chapter 1). Results also suggest that alterations in the *Fgf/Mapk* pathway are capable of generating differences in cell proliferation that mirror those observed between wild-type fore- and hind limbs of *M.*

domestica, and that manipulation of *Fgf/Mapk* signaling affects FA but not *Wnt* signaling in *M. domestica* limbs (Chapter 2). Taken together, these findings suggest that evolutionary changes in the *Fgf/MAPK* pathway could be driving the observed differences in cell behaviors in *M. domestica* fore- and hind limbs through the FA-ECM pathway (i.e., adhesion). However, these findings also suggest that the evolutionary divergence that led to the differential expression of the *Wnt* pathway in the limbs of *M. domestica* likely occurred independently.

The findings of this thesis advance our understanding of the regulation of differential limb, and, thereby, organ growth in mammals. Because of the importance of differential organ growth to organismal development, evolution, and health, these findings have the potential to positively impact diverse areas of biological research.

ACKNOWLEDGEMENTS

Thank you ever so much to my advisor, Dr. Karen Sears, for allowing me to work in her lab and complete my Master's thesis under her direction. Thank you for your relentless support and guidance throughout my time in your lab as well as your patience and knowledge as I completed my project.

Thank you to Dr. JoAnn Cameron for serving as my second thesis reader. I appreciate you taking time out of your busy schedule to help me in my thesis process.

Thank you to Dr. Andrew Suarez, Dr. Carla Cáceres, the Cheviron Lab, and the Robinson Lab for allowing me permission to utilize their lab's instruments and equipment.

Thank you to the Sears lab members, Daniel Urban, Anna Molineaux, and Jennifer Maier, for giving me their time, knowledge, and advice on protocols. Thank you to our lab's animal technician, Teresa Schecker, for her time and patience while guiding me through the animal protocols and dissection methods.

Thank you to the University of Illinois' Masters in Biology Department for their supervision and the National Science Foundation for funding my Master's project.

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CHAPTER 1 – LIMB TISSUE SIGNALING...SYSTEMIC OR LOCAL AND CELL PROLIFERATION AND DEATH

1.1 Introduction

Proper regulation of growth is essential to all stages of human life, from development of the egg into an embryo to the maintenance of normal cell cycle progression in adults. As a result, when something goes wrong with the regulation of growth it is often highly detrimental to human health. Improper growth *in utero* can result in conditions ranging in severity from relatively mild (e.g., low or high birth weight) to extreme (e.g., embryonic death), and uncontrolled cell growth is a defining characteristic of cancer. However, despite the severe consequences of errors in growth regulation, they often occur. About 10% of fetuses are growth-restricted (March of Dimes), and in 2007 cancer killed ~8 million people (American Cancer Society). Therefore, understanding the mechanisms by which mammalian growth is regulated is of great importance to human health.

Despite this, many of the basic features of growth regulation (e.g., which cell processes and genes regulate growth, and how they do so) remain virtually unknown for most systems [1-4]. This is definitely true for most mammalian organs. Mammalian organs achieve their correct relative sizes through the proper regulation of differential growth during organogenesis. When this differential growth is disrupted, structural birth defects (i.e., abnormally formed or missing organs) can result. The percentage of children displaying structural birth defects is high, and ranges from 3.5% to 16% (March of Dimes). The lack of knowledge of the mechanisms regulating differential organ growth severely hinders our ability to diagnose the causes of and treat these birth defects, and the many other human health problems that result from the disruption of proper organ growth (e.g., cancer).

Significant insights in the regulation of differential organ growth can be achieved by investigating the mechanisms by which serially homologous body parts of a single animal grow at different rates and thereby achieve different sizes (e.g., the *Drosophila* haltere and wing [5-10]). However, this approach has not been taken in mammals, largely because the phenotype of the

traditional model, mouse, does not lend itself to this type of assay. However, the phenotype of a relatively new mammalian research model, the opossum (*Monodelphis domestica*), does [11,12]. The forelimbs of opossums grow twice as fast as their hind limbs *in utero* [13]. This results in newborn opossums with forelimbs twice as large as their hind limbs (Fig. 1), and makes opossum an exceptional mammalian model with which to study the regulation of differential organ growth. The limbs of mice and other placental mammals grow at similar rates through pre-birth development.

In my thesis, I took advantage of the opossum limb system to investigate the cellular and molecular basis of differential organ growth in mammals. Specifically, I first leveraged the great differences in opossum fore- and hind limb growth to clarify cellular processes (e.g., proliferation, death, focal adhesions) that underlie differential limb growth (Chapter 1). I also performed tissue culture experiments to determine whether the signals driving the differential growth of opossum limbs arise within the limb itself, or come to the limb from elsewhere in the body (Chapter 1). I then capitalized on the advantages of the opossum system to elucidate the role of the Fgf/MAPK signaling pathway in these sub-cellular and cellular processes (Chapter 2).

Armed with knowledge generated by this project, we will be better able to diagnose the genetic causes of and thereby treat the many health problems caused by abnormal growth of the limb and other organs (e.g., cancer, structural birth defects, etc.). Furthermore, my results will have the potential to highlight possible avenues for research into mammalian limb regeneration, which relies on the ability to stimulate growth. Beyond human health, this project will also provide insights into the evolutionary modifications that may underlie the great natural diversity in mammalian limb size (e.g., from the long limbs of the giraffe to the short limbs of the otter).

