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Molecular and Cellular Mechanisms Whereby the Apical Ectodermal Ridge (AER), Via Wnt5a, Mediates Directional Migration of the Adjacent Mesenchyme During Vertebrate Limb Development

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MOLECULAR AND CELLULAR MECHANISMS WHEREBY THE APICAL
ECTODERMAL RIDGE (AER), VIA WNT5A, MEDIATES DIRECTIONAL
MIGRATION OF THE ADJACENT MESENCHYME DURING
VERTEBRATE LIMB DEVELOPMENT

by

Kate E Kmetzsch

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

Master of Science

Department of Physiology and Developmental Biology

Brigham Young University

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ABSTRACT

MOLECULAR AND CELLULAR MECHANISMS WHEREBY THE APICAL ECTODERMAL RIDGE (AER), VIA WNT5A, MEDIATES DIRECTIONAL MIGRATION OF THE ADJACENT MESENCHYME DURING VERTEBRATE LIMB DEVELOPMENT

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Department of Physiology and Developmental Biology

Master of Science

The vertebrate embryonic limb is a key model in elucidating the genetic basis underlying the three dimensional morphogenesis of structures. Despite the wealth of insights that have been generated from this model, many long-standing questions remain. For example, it has been known for over 70 years that the apical ectodermal ridge (AER) of the embryonic limb is essential for distal outgrowth and patterning of the adjacent limb mesenchyme. The mechanisms whereby the AER does accomplish outgrowth and patterning are still poorly understood. We propose that secreted FGFs from the AER activate *Wnt5a* expression in gradient fashion, which in turn provides an instructional cue to direct outgrowth in the direction of increasing *Wnt5a* expression (i.e. toward the distal tip of the limb). *In vivo* and *in vitro* models were used to test this hypothesis. We placed

Wnt5a expressing L-cell implants into stage 23 chick limb buds and demonstrate that labeled mesenchyme cells grow toward the source of Wnt5a. Purified Wnt5a soaked heparin bead implants have only a marginal effect on directed growth of the adjacent mesenchyme, whereas a greater effect was seen with beads soaked in Wnt5a conditioned media. Using an in vitro model where cultured limb mesenchyme cells were subjected to a gradient of conditioned Wnt5a media or purified Wnt5a, we show no specific migratory direction. However, clusters of cells tended to move toward the source of Wnt5a indicating that it might be necessary for the cells to be in complete contact to respond to the Wnt5a signal. Taken together, our results suggest that Wnt5a is sufficient to direct limb mesenchyme. This finding has given support to a new model of limb development proposed by our lab and referred to as the Mesenchyme Recruitment Model.

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“Our bodies are sacred. They were created in the image of God. They are marvelous, the crowning creation of Deity. No camera has ever matched the wonder of the human eye. No pump was ever built that could run so long and carry such heavy duty as the human heart. The ear and the brain constitute a miracle. . . . These, with others of our parts and organs, represent the divine, omnipotent genius of God.”

President Gordon B. Hinckley

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INTRODUCTION

Developmental biology is becoming an increasingly important area of study, with research only scratching the surface of the many complex processes involved in the process of making an ordered form. There are many facets of developmental biology; some focusing on cellular mechanisms and behavior with others focusing on organogenesis and the development of entire systems. The ultimate question underlying the study of development is how are structures of various shapes and sizes formed? Vertebrate limb development is a long standing model to understand how body parts of specific sizes and shapes are assembled, yet mechanisms that govern outgrowth of the limb are poorly understood. Limb abnormalities occur in approximately 0.7 to 1 per 1000 human births (McGuirk et al., 2001), therefore, knowledge of the developmental processes involved in patterning and outgrowth of the limb are of great interest to the medical community. Previously accepted models on vertebrate limb development have currently been put into question and new hypotheses must be proposed to explain this complex process. My research, using a chick model, focused on the molecular and cellular mechanisms regulating directional migration of limb mesenchyme cells.

BACKGROUND

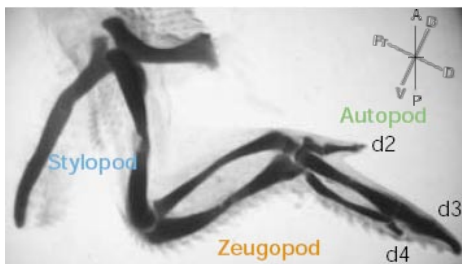


Figure 1: Sections and axes of the vertebrate limb. Pr=proximal, D=distal; D=dorsal, V=ventral; A=anterior, P=posterior (Image modified from Niswander et al., 2003).

The vertebrate limb is classified into three separate sections: the stylopod (humerus), the zeugopod (radius and ulna), and the autopod (digits) (**Figure 1**). The limb can also be classified along three different axes, proximal to distal (proximodistal),

dorsal/ventral (dorsoventral), and anterior to posterior (anteroposterior) (**Figure 1**). The humerus forms the most proximal element followed by the radius and ulna, with the digits comprising the most distal elements. Dorsally, there is the back of the hand with the palm defined as ventral. Digit 1 of the hand (the thumb) is designated as anterior and digit 5 of the hand (the pinky) is posterior.

In the chick, limb bud induction occurs progressively between stages 13 and 15 (roughly 48-55 hours of incubation) (Hamburger and

Hamilton, 1951). The limb develops from a small group of lateral plate mesoderm cells and overlying surface ectoderm (**Figure 2**). The mesoderm consists of loosely packed, unconnected cells termed

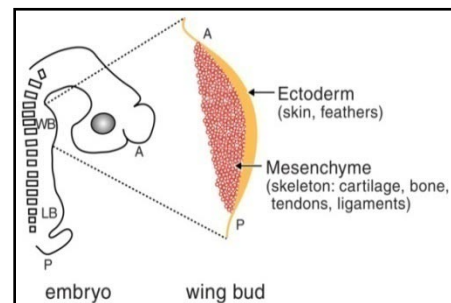


Figure 2: Components of a chick limb bud

mesenchyme, which eventually give rise to the skeleton: cartilage, bone, tendons, and ligaments. Ectoderm cells give rise to the skin and feathers. It has been shown that the limb develops in a proximal to distal fashion, beginning with the formation of the stylopod and ending with formation of the autopod (Saunders, 1948). As the limb grows outward from the body wall, differences along each axis can be observed which allow for normal function of the vertebrate limb: the proximal bone structure is much different than the distal bone structure, the formation of tendons on the dorsal side of the autopod compared to the ventral side allow for autopod movement, etc. These differences that are created along the different axes of the limb during development are termed ‘patterning.’ Many signaling processes are involved in generating molecular differences across axes, and these molecular patterns presage morphological pattern. However, only a very small number of these processes are fully understood. Currently, the mechanisms whereby the

limb becomes patterned along the proximodistal (shoulder to fingers) axis are being debated.

PREVIOUS RESEARCH

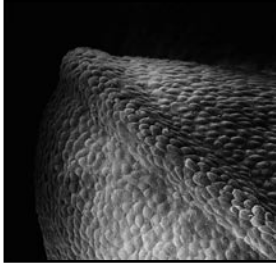


Figure 3: Scanning electron micrograph of the AER.

Along the dorsoventral boundary, running anterior to posterior on the vertebrate limb bud, lies a thickened piece of epithelium called the apical ectodermal ridge (AER) (**Figure 3**). The presence of the AER had been observed for some time and the

question arose as to what role the AER plays in the development of the limb. In 1948, John Saunders Jr. performed AER removal studies to elucidate the AER's role. He removed the AER from limb buds of chick embryos at different developmental stages. He observed that when the AER is removed at early stages of development only the most proximal

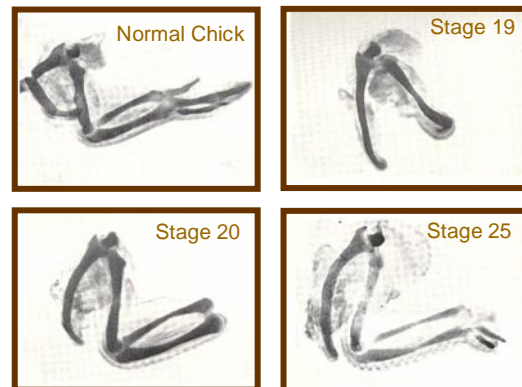


Figure 4: AER removal experiments (Saunders, 1948).

structures form, resulting in truncation of the limb, whereas when the AER is removed at later stages more distal elements were seen (**Figure 4**). These experiments show that the AER is required for normal outgrowth of the limb and also that the limb develops in a proximal to distal fashion. The mechanism whereby the AER regulated distal outgrowth was then investigated.

The first hypothesis tested was that the AER plays an instructive role during limb development, sending out different signals over time to instruct the cells to become

proximal or distal structures (i.e., different signals are required to make the different elements of the limb along the proximodistal axis). To test this hypothesis ectoderm recombination experiments were performed (Ruben and Saunders, 1972). In these experiments AERs from young limb buds were removed and grafted onto older limb mesenchyme (**Figure 5a**). The reverse experiment was also performed (**Figure 5b**). When a young AER was placed on older limb mesenchyme distal elements formed. While older ectoderm placed on younger limb mesenchyme resulted in normal formation of skeletal structures, which were present in their proper sequence. These results lead to the conclusion that the signals being secreted from the AER, were in fact, not changing over time. Ruben and Saunders (1972) state: “There issues from the AER not a level-specific sequence of different inductive signals but rather a constant signal that does not change qualitatively from level to level.” Thus, the AER might not be playing an instructive role, but a permissive role in limb development. This lead to experiments aimed at elucidating the factor/s responsible for directing limb patterning.

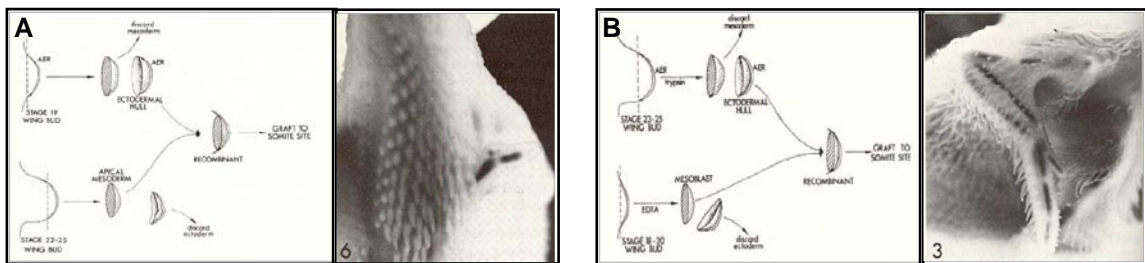


Figure 5: Ectoderm recombination experiments. (a) Young AER grafted onto old mesenchyme (b) Old AER grafted onto young mesenchyme (Ruben and Saunders, 1972).

One hypothesis proposed was that the information for patterning the limb might lie in the limb mesenchyme and this was tested by performing mesenchyme recombination experiments. In 1975, Summerbell and Lewis reported an experiment where they took a young chick AER and distal mesenchyme and grafted it onto an older chick limb bud.

