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Role of Wnt5a and Possible Pathway of Action through Ror2 in Proximodistal Outgrowth of the Limb

Tiffanie M. Dahl

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

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ABSTRACT

Role of Wnt5a and Possible Pathway of Action through Ror2 in Proximodistal
Outgrowth of the Limb

Tiffanie M. Dahl
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Master of Science

Despite over 60 years of study, the molecular pathways and mechanisms governing limb outgrowth and patterning remain poorly understood. Fgfs expressed in the AER are known to be necessary and sufficient for proximodistal limb outgrowth and have been proposed to have a chemoattractive role. Wnt5a is a secreted factor which is expressed in a gradient in the distal limb with the highest concentration next to the AER. The presence of the AER is necessary to establish this gradient. Expression of Wnt5a in a concentration dependant manner can be induced in the limb through the implantation of a bead soaked in recombinant Fgf4 protein. This indicates that Fgfs from the AER may establish the gradient of Wnt5a in the limb mesenchyme. $Wnt5a^{-/-}$ mutants exhibit severe shortening of the face, limbs, and body axis, with limbs being progressively truncated proximally to distally. In normal limb proximodistal outgrowth, cells are seen to grow directionally toward the AER. Previous studies done in the Barrow lab, as well as those done by myself, have shown that if a portion of the AER is removed and the cells proximal to this area are labeled, those which are close enough to intact AER will redirect their growth toward this intact AER. When Wnt5a secreting cells are implanted in the limb mesenchyme of the chick this ectopic source of Wnt5a is sufficient to redirect the growth of the mesenchyme cells toward the Wnt5a source. This indicates that the AER may mediate directed growth of limb mesenchyme cells through the establishment of the Wnt5a gradient which provides positional information to the cells. This Wnt5a gradient results in the recruitment of the mesenchyme cells toward the AER. The Ror2 receptor has been found to be involved in several different pathways involving Wnt5a which are involved in changes in polarity and migration. This makes Ror2 a likely candidate for causing changes in cell polarity and migration during distal outgrowth in the limb. To test whether Ror2 is necessary for the polarizing response of limb mesenchyme cells to the Wnt5a gradient *in vivo* I co-transfected a dominant-negative Ror2 (Ror2ΔC) and a GFP expression vector in the embryonic chick limb using sonoporation. Limb mesenchyme cells transfected with dominant-negative Ror2 grew as radial clones in contrast to the directional outgrowth of the control limb mesenchyme cells along the proximodistal axis. This indicates that cells expressing the dominant-negative Ror2 could no longer respond to the Wnt5a gradient in the limb mesenchyme. This supports a role for Ror2 as a receptor or co-receptor for Wnt5a in mediating directional growth and movement during proximodistal outgrowth and patterning in the limb.

Keywords: AER; Fgf; Wnt5a gradient; Ror2; DiI; sonoporation; directional outgrowth; limb mesenchyme; chicken

ACKNOWLEDGMENTS

I would like to thank my friend and mentor Dr. Jeffery Barrow for the help and support he has given me during the course of both my graduate and undergraduate education. He has been patient and understanding when I have done things wrong and when things just weren't working out. I would like to thank Aaron Smith for the time and effort put into assisting me in creating the plasmids needed and in helping with the sonoporation experiments. I am grateful to Dr. Sho Ohta for taking the time to teach us how to do sonoporations in the first place and to Dr. William Pitt and Marjan Javadi for the donation of their time, knowledge, and microbubbles, as well as their help in figuring out the more technical aspects of sonoporation. I would also like to thank Carl Ellingson for his assistance in weekly sonoporation experiments. I could not have carried out all the sonoporation and AER removal experiments without the help of a small army of pictures takers: Aaron Smith, Avery Hill, Carl Ellingson, Chris Robertson, Jorgen Goodman, Nathan Bodily, Sam Taylor, Scott Malcom, and Tyler Owens. I am especially grateful to those who were willing to take pictures at 2:00 or 3:00 a.m. and did so reliably. I am also grateful for the help of Jennifer and Ryan Widdison in determining the direction of cell growth following the sonoporation experiments. The AER removal experiments were made possible by the AER removal talent of Bryant Johnson. I would also like to thank the members of my committee: Dr. Michael Stark and Dr. Joel Griffitts for their support and knowledge. Dr. Stark and his lab have been especially helpful in teaching me about working with chick embryos.

Lastly, I would like to thank my family for their help and support while I have been working on my Masters: from listening to my complaints about how the chickens just aren't cooperating or that I had to go up to campus in the middle of the night again, to their words of encouragement and support for both me and the chicken embryos I spent so much time thinking about.

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INTRODUCTION

Developmental biology is an increasingly important field of study; providing insights into the molecular and morphological processes that allow for the creation of ordered form. A relatively small number of developmental processes and molecular mechanisms are responsible for the formation of all body structures. Some of these same processes are also involved in other events such as cancer. Developmental biologists seek to study how a small number of processes can create vastly different structures. The limb provides a good model for the study of how a structure can develop in three dimensions. In fact, a large amount of knowledge of how outgrowing tissue becomes patterned in vertebrates has come from studying the limb. Despite over 60 years of study however, the molecular pathways and mechanisms responsible for distal limb outgrowth and patterning still remain poorly understood. Limb anomalies occur in approximately 0.7 to 1 of 1000 human births (McGurik et al., 2001). Understanding the developmental and molecular processes underlying patterning and outgrowth of the limb is therefore of interest to the medical community as well. Previous models of vertebrate limb proximodistal outgrowth do not account for all of the current data. My research is part of the development of a new proposed model, the mesenchyme recruitment model. This model proposes that mesenchymal cells of the limb bud are recruited toward the apical ectodermal ridge (AER) of the limb bud through the establishment and action of a gradient of Wnt5a. If the AER is recruiting the mesenchyme toward it, then the dimensions of the AER will be critical in shaping the outgrowing mesenchyme. The recruitment of different shaped mesenchyme is predicted to give rise to the different shaped elements of the limb. I will be using a chick model to focus on the role and possible mechanism of action of Wnt5a.

Background

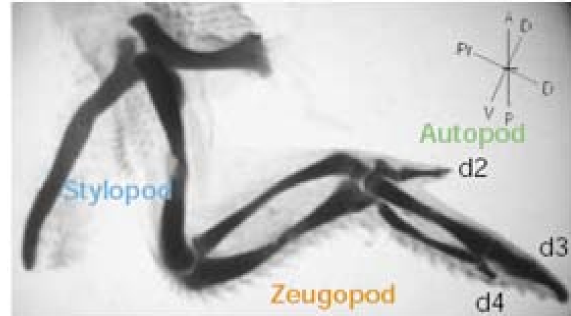

Figure 1: Axes of the vertebrate limb and sections along the proximodistal axis. Pr=proximal, D=distal; D=dorsal, V=ventral; A=anterior, P=posterior (Niswander, 2003).

Patterning is the process by which morphological or molecular differences are created along an axis. There are four basic steps involved in patterning. Initially all cells within the field to be patterned are equipotential. A signaling center is then created and this signaling center sends out positional information. This positional information creates molecular differences across the axis which tells the various cells within the field what to become. The cells will then differentiate according to the information they have received via cell signaling. There are three axes within the limb; patterning occurs along all three axes (**Figure 1**). The dorsal-ventral axis goes from the "back of the hand" (dorsal) to the palm (ventral). The anterior-posterior axis goes from digit 1 or the thumb (anterior) to digit 5 or the pinky (posterior). Lastly, the proximal-distal axis goes from the shoulder (proximal) to the digits (distal). The limb is divided into three sections along this axis (proceeding from proximal to distal): the stylopod (humerus), the zeugopod (radius/ulna), and the autopod (carpals, metacarpals, and phalanges) (see **Figure 1**).

Induction of the limb in chick occurs between Hamburger-Hamilton (HH) stages 13 and 15 (typically 48-55 hours of incubation) (Hamburger and Hamilton, 1951). The limb bud is essentially an outpocketing of mesenchymal cells derived from lateral plate mesoderm surrounded by overlying surface ectoderm (**Figure 2**). The lateral plate mesenchymal cells will give rise to the skeletal structures of the limb (bones, cartilage, tendons, and ligaments). The overlying ectoderm will give rise to the skin and feathers. Previous research has shown that limb development occurs proximally to distally (Saunders, 1948). The differences that are generated

along the proximodistal axis, such as the shape of the different bones, are important for normal functioning of the limb. Despite many years of study, the specific mechanisms underlying proximodistal patterning and outgrowth of the limb are still not well understood. There are currently several different theories for the regulation of this patterning and outgrowth which are being studied.

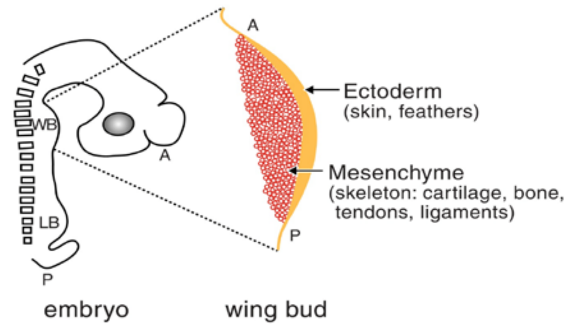

Figure 2: Chick limb bud.

Previous Research and Limb Outgrowth Models

The apical ectodermal ridge (AER) is a thickened piece of ectoderm running along the anterior-posterior axis of the distal tip of the limb bud, separating the dorsal and ventral sides (**Figure 3**). In 1948 in an effort to learn what possible role the AER plays in limb development, John Saunders, Jr. preformed a series of experiments in which he removed the AER at different stages during development. In these experiments he observed that the stage of development attained before removal of the AER was directly related to the extent of distal outgrowth and patterning of the embryonic chick limb. Hence, removal of the AER at early stages resulted in formation of only proximal structures (and a truncated limb) while removal at later stages resulted in formation of

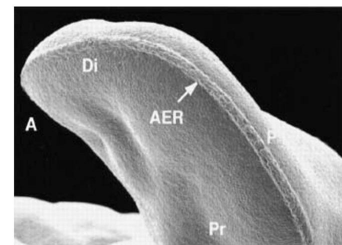

Figure 3: Apical Ectodermal Ridge (AER).

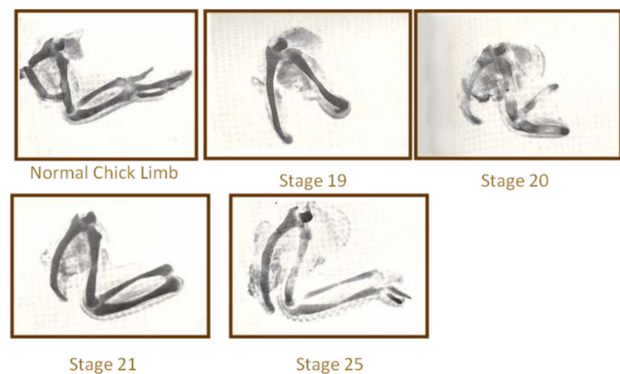

Figure 4: Removal of the AER at earlier stages results in more proximal limb truncations (Saunders, 1948).

increasingly more distal structures (Saunders, 1948) (**Figure 4**). From these experiments Saunders concluded that the AER was required for normal limb outgrowth and that proximodistal patterning occurs as a function of time (i.e. proximal elements forming first followed by more distal elements).

The next question researchers faced, and one that researchers are still trying to answer today, was how the AER is capable of patterning the limb in a proximal to distal fashion. The first theory was that the AER had an instructive role and released different signaling factors (or different levels of the same signaling factor) with time as development progressed (Rubin and Saunders, 1972). These different signaling factors then translated into different structures developing along the proximodistal axis. To test this theory ectoderm recombination experiments were performed. In these recombination experiments AERs from young limb buds were grafted in place of AERs on old limb buds. Converse experiments were also conducted by replacing AERs from young limb buds with those of old limb buds. Regardless of how the experiment was performed, the result was a normal limb (Rubin and Saunders, 1972). This demonstrates that the AER does not send out different instructive signals, but rather appears to send out a permissive signal. This also meant that something other than the AER must be directing patterning of the limb.