1.2 Materials and Methods

1.2.1. Limb percent growth in culture

I first set out to determine whether the signals driving the differential growth of opossum fore- and hind limbs arise within the limbs themselves or from the body proper. To address this hypothesis I removed fore- and hind limbs at equivalent stages of development (paddle stage; Stage 29 for the forelimb and 32 for the hind limb) from the body and monitored their growth in culture. If signals from the limb itself are driving the differential limb growth, then I would expect the forelimbs to grow significantly faster in culture than the hind limbs. In contrast, if signals from the body are regulating the differential limb growth, then I would expect to find that the limbs would grow at comparable rates *in vitro*. I also expect to find that comparably staged mouse fore- and hind limbs grow at similar rates in culture.

M. domestica females were dissected when their embryos were at either Stage 29 (for forelimbs; 11.7 days) or Stage 32 (for hind limbs; 13.4 days) [13-15]. To obtain timed matings, mating pairs of opossums were videotaped and observed copulatory events taken as the start of embryonic development [13]. Upon euthanization of the female by means of carbon dioxide inhalation, embryos were dissected from the uterus, and limbs severed from the rest of the embryo at the axillary region. Limbs were then transferred to Transwell Permeable Support Polyester (PET) Membrane Transwell-Clear Inserts (Corning Incorporate, Cat. No. 3470) for tissue culture. The membrane diameter measured 6.5mm, membrane pore size 0.4 μm , and growth surface area 0.33 cm^2 . The inserts were placed in wells that contained 300 μL of a conducive growth medium. The growth medium included BGJb Medium (1x) + L-Glutamine (GIBCO Invitrogen Corporation, Cat. No. 12591, Lot No. 1087572), MEM Nonessential Amino Acid 100x Solution (Corning, Ref. No. 25-025-C1, Lot No. 25025104), 10,000 I.U./mL Penicillin; 10,000 $\mu\text{g}/\text{mL}$ Streptomycin (BioWhittaker, Cat. No. 17-602E, Lot No. 0213M06226), and Heat-Inactivated Fetal Bovine Serum (Gibco, Ref. No. 16140-071, Lot No. 1399425). Digital images were

captured of the limbs to document initial limb morphology. Limbs were then incubated in a vacuum-sealed incubator at 37°C for a period of three days, with growth media being changed every twenty-four hours. A second set of digital images were taken after the three-day growth period. The length of the limbs before and after culturing was quantified using ImageJ, and the percent growth from Day 1 and Day 3 was calculated. Percent growth was statistically compared between limbs using Wilcoxon tests [17]. After images were taken, limbs were fixed in a 4% paraformaldehyde solution and then rinsed through a methanol/phosphate buffered saline (MeOH/PBS) series to prepare the limb tissue for sectioning. The same protocol was utilized for *Mus musculus* hind limbs at embryonic day 11, with the exception that mouse matings were determined from plugs (noon on the day of plug was taken to be 0.5 days of embryonic development).

1.2.2. Cell Proliferation and Cell Death

To begin to investigate the cellular processes that differ in the developing fore- and hind limbs of opossums, I quantified and compared cellular proliferation and death at Stages 28 and 29 (forelimb) and 30 and 32 (hind limb). These stages represent the bud and paddle stages of limb development, respectively.

Embryos were obtained as described in 1.2.1, and limbs fixed in a 4% paraformaldehyde solution diluted in 1x diethylpyrocarbonate phosphate buffered saline (depPBS) for twenty-four hours at 4°C. Limbs were then sunk in 30% sucrose for twenty-four hours at 4°C, and subsequently equilibrated in O.C.T. Compound (Tissue-Tek, No. 4583) at room temperature. Limbs were then embedded in O.C.T. and stored at -80°C until they were cryotome sectioned and mounted to slides using a motorized Leica CM3050 S Cryostat (Institute for Genomic Biology, University of Illinois Urbana-Champaign). Sections were cut at 10 microns and mounted onto Superfrost Plus Glass Slides 1 mm thick (Electron Microscopy Sciences, Cat. No. 71869-10) for staining.

Cell proliferation and death were visualized using immunohistochemical (IHC) methods. Proliferating cells were detected using antibodies against phospho-histone H3 (PHH3; Cell Signaling Technology, Ref. No. 97015, Lot No. 13). PHH3 detects the Ser12 phosphorylated histone H3 during the M-phase of mitotic cell proliferation. To perform IHC, limb sections were equilibrated to room temperature and rehydrated in Blocking Buffer. PHH3 was applied at 1:100 dilution in Blocking Buffer at 4°C in a humidified chamber for twenty-four hours. Slides next went through a series of washes in Blocking Buffer (1% heat-inactivated goat serum (HIGS) with 0.1% Triton X-100 in 1x phosphate buffered saline) in preparation for the secondary antibody, Alexia Fluor 488 goat anti-rabbit antibody (Invitrogen A31627), which was applied at a dilution of 1:250. Fluorescent TO-PRO-3 iodide (Invitrogen T3605) was applied in conjugation with the secondary antibody at a 1:1,000 dilution to visualize non-proliferating cells. Slides were then again washed with Blocking Buffer and mounted utilizing Vectashield Hard-Set™ Mounding Medium with DAPI (Vector Laboratories, Cat. No. H-1500).

Cell apoptosis was detected utilizing an APOP Tag Staining Kit (Millipore Lot: 2368741). Slides were washed in a series of 1x phosphate buffered saline and ethanol acetic acid. After the Equilibration Buffer was utilized, TdT enzyme was applied and slides were coverslipped and stored at 37°C for one hour. After the hour incubation, antidigoxigenin conjugate was administered and slides were incubated at room temperature for thirty minutes in a dark and humidified space. Vectashield® Hard-Set Mounding Medium with DAPI was applied to the slides and mounted with coverslips. The cells stained for proliferation and apoptosis were visualized using a standard fluorescent microscope and Image Pro Plus 7.0 software. ImageJ 1.46r was used to count those cells that positively stained for cell proliferation within a standard area. The number of proliferating cells in different samples and stages was statistically compared using a series of Wilcoxon tests [17]. Cell death was also visualized in freshly dissected whole-mount limbs by staining with neutral red for 40 minutes [16].