The results of this experiment revealed that proximal structures were recapitulated in the grafted limb buds (**Figure 6a**). When they exchanged an older limb bud tip (containing the AER and distal mesenchyme) with a young limb bud they saw that distal structures formed at the expense of proximal structures (**Figure 6b**). The conclusion from this work is that the information for patterning the proximal-distal axis in the chick limb bud is determined by the mesenchyme. These observations lead to the development of the “Progress-Zone Model.”

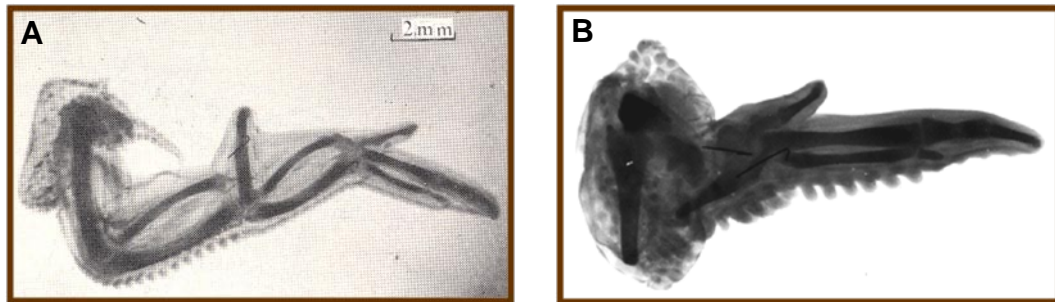


Figure 6: Mesenchyme recombination experiments. (a) Results of a young AER and mesenchyme grafted onto an older limb bud (b) Results of an old AER and mesenchyme grafted onto a young limb bud (Summerbell and Lewis, 1975).

The “Progress-Zone Model” was proposed by Summerbell, Lewis and Wolpert in 1973 to explain proximodistal patterning in the vertebrate limb. The idea is that the phenotype of each cell is determined by the position of the cell in relation to the AER. Cells receive information regarding their position in the limb and subsequently interpret those signals to differentiate into the appropriate structure (Summerbell and Lewis, 1975; Summerbell, Lewis and Wolpert, 1973). Stated in a different way, the cells of the limb bud establish their positional value by reference to an internal clock. The internal clock works as such, there is a signal secreted by the AER which establishes a ‘progress zone’, the size of roughly $200\mu\text{m}$, at the end of the limb bud. This signal, continually secreted by the AER, keeps the cells in the progress zone undifferentiated and proliferating. As cells fall

out of the progress zone, due to proliferation and overcrowding, they differentiate to a specified state. The amount of time that a cell ‘sees’ the signal emanating from the AER will eventually determine its fate. Thus, cells that fall out of the progress zone early on will form proximal elements, whereas the cells that are able to remain in the progress zone will eventually form distal elements (**Figure 7**).

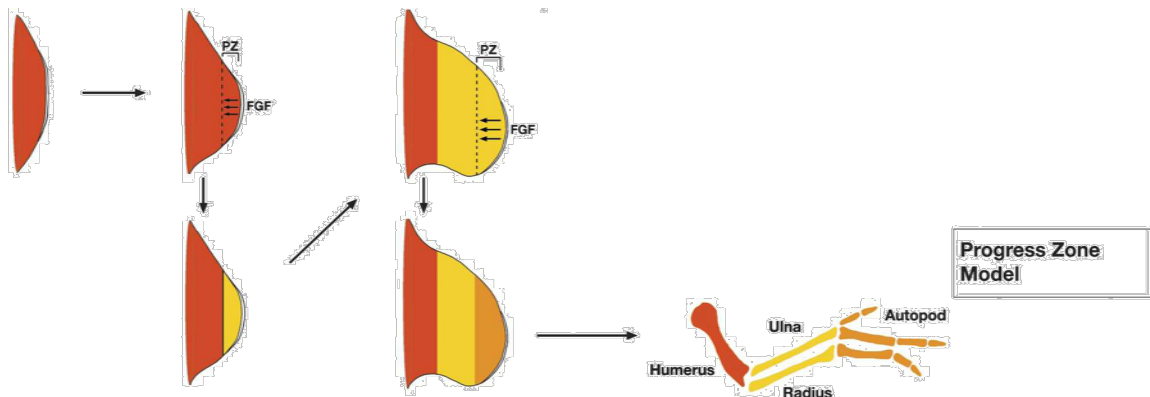


Figure 7: The Progress Zone Model. Initially the entire limb mesenchyme has a proximal identity. Left on its own it would develop into proximal, stylopod structures (red). However, the cells in the progress zone are exposed to the signal from the AER and become respecified at a slightly more distal, zeugopod (yellow) fate. As limb development proceeds, the progress zone cells divide, and as a result of this growth, not all of the cells remain within range of the signal. Those too far from the tip maintain their already specified fate, whereas those still in close proximity to the tip are once again respecified to a still more distal, autopod (orange) fate (Tabin and Wolpert, 2007).

For over thirty years, the Progress Zone Model has been the prevailing model for explaining patterning of the vertebrate limb. Much of limb development research has focused on proving the validity of this model. Although embryonic transplant experiments strongly support the Progress Zone Model, there has never been any molecular evidence to substantiate it. Thus, new models have been and are currently being proposed to explain the molecular mechanisms of embryonic limb development.

Fibroblast Growth Factor

It is established that the AER sends out a permissive signal, to which the limb mesenchyme responds, resulting in limb patterning. But, what signal the AER is producing was not known until the early 1990s. In 1992 it was discovered that Fibroblast Growth Factor (FGF) 4 and FGF2 are expressed in the AER (Niswander and Martin, 1992; Crossley and Martin, 1995). Experiments were then performed to elucidate the role of FGFs in limb outgrowth; AERs were removed from stage 20 limb buds and a bead soaked in endogenous FGF2 or FGF4 was positioned in place of the AER. It was shown that FGF2 and FGF4 can substitute for the AER to maintain limb outgrowth (Niswander et al., 1993; Fallon et al., 1994; Cohn et al., 1995).

Other FGFs, such as FGF8 have been shown to play a role in the initiation of limb bud outgrowth and the establishment of the signaling system that regulates limb development (Crossley and Martin, 1995). Targeted AER FGF gene knockout studies produce mice lacking elements of the limb. However, this phenotype is not a result of reduced mesenchyme cellular proliferation or increased cell death (Saxton et al., 2000; Sun et al., 2002; Boulet et al., 2004). The limb mesenchyme in FGF4 and FGF8 double mutants fails to survive, leading to the elimination of limb buds (Boulet et al., 2004). However, mutants do exhibit normal AER morphogenesis suggesting that the limb mesenchyme has lost the ability to respond to the AER.

FGF4 has been proposed to act as a chemoattractant for mesenchymal cells of the limb bud. Analysis of cell

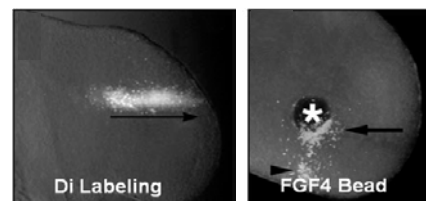


Figure 8: Cell behavior in response to an FGF4 soaked bead (Li and Muneoka, 1999).

behavior in response to a FGF4 soaked bead showed clones of cells dividing and/or migrating toward the source of the FGF4 signal (**Figure 8**) (Li and Muneoka, 1999; Saxton et al., 2000). However, the mechanism whereby the AER recruits the mesenchyme toward it is not currently understood.

Wnt5a

FGFs most certainly play a role in patterning the embryonic limb; however, they are not the only signaling factor involved. The Wnt/planar cell polarity (PCP) signaling pathway has recently been implicated to play an important role in tissue patterning and cell behavior, giving structures shape and function. One process this pathway has been shown influence is that of convergent extension, the process of oriented cell divisions and intercalation of cells in the neural tube resulting in lengthening of the embryo (Copp et al., 2003). The PCP pathway has also been shown to play a role in wing and eye development in *Drosophila* (Klein and Mlodzik, 2005). Zebrafish *Wnt5a/Wnt11* double mutants exhibit the same phenotype as PCP mutant embryos, suggesting that these ligands signal through the PCP pathway (Kilian et al., 2003).

Several reports have demonstrated that *Wnt5a* and its related family member *Wnt11* play

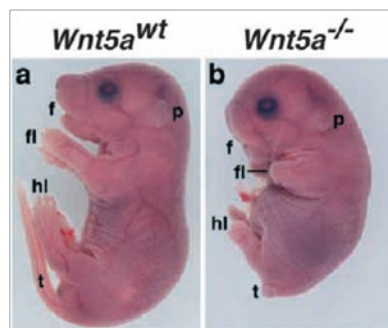


Figure 9: Phenotype of wild type and *Wnt5a* mutants (Yamaguchi et al., 1999).

a role in directed cell movements and oriented cell divisions (Gong et al., 2004; He et al., 2008; Heisenberg et al., 2000; Kilian et al., 2003; Kim et al., 2005; Qian et al., 2007; Rauch et al., 1997; Sakahuchi, et al., 2006;

Westfall et al., 2003). It is therefore possible that Wnt5a mediates the same events during limb development through signaling via the Wnt/PCP pathway (Barrow, 2006). Wnt5a mutant mice exhibit a truncated body axis and an open neural tube, reminiscent of the phenotype of zebrafish embryos lacking components of the PCP pathway (Qian et al., 2007; Yamaguchi et al., 1999). Wnt5a mutant mice also show severe shortening of the face and limbs (**Figure 9**). Despite the shortening of the limb, mutants exhibit normal AER morphogenesis and their limbs exhibit a recognizable proximodistal pattern. The data suggest that limbs fail to lengthen in these mutants due the limb mesenchyme losing the ability to respond to the AER.

Additionally, expression of Wnt5a in the limb depends on the presence of the AER.

Wnt5a is expressed in gradient fashion, with the highest concentration in the apical ectoderm at the distal tip of the limb

(Yamaguchi et al., 1999). In *Wnt3^{n/c}; Msx2Cre* mutant, which exhibit variable loss of AER,

expression of Wnt5a is seen as a function of the amount of AER present (**Figure 10**) (Barrow,

unpublished data). These data show that AER signals are necessary to activate Wnt5a expression in the limb mesenchyme.

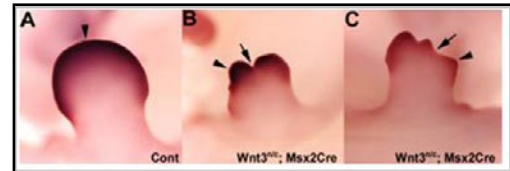


Figure 10: Expression of Wnt5a in response to the presence of the AER. Arrow heads and arrows denote regions where the AER is or is not present (Barrow, unpublished data).