The next theory researchers tested was that the instructive patterning information for the limb could be found in the limb mesenchyme. To test this theory, mesenchyme recombination experiments were performed. In these experiments the AER and distal mesenchyme from a young chick limb bud was grafted onto an older limb bud. This resulted in recapitulation of the proximal limb structures (**Figure 5**). The converse experiment was then performed (old AER and distal mesenchyme grafted onto a young limb bud) and distal structures formed without the

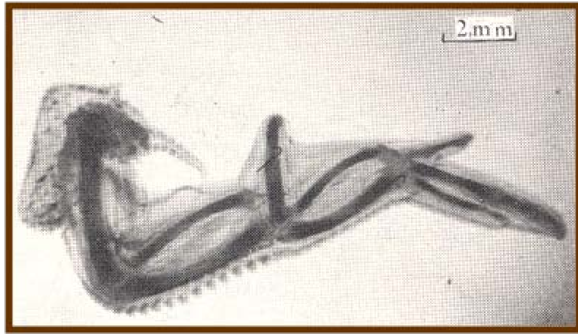

Figure 5: Mesenchyme recombination experiments. (Left) Young AER and mesenchyme on an older limb bud. (Right) Old AER and mesenchyme on young limb bud (Summerbell and Lewis, 1975).

formation of proximal structures (Summerbell and Lewis, 1975) (**Figure 5**). From these experiments it was concluded that

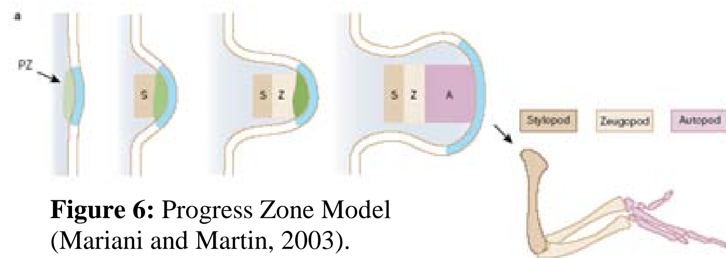

Figure 6: Progress Zone Model (Mariani and Martin, 2003).

patterning information was in fact found in the mesenchyme. The Progress Zone model of proximodistal patterning was developed based on these findings.

The Progress Zone model (**Figure 6**) proposes that the fate of cells along the proximodistal axis is specified by how long the cells are found within an area (the progress zone, PZ, **Figure 6**) at the distal tip of the limb bud. This zone is about 200 μm in size. While cells are within the progress zone they are able to receive a signal which is continually secreted from the AER which allows the cells to remain undifferentiated and keep proliferating. Once the cells fall out of this progress zone (due to proliferation and outgrowth of the limb) the cells will differentiate. The longer the amount of time a cell is within the progress zone, the more distal the fate of that cell will be. Cells which fall out of the progress zone early on will take on proximal fates since they were not influenced by AER signaling for very long. Cells which remain within the progress zone until the end of limb outgrowth will take on distal fates (such as digits) since they were influenced by the signal from the AER for a longer amount of time

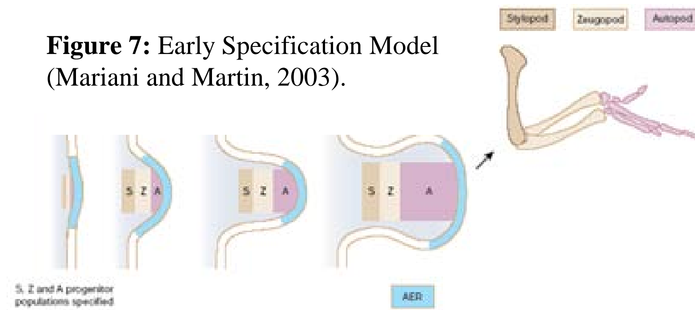

Figure 7: Early Specification Model (Mariani and Martin, 2003).

(Summerbell and Lewis, 1975; Summerbell et al., 1973). Essentially this model proposes that the positional identity of cells within the limb bud is established by the AER acting as an internal clock. The Progress Zone model has been the prevailing model of proximodistal patterning and outgrowth for over 30 years.

The next model that was developed is called the Early or Pre- Specification model (**Figure 7**). It proposes that cells are specified to take on a stylopod, zeugopod, or autopod fate early in development. Regardless of when the AER is removed, cell death occurs in a zone that extends 200 µm from the AER. Further, the three pre-specified regions expand in proximal to distal fashion. The time when the AER is removed dictates which of the three pre-specified zones will be within the 200 µm apoptotic window. Early on, all three zones will be within the 200 µm area whereas at later stages only the autopod region will be subject to apoptosis upon AER removal. This model is supported by the finding that when cells at different distances from the AER are labeled early in limb outgrowth the cells are restricted to a single segment of the limb (Dudley et al., 2002).

Neither the Progress Zone model nor the Early Specification model can fully account for all of the experimental data. There is also no molecular evidence that directly supports these models (Tabin and Wolpert, 2007). This indicates the need for further experimentation and the development of a new model.

FGFs

In 1992 several fibroblast growth factors, Fgf2 and Fgf4, were found to be expressed in the AER (Crossley and Martin, 1995; Niswander and Martin, 1992). In order to determine whether Fgfs alone were sufficient to promote distal outgrowth and patterning, experiments were done in which the AER was removed from stage 20 chick limb buds and replaced with beads soaked in recombinant Fgf2 or Fgf4 (**Figure 8**). These experiments revealed that the Fgf2 and Fgf4 beads could replace the AER in regulating outgrowth and patterning of the limb along the proximodistal axis (Cohn et al., 1995; Fallon et al., 1994; Niswander et al., 1993). It should be noted however that when this is done the shape of the various elements along the anterior-posterior axis and dorsal-ventral axis do not occur correctly. If one bead was applied distally the limb was truncated at the zeugopod (**8-C**), if two beads were applied distally the autopod did not form (**8-D**). When two beads, one distal and one posterior, were applied the autopod formed as a cluster of digits that were arranged dorsoventrally rather than anteroposteriorly (**8-F**). If only a single bead was applied to the posterior the radius also did not form (**8-E**) (Niswander et al., 1993).

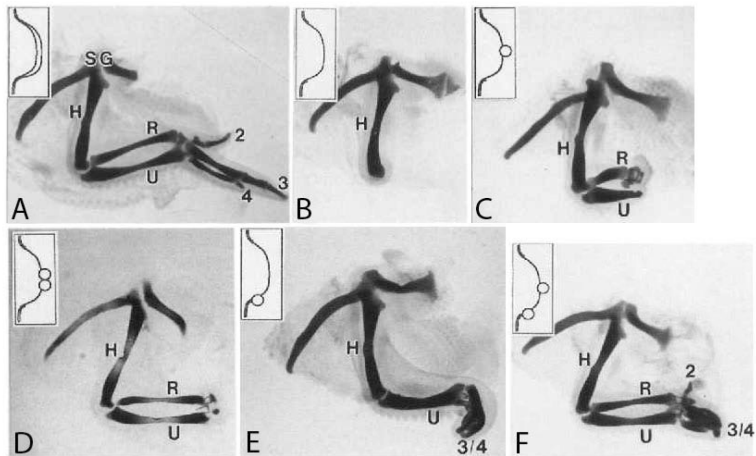

Figure 8: A) No treatment. B) AER removed at stage 20. C-F) AER removed at stage 20, followed by application of FGF bead(s) as indicated in inset. In E and F the cluster of four posterior digit-like elements is labeled 3/4 . (Niswander et al., 1993).

In addition to Fgf2 and Fgf4, other Fgfs have also been shown to play a role in limb bud outgrowth, both in the initiation of outgrowth and in the establishment of the signaling system regulating limb development (Crossley and Martin, 1995). Providing further support for the role

of Fgfs as the signaling factor released by the AER to control outgrowth of the limb is the fact that when Fgf genes are knocked out in the AER of mice, elements of the limb are missing. This was thought to be due to cell death since removal of the AER results in rapid death of the underlying mesenchyme. When cell death was assayed in Fgf4/Fgf8 double knockout mice however, increased cell death was not detected until later stages (39 somites in the hindlimb and 32 somites in the forelimb) and only in the proximal mesenchyme (not the distal mesenchyme as was expected) (Sun et al., 2002). Additionally, despite the failure of the limb mesenchyme in Fgf4 and Fgf8 double mutants to survive (leading to the elimination of the limb buds) these mutants do have normal AER morphogenesis (Boulet et al., 2004; Sun et al., 2002). This suggests that the AER of these mutants has lost the ability to signal for the outgrowth of the limb mesenchyme.

Fgf4 has previously been proposed to act as a chemoattractant for the mesenchymal cells of the limb bud (Niswander et al., 1993). Support for this comes from experiments where an Fgf4 soaked bead has been implanted in the limb. In these experiments cells were shown to divide and/or migrate toward the ectopic source of Fgf4 (Li and Muneoka, 1999; Niswander et al., 1993; Saxton et al., 2000). This suggests that the Fgfs of the AER may be acting to attract the mesenchymal cells toward them. The molecular mechanism whereby the AER recruits the mesenchyme toward it however is not currently understood.

Wnt5a

Wnt5a is a secreted factor which is expressed in a gradient fashion at the distal end of the limb. Studies of Wnts such as Wnt5a and Wnt11 have indicated that these secreted factors play a role in polarizing cells. The polarization of cells is crucial for directed cell movements and oriented cell divisions (Heisenberg et al., 2000; Kilian et al., 2003; Qian et al., 2007; Rauch et

al., 1997; Westfall et al., 2003; Ying et al., 2004). Wnt5a has been demonstrated to cause directional cell movement through the reorientation of the cytoskeleton in response to a chemokine gradient *in vitro* (Witze et al., 2008). Wnt5a is expressed in a gradient at the caudal end of the growing embryo as well as at the distal end of several structures which extend from the embryo later on such as the limb and tail buds (**Figure 9**) (Gavin et al., 1990; Yamaguchi et al., 1999). Wnt5a mutants exhibit severe shortening of the face, limbs, and body axis. In the limbs, the skeletal elements are progressively truncated proximally-to-distally. The distal phalanges are absent (Yamaguchi et al., 1999) (**Figure 10**). This indicates that the limb mesenchyme cells did not extend along the proximodistal axis. However these mutants still exhibit relatively normal anterior-posterior limb patterning (**Figure 10**). Further, the expression of other genes which are thought to have a role in proximodistal outgrowth and patterning (Distalless, Hoxd, and Fgfs) are not altered (Qian et al., 2007; Yamaguchi et al., 1999). What then results in the failure of these limbs to lengthen if the AER and expression of other proximodistal outgrowth genes is intact? It appears that the limb mesenchyme cells have lost the ability to respond to the AER. Wnt5a may therefore be involved in mediating cell polarity events during limb outgrowth. This conclusion is supported by a study in which reduced expression of Wnt5a in fish fin buds was shown to impair cell migration (Sakaguchi et al., 2006).

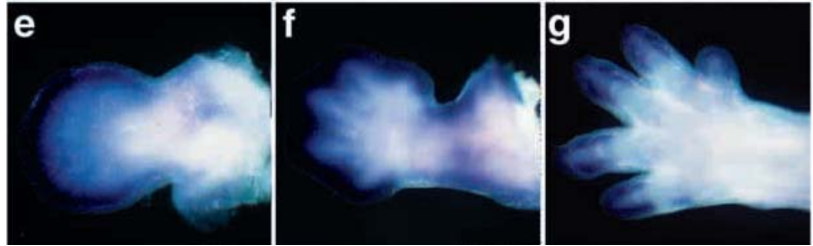

Figure 9: Wnt5a expression in the limb over time (Yamaguchi et al., 1999).

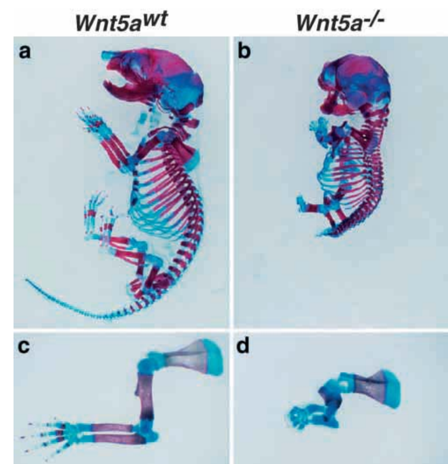

Figure 10: Wnt5a WT and Wnt5a mutant (Yamaguchi et al., 1999).