1.2.3. Focal Adhesions

Embryos were obtained as described in 1.2.1, and fixed and sectioned as described in 1.2.2. Focal adhesions were visualized on sectioned tissues with the Actin Cytoskeleton and Focal Adhesion Staining Kit (Millipore Cat. No. FAK100) consisting of TRITC-conjugated Phalloidin, anti-Vinculin, and DAPI for the immunofluorescent staining of actin filaments in the cytoskeleton, focal contacts, and nucleus of the cells, respectively. Slides were washed in a 1x wash buffer (1x PBS containing 0.05% Tween-20 at 2000 uL:1uL) followed by 0.1% Triton X-100 in 1x PBS (1:1000) to permeabilize the cells. Additional washes with 1x wash buffer and blocking solution (1% BSA in 1x PBS at 1000:10) were applied to the slides. The primary antibody (anti-Vinculin) was diluted in blocking solution (1:100) and applied. Slides were mounted with coverslips and incubated for one hour at room temperature. Slides went through a series of washes with 1x wash buffer in preparation for the secondary antibody, goat anti-mouse (diluted in 1x PBS (1:250) just before use). Slides were incubated for one hour at room temperature. For double labeling, TRITC-conjugated Phalloidin (1:100) was applied and incubated simultaneously with the secondary antibody. Slides were then again washed with 1x wash buffer, nuclei were counterstained by incubating cells with DAPI (1:1000), and slides were coverslipped with Aquamount (Fisher 14-390-5). The cells stained were visualized using a standard fluorescent microscope and Image Pro Plus 7.0 software. ImageJ 1.46r was used to quantify the number of focal adhesions present in a standard area of each sample. The number of focal adhesions was statistically compared among stages and limbs using a series of Wilcoxon tests [17].

1.3 Results

1.3.1. Limb percent growth in culture

Limbs of both *M. domestica* and *M. musculus* successfully grew in culture (Figure 2). *M. domestica* forelimbs grew more in culture (average percent change= 62.74%) than *M. domestica* hind

limbs (average percent change = 23.4%) ($P = 0.01$; Figure 3). In contrast, *M. musculus* fore- (average percent change = 100.87%) and hind (average percent change = 123.75%) limbs exhibited statistically indistinguishable levels of growth ($P = 0.67$; Figure 3).

1.3.2. Cell Proliferation and Cell Death

M. domestica forelimbs display significantly more proliferating cells (average = 69) in a standard area than do *M. domestica* hind limbs (average = 37) at the bud stage of development (P -value < 0.001; Table 1). However, the number of proliferating cells is similar in *M. domestica* (average = 69) and *M. musculus* (average = 67) forelimbs at the bud stage of development (P -value = 0.595). At the club stage of *M. domestica* development (St 29 for the forelimb and St 32 for the hind limb), the number of proliferating cells is similar in the fore- (average = 47) and hind (average = 41) limb (P -value = 0.305).

M. domestica fore- and hind limbs display similar, concentrated regions of cell death. The first of these is characterized by a diffuse group of dying cells located in the anterior of the limb, and corresponds to the “foyer préaxiale primaire” that has been previously documented in mouse [16] (Figure 3). A second region of cell death is located around the AER of both the fore- and hind limbs. This region of cell death has also been previously documented in mouse [16].

1.3.3. Focal Adhesions

The number of focal adhesions is significantly higher (P -value < 0.01) in *M. domestica* fore- than hind limbs at each developmental stage examined (bud, club and paddle).

1.4 Discussion

The goals of the research comprising this chapter were to (1) clarify cellular processes (e.g., proliferation, death, focal adhesions) that underlie differential limb growth, and (2) determine whether

signals driving the differential growth of opossum limbs arise within the limb itself, or come to the limb from elsewhere in the body. In regard to the first goal, results support the hypothesis that differences in cell proliferation and focal adhesion levels (which may indicate differences in cell migration) underlie the differential growth of *M. domestica* limbs. I found no evidence for differences in patterns of cell death between *M. domestica* fore- and hind limbs. It is possible that the *M. domestica* hind limb displays slightly less cell death, but this would not be expected to contribute to its lesser growth. In regard to the second goal, results suggest that the signals that drive the differential growth of *M. domestica* limbs are arising from within the limb themselves.

Taken together, results suggest that the molecular signals from within the limb drive differences in cell proliferation and focal adhesions that contribute to the differential growth of *M. domestica* fore- and hind limbs. Results of this chapter therefore provide a good foundation for the studies to identify these molecular signals, which form the basis of Chapter 2.

1.5 Figures



Fig 1. An opossum shortly before birth. At birth, the opossum forelimb is much larger and more developed than the opossum hind limb.

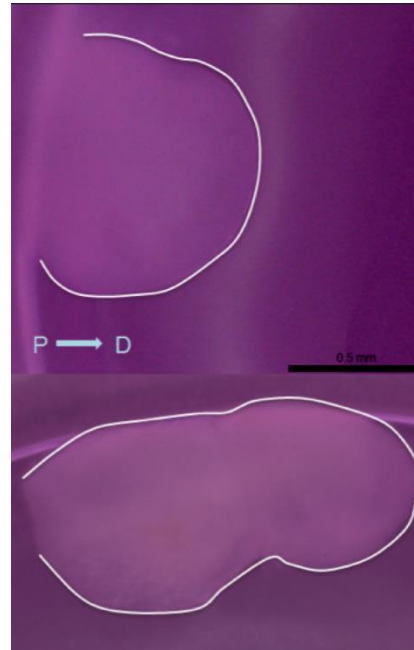


Figure 2. (Above) Day 1 – Initial photo of *M. domestica* Stage 32 hind limb. 50x magnification. (Below) Day 3 – Photo after three-day incubation in growth media of *M. domestica* Stage 32 hind limb. 50x magnification.