A NEW MODEL

Clonal analysis in mouse embryos chimerized with the tamoxifen inducible reporter cell line YFP3 has revealed that clones in the limb are organized into thin columns along the

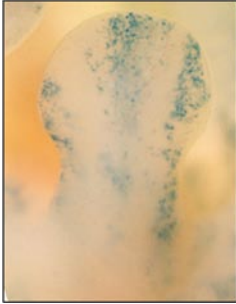


Figure 11: Clonal analysis of labeled mouse limb mesenchyme cells (Mao, et al., 2005).

proximal distal axis (**Figure 11**) (Mao et al., 2005). This finding is consistent with the hypothesis that limb outgrowth is mediated by directed migration and/or cell division of the limb mesenchyme along the proximodistal axis. These results correspond to dye labeling studies in chick limbs where clones are also restricted into thin columns (Dudley, et al., 2002; Li and Muneoka, 1999;

Vargesson et al., 1997). These linear columns of cells correlate with

the presence of the AER. Chick limb buds with small portions of the AER removed continue outgrowth over 24-26 hours with slight indentations in the mesenchyme subadjacent to the surgically removed AER. Over the next 24-48 hours large indentations occur. Previous models for the function of the AER would explain this observation by attributing the indentations to a decreased in cellular proliferation or an increase in apoptosis (Dudley et al., 2002). However, our lab has shown that labeled cells adjacent to the removed AER change their course of direction and are recruited toward sections of the AER (Barrow, unpublished data).

It has been established that emanating from the AER are FGF signals, which recruit mesenchyme cells toward the AER. Additionally, Wnt5a expression is activated in gradient fashion, due to the presence of the AER. Therefore, it is possible that FGFs are inducing expression of Wnt5a in gradient fashion which is in turn recruiting mesenchyme cells toward

the AER. To test this hypothesis, FGF4 soaked beads were implanted into the forelimbs

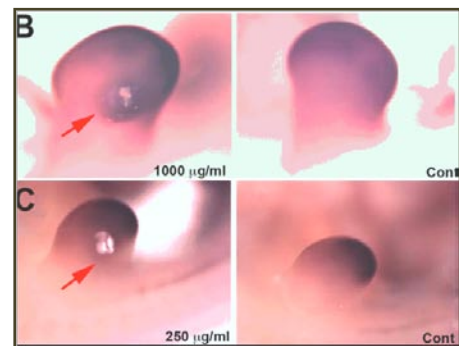


Figure 12: Wnt5a expression in response to an FGF4 soaked bead implanted into a chick limb bud (Low and Barrow, unpublished data).

of stage 20 chick embryos and expression of Wnt5a was examined. It was determined that FGF4 protein is sufficient to induce Wnt5a in a concentration dependent manner (**Figure 12**) (Low and Barrow, unpublished data).

PROPOSAL

HYPOTHESIS

In the developing limb bud FGF signaling from the AER activates Wnt5a in a gradient fashion which polarizes mesenchyme cells to migrate and/or proliferate in a directional fashion, resulting in limb outgrowth.

OBJECTIVES

1. Show that ectopic Wnt5a regulates directional growth of limb mesenchyme *in vivo*.
 - a. Demonstrate that Wnt5a is sufficient to attract limb mesenchyme
2. Perform live cell imaging experiments to show directional migration toward a source of Wnt5a.

MATERIALS AND METHODS

IMPLANTS

Fertilized White Leghorn chicken eggs were incubated at 37°C for 80 hours (Hamburger/Hamilton stage 23). Eggs were then removed from the incubator and sprayed with 70% isopropanol and allowed to air dry. 6-7ml of albumin was removed from the egg via a needle and syringe. The eggs were taped with Scotch® tape to prevent the egg from cracking and a hole was cut in the shell to expose the embryo (termed windowing). 3-4 drops (from a 10mL syringe) of penicillin-streptomycin-glutamine was placed on each embryo to protect it from bacteria. Next, India ink was injected underneath the embryo to allow better visualization of the limb bud while performing implants. After cell boluses or beads were implanted into the limb bud DiI was injected, by use of a picospritzer, roughly 50-100µm above and below the implant to observe the direction of migration and/or division of clones of cells. After implant surgeries were completed, the windowed eggs were taped shut using Scotch® tape and placed back in the incubator.

Bright field and dark field images were taken, at a magnification of 40X, every 4 hours over the course of 30 hours. Images were overlaid using Adobe® Photoshop® CS3 so as to track cell movements relative to the implanted bolus.

Dil

19 μ L 100% Ethanol

1 μ L stock DiI (Invitrogen #C7000)

180 μ L 0.3M Sucrose (EMD #SX1075-1)

2 μ L Vaz green

Cell Boluses

L-cell and Wnt5a expressing L-cell lines (ATCC) were cultured and used to create cell boluses used for implants. Both cell lines were cultured to confluency in LM media.

Cells were then collected (0.5% Trypsin-EDTA, Invitrogen # 25300062), concentrated to 7 million cells/mL, and dispensed in 2, 3 and 4 μ L drops onto lid of a culture dish. The lid was then inverted over a culture dish containing 10mL of Dulbecco's Phosphate-Buffered Saline (Invitrogen #14190-235). Hanging drops were incubated at 37°C + 5% CO₂ for 24 hours.

The following day, 18 eggs were windowed as outlined previously. Distal, horizontal incisions were made using a pulled pipette, in Hamburger/Hamilton stage 23 chick limbs. Cell boluses were removed from the culture dish lid by using a 10 μ L pipette and were then placed on the limb bud. The bolus was pushed into the incision using a blunt pulled pipette. A single cell bolus, control or Wnt5a, was placed in each incision.

LM media

Dulbecco's Modified Eagle Medium (Invitrogen #11965126) supplemented with:

15% Fetal Bovine Serum (Fisher Scientific # SV3001403P)

1% Non-Essential Amino Acids (Invitrogen # 11140-050)

1% Nucleotides

80mg Adenosine (Sigma #A4036-5G, Lot #022K12705)

73mg Guanosine (Sigma #G6264-5G, Lot #053K2507)

73mg Cytosine (Sigma #C4654-5G, Lot #073K1144)

73mg Uridine (Sigma #U-3003, Lot #112K10245)

24mg Thymidine (Sigma #T-1895-5G, Lot #033K1144)

100ml ddH₂O

1% β-mercaptoethanol (Sigma #033K0080)

1% Penicillin-Streptomycin-Glutamine (Invitrogen # 10378-016)

1% 20mM Calcium Chloride

Heparin beads

Purified protein. 30-40 heparin acrylic beads (Sigma #H5263) were selected and placed in a 0.2mL Eppendorf tube. The beads were rinsed twice in PBS + 0.1% BSA and all PBS was removed by aspiration. Beads were then soaked in 2μL of PBS + 0.1% BSA or recombinant mouse Wnt5a protein (R&D Systems # 645-WN-010) at loading concentrations of 250μg/mL, 500μg/mL, and 1000μg/mL. Tubes were placed in a 37°C + 5% CO₂ incubator for one hour as previously described (Chen et al., 1996; He et al., 2008). After the one hour incubation time the beads were stored overnight at 4°C.

Conditioned media. 30-40 heparin acrylic beads (Sigma #H5263) were selected and placed in a .2 mL Eppendorf tube. The beads were rinsed twice in PBS + 0.1% BSA. Beads were then soaked in 2 μ L of L-cell conditioned media or Wnt5a expressing L-cell conditioned media. Conditioned media was obtained from L-cells or Wnt5a expressing L-cells that had been cultured for 3 days in LM media (recipe as previously stated).

Media was removed from the cells after 3 days and filter sterilized using a Minisart® syringe filter unit, 2 μ m, PES, 26mm (ISC Bioexpress #F-2754-2). The filtered media was centrifuged for 15 minutes at 3019 x g in an Amicon Ultra-15 centrifugal filter unit with ultracel-10 membrane (Millipore # UFC901024) to concentrate the protein. Beads were placed in the concentrated protein obtained from the centrifuged media. The exact concentration of protein was not known. Protein obtained from L-cells was used as a control.

Affi-gel blue beads

Affi-Gel Blue Beads (BioRad #153-7301), size 200–250 μ m, were selected and prepared as the heparin beads soaked in conditioned media.

LIMB BUD REMOVAL

Fertilized White Leghorn chicken eggs were incubated at 37°C for 75 hours (Hamburger/Hamilton stage 21). Embryos were dissected out and placed in PBS. Limb buds were removed using a sharp pair of forceps and placed into a 15mL conical tube via

a Pasteur pipette. The limb buds were washed 3X with 2mL PBS, after which 2mL of 2% cold trypsin was added to remove ectoderm surrounding the limb. Limbs buds were incubated in the trypsin for 30 minutes at 4°C. After incubation the trypsin was removed and the limb mesenchyme cells were washed 3X with PBS. The PBS was removed, along with the ectoderm, and the cells were resuspended in 5mL of Wnt3a conditioned media.

Limb mesenchyme cells were cultured in Wnt3a conditioned media to prevent chondrogenesis (Berge et al., 2008). The cells were plated on a 60mm tissue culture dish at a density of 6 million cells and cultured at 37°C + 5% CO₂ for 2 days.

Cells were removed from the culture dish by trypsinization for 5 minutes. The removed cells were then centrifuged for 5 minutes at 4000 RPM. 500,000 cells were plated on a cover slip, placed inside a 35mm tissue culture dish, and cultured in Wnt3a conditioned media overnight. The following day the cells were imaged for 12 hours using time-lapse microscopy. Cover slips used in these experiments were cleaned and reused.

Wnt3a conditioned media

Wnt3a conditioned media was prepared by removing media from Wnt3a expressing L-cells, cultured for three days in LM media, and filter sterilizing it. The media was then centrifuged in an Amicon Ultra centrifuge tube (Millipore # UFC901024) for 15 min at 3019 X g. The protein still left in the filter was diluted 1:10 with LM media (recipe follows).

LM media

DMEM (Invitrogen #11965126) supplemented with:

15% Fetal Bovine Serum (Fisher Scientific # SV3001403P)

1% Non-Essential Amino Acids (Invitrogen # 11140-050)

1% Nucleotides

80mg Adenosine (Sigma #A4036-5G, Lot #022K12705)

73mg Guanosine (Sigma #G6264-5G, Lot #053K2507)

73mg Cytosine (Sigma #C4654-5G, Lot #073K1144)

73mg Uridine (Sigma #U-3003, Lot #112K10245)

24mg Thymidine (Sigma #T-1895-5G, Lot #033K1144)

100mL ddH₂O

1% β -mercaptoethanol (Sigma #033K0080)

1% Penicillin-Streptomycin-Glutamine (Invitrogen # 10378-016)

Cleaning cover slips

Each cover slip was individually washed with Alconox[®] detergent and rinsed six times with distilled water. After washing, the cover slips were allowed to soak for 10 minutes in concentrated HCl. Each cover slip was then washed with distilled water 6X and stored in 100% ethanol until use. Before use, the ethanol was removed from each cover slip by sweeping it through a naked flame using tweezers until all the ethanol had evaporated. This also served to sterilize the cover slip.

Alternative protocol

Limb mesenchyme cells were prepared as stated above. However, the cells obtained from the limb buds were plated directly on the a cover slip, previously coated with collagen, in a 35 mm tissue culture dish at a density of 10 million cells. The cells were cultured in Wnt3a conditioned media for 24 hours previous to imaging for 12 hours using time lapse microscope.

Coating cover slips with collagen

Cover slips were soaked in 100% ethanol. The ethanol was allowed to evaporate off the covers lips in the tissue culture hood for 15 minutes. When the cover slips were dry they were placed in 35 mm tissue culture dishes and covered with 1mL of a collagen I solution and left to sit for 1 hour. After 1 hour the collagen was aspirated off and the cover slips were left under UV light for 15 minutes. Dishes were then closed, sealed with Parafilm[®] and stored at room temperature until use for plating cells. After use, cover slips were discarded.