Expression of Wnt5a in the mouse limb is first observed around 20 – 22 somites, just after the forelimb begins to bud (Yamaguchi et al., 1999). In the chick limb Wnt5a expression is first detected in the limb forming region at Hamburger-Hamilton stage 14 (which is also just after induction of the limb begins) (Kawakami et al., 1999). As the limb bud grows, a gradient of Wnt5a is created with the highest concentration of Wnt5a at the distal tip of the limb next to the AER. This indicates that Wnt5a expression may be activated by and depend on the presence of the AER. The Wnt3$^{n/c}$; Msx2Cre mouse mutant exhibits a variable loss of the AER (**Figure 11**) (Barrow et al., 2003). In this mutant, expression of Wnt5a appears to be a function of the amount of AER present. This finding suggests that AER signals are necessary to establish Wnt5a expression in the limb mesenchyme.

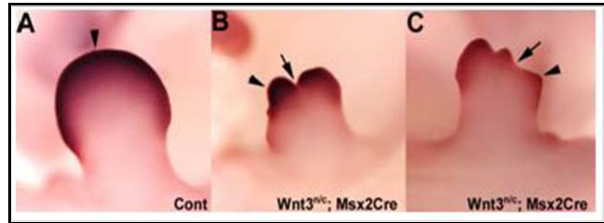

Figure 11: Wnt5a expression in response to the presence of the AER. Arrow heads denote regions where the AER is present while arrows denote regions where the AER is not present (Barrow et al., 2003).

Ror2

Receptor tyrosine kinases (RTKs) play a vital role in many developmental processes. Ror-family RTKs have an intracellular tyrosine kinase domain, an extracellular Frizzled-like cysteine-rich domain (CRD), an Ig-like domain, and a membrane-proximal Kringle domain. These domains are assumed to mediate protein-protein interactions (**Figure 12**). Members of the Frizzled family of genes encode proteins which are thought to act as cell-surface receptors for members of the Wnt family. The Frizzled-like CRD

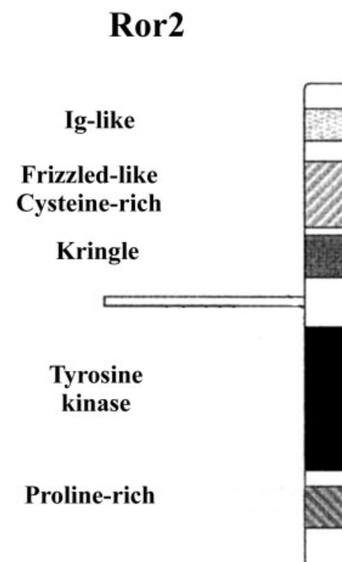

Figure 12: Structure of Ror2 (Yoda et al., 2003).

domain of the Ror-family may thus serve the same function and can be expected to be the Wnt binding domain (Yoda et al., 2003).

In C. elegans, mutations in Ror cause defects in cell migration and asymmetric cell division. In mice, Ror1 and Ror2 are expressed in migrating neural crest cells and mesenchymal cells (Yoda et al., 2003). Wnt5a and Ror2 are required for convergent extension and for changes in polarity and migration of several cell types during development (He et al., 2008; Nomachi et al., 2008). In mice both Ror1 and Ror2 are strongly expressed in the mesenchyme (but not the ectoderm) of the limbs from E9.5 and onward. By E10.5 Ror1 is restricted to the proximal regions of the limb buds while Ror2 is expressed throughout the limbs. At E12.5 and E13.5 Ror2 is detected in the perichondrium of the digits and the marginal regions of the limbs, while Ror1 is expressed in the anterior and posterior portions of the limb and the interdigital regions (Al-Shawi et al., 2001; Matsuda et al., 2001). Expression of Ror2 in the chick is very similar to that seen in the mouse with strong expression observed in the limb. In the early limb bud this expression is found throughout the limb with stronger expression in the anterior and posterior areas. As development continues expression in the central mesenchyme fades while anterior and posterior expression remains as in mice. At HH 27 Ror2 is found in early stylopod and zeugopod cartilage condensations. By HH 30 expression is seen in condensations of the autopod (Stricker et al., 2006). Ror2-deficient mice have skeletal abnormalities, including abnormally short limbs/tail and abnormal vertebrae and facial structures. Defects are more severe distally. Ror2 mutants also exhibit significant or complete loss of the radius, ulna, tibia, and fibula, providing evidence for a mechanism involving Ror2 in proper limb development (DeChiara et al., 2000; Takeuchi et al., 2000). This phenotype is more severe in Ror1/Ror2 double mutant mice, with dysplasia of the humerus and femur occurring as well. This indicates that the two

Rors are functionally redundant in areas of skeletal development and that the two interact during morphogenesis (Nomi et al., 2001).

Ror2 is mutated in the human syndromes Brachydactyly type B (BDB) and recessive Robinow syndrome (RS) (Stricker et al., 2006). BDB is characterized by hypoplasia/aplasia of the distal phalanges while RS is characterized by short stature, mesomelic limb shortening, hemivertebrae, genital hypoplasia, and characteristic facial features. When constructs containing mutations/truncations to Ror2, like those responsible for BDB and RS, were overexpressed in the chick using replication-competent retroviral vector-mediated overexpression the result was limb cartilage which appeared shorter and thicker (Stricker et al., 2006).

The mechanism (specific pathway of action) used by signaling involving Ror2 is not well understood. Evidence has been found for several different pathways involving Ror2 (DeMorrow et al., 2008; He et al., 2008; Hikasa et al., 2002; Mikels et al., 2009; Nomachi et al., 2008; Qian et al., 2007; Sato et al., 2010). In these pathways it is thought that Wnt5a acts as a ligand for Ror2. A study in Xenopus showed that the extracellular region of the Xenopus ortholog of Ror2 binds to Xenopus Wnt proteins *in vitro* and that the CRD domain is needed for this interaction (Hikasa et al., 2002). This provides evidence that Wnt proteins may be ligands for Ror-family RTKs. Wnt5a has been shown to induce Ror2 homo-dimerization *in vitro*. Wnt5a induced phosphorylation of Ror2 on the tyrosine residues also results in increased phosphorylation of the Ror2 substrate 14-3-3β scaffold protein. This indicates that Wnt5a binding results in the activation of the Ror2 signaling cascade *in vitro* (Liu et al., 2008). The Xenopus Ror2 ortholog has been shown to function in the planar cell polarity pathway (PCP) of Wnt signaling which is involved in convergent extension movements. Overexpression of the Ror2 ortholog in Xenopus inhibits these movements (Hikasa et al., 2002). Experiments done in mice also support the

involvement of Ror2 in the PCP pathway (Yoda et al., 2003). These findings indicate that Ror RTK may act either as a receptor or co-receptor in the PCP pathway. Other studies have indicated that Wnt5a functions in the PCP pathway as well (Qian et al., 2007).

Other studies of Wnt5a and Ror2 however, such as those involving cholangiocarcinomas, have shown that Wnt5a is able to signal through a noncanonical Ca^{2+}-independent pathway involving Ror2 and subsequent activation of Jun NH_2-terminal kinase (JNK) (DeMorrow et al., 2008). Through this pathway, Wnt5a has been shown to promote cell migration. This migration appears to be mediated by Ror2 which is in association with filamin A (FLNa) (Nomachi et al., 2008). This pathway has been further studied in wound-healing assays of cultured cells. It is not known whether Ror2 acts as a receptor or a co-receptor for Wnt5a in this pathway. During wound healing however, Wnt-c-Jun N-terminal kinase (JNK) is activated at the wound edge in a Ror2-dependant manner following Wnt5a stimulation (Nomachi et al., 2008). Through the Wnt5a/Ror2/JNK pathway the formation of lamellipodia and reorientation of the microtubule-organizing center is controlled. This regulation of polarized cell migration can be suppressed by an inhibitor of PKCζ (aPKC). PKCζ is activated in response to wound-induced loss of cell-cell contacts. The suppression of Wnt5a induced polarized cell migration by an inhibitor of PKCζ indicates that PKCζ activity is involved in Wnt5a-induced microtubule-organizing center reorientation, providing a link between the Wnt5a/Ror2/JNK pathway and the Par/aPKC pathway (Nomachi et al., 2008).

Canonical Wnt signaling can also be inhibited by Wnt5a Ror2 signaling. This inhibition requires the extracellular Ror2 CRD and Ig-like domains and the intracellular tyrosine kinase and proline domains. Wnt5a was shown to directly modulate Ror2 tyrosine kinase activity but downstream events in this pathway were not studied (Mikels et al., 2009). Sato et al. showed

that Wnt5a can induce the internalization of Frizzled2 (Fz2) in HeLaS3 cells. Knockdown of either Ror1 or Ror2 suppresses this internalization. Knockdown of both Ror1 and Ror2 further inhibits internalization of Fz2 (Sato et al., 2010). This indicates that Wnt5a induces internalization of Fz2 through a process which involves Ror1 and/or Ror2. This endocytosis is clathrin-mediated and also involves Disheveled2 (Dvl2) and β-arrestin. Through this process canonical Wnt signaling could be inhibited and Rac could be activated. This process appears to be involved in convergent extension. Ror1 or Ror2 may also be internalized in response to Wnt5a (Sato et al., 2010).

In the developing palate Wnt5a is expressed in a gradient along the anterior-posterior axis. Mesenchymal cells of the palate exhibit directional migration with Wnt5a providing a chemotactic role (migration is disrupted in Wnt5a$^{-/-}$ palates). The cell proliferation and cell migration occurring during formation of the palate appears to be mediated by Wnt5a signaling through a noncanonical pathway involving Ror2 (He et al., 2008). Ror2$^{-/-}$ and Wnt5a$^{-/-}$ mutants have very similar phenotypes which include shortened limbs (Oishi et al., 2003). Therefore in the limb Wnt5a may be signaling through a pathway which involves Ror2 as either a receptor or a co-receptor to cause changes in cell polarity and the direction of migration and/or division needed for growth along the proximodistal axis.

Previous Research from the Barrow Lab

Limb Mesenchyme Cells Grow Directionally Toward the AER

When a small cluster of cells is labeled in the mouse embryonic limb, the cells grow directionally toward the AER rather than growing in all directions (as would be evidenced by a large circular clone) (**Figure 13**) (Mao et al., 2005; Sowby and Barrow, Unpublished). This finding is consistent with dye labeling studies performed in chick limbs where labeled cellular

clones are found to elongate toward the AER. This suggests that directed outgrowth is occurring (Dudley et al., 2002; Li and Muneoka, 1999; Vargesson et al., 1997).

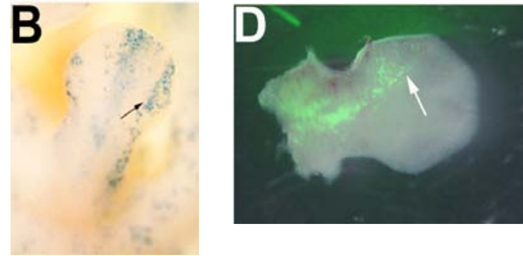

Figure 13: Labeled mouse limb cells showing outgrowth along the proximodistal axis (Sowby and Barrow, Unpublished).

If the AER is recruiting cells toward it then removing a portion of the AER might influence the outgrowth behavior of adjacent mesenchymal cells. In support of this hypothesis labeled cells residing next to regions of denuded ectoderm have been observed to redirect their growth toward portions of the AER which remain (**Figure 14A**). If the labeled cells are too far from the remaining AER however, they continue to grow but do so in all directions as evidenced by a large circular clone (**Figure 14B**) (Kendall et al., Unpublished). As seen in **Figure 14**, slight indentations in the mesenchyme next to where the AER has been removed can be observed beginning at 24 to 26 hours. These indentations become larger over the next 48 hours (see **Figures 26**, **27**, **28**, **29**). Previous models for limb outgrowth would account for these findings by hypothesizing that the indentations are due to a decrease in cellular proliferation or an increase in apoptosis (Dudley et al., 2002). Our data show that one of the reasons for this indentation is that cells no longer grow toward the denuded ectoderm but rather grow toward the adjacent AERs.

