Fgf4 and Wnt5a/Pcp Signaling Promote Limb Outgrowth by Polarizing Limb Mesenchyme

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FGF4 AND WNT5A/PCP SIGNALING PROMOTE LIMB OUTGROWTH
BY POLARIZING LIMB MESENCHYME

by

Keri Lynn Low

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

Master of Science

Department of Physiology and Developmental Biology
Brigham Young University
December 2006
of a thesis submitted by

Keri Lynn Low

This thesis has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

Date ___________________________  Jeffery R. Barrow, Committee Chair

Date ___________________________  Michael R. Stark, Committee Member

Date ___________________________  Daniel Simmons, Committee Member
As chair of the candidate’s graduate committee, I have read the thesis of Keri Lynn Low in its final form and have found that (1) its format, citations, and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the graduate committee and is ready for submission to the university library.

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ABSTRACT

FGF4 AND WNT5A/PCP SIGNALING PROMOTE LIMB OUTGROWTH BY POLARIZING LIMB MESENCHYME

Keri Lynn Low
Department of Physiology and Developmental Biology
Master of Science

The focus of this study was to elucidate the molecular and cellular mechanisms whereby fibroblast growth factors (FGFs) mediate outgrowth of the limb. Specifically, we examined the epistatic relationship between FGF and Wnt/Planar cell polarity (PCP) signaling in establishing cell polarity as a mechanism for outgrowth. By implanting beads into embryonic limbs and lateral plate mesoderm, we established that FGF activates Wnt5a in a gradient fashion. Once it was established that Wnt5a was expressed at the right time and place to turn on PCP signaling, we investigated the ability of Wnt5a to influence cell migration and/or cell polarity.

Our analysis revealed that there was no difference in cell migration when cells were exposed to an exogenous Wnt5a source. However, this did not rule out the possibility that cells were responding in a more mild fashion and polarizing toward a Wnt5a source. Live cell imaging was performed to observe the movement and morphology of limb mesenchyme cell cultures in the presence or absence of a Wnt5a expressing cell bolus. It appears as though the cells orient and move in a random fashion regardless of Wnt5a. However, this in vitro method
may not truly recapitulate in vivo events. Future studies aim to develop better methods of observing cell polarization in vitro, including developing better methods to trac the movement of cells and observe “PCP” events.

Due to the lack of information gathered from our in vitro studies, an in vivo study was conducted to test if FGF is necessary to polarize limb mesenchyme cells. If FGF is turning on Wnt5a and Wnt/PCP signaling is directing cell polarization, then FGF mutant clones will not migrate toward the AER. Therefore, it is expected that these mutant clones would be unable to undergo directed cell movement and/or cell divisions. Early clonal analysis indicates that a response to FGFs appears to be necessary to direct polarized outgrowth of limb mesenchyme.
ACKNOWLEDGMENTS

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INTRODUCTION

Embryonic Limb Outgrowth

The early embryonic limb is a simple bud-like structure composed of two tissues: limb mesenchyme (sometimes called lateral plate mesoderm or LPM) and limb ectoderm (Fig. 1). The loosely packed cells of the limb mesenchyme give rise to the limb skeleton, whereas the sheet-like epithelium of the ectoderm encases the mesenchyme and will give rise to the skin and epidermal appendages (i.e. hair, feathers) covering the limb.

Models of Limb Development

For many years, embryologists have known that outgrowth of the limb is dependent on cross-signaling between limb mesenchyme and ectoderm. In 1948, John Saunders demonstrated that a small ridge of ectodermal tissue located on the distal end of the limb called the apical ectodermal ridge (AER) is required for limb outgrowth (Fig. 1). By removing the AER at various developmental stages, he also established that the limb develops in proximal to distal fashion (i.e. humerus, ulna/radius, and then digits).

Saunders’s discovery prompted an intense investigation into the molecular details regulating limb outgrowth. In the chick, it was discovered that members of the fibroblast growth factor (FGF) family are expressed in the AER and are sufficient to restore normal limb development to limbs lacking the AER (Fallon et al., 1994; Niswander et al., 1993). Additionally, knockout mice with mutant FGF genes in the AER lack limbs.

Despite these significant findings, the mechanism by which FGFs mediate outgrowth is still under dispute. Some studies provide evidence that FGFs may mediate outgrowth by promoting proliferation or survival of limb mesenchyme (Dudley et al., 2002; Martin, 1998;
Rowe et al., 1982). Other studies, such as the FGF gene knockouts described above, do not attribute the absence of limbs to reduced proliferation or increased cell death of the limb mesenchyme (Sun et al., 2002).