Collagen I solution

Collagen I from rat tail (BD Biosciences #354236) was diluted in 0.02 N acetic acid to a concentration of 50µg/mL.

Homogenous cell population

In order to obtain a homogenous mix of limb mesenchyme cells, 0.5mm of the distal tip of stage 25 limb buds were removed (Gay and Kosher, 1984; Kosher et al., 1979). Limb mesenchyme cells were cultured as previously outlined.

DUNN CHAMBER

Eight hours prior to the Dunn chamber set up, media was placed in a 24 well plate and incubated at 37°C + 5% CO₂ to allow the media to become warmed and gassed.

A 1:1:1 mixture of beeswax (Sigma #243221): paraffin : Vaseline[®] was placed in a glass beaker and melted slowly using a low setting on a heated plate. The Dunn chamber, which had been stored in 100% ethanol, was placed on a Kimwipe[®] in a tissue culture hood until the storage ethanol has evaporated. The chamber was completely dry before proceeding. No attempt was made to wipe the chamber as contact with the bridge can result in damage that may affect the gradient. Using 150µL of sterile D-PBS, the chamber was washed 6X to remove any residual traces of ethanol. Careful pipeting of 100-150µL of control media was done to place the media over the center of the chamber without letting the tip touch the bridge. Both wells were filled but were not allowed to overflow. The cover slip was quickly, but carefully, inverted over the two wells in the center of the chamber. It was placed in an offset position in order to leave a narrow filling slit at one edge for access to the outer well. However, the cover slip completely covered the inner well so that the outer well could be drained without any loss of medium

from the inner well. It was important that inverting the cover slip over the chamber did not incorporate any bubbles into the chamber as these can affect the establishment of a gradient. Light pressure was applied to the cover slip around the edges and excess medium was mopped up with Fisher filter paper. Care was taken not to apply direct pressure over the bridge as to not crush the cells.

Using a sable paintbrush, wax was applied to three sides of the cover slip leaving the side with the outer well gap unwaxed. A small corner of Fisher filter paper was placed just inside the outer well gap until it started to absorb the medium. The filter paper was left in the well until all of the medium in the outer well was absorbed. It was important not to move or lift the paper as this can introduce air into the chamber. 100 μ L of the Wnt5a media was added into the outer well through the gap, making sure that it was bubble free, until the well was full. For control experiments 100 μ L of L-cell media, or LM media, was added to the outer well in place of the Wnt5a media. The remaining side was quickly waxed, ensuring that the gap was completely sealed. Immediately, the Dunn chamber was placed on the microscope and time lapse microscopy was begun.

Cleaning the Dunn chamber

Each Dunn chamber was cleaned after use. The most important thing taken into consideration during cleaning was to avoid touching the bridge. Wax was removed from the cover slip by scraping with a gloved finger in the opposite direction to the bridge. Residual wax was removed by rubbing under distilled water. Then, the chamber was washed with Alconox[®] detergent and rinsed six

times with distilled water. A glass Petri dish was filled with 100% acetone and the chamber was placed in the acetone and allowed to soak for 10 minutes, after which it was rinsed 6X with distilled water. Each Dunn chamber was placed in a different glass Petri dish, grooved side upwards, in 30% hydrogen peroxide for 10 minutes. Once again, the chamber was rinsed 6X in distilled water and then stored in 100% ethanol until use.

Media conditions for the Dunn chamber

Conditioned Media (1:10). Wnt5a conditioned media was prepared by removing media from Wnt5a expressing L-cells, cultured for three days in LM media, and filter sterilizing it. The media was then centrifuged in an Amicon Ultra centrifuge tube (Millipore # UFC901024) for 15 min at 3019 X g. The protein still left in the filter was diluted 1:10 with LM media (recipe as previously stated).

Control conditioned media was prepared by removing media from L-cells, cultured for three days, in LM media and filter sterilizing it. The media was then centrifuged in an Amicon Ultra centrifuge tube (Millipore # UFC901024) for 15 min at 3019 X g. The protein still left in the filter was diluted 1:10 with LM media (recipe as previously stated).

Conditioned Media (40%). Wnt5a conditioned media was prepared by removing media from Wnt5a expressing L-cells, cultured for three days in LM media, and filter sterilizing

it. The media was then centrifuged in an Amicon Ultra centrifuge tube (Millipore # UFC901024) for 15 min at 3019 X g. 80 μ L of the concentrated Wnt5a protein, obtained from centrifugation, was mixed with 120 μ L of LM media (recipe as previously stated).

Control conditioned media was prepared by removing media from L-cells, cultured for three days, in LM media and filter sterilizing it. The media was then centrifuged in an Amicon Ultra centrifuge tube (Millipore # UFC901024) for 15 min at 3019 X g. 80 μ L of the concentrated L-cell protein, obtained from centrifugation, was mixed with 120 μ L of LM media (recipe as previously stated).

Wnt5a Purified Protein (10%). Recombinant mouse Wnt5a protein (R&D Systems # 645-WN-010) was reconstituted to a final concentration of 150ng/mL in sterile D-PBS containing 0.1% BSA. To make 10% Wnt5a media, 20 μ L of the purified Wnt5a protein (at a concentration of 150ng/mL) was added to 180 μ L of LM media (recipe as previously stated). The media was mixed by pipetting.

LM media (recipe as previously stated) was used as the control media in these Dunn chamber experiments.

Wnt5a Purified Protein (20%). Recombinant mouse Wnt5a protein (R&D Systems # 645-WN-010) was reconstituted to a final concentration of 150ng/mL in sterile D-PBS containing 0.1% BSA. To make 20% Wnt5a media, 40 μ L of the purified Wnt5a protein

(at a concentration of 150ng/mL) was added to 160 μ L of LM media (recipe as previously stated). The media was mixed by pipetting.

LM media (recipe as previously stated) was used as the control media in these Dunn chamber experiments.

Wnt5a Purified Protein (40%). Recombinant mouse Wnt5a protein (R&D Systems # 645-WN-010) was reconstituted to a final concentration of 150ng/mL in sterile D-PBS containing 0.1% BSA. To make 20% Wnt5a media, 80 μ L of the purified Wnt5a protein (at a concentration of 150ng/mL) was added to 120 μ L of LM media (recipe as previously stated). The media was mixed by pipetting.

LM media (recipe as previously stated) was used as the control media in these Dunn chamber experiments.

Microscope

Once the Dunn chamber was assembled it was placed on an Olympus IX-81 inverted microscope equipped with a motorized, heated (37°C) stage, and digital image capture system. Cells along the bridge of the Dunn chamber were chosen and photographed at 30 minute intervals over a 12 hour period. Each image was taken in bright field at 10X magnification. Individual images for each data point were compiled into time-lapse movies using Slidebook[®] software. The time-lapse images were then exported as QuickTime[®] movies for further analysis.

Table 1: Dunn Chamber Experiments

Date	Media Conditions	Wnt5a or Control	Number of Data Points	Notes
9-18-08	Conditioned Media (1:10)	Wnt5a	12	500,000 cells plated on cover slip
10-2-08	Conditioned Media (1:10)	Wnt5a	20	100,000 cells plated on cover slip
10-10-08	Conditioned Media (1:10)	Control	8	800,000 cells plated on cover slip
10-16-08	Conditioned Media (1:10)	Control	17	500,000 cells plated on cover slip
10-17-08	Conditioned Media (1:10)	Wnt5a	14	500,000 cells plated on cover slip
11-6-08	Conditioned Media (1:10)	Control	24	500,000 cells plated on cover slip
11-13-08	Wnt5a Purified Protein (10%)	Wnt5a	42	500,000 cells plated on cover slip
11-20-08	Wnt5a Purified Protein (20%)	Wnt5a	38	500,000 cells plated on cover slip
12-11-08	Wnt5a Purified Protein (20%)	Wnt5a	2	300,000 cells plated on cover slip
12-18-08	Wnt5a Purified Protein (20%)	Wnt5a	13	500,000 cells plated on cover slip
1-16-09	Wnt5a Purified Protein (20%)	Control	12	1 million cells plated on cover slip
1-24-09	Wnt5a Purified Protein (20%)	Wnt5a	5	1 million cells plated on cover slip
1-31-09	Wnt5a Purified Protein (20%)	Wnt5a	51	1 million cells plated on cover slip
2-6-09	Wnt5a Purified Protein (20%)	Wnt5a	13	1 million cells plated on

				cover slip
2-7-09	Wnt5a Purified Protein (20%)	Wnt5a	19	1 million cells plated on cover slip
2-13-09	Wnt5a Purified Protein (20%)	Wnt5a	13	1 million cells plated on cover slip
2-14-09	Wnt5a Purified Protein (20%)	Wnt5a	7	1 million cells plated on cover slip
3-5-09	Wnt5a Purified Protein (40%)	Wnt5a	7	3 million cells plated on cover slip
3-12-09	Wnt5a Purified Protein (40%)	Wnt5a	17	10 million cells plated on cover slip
3-13-09	Wnt5a Purified Protein (40%)	Wnt5a	17	10 million cells plated on cover slip
3-19-09	Wnt5a Purified Protein (40%)	Wnt5a	17	10 million cells plated on cover slip
3-26-09	Wnt5a Purified Protein (40%)	Control	21	10 million cells plated on cover slip
4-2-09	Wnt5a Purified Protein (40%)	Wnt5a	26	10 million cells plated on cover slip; collagen on cover slip
4-3-09	Wnt5a Purified Protein (40%)	Control	17	10 million cells plated on cover slip; collagen on cover slip
4-9-09	Conditioned Media (1:10)	Wnt5a	23	10 million cells plated on cover slip; collagen on cover slip
4-10-09	Conditioned Media (1:10)	Control	14	10 million cells plated on cover slip; collagen on cover slip
4-16-09	Conditioned	Wnt5a	28	10 million

	Media (40%)			cells plated on cover slip; collagen on cover slip
4-17-09	Conditioned Media (40%)	Control	11	10 million cells plated on cover slip; collagen on cover slip
5-1-09	Conditioned Media (40%)	Control	11	10 million cells plated on cover slip; collagen on cover slip
5-8-09	Conditioned Media (40%)	Wnt5a	8	10 million cells plated on cover slip; collagen on cover slip
5-21-09	Conditioned Media (40%)	Control	22	6 million cells plated on cover slip; collagen on cover slip; limb buds from stage 25 embryos (distal tip only)
5-22-09	Conditioned Media (40%)	Wnt5a	25	6 million cells plated on cover slip; collagen on cover slip; limb buds from stage 25 embryos (distal tip only)

RESULTS

CELL BOLUS IMPLANTS

Previous studies have shown that Wnt5a is expressed in the distal limb mesenchyme of the vertebrate embryonic limb bud (Yamaguchi et al., 1999). Its expression appears to be dependent on the presence of the AER, as the highest levels of Wnt5a expression are seen closest to the AER.