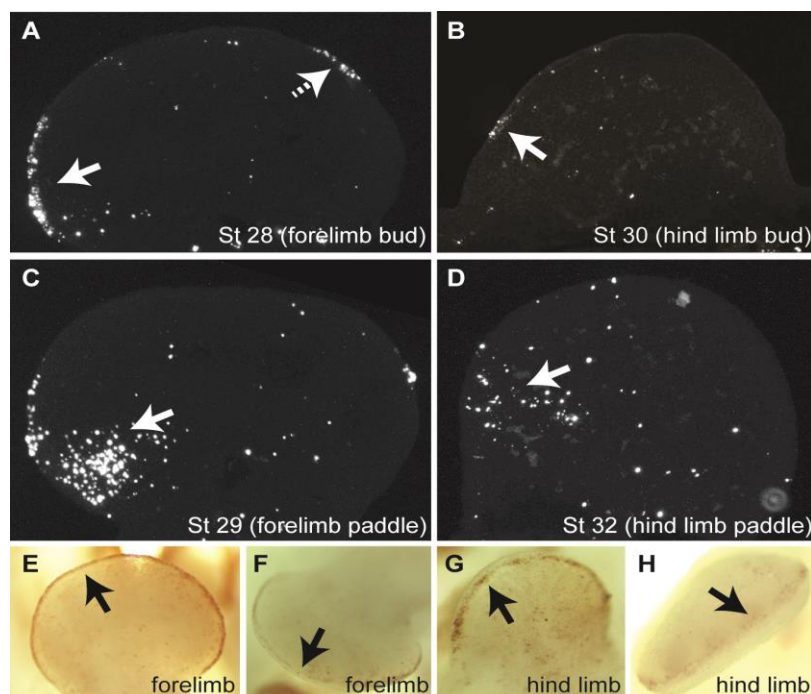


Figure 3. Cell death in *M. domestica* fore- (A, C, E and F) and hind (B, D, G and H) limbs. (A, B, C, and D) Cell death visualized with TUNEL IHC, limbs in frontal section with anterior on the left. Solid arrows indicate the "foyer préaxiale primaire", and the dashed arrow indicates the cell death associated with the AER. (E, F, G, H) Cell death visualized in whole-mount limbs with neutral red. Black arrows indicate cell death in the AER. Although only (A) shows evidence of AER-related cell death in section, both fore- and hind limbs

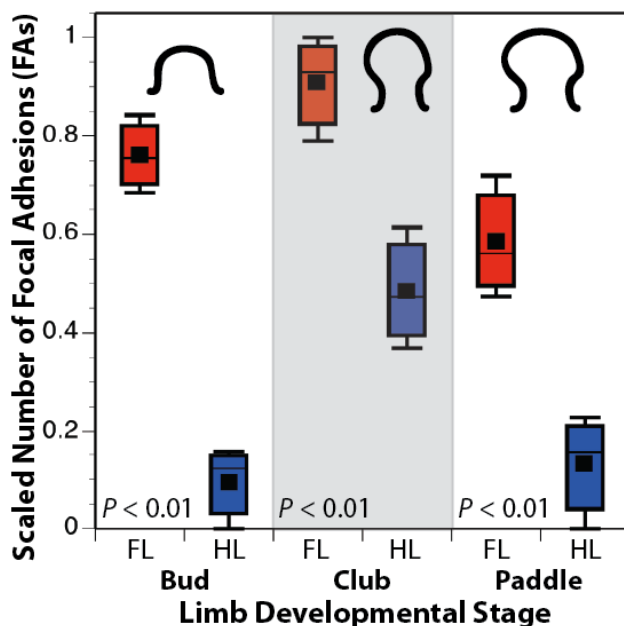


Figure 4. Number of focal adhesions present per standardized sample area in *M. domestica* fore- and hind limbs at three stages of development: bud, club, and paddle. FL bud (St. 28), FL club (St. 29), FL paddle (St. 30), HL bud (St. 30), HL club (St. 31), HL paddle (St. 32).

1.6 Tables

	Average		P-Values
<i>Md</i> 10.5d FL	67.425	<i>M. musculus</i> FL (10.5) and <i>M. domestica</i> FL (St 28) buds	p = 0.5954
<i>Md</i> St. 28 FL	69.38889	<i>M. domestica</i> FL (St 28) and HL (St 30) buds	p = 0.0009*
<i>Md</i> St. 29 FL	47.175	<i>M. domestica</i> FL (St 29) and HL (St 32) clubs	p = 0.3049
<i>Md</i> St. 30 HL	36.69444		
<i>Md</i> St. 32 HL	41.25		

Table 1. Number of proliferating cells in *M. musculus* and *M. domestica* fore- and hind limbs.