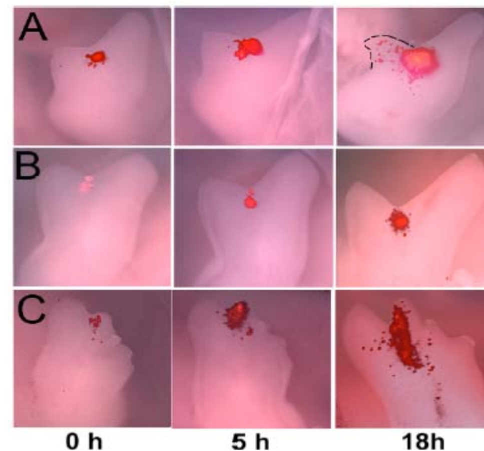

Figure 14: Labeling of cells directly proximal to a section of AER which has been removed (Kendall et al., Unpublished).

Wnt5a Mediates Directed Growth of the Limb Mesenchyme

The question of how the AER recruits mesenchyme cells in its direction is a crucial one. As discussed above, Wnt5a is expressed in the distal limb mesenchyme in a gradient fashion (**Figure 11**). Wnt5a and its receptor Ror2 have previously been shown to play a role in directed migration and oriented cell division (He et al., 2008; Yamaguchi et al., 1999). Therefore it is possible that the AER mediates directed growth of the adjacent mesenchyme through the secreted protein, Wnt5a. We have shown that removal of the AER results in loss of Wnt5a expression (**Figure 11**). Additionally, we have demonstrated that a bead soaked in recombinant Fgf4 protein induces the expression of Wnt5a in adjacent cells in a concentration dependant manner (Low and Barrow, Unpublished) (**Figure 15**). Hence, the Wnt5a gradient appears to be induced by the gradient of Fgfs which are secreted from the AER.

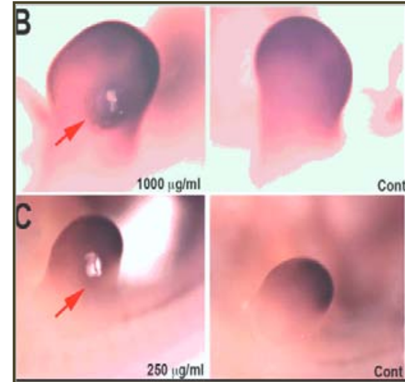

Figure 15: Wnt5a expression in response to an Fgf4 soaked bead implanted into a stage 20 chick forelimb bud (Low and Barrow, Unpublished).

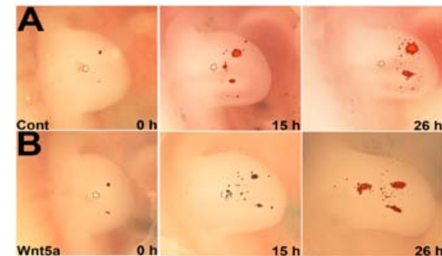

Figure 16: Implantation of Wnt5a secreting cells in limb mesenchyme results in redirection of cell outgrowth (Kmetzsch and Barrow, Unpublished).

We next sought to determine if the gradient of Wnt5a was crucial in providing a positional cue to the limb mesenchyme which resulted in cells growing in the direction of the increasing Wnt5a concentration. Consistent with this hypothesis, we found that transplanting Wnt5a secreting cells in the limb mesenchyme of chick embryos was sufficient to redirect the growth of adjacent limb mesenchyme cells toward the source of Wnt5a (**Figure 16**). Therefore, the AER mediates directed outgrowth of the adjacent limb mesenchyme, through its induction of a gradient of Wnt5a protein.

In support of a role for Wnt5a in providing directional information which results in limb mesenchyme cells being recruited toward the AER during outgrowth are recent findings that Wnt5a has a chemoattractive role in drawing mesenchyme cells into the future limb bud during initiation of limb bud formation. Here Wnt5a is thought to act in establishing mesodermal cell orientation and in signaling the transition in cell polarity which is necessary to generate a limb bud (Wyngaarden et al., 2010).

The Limb Mesenchyme Recruitment Model

As discussed above, John Saunder's AER removal experiments demonstrated that the AER is required for distal outgrowth and patterning of the limb. Others have proposed several models to describe how the AER accomplishes this task (i.e. promotes distalization of the mesenchyme as a function of time, prevents apoptosis of pre-specified mesenchymal zones). There have however, been serious uncertainties about the ability of both of these models to account for the various events and observations during limb outgrowth and patterning (Tabin and Wolpert, 2007). Our data suggest a different model for how the AER mediates outgrowth as well as patterning of the mesenchyme along the proximodistal axis. We propose that Fgfs from the AER induce a gradient of Wnt5a expression in the adjacent limb mesenchyme. The gradient of Wnt5a provides a directional cue to the mesenchymal cells via Wnt5a signaling through a pathway involving Ror2. This directional cue promotes distal growth (via directed migration or oriented cell divisions) of the mesenchyme toward the AER. This information results in the recruitment of mesenchymal cells toward the AER.

In this model the shape of the AER is expected to be important because it dictates the shape of the field of mesenchymal cells which are recruited toward it. During normal limb development the AER can in fact be observed to change its shape over time. It is more circular

(similar to the shape of the stylopod) early in limb development but becomes long and thin as development progresses (similar to the shape of the paddle which will become the digits) (**Figure 17**) (Barrow, Unpublished; Barrow et al., 2003; Ying et al., 2002). This indicates that the shape of the AER and the ability of the AER to change its shape may be important in proper patterning of the limb. If this is true it may mean that the AER is recruiting cells toward it and changes its shape to recruit differently shaped fields of cells. This prediction is in accordance with the mesenchyme recruitment model.

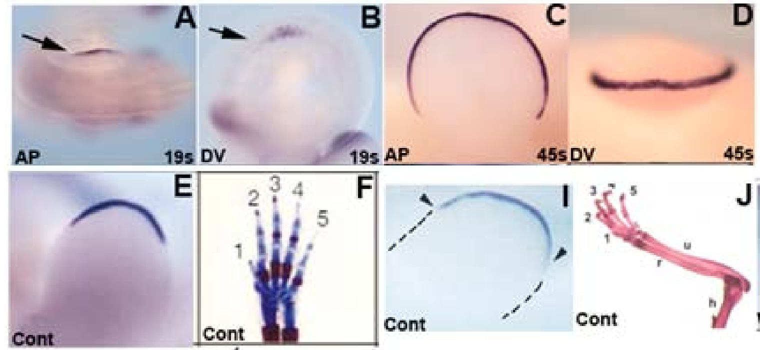

Figure 17: Changing shape of the AER over time (Barrow, Unpublished; Barrow et al., 2003; Ying et al., 2002).

PROPOSAL

Hypothesis

Proximodistal outgrowth and patterning of the developing limb bud is mediated by Fgf signaling from the AER which activates Wnt5a in a gradient fashion. Wnt5a then signals through a pathway involving Ror2 to polarize the mesenchymal cells to migrate and/or proliferate in a directional fashion.

Objectives

Test whether Ror2 is necessary for the polarizing response of limb mesenchyme cells to the Wnt5a gradient *in vivo*.

I will also be recreating the AER removal experiments (see **Figure 14**) done by previous graduate students in the lab as a confirmation of their results.

Material and Methods

Plasmid Construction

A major goal of my thesis work was to block the Wnt5a/Ror2 signaling pathway in select limb mesenchyme cells of the developing chick limb. In order to accomplish this I co-transfected dominant-negative Ror2 and GFP expression vectors into embryonic chick limbs *in ovo* using sonoporation (see below). I used the pCAGGS-AFP expression vector which expresses a mutant green fluorescent protein (GFP) at high levels (Ogawa et al., 1995) (used by permission from Hidesato Ogawa) as a lineage tracer to positively label cells which had taken up the plasmid. The dominant-negative Ror2 plasmid was created using a pCIG backbone (Megason and McMahon, 2002). The pCIG vector contains a β-actin/CMV promoter which was used to drive expression of genes. Co-transfection of pCAGGS-AFP and an empty pCIG vector served as a control to observe the normal behavior of mesenchyme cells during outgrowth of the limb. To test whether Ror2 is necessary in the polarizing response of cells to the Wnt5a gradient I inserted Ror2 missing the C terminus (Ror2ΔC) in the multiple cloning site of pCIG. Ror2ΔC has been demonstrated to have a dominant negative effect on Ror2 signaling *in vitro* (Nishita et al., 2006). Thus overexpression of this Ror2 construct can be predicted to result in the cells being incapable of responding to Wnt5a signals. Further, similarly truncated Ror2 alleles in humans cause shortening of the limbs (Stricker et al., 2006).

The constructs were then introduced into the limb mesenchyme of chick embryos *in ovo* using sonoporation. Chicks are useful for studying limb development because the living embryo can be manipulated and then observed during development. This allows the movements of cells in the limb to be observed as the limb is still developing.

Windowing

Once the proper stage of development was attained a hole was cut in the top of the egg, a process referred to as "windowing." Prior to windowing, the top of each egg was wiped with isopropanol. Using an 18 gauge 1 ½ inch needle (Becton Dickinson) 5 mL of albumen was removed from the eggs. The top of the egg was then covered with Scotch® Super 33+ Vinyl Electrical Tape and an oval shaped piece of shell was cut out of the top of the egg. Five drops of 1% penicillin-streptomycin-glutamine (PSG) in phosphate buffered saline (PBS) was dropped on top of the embryo to keep the embryo hydrated and to prevent bacterial infection. The eggs were then re-taped and placed back in the incubator until needed. Using electrical tape, rather than clear tape which is more typically used when windowing eggs, allowed for smoother adherence of the tape to the surface of the egg prior to windowing. It also allowed for the easy removal and re-adherence of the tape covering the hole in the top of the egg. The electrical tape cover sealed tighter to the egg than a cover made of clear tape, further reducing the number of embryos lost to dehydration. Immediately prior to experiments a bent 25 gauge 1 ½ inch needle (Becton Dickinson) was used to add a 1:10 dilution of India ink (Pelikan Fount India) with Dulbecco's Phosphate Buffered Saline 1X (GIBCO) underneath the embryo to make visualization of the limb bud easier.

Microbubble Preparation

Gene transfection in the sonoporation experiments was enhanced by using phospholipid-stabilized microbubbles. The phospholipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (DPPA), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DPPE) in chloroform were purchased from Avanti polar lipids, Inc. (Alabaster, AL). NaCl, Na_2PO_4 (anhydrous) and $NaHPO_4 \cdot H_2O$ were purchased from

Mallinckrodt (Phillipsburg, NJ). Glycerol and propylene glycol (PG) were purchased from EMDTM (Gibbstown, NJ) and Spectrum Chemical (New Brunswick, NJ) respectively. Buffer was prepared by adding PG (0.828 g), glycerol (1.010g), NaCl (0.054 g), Na_2PO_4 (1.1445 mg), and $NaHPO_4 \cdot H_2O$ (2.34 mg) to 7.4 mL H_2O and sterilized by autoclave. DPPA (18 µL, 20 mg/mL), DPPC (160 µL, 20 mg/mL) and DPPE (97.3 µL, 25 mg/mL) were mixed and dispensed equally into eight sterile 2-mL vials (Xpertek, Saint Louis, MO). The chloroform was evaporated by N_2 gas flow, following which 1 mL of buffer was added to each vial. Sterile caps with rubber septums were crimped on each vial, and the head space of each vial was flushed and filled with perfluoropropane gas (Advanced Specialty Gases, Reno, NV) through 25 gauge needles. The vials were stored upside down at 4°C until activated for use.

Sonoporation

Sonoporation is a technique which allows for the introduction of expression vectors into chick cells *in ovo*. Ultrasound generated by a sonoporator can be used to increase the permeability of the cell membrane to DNA. This is accomplished by mixing a DNA solution with microbubbles. The solution is then introduced into chick tissues where it is subjected to strong pulses of ultrasound. Ultrasound causes the collapse of cavitated microbubbles, creating small transient holes in the cell membrane which allow for the entry of DNA (Ohta et al., 2008; Ohta et al., 2003).