Earlier work has demonstrated that labeled limb mesenchyme cells migrate toward the AER (Li and Muneoka, 1999; Kendal, J.J, Barrott, J.J., and Barrow, J.R., unpublished data). Further, these studies and others more specifically demonstrate that members of the Fibroblast Growth Factor family mediate this directional migration (Li and Muneoka, 1999; Saxton et al., 2000). As Wnt5a expression in the limb mesenchyme is dependent on FGF expression from the AER and because Wnt5a has been previously demonstrated to play a role in directional outgrowth (He et al., 2008), we hypothesized that the AER (FGFs) may regulate directional outgrowth of the limb mesenchyme via Wnt5a. In order to examine this question, we applied an ectopic source of Wnt5a to the limb mesenchyme of chick embryos and examined the behavior of nearby mesenchyme cells. Briefly, we implanted boluses of Wnt5a secreting L-cells (nontransformed L-cells were used as a negative control) into stage 23 chick wing limb buds. In addition, the lipophilic lineage tracer, DiI was injected above and below the implant site so as to track the migration of the limb mesenchyme cells in relation to the bolus with respect to time. We imaged the cells at 4 hour intervals over a 30 hour period. Migration toward the L-cell implant was

quantitated by counting the number of labeled pixels within a radius of 250 μ m relative to the bolus.

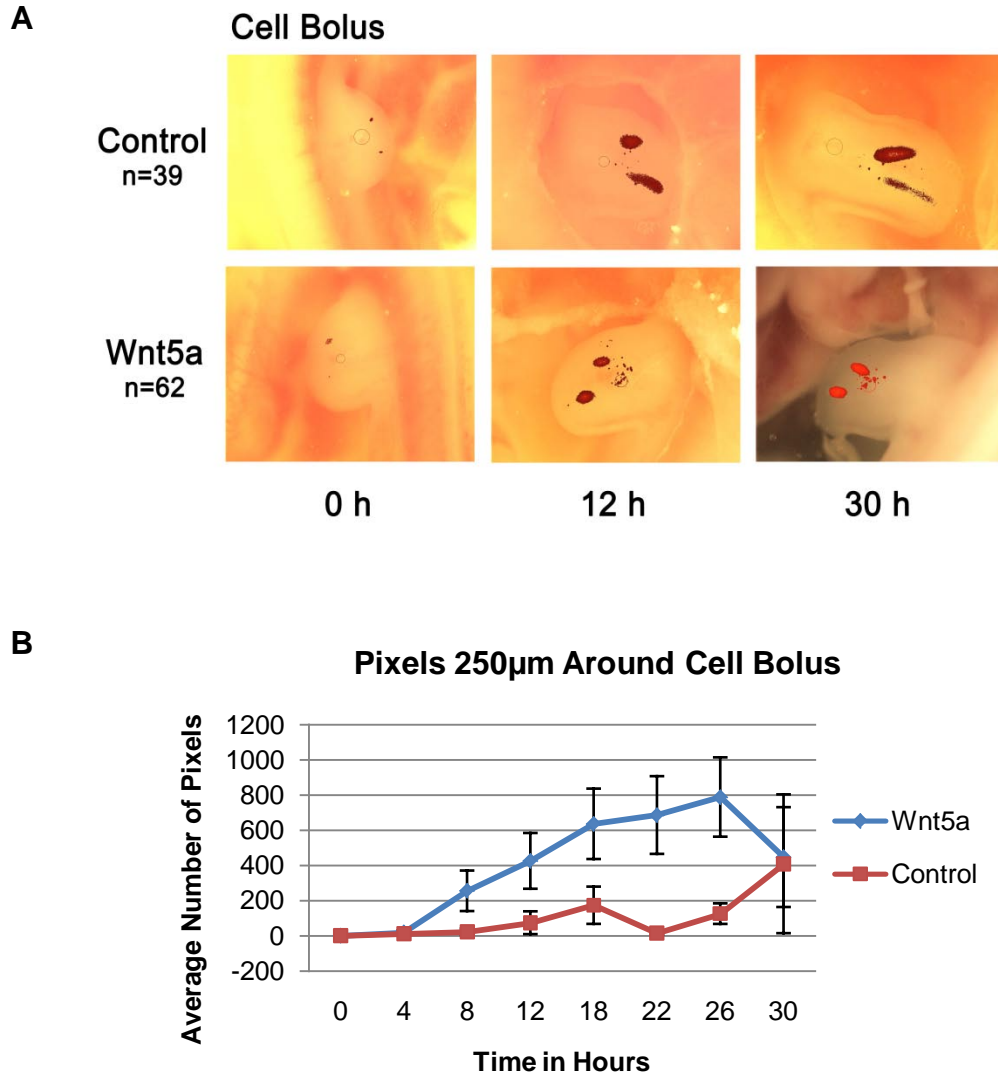


Figure 13: Cell bolus implants. (a) Chick limb buds with Wnt5a or control cell bolus implants. Images are of one embryo limb bud over time and are representative of all cell bolus implant experiments. The circle in each image indicates the location of the cell bolus. (b) Graph showing the average number of pixels in a 250 μ m radius around the cell bolus in all performed experiments. Error bars represent the standard error of the mean.

Images of these experiments showed that labeled cells proliferated/migrated in a linear fashion toward the ectopic Wnt5a gradient, whereas no directional migration toward the

implant was seen in control limbs (**Figure 13**). Control limbs showed clones migrating in a posterior/distal fashion. Based on these results we conclude that Wnt5a, from Wnt5a expressing L-cells, was able to attract limb mesenchyme cells towards it. The AER, which was left intact on the implanted limbs, also attracted a portion of the labeled limb mesenchyme. This result was expected considering the intact AER allowed for an endogenous Wnt5a gradient to be established.

It was also observed that the clone cell shape over time was indicative of convergent extension movements. Cell clones were initially round in shape, but over time showed a trend of converging along the anteroposterior axis and lengthening along the proximodistal axis. This finding supports the proposal that Wnt5a mediates directed cell movements via the Wnt/PCP pathway during limb development (Barrow, 2006).

BEAD IMPLANTS

The bolus of Wnt5a expressing cells, in addition to producing Wnt5a, also produces many other secreted proteins that, in cooperation with Wnt5a, may have the ability to attract limb mesenchyme. To determine whether Wnt5a alone is sufficient to attract limb mesenchyme, heparin beads soaked in purified Wnt5a protein (at loading concentrations of 250 μ g/mL, 500 μ g/mL and 1000 μ g/mL) were implanted into chick wing limb buds. Beads soaked in PBS + 0.1% BSA served as negative controls. Similar to the previous experiment, DiI injections were placed above and below the implant site so as to track the migration of the limb mesenchyme cells in relation to the bead.

Migratory cell number was calculated by counting the number of pixels within a 250 μ m radius from the bead.

Bead soaked in 250 μ g/mL purified Wnt5a

The concentration of secreted Wnt5a protein produced by the AER is currently unknown, so we employed various concentrations of purified Wnt5a protein (R&D Systems) to load heparin beads in an effort to determine the minimal concentration required to redirect limb mesenchyme. Overall 250 μ g/mL Wnt5a beads were not sufficient to direct growth of adjacent mesenchyme (n=19). We did observe a slight increase in the number of cells growing toward the bead at earlier time points, suggesting that the beads charged with protein are capable of recruiting mesenchyme early on but as the protein diffuses away and presumably degrades over time, cells are no longer attracted to the bead. Pixel analysis of control limbs is consistent with image results, showing no labeled clones directly on the bead at any time point as well as the greatest increase in clone number occurring at the earliest time points. Wnt5a pixel analysis illustrates the small number of clones located near the bead indicating a small attraction, but not a significant one (**Figure 14**).

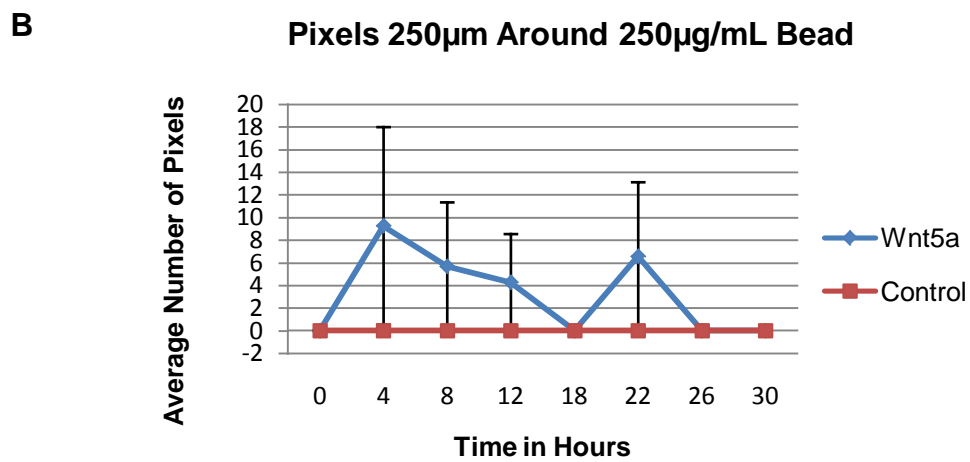
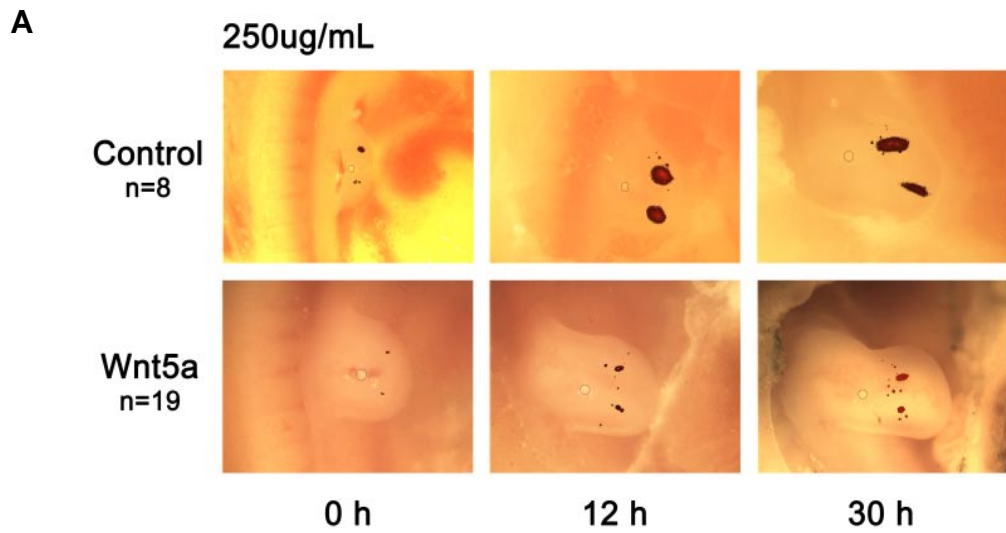


Figure 14: Heparin beads soaked in 250 μ g/mL purified Wnt5a or PBS + 0.1% BSA. (a) Chick limb buds with Wnt5a or control bead implants. Images are of one embryo limb bud over time and are representative of all bead implant experiments. The circle in each image indicates the location of the bead. (b) Graph showing the average number of pixels in a 250 μ m radius around the bead in all performed experiments. Error bars represent the standard error of the mean.

Bead soaked in 500µg/mL purified Wnt5a

Next, limbs buds were implanted with a Wnt5a bead at a concentration of 500µg/mL.