CHAPTER 2 – FGF PATHWAY IN *MONODELPHIS DOMESTICA* HINDLIMBS

2.1 Introduction

The goal of my thesis is to clarify the cellular and molecular basis of differential organ growth in mammals using opossum limbs as a model system. Results presented in Chapter 1 suggest that molecular signals from within *Monodelphis domestica* fore- and hind limbs drive differences in cell proliferation and adhesion, which likely contribute to their differences in growth. The goal of Chapter 2 is to elucidate the molecular signals that generate these differences in cellular behavior. Previous microarray assays in the lab [19] identified the *Fgf/MAPK*, *Wnt* (non-canonical planar cell polarity) and Focal Adhesion (FA) pathways as the most differentially expressed in the developing fore- and hind limbs of *M. domestica*. Of these I chose to focus primarily on the *Fgf/MAPK* pathway as a first step toward understanding the molecular basis of differential limb growth in *M. domestica*.

Fgfs play crucial roles in many aspects of vertebrate organogenesis, including that of the limb [20], by promoting cellular proliferation and differentiation [21]. Within the limb, *Fgfs* are mainly expressed in the distal portion of the limb bud [20, 21]. *Fgf8*, for example, is expressed throughout the AER and mediates AER activity [22, 23]. A continuous feedback loop between *Fgfs* in the mesenchyme and AER helps to maintain cell proliferation and proximal-distal limb outgrowth. Studies in mouse have demonstrated that the *Fgf/MAPK* pathway also helps regulate cell adhesion and migration during limb growth [21, 22]. Therefore, it is possible that differences in *Fgf/MAPK* signaling are driving the differences in cellular proliferation and adhesion documented in *M. domestica* fore- and hind limbs in Chapter 1 (Hypothesis 1). It is also possible that differences in *Fgf/MAPK* signaling drive differences in *Wnt* and FA signaling in *M. domestica* limbs (Hypothesis 2; Figure 1). *Wnt* signaling has been shown to regulate the orientation and polarity of cell movements and divisions in mouse limbs, while the FA pathway has been linked to the regulation of cell migration and adhesion.

To test these hypotheses, I performed a series of limb culture experiments in which I experimentally manipulated *Fgf* levels using *Fgf8* protein and a known inhibitor of *Fgf* signaling, SU5402, in *M. domestica* limbs. I then documented the cellular behaviors in the treated limbs, and the levels of the *Wnt* and FA signaling pathways (Hypothesis 2).

2.2 Materials and Methods

2.2.1. Limb cultures with *Fgf-8b* Activator and SU5402 Inhibitor

M. domestica females were dissected when their embryos were at Stage 32 (13.4 days) [12, 13, 15]. To obtain timed matings, mating pairs of opossums were videotaped and observed copulatory events taken as the start of embryonic development [3]. Upon euthanization of the female by means of carbon dioxide inhalation, embryos were dissected from the uterus, and limbs severed from the rest of the embryo at the axillary region. Limbs were then transferred to Transwell Permeable Support Polyester (PET) Membrane Transwell-Clear Inserts (Corning Incorporate, Cat. No. 3470) for tissue culture. The membrane diameter measured 6.5mm, membrane pore size 0.4 μm , and growth surface area 0.33 cm^2 . The inserts were placed in wells that contained 300 μL of a conducive growth medium. The growth medium included BGJb Medium (1x) + L-Glutamine (GIBCO Invitrogen Corporation, Cat. No. 12591, Lot No. 1087572), MEM Nonessential Amino Acid 100x Solution (Corning, Ref. No. 25-025-C1, Lot No. 25025104), 10,000 I.U./mL Penicillin; 10,000 $\mu\text{g}/\text{mL}$ Streptomycin (BioWhittaker, Cat. No. 17-602E, Lot No. 0213M06226), and Heat-Inactivated Fetal Bovine Serum (Gibco, Ref. No. 16140-071, Lot No. 1399425). Growth media was supplemented with either Recombinant Human/Mouse FGF-8 b Isoform (R&D Systems, Lot: AFK2213011) or SU5402 (Tocris Bioscience, Cat. No. 3300, Batch No. 5). Reagents were reconstituted as recommended in PBS (*Fgf8* protein) or DMSO (SU5402), and added to growth media at the dilution of 10 μL reagent solution per 2 ml of growth media. Control assays were also performed in which 10 μL of PBS or DMSO alone was added to 2 ml of growth media.

For each culture assay, digital images were captured of the limbs to document initial limb morphology. Limbs were then incubated in a vacuum-sealed incubator at 37°C for a period of three days, with growth media being changed every twenty-four hours. A second set of digital images were taken after the three-day growth period. The length of the limbs before and after culturing was quantified using ImageJ, and the percent growth from Day 1 and Day 3 was calculated. After images were taken, limbs were fixed in a 4% paraformaldehyde solution and then rinsed through a methanol/phosphate buffered saline (MeOH/PBS) series to prepare the limb tissue for immunohistochemical (IHC) assays, or stored in RNALater until use for RT-PCR. Percent growth was compared among treatments using a series of Wilcoxon tests [17].

2.2.2. IHC for cell proliferation

After fixing, limbs were sunk in 30% sucrose for twenty-four hours at 4°C, and subsequently equilibrated in O.C.T. Compound (Tissue-Tek, No. 4583) at room temperature. Limbs were then embedded in O.C.T. and stored at -80°C until they were cryotome sectioned and mounted to slides using a motorized Leica CM3050 S Cryostat (Institute for Genomic Biology, University of Illinois Urbana-Champaign). Sections were cut at 10 microns and mounted onto Superfrost Plus Glass Slides 1 mm thick (Electron Microscopy Sciences, Cat. No. 71869-10) for staining.