Recent studies suggest that FGFs may mediate outgrowth by affecting cell behavior as opposed to proliferation and/or cell survival (Li and Muneoka, 1999; Saxton et al., 2000). For example, Li and Muneoka (1999) found that labeled mesenchyme cells in chick limbs migrate toward implanted beads soaked with FGF proteins. Furthermore, Saxton et al. (2000) discovered that distal mesenchyme of chimeric limbs composed of both wildtype cells and cells that lacked the ability to respond to FGF signals was composed solely of wildtype cells. These results suggest that ectodermal FGFs may be signaling outgrowth by recruiting limb mesenchyme cells toward the AER.

**Dependence of Wnt5a Expression on AER Function**

We have previously shown that Wnt3<sup>nc</sup>; Msx3Cre conditional mutants lack Wnt3 to variable extents in the limb ectoderm which results in patchy AER formation (Barrow et al., 2003). Where AER is present, strong Wnt5a expression is also observed (Fig. 2, unpublished observations), suggesting that Wnt5a may be activated in gradient fashion by FGFs secreted from the AER (Barrow et al. 2003, unpublished observations). In these mutants, we have also

![Figure 2: Wnt5a expression in control (A) and Wnt<sup>nc</sup>; Msx2Cre (B,C) hindlimbs at E11.5. Note the gradient of Wnt5a expression fading with increasing distance from the AER (A). In the Wnt<sup>nc</sup>; Msx2Cre mutants which have variable loss of the AER, Wnt5a levels are a function of the amount of AER present. Arrowheads and arrows denote representative areas possessing or lacking the AER, respectively.](image)
demonstrated that there is no change in cell death or proliferation in limb mesenchyme adjacent to the regions lacking the AER. This observation suggests that other mechanisms must be regulating the outgrowth of limb mesenchyme (Barrow et al., 2003 and unpublished observations), which may include Wnt5a and its downstream pathways.

**Wnt Signaling: β-catenin/TCF4 and PCP Pathways**

The Wnt pathway is initiated by secreted Wnt ligands that bind to a Frizzled/LRP receptor complex embedded in cell membranes. In vertebrates, the type of Wnt ligand dictates whether it will signal through β-catenin (canonical pathway) or activate other pathways (non-canonical pathway). Both pathways involve the activation of the Dishevelled protein.

In the canonical pathway, the ligand-receptor complex interacts with Dishevelled to stabilize the cytoplasmic protein β-catenin. In the absence of Wnt signaling, β-catenin is degraded. Once stabilized, β-catenin moves into the cell’s nucleus where it interacts with the DNA binding protein Lef/TCF and transcription factors to activate the expression of downstream genes.

In a non-canonical pathway, known as the Wnt planar cell polarity (PCP) pathway, Wnt through Dishevelled appears to activate the JNK pathway and Rho kinase which are known to have direct consequences on the cytoskeleton and on cell polarity (Veeman et al., 2003). The Wnt/PCP pathway is therefore thought to be important in mediating key molecular events such as directional migration and oriented cell division, which are required for proper morphogenesis. On the other hand, the canonical Wnt/β-catenin pathway is known to be critical for making cell fate decisions (Giles et al., 2003).

**The Wnt/PCP Pathway in Invertebrate and Vertebrate Development**

Studies initiated in Drosophila have demonstrated that Wnt/PCP signaling regulates cell polarity not cell fate (Axelrod and McNeill, 2002). When the Wnt/PCP pathway is disrupted in
Drosophila, orientation of individual ommatidia of the eye and trichomes on the body and wings are defective.

In vertebrate model systems, it has long been recognized that the Wnt5a ligand signals through β-catenin independent pathways. For example, Wnt5a misexpression in Xenopus laevis embryos does not duplicate the body axis. Additionally Wnt5a is incapable of transforming mouse mammary epithelial cell lines. Both of these events, however, can be induced with Wnts that are known to activate the β-catenin pathway (Du et al., 1995; Wong et al., 1994).

Furthermore, loss of Wnt5a function in zebrafish affects convergent extension movements during gastrulation, consistent with it signaling through the Wnt/PCP pathway (Myers et al., 2002; Rauch et al., 1997, Heisenberg et al., 2000). Convergent extension occurs when cells migrate and intercalate with similarly polarized cells along an axis, causing the tissue/embryo to extend (see Fig. 3).