Pixel analysis at each time point revealed little to no pixels on the bead in controls, compared to Wnt5a beads which showed a greater number of pixels near the bead (**Figure 15**). Similar to the 250µg/mL experiments, we observed that attraction of neighboring mesenchyme toward Wnt5a soaked beads was an early phenomenon; suggesting that after Wnt5a protein discharges from the beads there is no further attraction. Our results also demonstrate that beads soaked in 500µg/mL only have a marginal effect on directed growth of adjacent mesenchyme.

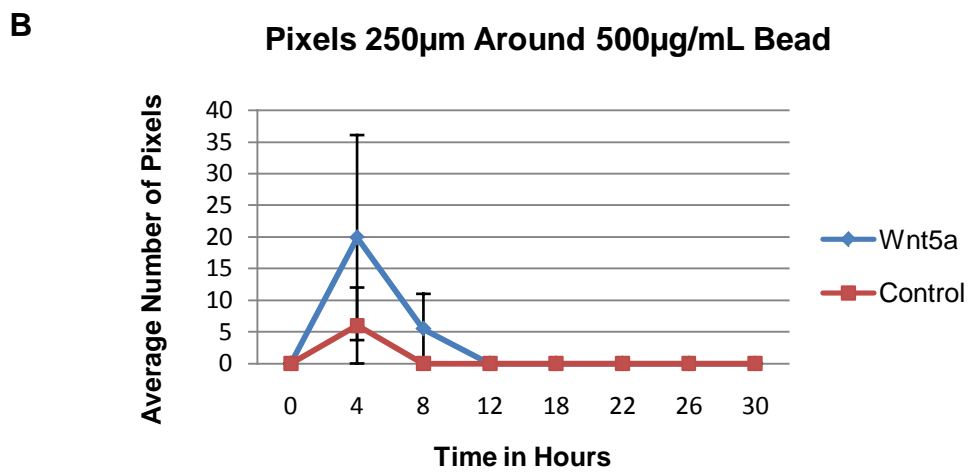
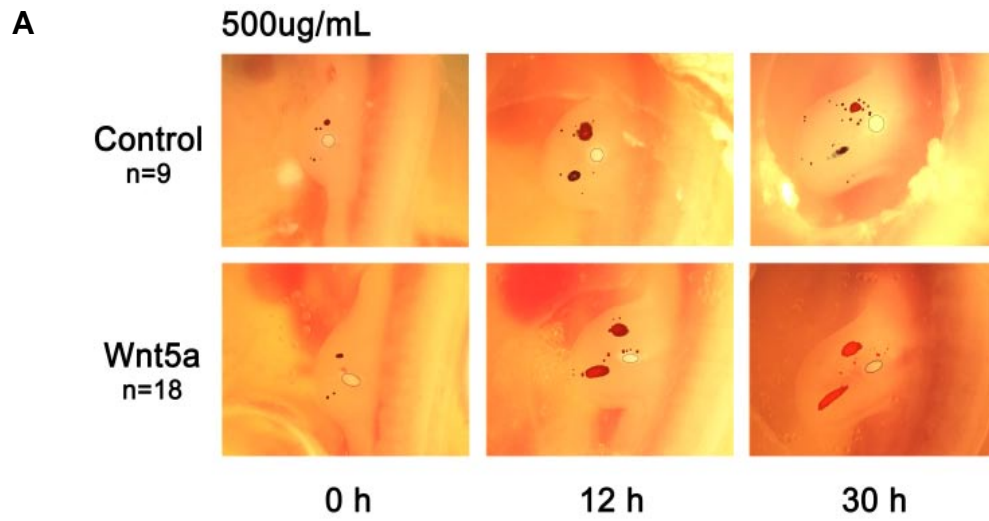


Figure 15: Heparin beads soaked in 500 μ g/mL purified Wnt5a or PBS + 0.1% BSA. (a) Chick limb buds with Wnt5a or control bead implants. Images are of one embryo limb bud over time and are representative of all bead implant experiments. The circle in each image indicates the location of the bead. (b) Graph showing the average number of pixels in a 250 μ m radius around the bead in all performed experiments. Error bars represent the standard error of the mean.

Bead soaked in 1000µg/mL purified Wnt5a

Finally, beads were soaked in purified Wnt5a at a concentration of 1000µg/mL. Once again, there was a difference seen between control and experimental limb buds. Labeled clones traveled toward the Wnt5a soaked bead whereas clones were not seen on the bead in control limbs (**Figure 16**). There appeared to be a significant effect on mesenchyme cell recruitment produced by Wnt5a at a concentration of 1000µg/mL, compared to previous experiments with lower concentrations of Wnt5a. However, the effect seen was not as great as the effect produced with the Wnt5a expressing L-cell bolus implants.

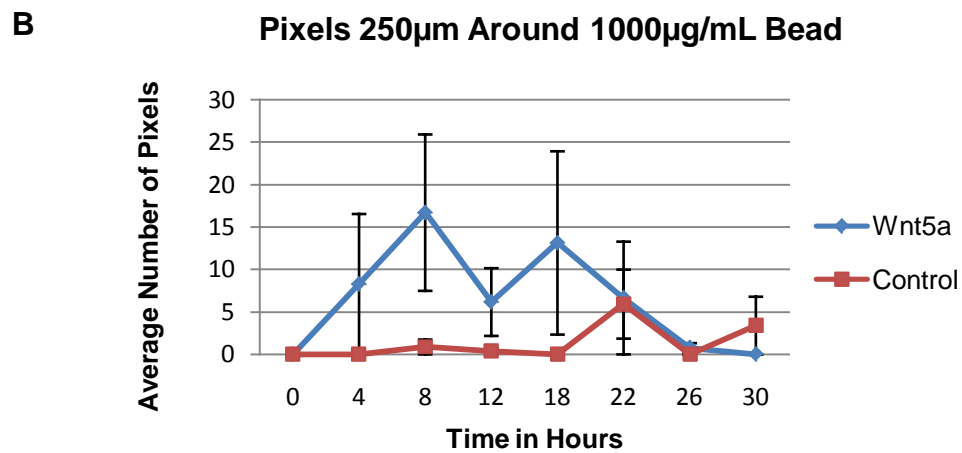
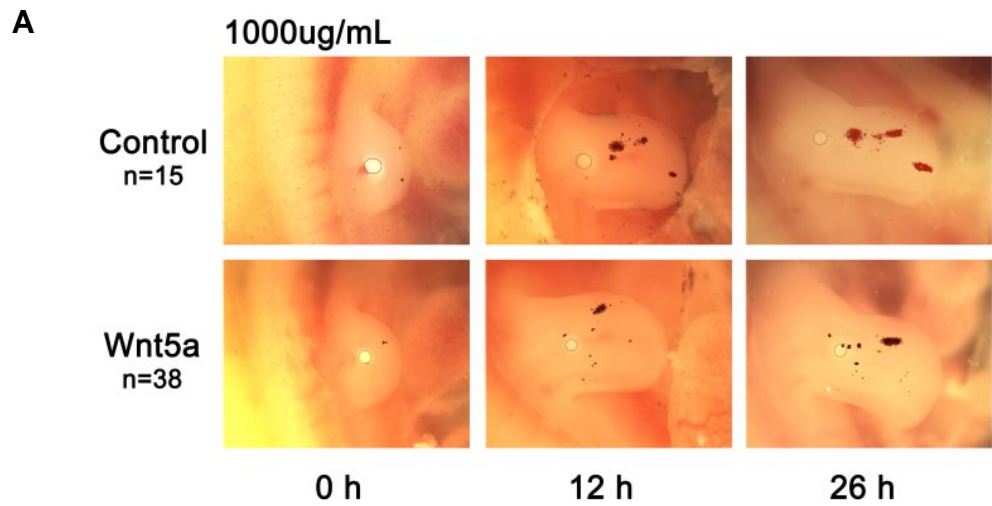


Figure 16: Heparin beads soaked in 1000 $\mu\text{g}/\text{mL}$ purified Wnt5a or PBS + 0.1% BSA. (a) Chick limb buds with Wnt5a or control bead implants. Images are of one embryo limb bud over time and are representative of all bead implant experiments. The circle in each image indicates the location of the bead. (b) Graph showing the average number of pixels in a 250 μm radius around the bead in all performed experiments. Error bars represent the standard error of the mean.

Affi-gel blue beads

To eliminate the possibility that the heparin beads were not accurately soaking up the protein, all experiments were performed using Affi-gel blue beads. The results obtained were essentially the same as those using the heparin beads (data not shown).

Conditioned media

Wnt5a expressing L-cells were able to induce migration of limb mesenchyme cells in a directed fashion, while purified Wnt5a protein could not produce the same effect.

Therefore, it was hypothesized that Wnt5a, in addition to a secondary factor, is causing migration. Alternatively, beads may not be as efficient as living cells to discharge the

Wnt5a to neighboring cells. To distinguish between these hypotheses, we took beads

soaked in Wnt5a or control L-cell conditioned medium. We surmised that if Wnt5a alone

poorly recruits adjacent cells and that other hypothetical secreted proteins produced by L-

cells that cooperate with Wnt5a will also be present in the conditioned medium, the

medium should robustly recruit adjacent mesenchyme. Alternatively, if beads are a poor

substitute for providing active Wnt5a, the beads charged with Wnt5a conditioned

medium should only weakly attract the mesenchyme as the purified Wnt5a experiments

demonstrated. The possible problem with this experiment was that it was not known how

long conditioned media remains potent and/or stable enough to produce an effect.

Additionally, the concentration of Wnt5a in the conditioned media was unknown. It was

observed that the Wnt5a conditioned media was able to direct more cell movement

compared to controls (**Figure 17**). Cell recruitment was much greater compared to beads

soaked in purified protein. However, the effect seen with the Wnt5a conditioned media was not as robust as that seen with the Wnt5a expressing L-cell boluses.