Cell proliferation was visualized using IHC. Proliferating cells were detected using antibodies against phospho-histone H3 (PHH3; Cell Signaling Technology, Ref. No. 97015, Lot No. 13). PHH3 detects the Ser12 phosphorylated histone H3 during the M-phase of mitotic cell proliferation. To perform IHC, limb sections were equilibrated to room temperature and rehydrated in Blocking Buffer. PHH3 was applied at 1:100 dilution in Blocking Buffer at 4°C in a humidified chamber for twenty-four hours. Slides next went through a series of washes in Blocking Buffer (1% heat-inactivated goat serum (HIGS) with 0.1% Triton X-100 in 1x phosphate buffered saline) in preparation for the secondary antibody, Alexia

Fluor 488 goat anti-rabbit antibody (Invitrogen A31627), which was applied at a dilution of 1:250. Fluorescent TO-PRO-3 iodide, (Invitrogen T3605) was applied in conjugation with the secondary antibody at a 1:1,000 dilution to visualize non-proliferating cells. Slides were then again washed with Blocking Buffer and mounted utilizing Vectashield Hard-Set™ Mounding Medium with DAPI (Vector Laboratories, Cat. No. H-1500). Cells stained for proliferation were visualized using a standard fluorescent microscope and Image Pro Plus 7.0 software. ImageJ 1.46r was used to count those cells that positively stained for cell proliferation within a standard area. The number of proliferating cells in treated and control tissues was compared using a series of Wilcoxon tests [17].

2.2.3. Semi-quantitative RT-PCR for pathway levels

RNA later preserved limbs were homogenized using a hand-held Fisher Scientific TissueMiser, and RNA extracted using an Omega E.Z.N.A. Total RNA Kit (Omega Bio-Tek, Ref. No. R6834-01). RNA concentrations for resulting samples concentrations were obtained using a NanoDrop® Spectrophotometer (ND-1000), and RNA samples were stored at -80°C. cDNA was synthesized from the resulting RNA samples with SuperScript™ III First-Strand Synthesis System (Invitrogen™, Ref. No. 18080-044, Lot No. 1381306). Nucleotide concentrations were obtained using a NanoDrop® Spectrophotometer (ND-1000), and cDNA samples were stored at -20°C.

Oligonucleotide primers were designed using NCBI Primer Blast and manufactured by Sigma-Aldrich™, for several genes (Table 1). These genes were selected for study because they are representatives of the Fgf/MAPK, Wnt and FA pathways, or control housekeeping genes

Fgf Pathway → Fgf8, Fgf10, Spry2, Mapk3

Wnt Pathway → Wnt3, Wnt5a, Rac1, Mapk8

FA Matrix Pathway → FAK (PTK2), Shc1

Housekeeping gene → GAPDH

For RT-PCR, samples were amplified using 5uL cDNA, 2uL 10ng/uL primer mix, and primer master mix (0.5 uL 10x Buffer, 0.1 uL dNTP (New England BioLabs, Cat. No. N0447S, Lot No. 0681109), 0.15 uL MgCl₂, 0.02 uL Taq DNA polymerase (Invitrogen™, Ref. No. 18038-042, Lot No. 1296505), 4.13 uL sterile, double-distilled RNase-free water). Reactions were performed in an Eppendorf Thermal Cycler at the same denaturing (45 seconds at 94°C) and extension (1 minute at 72°C) cycles but had various cycles and annealing temperatures (Table 1). Cycle number was experimentally determined for each gene, and was always within the exponential phase of amplification. PCR products were then run on 1.5% agarose gels. Band peak intensities and average intensities were quantified using Quantity One Analysis software (BioRad, Version 4.6.5, Build 094). GAPDH values were used to standardize the average intensities (i.e., intensities for each gene were divided by the GAPDH value for that gene). Differences in intensities between treatments were statistically analyzed using the Wilcoxon test [17].

2.3 Results

2.3.1. Limb cultures with *Fgf-8b* Activator and SU5402 Inhibitor.

M. domestica limbs treated with *Fgf8* protein (N = 18) grew significantly more in culture than *M. domestica* limbs treated with SU5402 (N = 21) ($\chi^2 = 26.58$; *P*-value < 0.001). *M. domestica* limbs treated with SU5402 also grew significantly less than did control limbs treated with just DMSO (N = 8) ($\chi^2 = 15.24$; *P*-value < 0.001). Too few samples were obtained to statistically test the difference in growth between limbs treated with *Fgf8* protein and control limbs treated with just PBS (N = 1).

2.3.2. IHC for cell proliferation

M. domestica limbs treated with *Fgf8* protein (N = 4) displayed significantly more proliferating cells per standard area (average = 76) than *M. domestica* limbs treated with SU5402 (N = 6; average = 32) ($\chi^2 = 6.00$; *P*-value = 0.014) (Figure 1). *M. domestica* limbs treated with SU5402 also exhibited

significantly fewer proliferating cells per standard area than did control limbs treated with just DMSO (N = 7; average = 69) ($\chi^2 = 8.08$; P -value = 0.005). Too few samples were obtained to statistically test the difference in growth between limbs treated with *Fgf8* protein and control limbs treated with just PBS (N = 1).

2.3.3. Semi-quantitative RT-PCR for pathway levels

Several genes in the *Fgf* (*Fgf10*, P -value = 0.034; *Spry2*, P -value = 0.034; *Mapk3*, P -value = 0.034) and FA pathways (*Rac1*, P -value = 0.034; *FAK* (*PTK2*), P -value = 0.034; *Shc1*, P -value = 0.034) are significantly differentially expressed in SU5402 and control (DMSO treated) limbs (Figure 1). *Fgf8* itself is not differentially expressed in SU5402 and control limbs (P -value = 1), which suggests that only genes downstream of *Fgf8* are affected. In contrast to the general trend displayed by *Fgf* and FA signaling genes, genes in the *Wnt* pathway are expressed at similar levels in SU5402 and control limbs (*Wnt3*, P -value = 0.823; *Wnt5a*, P -value = 0.439; *Mapk8*, P -value = 0.077). The exception to this is *Rac1*, which although involved with FA signaling also has a role in *Wnt* signaling. Sample size for *Fgf8*-treated limbs was insufficient to allow for statistical comparisons of gene expression.