To accomplish sonoporation in the chick, stage 20 – 21 (85 hours incubation) White Leghorn chicken embryos were windowed in preparation for injection of a plasmid and microbubble solution into the limb bud. Sonoporation was then utilized in getting the limb bud cells to take up the plasmids as described in Ohta et al., 2003 and Ohta et al., 2008. To create the plasmid and microbubble solution the following protocol was used. Microbubbles were prepared

as described above. The microbubbles were activated by 30 seconds of shaking in a dental amalgamator (TP-103, GC America, Alsip, IL). Immediately following shaking, 5 μL of microbubbles were withdrawn from the vial and added to the plasmid solution. For control experiments 2.8 μL of 6.5 μg/μL pCIG and 1.8 μL of 10 μg/μL pCAGGS-AFP were combined in a 0.7 mL microfuge tube and the volume was brought up to 20.5 μL by the addition of 15.4 μL of 1% PSG in PBS and 0.5 μL of a 1:5 dilution of fast green for color. Microbubbles were then added. The final concentration of both pCIG and pCAGGS-AFP was 0.71 μg/μL. The majority of the dominant-negative Ror2 experiments were done using a solution which contained 0.7 μL of 8.8 μg/μL Ror2ΔC in pCIG added in place of the 2.8 μL of 6.5 μg/μL pCIG. The total volume of solution was brought to 20.5 μL with the addition of 17.5 μL of 1% PSG in PBS and 0.5 μL of a 1:5 dilution of fast green. Microbubbles were then added. The final concentration of Ror2ΔC was 0.24 μg/μL. This 3:1 ratio of pCAGGS-AFP to Ror2ΔC was used for the majority of sonoporation experiments because in initial experiments where a 1:1 or 2:1 ratio of pCAGGS-AFP to Ror2ΔC was used there appeared to be an excess of cell death of the transfected cells. The microbubble/plasmid solution was stored upside-down on ice, allowing the microbubbles to collect at the tip of the tube. Just prior to injection into the limb bud, the solution was gently pipetted in and out of a micropipettor until the bubbles were uniformly distributed throughout the plasmid solution. A 1-2 μL drop of solution was pipetted onto a 35mm Petri dish lid. Injections were accomplished by drawing a small amount (0.25-0.50 μL) of solution into a pulled glass capillary pipette on which the tip was broken off with forceps to create a glass needle. The glass needle was then used to inject a small amount of solution into the limb bud of a stage 20 HH chick embryo (**Figure 18, Step 1**). Injections were done along the anterior-posterior axis and toward the distal tip of the limb. A slight proximal-posterior to distal-anterior angle was used to

get the injection as distal as possible. Ultrasound was then applied using a 3.0 mm probe on a Sonitron 2000N (Protech International, San Antonio, Texas) (**Figure 18, Step 2**). The parameters were as follows: ultrasound intensity was 3 W/cm^2, duty cycle (rate of pulse wave irradiation) was 50%, frequency was 1 MHz, and the duration of exposure to ultrasound was 60 seconds. The probe was placed above the limb bud directly but gently in contact with the limb bud itself. Gene expression was then observed and photographed using an Olympus SZX12 microscope, Olympus U-CMAD3 camera, and PictureFrame™ Application 2.3 software at 4 hour intervals beginning 2 hours after sonoporation and continuing until the embryo died or GFP expression was no longer seen. Both bright field and fluorescent pictures were taken and then superimposed in Adobe Photoshop CS4 allowing for the direction of growth of the cells which had taken up the plasmids to be followed over time.

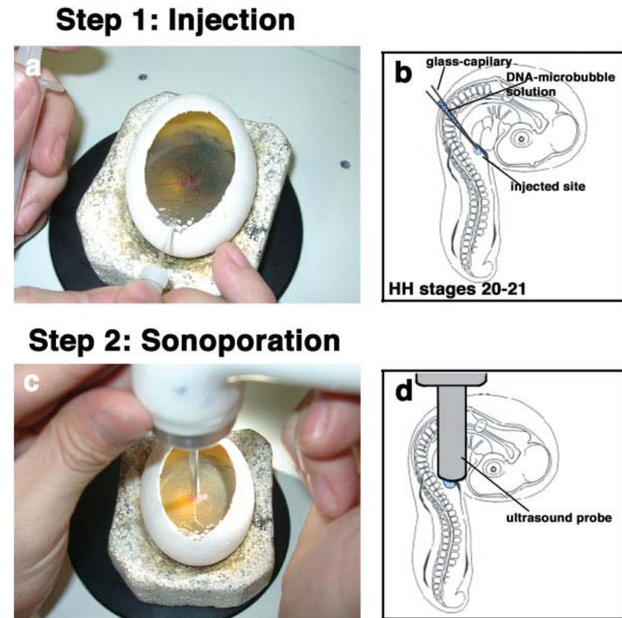

Figure 18: Sonoporation technique (Ohta et al., 2003).

Skeletal Preparation

Day 6 to 7.5 embryos were removed from the eggs and membranes were removed in PBS. Embryos were then placed in 17 X 100mm polypropylene tubes (Fisherbrand 14-956-1J) and fixed in Bouins solution for 2 hours. Six to eight washes in 70% EtOH/0.1% NH_4OH were done on a rocker over a period of 24 hours until the embryos were white. Embryos were then equilibrated in 5% acetic acid twice for one hour each. Following this the embryos were stained

overnight in fresh 0.05% Alcian blue 8GX in 5% acetic acid. Alcian blue was prepared the night before by mixing 0.015g Alcian blue 8GX, 5 mL acetic acid, 75 mL 95% ethanol, and 20 mL double distilled H_20. The solution was placed on a stir plate overnight. The next day the solution was filtered prior to addition to the embryos. The embryos were then washed twice in 5% acetic acid for an hour each time. Embryos were then dehydrated through two one hour changes of 100% methanol. Finally embryos were cleared in 2:1 benzyl benzoate to benzyl alcohol overnight in a glass vial. Embryos were stored long term in benzyl benzoate. Embryos were then photographed and analyzed under a microscope.

DiI Injection

DiI (Invitrogen C700) was prepared as follows: Stock DiI was prepared at a concentration of 1.5 µg/µL by the addition of 33.3 µL of 100% ethanol. To make the DiI solution used in experiments 1 µL of stock DiI, 19 µL of 100% ethanol, 180 µL of 5% sucrose, and 2 µL of fast green were combined. DiI was injected using a pulled glass capillary on which the tip had been broken off.

AER Removal Experiments

Eggs were windowed and portions of the AER were removed at 86 hours incubation. Five drops of 1% PSG in PBS was added on top of the embryo and the eggs were taped back up with Scotch® Super 33+ Vinyl Electrical Tape and put back in the incubator for 24 hours. After 24 hours of growth, valleys are created in the limb bud proximal to where the AER had been removed. DiI was used to label cells at the distal edge of the valley and pictures were immediately taken using an Olympus SZX12 microscope, Olympus U-CMAD3 camera, and PictureFrame™ Application 2.3 software. Subsequent pictures were taken starting at 2 hours after DiI labeling and then every 4 hours until the embryo died or 58 hours post injection was

obtained. Both a bright field picture and a dark field picture showing the fluorescence of the DiI were taken at each time point. Overlays of the dark field and bright field images were made using Adobe Photoshop CS4. As controls, DiI was injected at the distal end of the limb bud of chicks at 110 hours (equivalent to the incubation time of the AER removal eggs when DiI injection was done) and pictures were taken every four hours and overlays made as above.

Results

Clonal Analysis after Sonoporation

My hypothesis is that Ror2 acts as the receptor (or a co-receptor) for Wnt5a in a non-canonical signaling pathway in the limb and that this signaling provides positional information which directs the outward growth and migration of limb mesenchyme. If this is correct I expect that control limb mesenchyme cells will respond to the Wnt5a gradient and divide/migrate in a linear pattern along the proximodistal axis while limb mesenchyme cells where Ror2ΔC is acting in a dominant negative manner will not be able to respond to the Wnt5a gradient. Without this polarizing cue, I predict that these limb mesenchyme cells will lose their sense of directionality and grow in random directions (as evidenced by a radial or circular clone rather than a linear clone of labeled cells). These results will suggest that Ror2 is required as a receptor (or co-receptor) in the pathway which directs changes in cell polarity in response to the Wnt5a gradient.

As indicated in **Figure 19**, cells which were transfected with the 3:1 ratio of Ror2ΔC consistently showed radial growth (**Figure 20**) while those transfected with control pCIG exhibited linear growth (**Figure 21**). Radial growth of the Ror2ΔC transfected cells was seen with all ratios of plasmid (**Figure 22**). If the injection of the plasmid/microbubble solution was too far proximal either radial growth or linear growth in which the cells stayed in the proximal limb bud resulted. This indicates that Wnt5a must be at a high enough concentration for the

polarizing signal to have its effect. The direction of growth of the transfected cells was determined by individuals without a knowledge of which plasmids were co-sonoporated in each instance. The controls included in both of the graphs are those which were done during the same time period as the Ror2ΔC transfected embryos. The embryos represented in **Figure 19** were all done during a month long period while those represented in **Figure 22** were done during a five month period (which includes the month during which the **Figure 19** embryos were done). Since during this five month period I was still experimenting with different injection angles and directions in order to determine the best way to get a distal injection, some of these injections

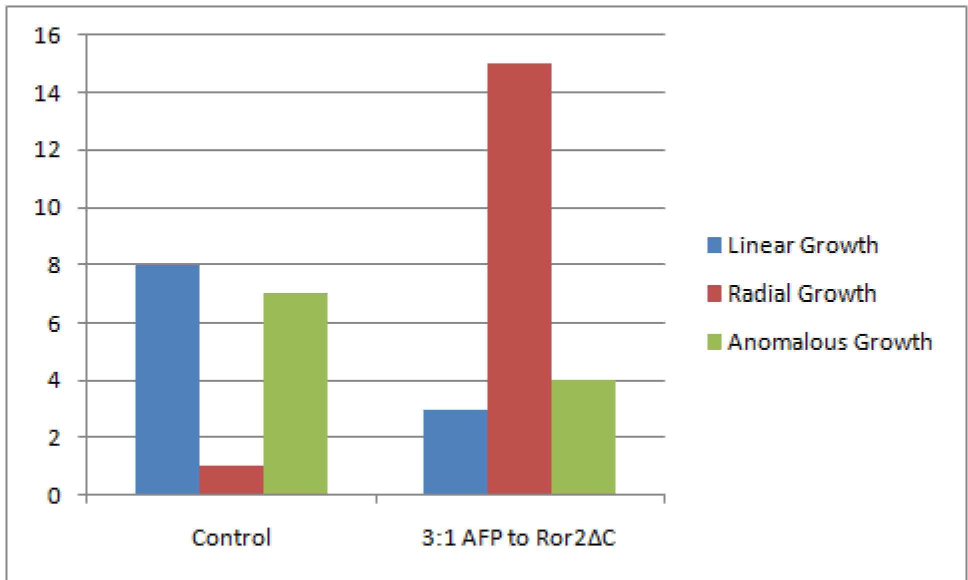

Figure 19: Outgrowth patterns of cells co-transfected with either pCAGGS-AFP and pCIG (control) or with a 3:1 ratio of pCAGGS-AFP and pCIG containing Ror2ΔC within the multiple cloning site. Controls were done during the same time period as the Ror2ΔC experiments. Direction of growth was deter-mined by individuals without a knowledge of which plasmids were transfected.