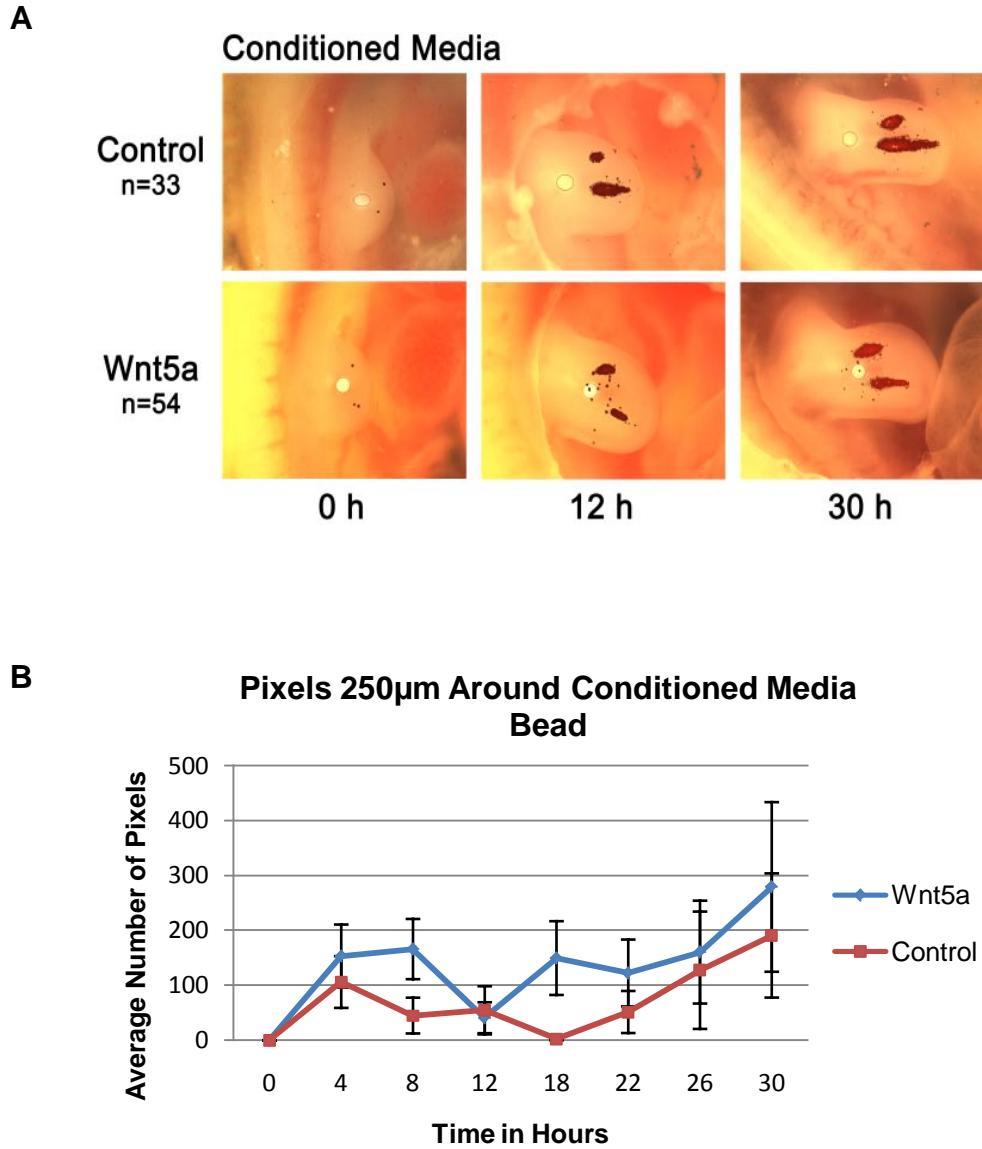


Figure 17: Heparin beads soaked in Wnt5a conditioned media or L-cell conditioned media. (a) Chick limb buds with Wnt5a or control bead implants. Images are of one embryo limb bud over time and are representative of all bead implant experiments. The circle in each image indicated the location of the bead. (b) Graph showing the average number of pixels in a 250 μ m radius around the bead in all performed experiments. Error bars indicate the standard error of the mean.

IN VITRO MIGRATION

We developed an *in vitro* model to study the effect of Wnt5a on cell migration. In whole mount embryos, it is difficult to examine the exact movement of one cell to convincingly determine if the cell is polarized toward a protein gradient. Therefore, we used a two dimensional limb mesenchyme culture model to examine the effect of Wnt5a on cell migration.

Limb mesenchyme cells were obtained from Hamburger/Hamilton stage 21 chick limb buds and cultured on a cover slip. The cover slip was then placed on a Dunn chamber (**Figure 18**). It is important to note that collagen was placed on the

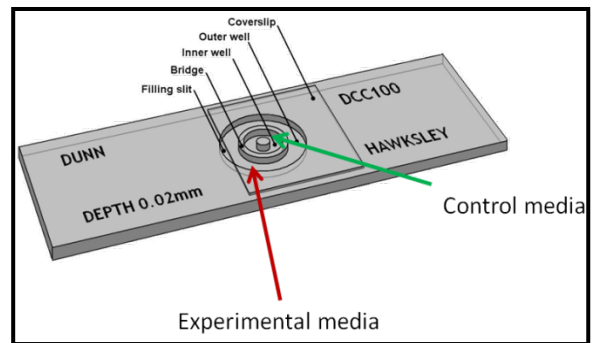


Figure 18: Dunn Chamber

cover slips to facilitate migration of the cultured cells in these experiments. The Dunn chamber has been previously reported as a means to establish protein concentration gradients and for cells to sense the gradient (Witze et al., 2008). We used Wnt5a condition media as well as Wnt5a purified protein to produce a gradient of Wnt5a. Time lapse microscopy, using the Dunn chamber, allowed us to observe the migration of limb mesenchyme cells in response to the established gradient.

The initial hypothesis was that limb mesenchyme cells should respond to the gradient by moving toward the source of Wnt5a. Results showed that despite the source of Wnt5a, migration of cells dispersed on the cover slip was not seen in any specific direction; there

was no promising migratory effect (**Figure 19a**). However, preliminary analysis has shown that cells which were in contact with each other, such as cell clusters (**Figure 19b and Figure 19c**), tended to move toward the source of Wnt5a compared to controls which moved in every direction (**Table 2**).

Table 2: Cell Cluster Movement Analysis

	Wnt5a	Control
Number of cell clusters moving toward the outer well	14	2
Number of cell clusters moving toward the inner well	6	3
Number of cell clusters that spread out in every direction	7	23
Number of cell clusters that stayed in the same place	7	3

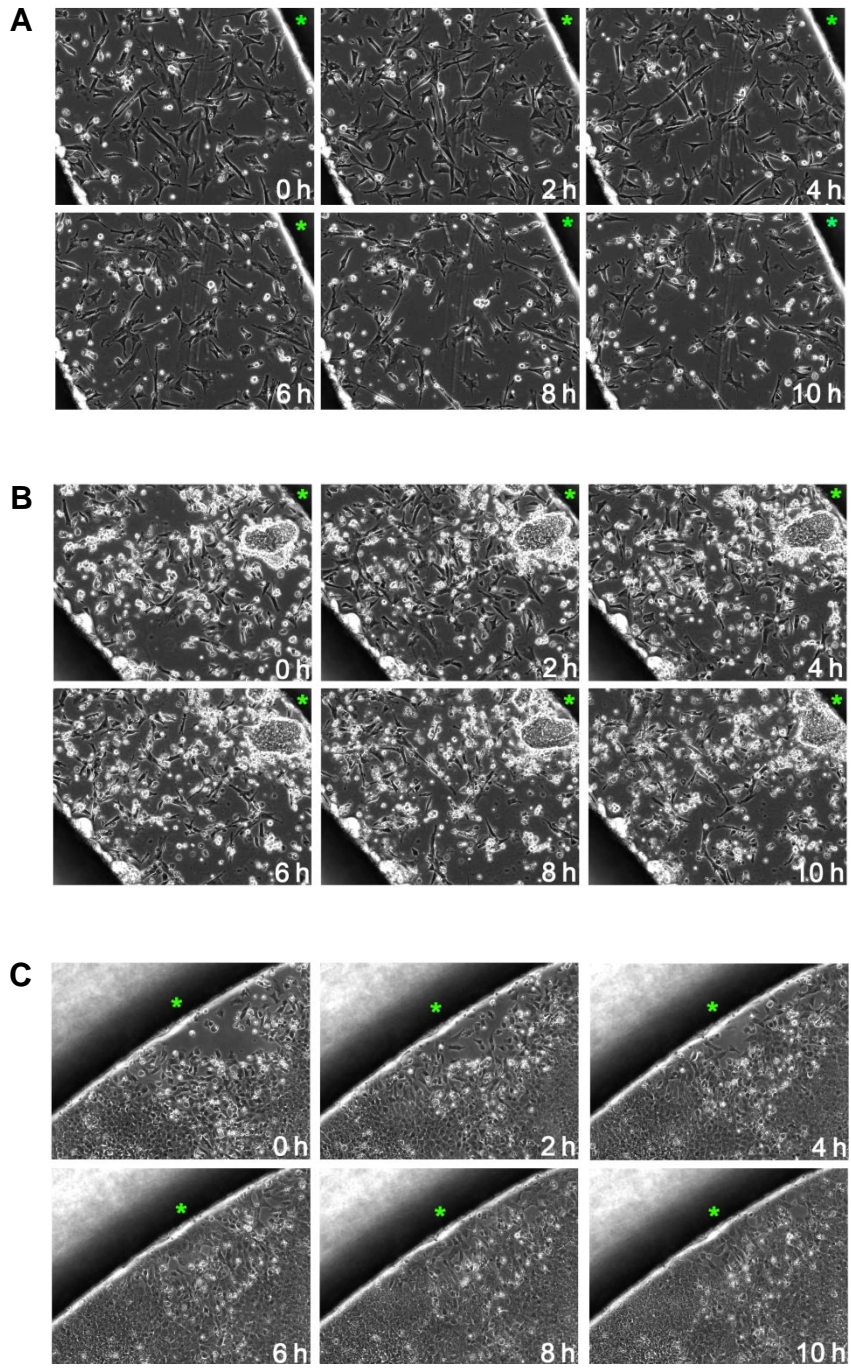


Figure 19: Stills from time lapse microscopy movies. (a) Wnt5a purified protein media (b) Wnt5a purified protein media showing a cluster of cells (c) Wnt5a conditioned media showing cells in contact moving toward the source of Wnt5a. Asterisk indicates the location of the experimental media.

One potential problem with the Dunn chamber experiment is that the cells obtained from the limb buds may be a heterogeneous mix of cells. Some cells may be cells that normally do not respond to the AER FGF/Wnt5a signal. Thus, when pictured on the movies they may have moved in a randomized way. However, most cells should have been competent to respond to the signal and as such the majority of cells should have migrated toward the Wnt5a gradient. In light of the fact that this result was not seen, an effort was made to obtain a homogenous mix of mesenchyme cells.

To do this, 0.5mm of the distal tip of stage 25 limb buds were removed. This has been reported to represent a homogenous population of cells that will give rise to cartilage and are likely those that respond to AER signals (Gay and Kosher, 1984; Kosher et al., 1979). These cells were cultured and placed on the Dunn chamber as stated previously. The results obtained were the same as those using cells from entire limb buds (data not shown).

DISCUSSION

The process of embryogenesis depends heavily on polarized cell growth for the formation of many organs and structures. Specifically, we suggest that directed cell migration/proliferation is critical for correct limb patterning and outgrowth. Consistent with this hypothesis, limbs in which cell movement has been impaired or inhibited exhibit stunted growth and a disruption of normal limb bud shape (Li and Muneoka, 1999; Saxton et al., 2000). It is established that the AER is required for outgrowth and proximodistal patterning of the vertebrate limb, the mechanisms whereby this is accomplished are not clear (Saunders, 1948). Because the AER is ectodermal it must exert its effects on the precursors of the limb skeleton via a secreted signal. One protein shown to be secreted from the AER is FGF4, which in turn has been shown to direct cell recruitment in chick limb buds (Li and Muneoka, 1999). Recent data has led us to believe that FGF4 is working through an additional protein to recruit limb mesenchyme cells (Low and Barrow, unpublished data). This study reports the effect of a downstream target of FGF4, Wnt5a, on the directional migration and directional proliferation of limb mesenchyme cells.