2.4 Discussion

The research presented in Chapter 1 suggests that the differential growth of *M. domestica* fore- and hind limbs is driven by differences in cell proliferation and adhesion brought upon by signals within the limb itself. In Chapter 2 I investigated the possibility that the *Fgf*/*MAPK* pathway provides at least some of these limb-specific signals.

Results suggest that alterations in the *Fgf*/*Mapk* pathway are capable of generating differences in cell proliferation that mirror those observed between wild-type fore- and hind limbs of *M. domestica*. Results therefore support this chapter's first hypothesis. Results also suggest that manipulation of

Fgf/Mapk signaling affects FA but not *Wnt* signaling in *M. domestica* limbs. This suggests that evolutionary changes in the *Fgf/MAPK* pathway could be driving differences in cell behaviors through the FA-ECM pathway (i.e., adhesion), thereby supporting part of the second hypothesis. However, results also suggest that the evolutionary divergence that led to the differential expression of the *Wnt* pathway in the limbs of *M. domestica* likely occurred independently.

In summary, results of my thesis suggest that cell proliferation and focal adhesion levels are key cellular processes that likely contribute to the differential growth of the fore- and hind limbs in *M. domestica*. It is also probable that the differences in focal adhesion are linked to differences in rates of cellular migration in *M. domestica* fore- and hind limbs. Future research will use cell labeling in limb cultures to test the hypothesis that cells migrate more rapidly in *M. domestica* fore- than hind limbs during key stages of differential growth. Results also suggest that differences in *Fgf/MAPK* signaling contribute to the differences in the overall growth rates of *M. domestica* fore- and hind limbs, at least in part by contributing to greater rates of cell proliferation in the fore- than hind limbs. Results also suggest that differences in *Fgf/MAPK* signaling may also help generate the differences in FA signaling and focal adhesion levels that characterize *M. domestica* fore- and hind limbs, but not the observed differences in *Wnt* signaling levels. Future research will use culturing assays to directly assess the impact of *Fgf/MAPK* signaling on focal adhesion levels. In addition, future research will assess the role of the *Wnt* signaling on the differential growth of *M. domestica* fore- and hind limbs in a manner similar to that presented here for *Fgf/MAPK* signaling.

2.5 Figures

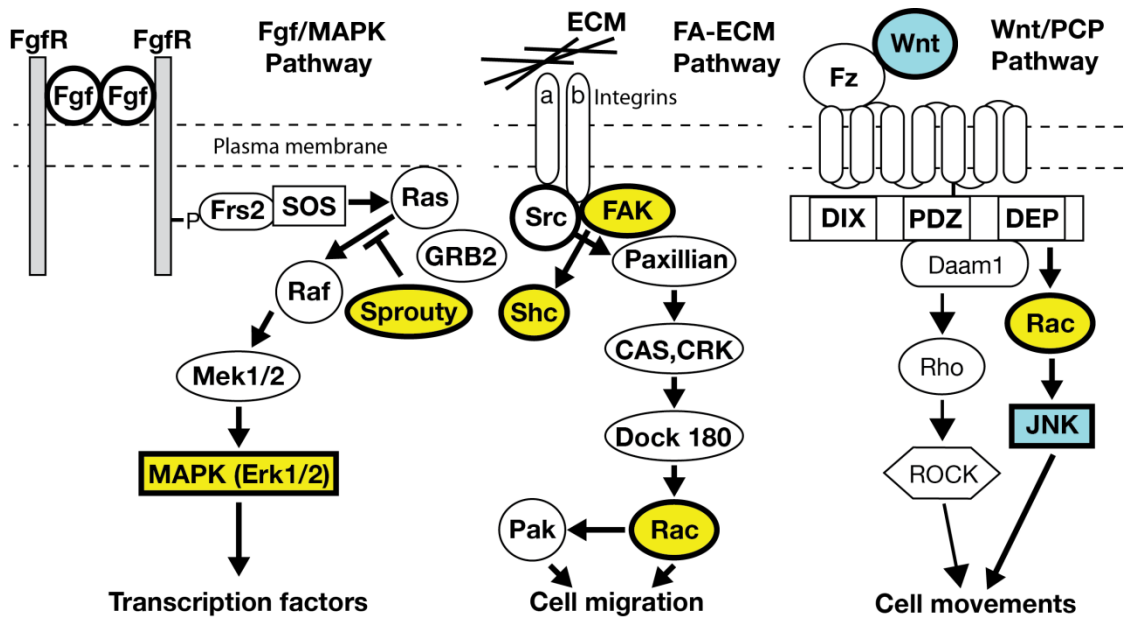


Figure 5. Schematic of the *Fgf/Mapk*, FA and Wnt pathways, with possible interactions shown. Genes highlighted with yellow were expressed at significantly different levels in control and *SU5402*-treated limbs. In contrast, genes highlighted with blue are not differentially expressed in control and *SU5402*-treated limbs.