![Figure 3: Schematic diagram of convergent extension beginning with cells without lamellipodium polarized, followed by polarized cells migrating and intercalating lateral to medial (mediolateral convergence). Such cell behavior results in an extended anteroposterior axis as diagramed.](image)

In the amphibian Xenopus, Wnt/PCP signaling also plays an important role in convergent extension by stabilizing actin rich filopodia along the mediolateral axis of the embryo (Wallingford et al., 2000). Disrupting this pathway results in random, unstable filopodia formation and thus randomized cell movements (see videos from Wallingford et al., 2000 at http://mcb.berkeley.edu/labs/harland/movies.html).

Wnt/PCP signaling has also been shown to affect the orientation of the mitotic spindle (Bellaiche et al., 2001; Gong et al., 2004; Walston et al., 2004). In Zebrafish, cleavage planes orient along the mediolateral axis of the embryo due to Wnt/PCP’s effects on the mitotic
spindle. As a result, daughter cells align parallel to the anteroposterior axis of the embryo resulting in a configuration much like the middle diagram in Figure 3 (Gong et al., 2004). Not surprisingly, disruption of Wnt/PCP signaling in the zebrafish causes complete randomization of the mitotic spindle and cleavage plane. As such, zebrafish Wnt/PCP mutants have a shortened and widened embryonic axis (reviewed in Veeman et al., 2003). Therefore, a combination of directed cell movements and cell divisions may very well underlie proper mediolateral convergence and anteroposterior extension of the vertebrate limb (see Gong et al., 2004).

Much like the Xenopus and zebrafish Wnt/PCP mutants, Wnt5a mutant mice have shortened body axes, defective craniofacial structures, and stunted limbs (Figures 4 and 5; Yamaguchi et al., 1999). These defects are not a result of a change in cell fate; in fact, the limbs of these mutants express all the appropriate markers and exhibit relatively normal limb patterning, suggesting a possible defect in morphogenesis. Regardless of the phenotypic similarities, it is not known if the repertoire of the mouse Wnt5a mutant anomalies is due to cell polarity defects mediated by Wnt/PCP signaling.

![Figure 4: Limb skeletons of E18.5 control and Wnt5a mutant limb buds.](image)

**Wnt5a and Wnt5b Mutant Analysis**

When Wnt5a mutants are compared to mutants lacking FGF function in the AER, the FGF mutants are much more severe (Boulet et al., 2004; Sun et al., 2002). These results suggest
that FGFs from the AER do not promote outgrowth solely through Wnt5a, and it is possible that other Wnt ligands cooperate with Wnt5a to stimulate outgrowth of the limb.

Consistent with this hypothesis, we have observed significant functional overlap between Wnt5a and its closely related family member Wnt5b in mediating outgrowth of the primary embryonic axis (Fig. 5); however, early embryonic lethality of Wnt5a and Wnt5b double mutants does not permit us to rigorously examine limb development. Notwithstanding this obstacle, we have observed that the antero-posterior length of the limb bud is dramatically extended suggesting a possible “convergent extension” defect (Fig. 5). It is possible that complete disruption of Wnt/PCP signaling in the limb might entirely disrupt limb outgrowth and result in phenotypes similar to those in which FGF signaling is blocked in the AER. We are constructing reagents that should disrupt the pathway in a clonal fashion to test this hypothesis.

**Figure 5: Comparison of axis elongation and limb defects in mutants lacking Wnt5 genes.** Mutants lacking Wnt5 family members exhibit shortening of the primary embryonic axis. The forelimb buds of Wnt5a/5b double mutants are poorly defined. Distance between arrowheads indicates width of forelimb bud.

**Limb Mesenchyme Polarity**

In addition to our analysis of Wnt5a mutants, we have performed clonal analyses in wildtype mouse limb buds and revealed that labeled mesenchyme clones are restricted to narrow columns that extend along the proximodistal axis of the limb (Fig. 6). Since Wnt5a is distributed in a gradient from the AER (observed in Fig. 2) and is capable of activating...
Wnt/PCP signaling, we hypothesized Wnt5a may be critical in directing polarized distal outgrowth of the limb. Without directed cell movement and/or cell divisions we would expect clones to be distributed in a random fashion.