A graded expression of Wnt5a is seen in organs and tissues that undergo outgrowth (Yamaguchi et al., 1999) and has thus been implicated as a driving force for cell migration/proliferation in the developing vertebrate embryo (He et al., 2008; Kilian et al., 2003; Kim et al., 2005; Qian et al., 2007; Rauch et al., 1997; Sakahuchi, et al., 2006; and Yamaguchi et al., 1999). Recently, He et al., (2008) demonstrated that Wnt5a is a potent and specific chemoattractant for palatal mesenchymal cells. It is expressed in an anterior

to posterior gradient which results in directional growth of the palatal mesenchyme toward the anterior. He et al., (2008) further show that applying an ectopic source of Wnt5a can redirect mesenchyme toward this source. Similar to the palate, Wnt5a is expressed in a proximal to distal gradient in the vertebrate limb (Yamaguchi et al., 1999). Consistent with our hypothesis, we have shown in this study that labeled limb mesenchyme cells grow directionally toward an ectopic source of Wnt5a in intact limb buds. Interestingly, we found that the source of Wnt5a correlated to the extent of directed outgrowth: Wnt5a expressing L-cells elicited a stronger directional response in neighboring mesenchyme cells compared to beads soaked in purified Wnt5a protein. The difference in biological activity of the Wnt5a bolus and the purified protein might be due to the loss of Wnt5a activity during the purification process. Alternatively, factors in addition to Wnt5a that are present in the secreted cocktail from the Wnt5a expressing bolus but absent in the purified sample might be responsible for the directed growth. Witze et al., (2008) provided support for this explanation by showing that Wnt5a promotes cell polarity only in the presence of a chemokine gradient. We show that beads soaked in conditioned media were able to attract limb mesenchyme to a greater extent than the beads soaked in purified protein. However, it is important to note that the attraction was not as robust as that seen with the Wnt5a expressing L-cell boluses. This lead to the possibility that Wnt5a alone might be sufficient to elicit directed growth of adjacent cells but the source of the signal must be sustained for long periods of time. Consistent with this observation we found that beads soaked in Wnt5a (purified or conditioned media) had a mild attractive effect on neighboring mesenchyme cells that was eventually lost. Additionally, Wnt5a may need to be present in its endogenous

concentration to elicit recruitment. The beads soaked in purified protein may have presented too high of a Wnt5a concentration to mimic the endogenous gradient. A gross over expression of Wnt5a in the developing limb may have the same effect as no Wnt5a at all, thus we would see little to no cell recruitment with beads soaked in high concentrations of purified Wnt5a.

Wnt5a has been also been shown to increase migration in Wnt5a treated cells (Nomachi et al., 2008). It was reported that Wnt5a conditioned media as well as Wnt5a purified protein, to a smaller extent, induces wound closure in a monolayer of NIH3T3 cells by 1 fold compared to controls (Nomachi et al., 2008). Here we show, using a Dunn chamber system, that limb mesenchyme cells migrate *in vitro*. The migration, however, was not directed toward the source of Wnt5a would have been predicted by our hypothesis. An explanation for this observation could be that the cells must be in contact with one another (i.e., a monolayer) rather than dispersed, as they were in the Dunn chamber experiments, in order for Wnt5a mediated directional migration/growth to occur. In *Drosophila* there is ample evidence that planar cell polarity requires cell/cell contact in order to function. Accordingly, we have observed a significant increase in polarized cell movements of clustered cells toward Wnt5a versus clusters in control media. These results are the motivation for future experiments in the lab. Further experiments will be performed by plating cells in a monolayer and observing cell movement over time in response to Wnt5a.

On the other hand, there is a possibility that the gradient did not exist for a long enough period of time to drive directional growth. We have also hypothesized that the source of Wnt5a may need to be from cells rather than beads, given that the source is continual and is likely to be in concentrations that more closely mirror conditions that exist among Wnt5a expressing cells in the distal mesenchyme. We will, therefore, be commencing experiments where a bolus of Wnt5a is placed in the center of a Dunn chamber to see if cellular Wnt5a can induce directional migration and/or proliferation in cultured limb mesenchyme cells.

Taking into account data obtained from both assays used in this study, we propose that Wnt5a is sufficient to direct limb mesenchyme. When secreted from Wnt5a expressing L-cells, Wnt5a provided strong a directional signal. To a smaller extent, conditioned media was also able to provide this signal and direct cell recruitment. However, due to the loss of bead potency over time, the recruitment is not as great as that seen with the constant source of Wnt5a secreted from the cell bolus. For years beads have dominated the development field for use in cell fate determination. From our work it appears that beads are not the answer to test for cell polarity. Cell fate simply depends on thresholds; if one is above a certain threshold, one gets a certain fate and as one drops below the threshold a different fate is taken on. Cell polarity, on the other hand, is in response to established gradient. We do not know how long the beads secreted Wnt5a and therefore, how long the cells were able to see a gradient of Wnt5a. The possibility that the beads soaked in purified Wnt5a lost potency before the cells were able to adequately respond to may account for the small response in cell recruitment. Thus, further experiments must

be conducted to show cell response to an exogenous source of Wnt5a. Additionally, it appears from the Dunn chamber experiments that the cells may need to be in direct contact to respond to the Wnt5a signal. Based on preliminary data, we predict that when cells are placed in a monolayer *in vitro* they will respond to Wnt5a by directed cell migration.

Over 70 years ago, John Saunders discovered that the AER was crucial for limb outgrowth and patterning. Since that time, countless studies have been devoted to understanding how the AER accomplishes these tasks. A significant breakthrough was made in 1993 and 1994 when it was shown that members of the FGF family are secreted molecules, expressed in the AER, and can functionally replace the AER (Niswander et al., 1993; Fallon et al., 1994). Further it was shown that loss of AER FGFs result in the complete absence of limbs (Sun et al., 2002; Boulet et al., 2004). Interestingly, Sun et al., (2002) also showed dramatically reduced limb buds in mutant embryos but that this reduction is not a result of a decrease in the rate of proliferation or an increase in apoptosis. These results suggest that the AER regulates outgrowth by mechanisms other than cell proliferation or cell survival. Several studies have provided evidence that the AER may regulate outgrowth by directing growth toward the source of FGF (Li and Muneoka, 1999; Saxton et al., 2000). The mechanism whereby FGF does this is not currently understood. Recent evidence from our lab has shown that an exogenous source of FGF can induce a gradient of Wnt5a in the developing limb bud (Low and Barrow, unpublished data). This data shows that the AER/FGFs are both necessary and sufficient for Wnt5a expression. Our work in this study has demonstrated that the gradient of

Wnt5a is likely important as cells appear to migrate toward increasing Wnt5a expression. In addition, we have shown that Wnt5a possesses the same properties of FGFs, in that Wnt5a was shown to be sufficient to recruit mesenchyme cells.

CONCLUSION

Over the years models have been proposed to explain the process of limb patterning and outgrowth. Two models of limb development have been longstanding, the Progress Zone Model and the Early Specification Model. While there is little molecular evidence to substantiate these models, my work provides molecular evidence for a new model of limb development.

Li and Muneoka, (1999) showed that FGF4, one of the secreted factors produced in the AER, is sufficient for directed outgrowth. Additionally, our lab has shown that FGFs from the AER are both necessary and sufficient for activating Wnt5a expression in the distal mesenchyme (Low and Barrow, unpublished data). This study shows that Wnt5a is sufficient to regulate directional growth of limb mesenchyme cells *in vivo* (**Figure 20**). We therefore, propose that FGFs, emanating from the AER, signal to the adjacent limb mesenchyme and activate a gradient of Wnt5a activity. This Wnt5a activity regulates cell recruitment, thereby regulating

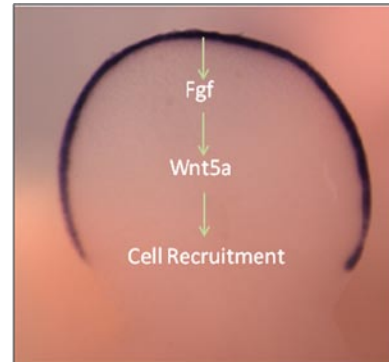


Figure 20: Mesenchyme Recruitment Model. Limb bud with FGF8 staining showing the AER. We propose that the AER secretes members of the FGF family which in turn activates Wnt5a in gradient fashion. Wnt5a then recruits cells toward the AER. The morphology of the AER then patterns the limb mesenchyme which has been recruited toward it via Wnt5a.

directional growth of the limb. The mesenchyme recruited toward the AER is shaped by the morphology of the AER. Our lab has shown that the AER is short along the anteroposterior axis but thick along the dorsoventral axis initially and is therefore almost circular in shape, which recruits a cylindrical mass of mesenchyme that prefigures the humerus (**Figure 21a**). Over time, the AER lengthens along the anteroposterior axis and thins along the dorsoventral axis recruiting a slightly wider mass of mesenchyme which condenses into the two bones of the zeugopod (**Figure 21b**). Later still, there is a dramatic change in the shape of the AER in that the AER lengthens extensively along the anteroposterior axis and thins along the dorsoventral axis recruiting a paddle shaped mass of mesenchyme which condenses into the five digits of the autopod (**Figure 21c**).

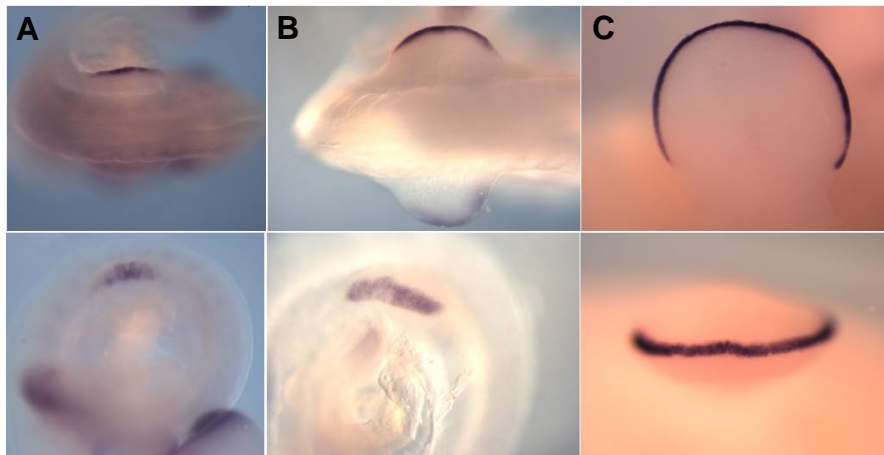


Figure 21: Morphology of the AER over time. The AER was stained using FGF 8. The shape of the AER when it is recruiting mesenchyme for the (a) stylopod, (b) zeugopod, (c) autopod (Barrow, unpublished data).

The findings in this study are novel and were critical to the establishment of the Mesenchyme Recruitment Model of limb patterning. It has never before been shown that Wnt5a is sufficient to recruit limb mesenchyme. This data, combined with data from previous experiments performed in our lab has provided evidence against the long

standing models of limb development. Further experiments must be done to provide additional evidence to support our proposed model. However, our findings, here presented, have paved a way to elucidate a model that will resolve the discrepancies in the current models of vertebrate limb development.

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ADVANCED DEGREE TOPIC

Molecular and cellular mechanisms whereby the apical ectodermal ridge (AER), via Wnt5a, mediates directional migration of the adjacent mesenchyme during vertebrate limb development.

TECHNICAL SKILLS

Chick Embryo Bead and Cell Bolus Implants

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Chick Limb Bud Removal

Cell Culture

Transfection

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Time Lapse Microscopy, including Dunn chamber assays

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REFERENCES

Available upon request