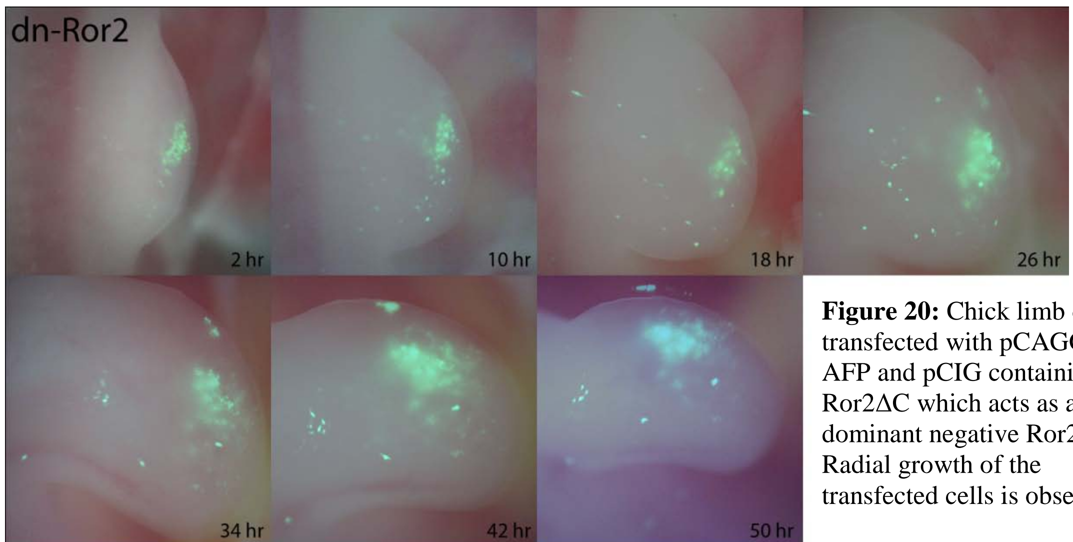

Figure 20: Chick limb co-transfected with pCAGGS-AFP and pCIG containing Ror2ΔC which acts as a dominant negative Ror2. Radial growth of the transfected cells is observed.

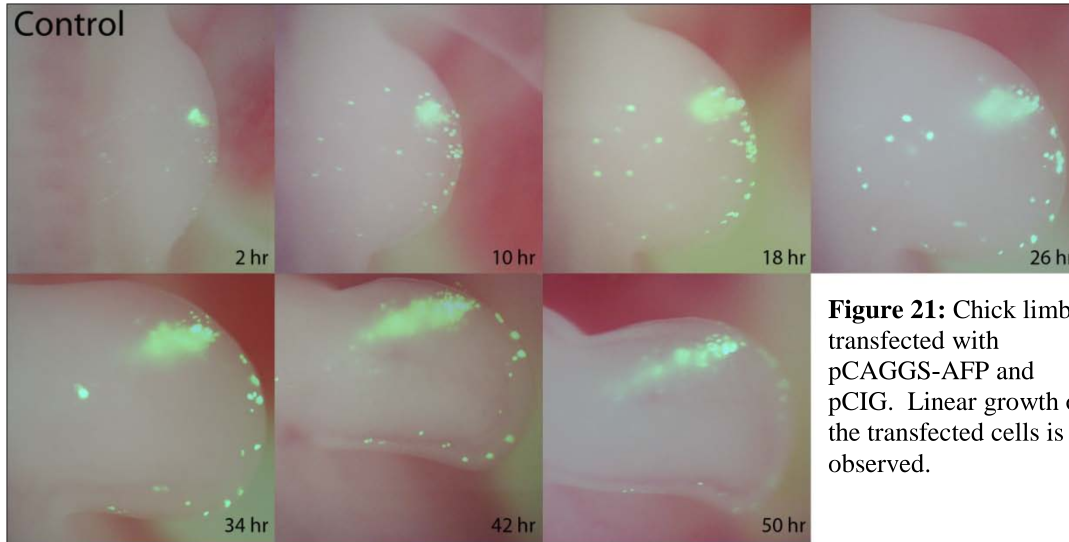

Figure 21: Chick limb transfected with pCAGGS-AFP and pCIG. Linear growth of the transfected cells is observed.

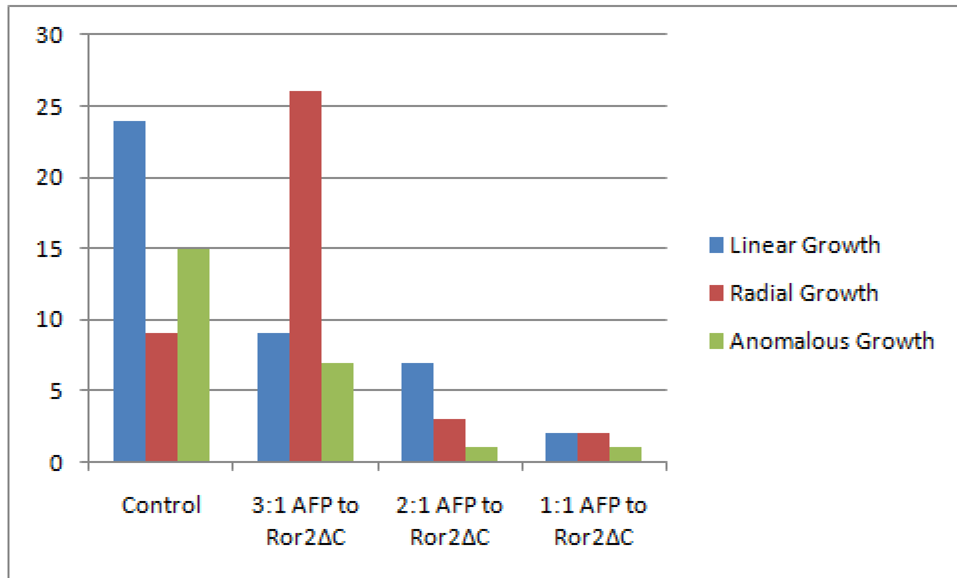

Figure 22: Outgrowth patterns of cells co-transfected with pCAGGS-AFP and pCIG (control) or a 3:1, 2:1, or 1:1 ratio of pCAGGS-AFP and pCIG containing Ror2ΔC within the multiple cloning site. The controls here were done during the same time period as the Ror2ΔC experiments.

were not distal enough to be in the area of the Wnt5a gradient. This likely accounts for the higher frequency of controls in which radial growth was seen (in these controls the transfected cells were too proximal to receive the polarizing signal and grew radially) (**Figure 23**). Additionally, during this time period some of the injections were done along the proximodistal axis. This type of injection resulted in a line of transfected cells along the proximodistal axis starting from the first data imaging time point two hours after sonoporation. Since these cells started out in a linear pattern along the proximodistal axis there are likely cases in which the

growth appeared to be linear but this directionality was actually just an artifact of the initial injection. In other cases there was a control or a dominant-negative Ror2 transfection in which linear outgrowth was seen even when the injection was proximal and outside the area of Wnt5a signaling (**Figure 24**). This can be explained by the proximal cells having a memory of which direction they are supposed to be growing in. It also shows that limb outgrowth continues in the proximal cells which are outside of the polarizing influence of the Wnt5a gradient and that the ability to respond to Wnt5a is no longer needed for this outgrowth. A possible explanation for the frequency of linear growth seen in the 1:1 and 2:1 ratio experiments comes from initial experiments where there appeared to be an excess of cell death of the transfected cells in experiments done with the 1:1 and 2:1 ratios. Since co-transfection of two plasmids (one serving as a lineage tracer and one with the dn-Ror2) was used it is possible for some cells to have taken up more of one of the plasmids than the other. If cells which took up a large amount of the Ror2ΔC in pCIG plasmid died off, cells which took up little or no Ror2ΔC may remain. Cells which have only taken up the pCAGGS-AFP plasmid exhibit linear outgrowth.

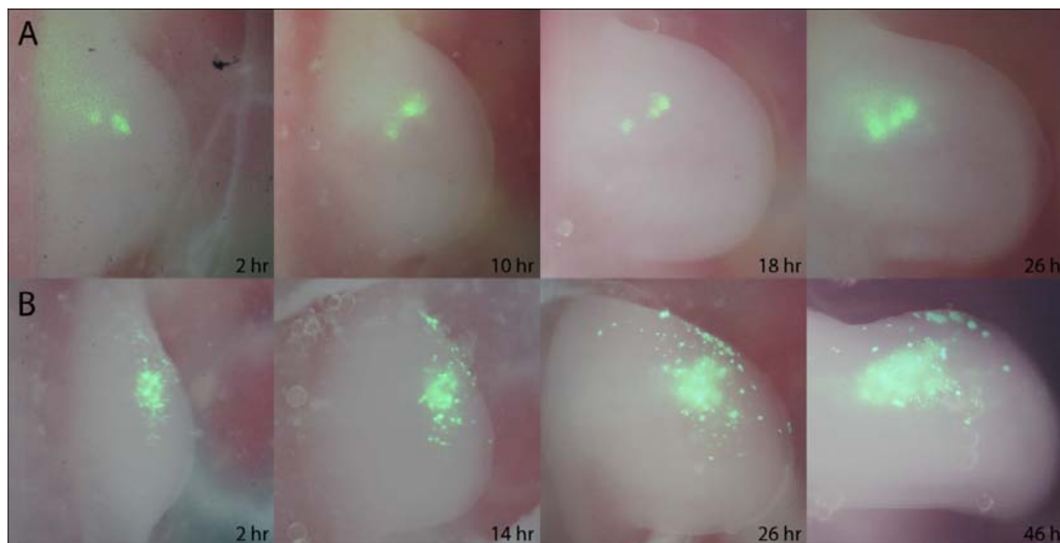

Figure 23: Sonoporation of control plasmids (pCAGGS-AFP and pCIG) where the transfected cells were too proximal to receive and respond to the polarizing signal. Radial growth of the transfected cells is observed.

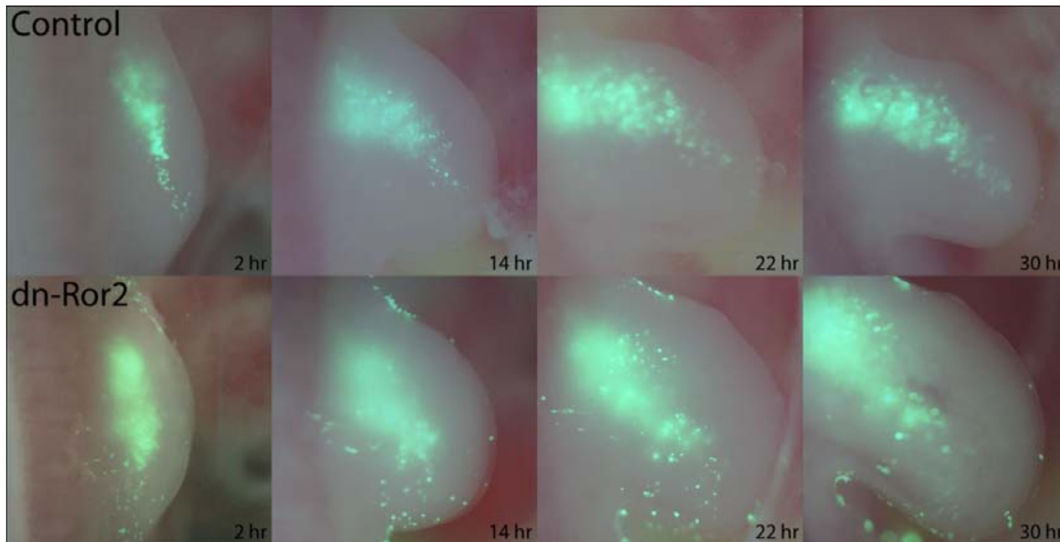

Figure 24: Proximal transfections in which cell memory of the direction of growth resulted in the cells continuing to grow in an outward and linear direction. This behavior was observed in both controls and dn-Ror2 indicating that the ability to respond to Wnt5a was no longer necessary for outgrowth of these cells. The dn-Ror2 pictured here had a 2:1 ratio of pCAGGS-AFP and pCIG containing Ror2ΔC.

Skeletal Preparations after Sonoporation

I wanted to see if sonoporation of Ror2ΔC could result in changes to the overall morphology of the skeleton due to cells not being recruited distally. In order to do this embryos were sonoporated as in the clonal analysis experiments; rather than taking pictures every four hours however embryos were instead photographed four hours after sonoporation and then re-taped and placed back in the incubator with the goal of keeping the embryo alive as long as possible. Once a day I opened the embryos up and added 1% PSG in PBS to keep the embryo hydrated and prevent bacterial infection in the egg. After 6 days of incubation was reached I checked the embryos twice a day and removed any embryos that had died in the last 12 hours for staining of the cartilage with Alcian blue. The skeletons of 6 and 6.5 day embryos were still too underdeveloped. By 7 or 7.5 days elements of the limb skeleton could be observed (I was not able to keep any embryos alive past 7.5 days of incubation).