**RESULTS**

**FGF Activates Wnt5a in a Gradient Fashion.**

In order to test the hypothesis that FGF signaling activates the expression of Wnt5a in gradient fashion, beads soaked in increasing concentrations of FGF-4 were implanted into the chick limbs or lateral plate mesoderm. After at least 24 hours of incubation, embryos were collected and Wnt5a in situ hybridization was performed. When a bead soaked in 1000 μg/ml or 250 μg/ml of FGF-4 was implanted into the distal portion of a chick limb (stage 20-23),
Wnt5a expression was induced in the limb mesenchyme surrounding the bead (Figs. 7A, C, & E).

This effect was not observed when FGF-4 beads were implanted into the proximal limb bud (Figs. 8A-B & 8E-F). Additionally, no induction was observed when control beads were implanted into the limb (data not shown).

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**Figure 8: Wnt5a in situ hybridization.** (A-B & E-F) Proximal implantation of bead into the limbs of stage 20-23 embryos; (C-D & G-H) Comparable opposing limb buds with no bead implanted.

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**Wnt5a is Not Sufficient to Induce Cell Migration In Vitro.**

Once it was established that Wnt5a was expressed at the right time and place to turn on PCP signaling, we investigated the ability of Wnt5a to influence cell migration by culturing primary limb mesenchyme cell in the presence or absence of Wnt5a conditioned media. Limb mesenchyme cells derived from Hamburger and Hamilton stage 19-23 chick embryos were plated onto a Thincert membrane (Fig. 9) and incubated for at least 24 hours with the following types of media: supernatant from Wnt-5a expressing L-cells, supernatant from non-altered L-cells, and regular limb

---

**Figure 9: Boyden chamber assay set-up**
mesenchyme media. The basic formulation for all three types of media was the same (DMEM plus 15% FBS non-essential amino acids, nucleotides, β-merc, and pen/strep glutamine).

Cells that had migrated to the bottom of the filter or through the filter to the bottom of the tissue culture dish were collected and counted using flow cytometry. Propidium iodide was used to classify cells as living or dead. Analyses revealed that regardless of treatment (Wnt5a conditioned media, L-cell media, or fresh cell culture media), there was no significant difference in the number of cells that had migrated. Any difference in cell number observed was attributed to cell death (see Appendix A).

**Wnt5a is Not Sufficient to Induce Polarization In Vitro.**

Although cells did not migrate to a greater degree in the presence of Wnt5a, this did not rule out the possibility that cells were responding in a more mild fashion. Because several studies have demonstrated that Wnt5a signals through the Wnt/PCP pathway, it was expected that Wnt5a cells would at least orient in the direction of a Wnt5a source and exhibit polarized behavior (Du et al., 1995; Wong et al., 1994; Myers et al., 2002; Rauch et al., 1997).

Such effects could not be measured with the Boyden chamber cell migration assay. Therefore, in order to view the polarity of cytoskeletal elements, two-dimensional live cell imaging of limb mesenchyme was implemented to examine the effect of FGFs or Wnt/PCP signals on cell behavior.

In this model, we isolated primary limb mesenchyme cells in the same manner used for the cell migration assay. Confluent monolayers of limb mesenchyme cells were transfected with plasmids capable of expressing tubulin-GFP or actin-GFP fusion proteins. To recapitulate the AER/mesenchyme interaction, transfected cells were placed in the presence of an L-cell or Wnt5a expressing L-cell bolus (Fig. 15). Cells were also observed without any bolus. After overnight incubation, the orientation and movement of cells relative to the boluses were
examined for up to twelve hours. Polarity was assessed by the presence of a ruffled leading edge, tapered trailing cell body, and cell migration.

It was expected that limb mesenchyme cells would only exhibit polarity when exposed to Wnt5a and extend actin rich filopodia and microtubules toward the Wnt5a bolus. However, regardless of position in the dish or type of bolus, the cells exhibited polarized behavior (Figure 10). Additionally, when the cells were assigned a vector indicating polarity, the orientation of all cells observed was random with respect to either the Wnt5a L-cell bolus or the L-cell bolus. (see Appendices C, E, & G).