2.6 Tables

Gene	PCR Cycle #	Forward Primer (5' to 3')	Reverse Primer
<i>fgf8</i>	65	AAGCTCAGCATGTGAGGGAG	GGCCATGTACCAGCCTTCAT
<i>fgf10</i>	45	GAGAACTGCCCATACAGTATCC	GGGAAGAAAGTGAGCAGAGGT
<i>Spry2</i>	55	GGTCCAGCACTCACAGACAC	CGGGTAGGTGCACTCCTTAC
<i>mapk3</i>	55	GCCCCGAGGAGTTGACGGGA	CATGGCCTCCAGCGTGGGTG
<i>wnt3</i>	65	CTGTCAACAACAGCCTTGCC	GGTCAATGATGGCCAGCCG
<i>wnt5a</i>	65	ATTGGTCCACCAGGGAGGTA	TCTCCCTGCTCCCTATCTGC
<i>Rac1</i>	65	TGGGCGGGGAAAGGTCTA	GGCTGAGTAATTGTCTGAAGACAG
<i>mapk8</i>	65	TGCCATCATGAGTAGAAGCAAGCGT	GGACATTTCGTTTCATGGTCTAGCTCC
<i>FAK (PTK2)</i>	65	TTCGGAGGAAGTTCACTGGC	TCGGAGTGTTTTGGCCTTGA
<i>Shc1</i>	65	TCGGTACATGGGTTGTGTGG	TGGCAGGCTCTCTGATTGAC
<i>gapdh</i>	40	ATGCCGAAACGAGACGTCAT	GGTGGTTTCTTCAGTTTGGCC

Table 2. Primer sequences and number of PCR cycles used for each gene.

REFERENCES

1. Conlon, I. and M. Raff, Size control in animal development. *Cell*, 1999. 96(2): p. 235-44.
2. Crickmore, M.A. and R.S. Mann, The control of size in animals: insights from selector genes. *BioEssays*, 2008. 30(9): p. 843-53.
3. Stanger, B.Z., The biology of organ size determination. *Diabetes Obes Metab*, 2008. 10 Suppl 4: p. 16- 22.
4. Kango-Singh, M. and A. Singh, Regulation of organ size: insights from the Drosophila Hippo signaling pathway. *Developmental Dynamics*, 2009. 238(7): p. 1627-37.
5. Martin-Castellanos, C. and B.A. Edgar, A characterization of the effects of Dpp signaling on cell growth and proliferation in the Drosophila wing. *Development*, 2002. 129(4): p. 1003-13.
6. Crickmore, M. A. and R. S. Mann (2006). "Hox control of organ size by regulation of morphogen production and mobility." *Science* **313**(5783): 63-68.
7. Aegerter-Wilmsen, T., C.M. Aegerter, E. Hafen, and K. Basler, Model for the regulation of size in the wing imaginal disc of Drosophila. *Mechanisms of Development*, 2007. 124(4): p. 318-26.
8. Affolter, M. and K. Basler, The Decapentaplegic morphogen gradient: from pattern formation to growth regulation. *Nature Reviews Genetics*, 2007. 8(9): p. 663-74.
9. Hufnagel, L., A.A. Teleman, H. Rouault, S.M. Cohen, and B.I. Shraiman, On the mechanism of wing size determination in fly development. *Proc Natl Acad Sci U S A*, 2007. 104(10): p. 3835-40.
10. Rogulja, D., C. Rauskolb, and K.D. Irvine, Morphogen control of wing growth through the Fat signaling pathway. *Dev Cell*, 2008. 15(2): p. 309-21.
11. Miller, R.D. and K. Belov, Immunoglobulin genetics of marsupials. *Developmental and Comparative Immunology*, 2000. 24: p. 485-490.
12. Sears, K.E., Novel insights into the regulation of limb development from "natural" mammalian mutants. *BioEssays*, 2011. 33: 327-331.
13. Mate, K.E., E.S. Robinson, J.L. VandeBerg, and R.A. Pederson, Timetable of in vivo embryonic development in the gray short-tailed opossum (*Monodelphis domestica*). *Molecular Reproduction and Development*, 1994. 39: p. 365-374.
14. Hinchliffe, J.R. and P.J. Griffiths, Vital staining for cell death in chick limb buds: a histochemical technique in the analysis of control of limb development. *Acta Histochem*, 1986. Suppl 32: 159-164.
15. McCrady, E., The embryology of the Opossum. 1938, Philadelphia: Wistar Institute of Anatomy and Biology. 233.

16. Wanek, N., et al., A staging system for mouse limb development. *Journal of Experimental Zoology*, 1989. 249(1): p. 41-49.
17. Sokal, R.R. and F.J. Rohlf, *Biometry*. 1995, New York: W.H. Freeman and Company. 880.
18. Fernández-Terán, M.A., J.R. Hinchliffe, and M.A. Ros, Birth and death of cells in limb development: a mapping study. *Developmental Dynamics*, 2006. 235: p. 2521-2537.
19. Baker, J., J.P. Liu, E.J. Robertson, and A. Efstratiadis, Role of insulin-like growth factors in embryonic and postnatal growth. *Cell*, 1993. 75(1): p. 443-55.
20. Martin, G. R., The roles of FGFs in the early development of vertebrate limbs. *Genes Development*, 1998. 12: p. 1571-1586.
21. Gros, J., J. K. Hu, C. Vinegoni, P. F. Feruglio, R. Weissleder, and C. J. Tabin, Wnt5a/jnk and fgf/mapk pathways regulate the cellular events shaping the vertebrate limb bud. *Current Biology*, 2010. 20(22): p. 1993-2002.
22. Wyngaarden, L.A., K. M. Vogeli, B. G. Ciruna, M. Wells, A-K Hadjantonakis, and S. Hopyan, Oriented cell motility and division underlie early limb bud morphogenesis. *Development*, 2010. 137: 2551-2558.
23. Sun, X., F.V. Mariani, and G.R. Martin, Functions of FGF signalling from the apical ectodermal ridge in limb development. *Nature*, 2002. 418: p. 501-508.