Due to the transient nature of sonoporation (very few of the cells within the limb bud are transfected and the plasmids do not incorporate themselves into the genome) I was not able to

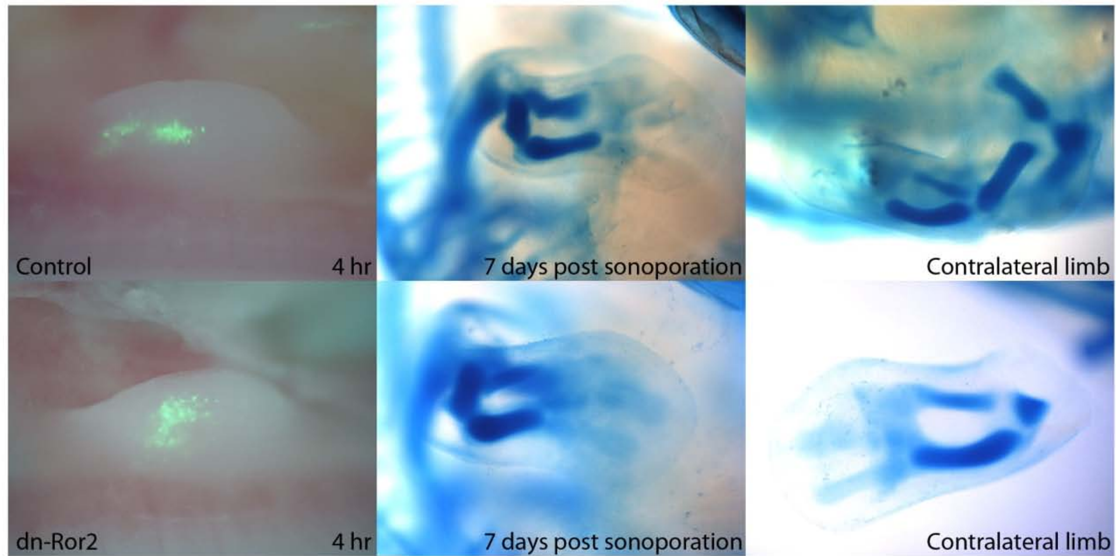

Figure 25: Limb buds were photographed 4 hours after sonoporation at 60X magnification. After 7 days of total incubation a skeletal preparation was done. The limb skeletons were then photographed. The control limb skeletons were photographed at 30X magnification while the Ror2ΔC (dn-Ror2) were photographed at 38X magnification.

conclude if sonoporation of the Ror2ΔC construct had an effect on the morphology of the limb. I did not observe any significant differences in size or shape of any of the skeletal elements of the limbs which were sonoporated versus those of the contralateral limb (**Figure 25**). Future work in the lab is planned to overexpress our Ror2ΔC using a stable transposon mediated transfection of the Ror2ΔC construct into the limb mesenchyme of intact limbs. When this is done I expect that the limbs in which Ror2ΔC is overexpressed will exhibit shortening of the skeletal elements. This expectation is supported by the finding that chicks in which replication-competent retroviral vector-mediated overexpression of C-terminally truncated forms of Ror2 has been performed exhibit shortening of the elements of the limb (Stricker et al., 2006).

AER Removal

My AER removal experiments confirmed the results of the AER removal experiments previously done in the lab. Labeled cells proximal to the area where the AER had been removed show one of three different growth patterns depending on the distance to the intact AER. If the intact AER is too far away, the cells grow radially (**Figure 26** and **Figure 27**). If the intact AER

is very close however, the cells grow toward the residual AER (**Figures 28** and **Figure 29**). In other experiments where the intact AER is at more of an intermediate distance from the labeled cells, some cells will grow toward the AER (presumably the cells at the top of the injected area) and some cells grow in a radial clone (presumably those at the bottom of the injected area) (**Figure 29**). Controls in which DiI was injected at the distal end of the limb bud of embryos in which the AER was left intact exhibited linear growth of the cells (**Figure 30**).

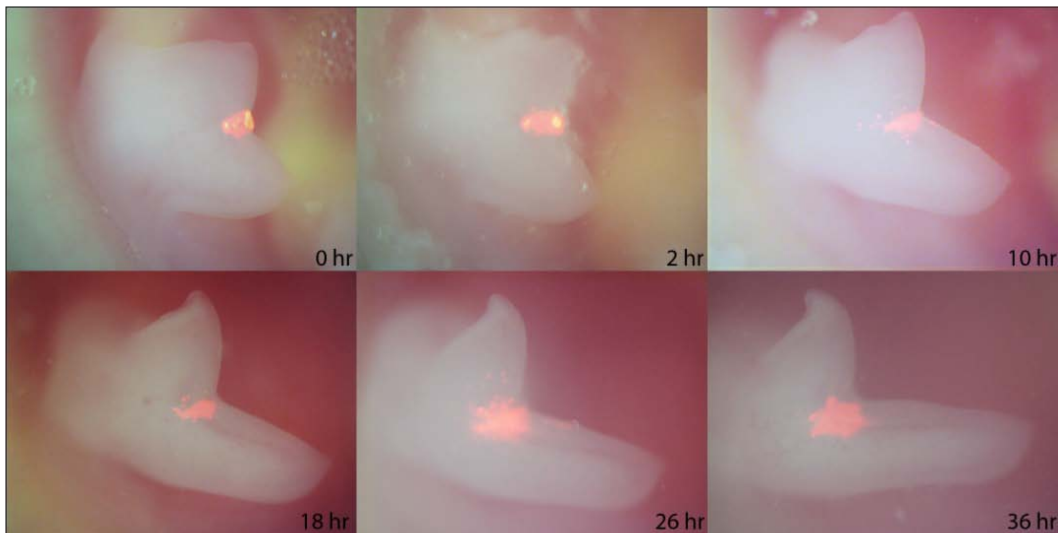

Figure 26: Cells were labeled with DiI 24 hours after a portion of the AER was removed. Labeled cells were too far away from the intact AER and exhibited radial growth.

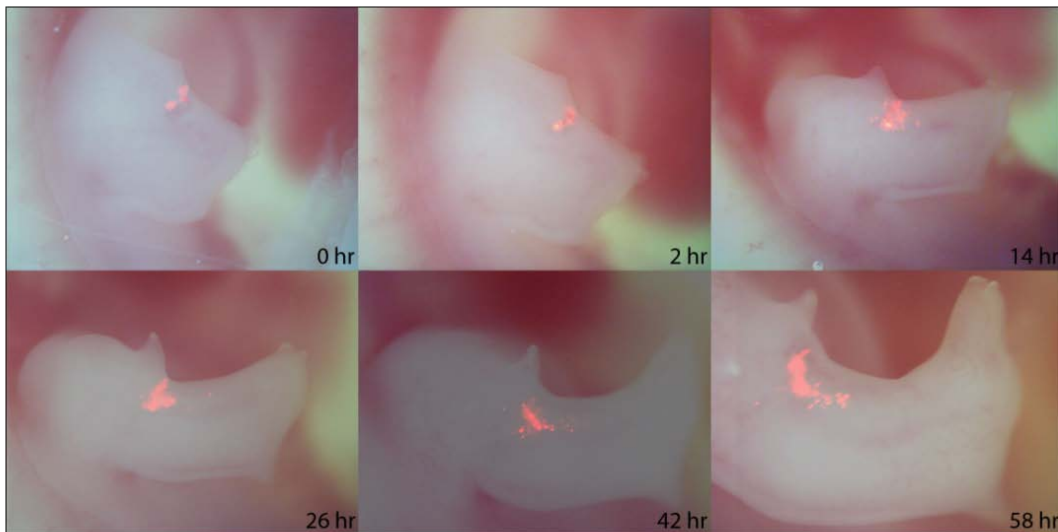

Figure 27: Cells were labeled with DiI 24 hours after a portion of the AER was removed. Labeled cells were too far away from the intact AER and exhibited radial growth.

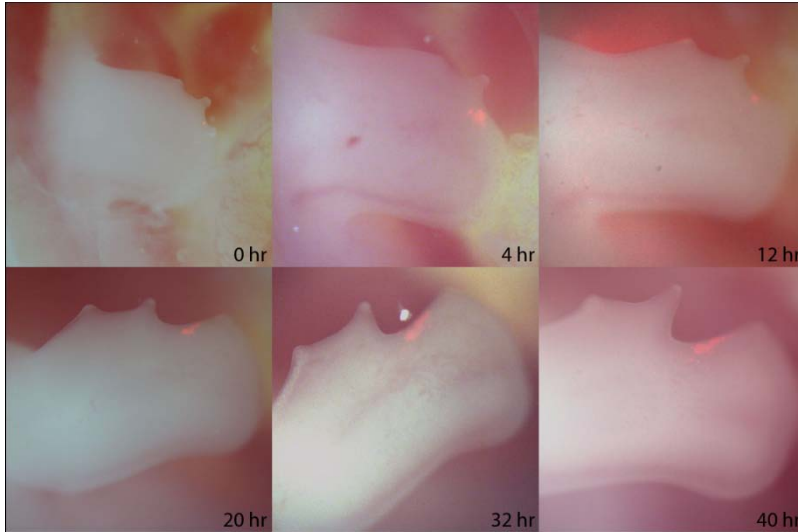

Figure 28: Cells were labeled with DiI 24 hours after a portion of the AER was removed. Residual AER was close enough to the labeled cells that the cells were able to exhibit directional growth toward the intact AER.

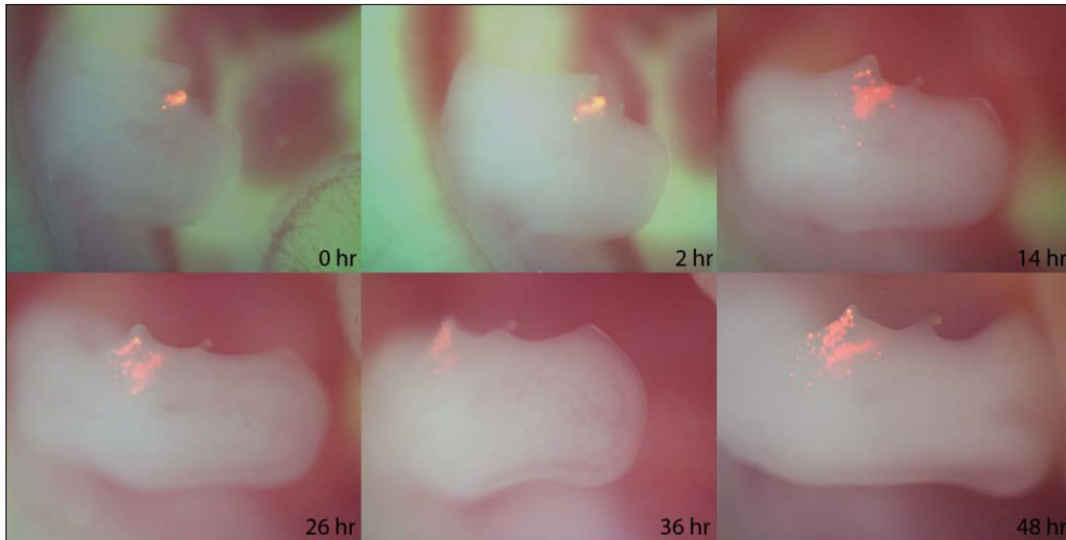

Figure 29: Cells were labeled with DiI 24 hours after a portion of the AER was removed. The DiI injection showed growth toward the intact AER of the distal-most labeled cells and radial growth of the proximal-most labeled cells.

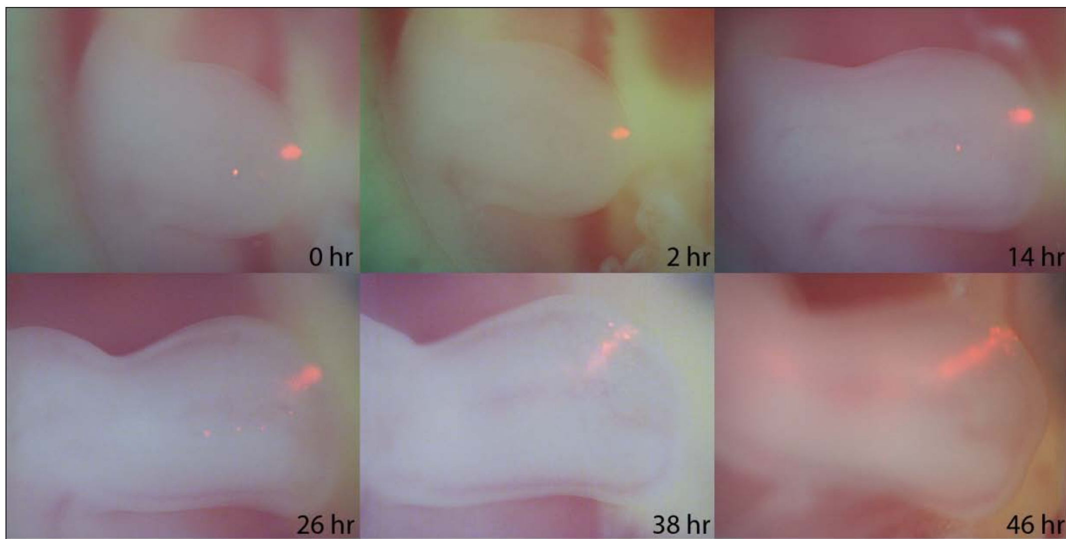

Figure 30: The AER was left intact and cells at the distal tip of the limb bud were labeled with DiI at 110 hours of incubation. Labeled cells exhibited linear growth toward the AER.

DISCUSSION

Together with previous work from the Barrow lab my results support the conclusions of the mesenchyme recruitment model. As predicted by the model, cells proximal to where the AER has been removed are able to be recruited toward intact AER if they are within a close enough distance to the AER. If the cells are too far away from the AER (and the source of the polarizing signal) the cells grow in a radial pattern. Previous outgrowth models would have predicted that these cells would have undergone apoptosis. The mesenchyme recruitment model proposes that the Wnt5a gradient, set up along the distal-to-proximal axis due to the action of Fgfs from the AER, provides the polarizing cue to the mesenchyme cells which directs this outgrowth toward the intact AER. This proposed role of the Wnt5a gradient in directing outgrowth of the cells is supported by the Wnt5a secreting L-cell implant experiments. My data supports the role of Ror2 as the receptor (or a co-receptor) for Wnt5a in directing this polarized outgrowth. Mesenchyme cells transfected with a dominant-negative acting Ror2 exhibited the same outgrowth behavior as labeled cells which were too far away from the intact AER in the AER removal experiments. In both of these cases cells grew in a radial pattern rather than with linear outgrowth toward the AER. Additionally, radial growth was also seen in some control sonoporation experiments in which the transfected cells were too proximal (and thus too far away from the Wnt5a polarizing signal) (see **Figure 23**).

The limb mesenchyme recruitment model prescribes an even more active role in limb patterning for the AER than was previously thought. According to this model the size and shape of the AER itself will determine the shape of the field of mesenchyme cells which are recruited distally. This role of the AER is carried out in the following manner: the shape of the AER determines the shape of the Fgf signal, the shape of the Fgf signal determines the shape of the

Wnt5a gradient, and the shape of the Wnt5a gradient determines the shape of the field of mesenchyme cells which receive the polarizing signal to grow distally toward the AER. This proposed effect of the size and shape of the AER on limb patterning is supported by observations about limb patterning from both normal and mutant embryos. During normal limb patterning the size and shape of the AER is seen to change with time: circular at earlier stages when the proximal elements are being patterned and long and thin at later stages when the distal elements are being patterned (see **Figure 17**). Additionally, mutants which exhibit an AER which is longer along the anterioposterior axis than normal have extra digits while those with a shortened AER along the same axis lack digits (**Figure 31**). The mesenchyme recruitment model would propose that the extra digits in panel **31-B** are due to a longer field of cells being recruited distally and the missing digits in panel **31-D** are due to only a small field of cells being recruited. Further evidence for the mesenchyme recruitment model comes from the AER removal experiments done by myself and previous members of the Barrow lab (see **Figures 14**, **26**, **27**, **28**, **29**, and **30**). Here growth of mesenchyme cells toward the residual AER tissue has a dramatic effect on the shape of the limb mesenchyme.

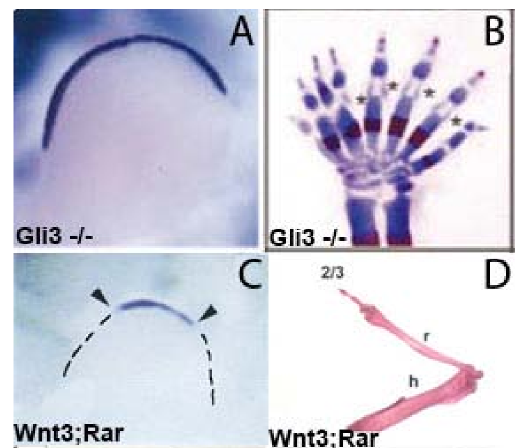

Figure 31: Mutants with defects in the size/shape of the AER and the resulting skeletal defects in the autopod (Barrow, Unpublished; Barrow et al., 2003; Ying et al., 2002).

In additon to providing insights into how Fgfs from the AER regulate outgrowth of limb mesenchyme, results of this and previous studies also provide insight into how any structure can develop in three dimensions. The same signaling pathways are used throughout the body to accomplish similar patterning and growth tasks. For example, it is likely that the same or a very closely related signaling

pathway is working in the palate and the limb to cause directed outgrowth of the respective cells. It has already been shown by He, et al. that Wnt5a is expressed in a gradient along the anterior-posterior axis of the palate. Additionally, directional migration occurs in the developing palate and this directional migration does not occur in the absence of Wnt5a (He et al., 2008). A Wnt5a gradient also exists along the proximal-distal axis of the developing limb. We have shown that in the limb an ectopic source of Wnt5a results in directional cell migration toward the source of Wnt5a. In the palate Ror2 was demostrated to have a role in this Wnt5a signaling (He et al., 2008). I have demonstrated that Ror2 is also needed for directional migration in response to the Wnt5a gradient in the limb. Wnt5a is expressed in a gradient in other tissues undergoing some type of outgrowth as well (including the caudal end of the embyo itself). A Wnt5a-Ror2 pathway of outgrowth may thus operate in other parts of the developing embryo as well. The information provided by this research may therefore be of strong interest to those studying other aspects of developmental biology as well as those in the medical community.

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B.S. in Physiology and Developmental Biology — GPA: 3.94
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Honors and Awards

Graduate Student Research Assistantship (2009 – 2011)
PDBio Graduate Student Funding (2010)
Mentoring Grant (2009-2010)
Graduate Student Teaching Assistantship (2008 – 2010)
Brigham Young University Scholarship (2004 – 2008)
College Dean's List (2004, 2007-2008)
The National Dean's List (2004 – 2007)
Washington State Honors Award - Top 10 percent of the Washington State High School Graduating Class of 2004
President's Education Award for Educational Excellence 2004

Professional Societies

Phi Kappa Phi Honor Society
Golden Key International Honor Society

Work Experience

Graduate Research Assistant – BYU, Provo, UT
September 2008 – Present

- Trained and supervised undergraduates working with me on my thesis project
- Coordinated lab meeting times and data imaging times
- Cloning techniques: restriction enzyme digests and ligations, gel purification, agarose gel electrophoresis, chemical and electrical bacterial transformation followed by mini, midi, or maxi preps
- Chick egg windowing
- Chick embryo extraction and dissection
- Tissue sectioning using cryomicrotome
- Microinjection using both a picospritzer and a syringe attached to a mouth pipette

- *In ovo* sonoporation
- *In ovo* electroporation
- Cell bolus implants in chick limb
- Cartilage staining with Alcian blue
- Tracking of cells labeled with fluorescent dye or protein using a light microscope and PictureFrame software
- Creation of overlays of data images in Adobe Photoshop

Teaching Assistant for Human Embryology (PDBio 484) – BYU, Provo, UT
August 2009 – December 2009 & August 2010 – December 2010

- Created Power Point slides to be used in teaching class lectures after adopting a new textbook
- Responsible for creating Blackboard quizzes over each chapter
- Responsible for inputting student grades and helped determine final grades
- Graded student presentations
- Helped in creating tests and made sure the test covered what had been taught
- Responsible for conducting a test review before the three midterms and final
- Answered student questions
- Taught class several times during each semester

Physiology Lab Instructor (PDBio 305)
January 2009 – April 2009

- Taught 20 minute lesson about the concepts covered at the beginning of each weekly two hour lab
- Answered student questions during class
- Administered student access to quizzes and grades in Blackboard
- Responsible for maintaining lab equipment and supplies
- Supervised undergraduate lab assistants
- Calculated final grades

Anatomy Lab Instructor (PDBio 220) – BYU, Provo, UT
January 2007 – December 2009

- Responsible for teaching the laboratory portion of the class: this included deciding how to present the material in ways the students would be able to more effectively remember
- Responsible for making half of the quiz each week covering the previous week's material
- Graded student quizzes every other week and input grades in Blackboard
- Worked in the open lab where students could come in to study and ask questions
- Set an example in proper cadaver care

Anatomy Lab Group Leader – BYU, Provo, UT
September 2008 – December 2008 & June 2009 – August 2009

- Helped run weekly lab meetings
- Instructed other TAs in how to effectively teach anatomy
- Evaluated TAs on how they were teaching
- Responsible for attendance of all TAs to lab meeting, sections, and open lab
- Helped pin questions on cadavers and bones for student midterm and final

Editing Educational Software – BYU, Provo, UT
March 2008 – April 2008

- Edited a CD on the Embryological Development of the Heart in preparation for an updated version to be released in the fall for use in PDBio 484

Chemistry Recitation Leader (Chem 105) – BYU, Provo, UT

August 2006 – December 2006

- Attended class lectures three times a week
- Responsible for running recitation class periods twice a week for two sections of the course with 25 students in each section
- Graded assignments for my sections twice a week and input grades
- Worked in the Chem. 105/106 tutorial lab six hours a week
- Conducted test reviews before each of the tests and the final

Leadership Experience

Research Project Leader 2008 – 2011
Anatomy Lab Group Leader 2008 – 2009
4-H – President, Vice-President, Junior Leader 1993 – 2005
Church Youth Group – President, First Counselor, Secretary 1998 – 2004

Research Labs Worked In

Jeffery Barrow, BYU – Limb Development Fall 2008 – Winter 2011
Robert Seegmiller, BYU – Fetal Growth Cartilage Defects Fall 2008
Michael Stark, BYU – Development of the Trigeminal Placode Winter 2008

Advanced Training Sessions Attended

Society for Developmental Biology 69th Annual Meeting
Training Meetings for Physiology Lab
Lab Meetings for the Basic Human Anatomy Lab
College of Physical and Mathematical Sciences TA Training Conference
Ignite Leadership Academy

Publications/Poster Presentation

Paper encompassing Mesenchyme Recruitment Model soon to be submitted.

Poster Presentation at Society for Developmental Biology 69th Annual Meeting
Program/Abstract # 94
The Limb Mesenchyme Recruitment Model for Patterning the Vertebrate Limb
Jeffery R. Barrow, Tiffany M. Dahl, Aaron P. Smith, Kate E. Kmetzsch, Jared J. Barrott, Jed J. Kendall and Keri L. Low
Dept. of Phys. and Developmental Biology, Brigham Young University, Provo, UT, 84602
Developmental Biology Volume 344, Issue 1, 1 August 2010, Pages 444-445.
Available online 21 July 2010.
*My name was spelled wrong in the publication.

PDBio Graduate Student Presentation: Role of Wnt5a and Possible Pathway of Action through Ror2 in Proximodistal Outgrowth of the Limb – BYU, Provo, UT, 2010

PDBio Graduate Student Presentation: Mesenchyme Recruitment Model for Proximal-Distal Limb Outgrowth – BYU, Provo, UT, 2009

Development of the Human Embryonic Heart, CD-ROM. BYU, Provo, UT, 2008