![Figure 10: (A-D) Limb mesenchyme cells transfected with actin-GFP. Solid arrows = polarized leading edge. Open arrows = direction of cell migration, how vectors were assigned to cells in Appendices C-E. See Appendices C-F for position of cell relative to bolus. (A) cell #2655, (B) cell #8180, (C) cell #1163, (D) cell #3904.](image)

**FGF is Necessary to Polarize Limb Mesenchyme Cells.**

Since our in vitro model did not reveal expected results, we attempted to visualize cell polarity in an in vivo setting. When sections of Wnt5a mutant mouse limbs were stained with fluorescent markers to detect cytoskeletal elements and nuclei, cell orientation with respect to
the AER was not readily apparent and polarized cell divisions were difficult to detect. Therefore, in order to examine individual cell behavior within a field of thousands of mesenchyme cells, clonal analysis was employed. Initially, we aimed to knockout Wnt5a and later FGF and examine the effects on cell migration and/or cell divisions. However, time constraints only permitted testing the necessity of FGF (Wnt5a’s upstream regulator).

Replication incompetent viruses were chosen to knockout FGF in a clonal fashion. These viruses are unable to spread to surrounding tissue once a cell is infected. Therefore, this system allows us to study mutant cell behavior in relation to adjacent wildtype cells.

To test the hypothesis that FGF is necessary to polarize limb mesenchyme cells, a replication incompetent retrovirus RISAP (see details in Methods) was used to inhibit FGF signaling. Murine Spry2 (mSpry2) has previously been shown to be a potent inhibitor of FGF signaling in chick limb buds and was cloned into RISAP (Minowada et al., 1999). It was hypothesized that if transduced limb mesenchyme cells were unable to respond to an FGF signal, Wnt5a would not be expressed in a gradient fashion. Therefore, without proper Wnt/PCP signaling, cells may not orient and move toward the AER. Thus, it was expected that Spry2 clones would be rounded rather than columnar as shown in Figure 6.

The viral vector RISAP-mSpry2 and a viral packaging plasmid were co-transfected into DF1 cells, a chicken fibroblast cell line, to produce pseudotyped viral particles. Concentrated viral supernatants were injected into the lateral plate mesoderm or limb bud of chick embryos. After 48 hours, embryos were harvested and viral infection was detected by PLAP (placental alkaline phosphatase) staining.

Figure 11: Chick embryo limb buds infected at stage 20, collected 48 hours after infection, and stained for PLAP activity. (A) Limb bud infected with RISAP, note the columnar shape of the clone outlined. (B) Limb bud infected with RISAP-mSpry2, note the more spherical shape of the outlined clone.
Despite widespread background infection, preliminary analysis revealed results consistent with our hypothesis. Clones of cells infected with RISAP-mSpry2 were round whereas clones infected with the control RISAP were more columnar (Fig. 11). Therefore, clones lacking the ability to respond to an FGF signal could not polarize correctly and directionally proliferate and/or migrate toward the AER.

**Wnt/PCP Signaling is Necessary to Polarize Limb Mesenchyme Cells.**

A great deal of time has been spent developing a method to study the consequences of improper Wnt signaling in the mouse limb. Much like the retroviral infections described above, this method generates clones of cells that can be compared to wildtype neighbors.

Standard cloning procedures have been used to create a construct (see Figure 12) that will express a dominant negative form of Dishevelled when injected with tamoxifern (a modified estrogen compound). These constructs will be electroporated into ES cells. Those ES cells that properly incorporate the construct will be selected. Chimeric aggregates composed of these modified ES cells and host embryos will be inserted into a surrogate mother. Because of the inducible feature of this method, cells can be sporadically induced at various times within the embryo or the adult by injecting the mothers with tamoxifan. Wildtype cells and mutant cells in the same limb of chimeric embryos will be compared, controlling for differences between individual embryos.

**Figure 12:** Rosa 26 5’ or 3’ = homology to the mouse Rosa 26 that will help in targeting the construct to the Rosa 26 locus (a site that is constitutively expressed in all mouse cells) in mouse embryonic stem cells; P = loxP sites; ts = transcriptional stops (poly A sequences); Dvl-DEP or PDZ = genes that encode dominant negative forms of Dvl, cells expressing it cannot respond to non-canonical Wnt signaling; IRES = allows for 5’ cap independent ribosomal entry onto an mRNA (in this case allows YFP to also be translated).
In order to knockout Wnt signaling, the downstream regulator of Wnt/PCP and Wnt/β-catenin signaling Dishevelled will be modified. The Dishevelled protein consists of three regulatory domains: DIX, DEP, and PDZ. The DIX domain has been demonstrated to play an important role in canonical signaling whereas DEP and PDZ are critical to Wnt/PCP signaling. The dominant negative Dvl construct that I will be using in my studies lacks the PDZ domain and contains the DEP and DIX domains (Dvl-ΔPDZ). Removing these domains only affects the non-canonical pathway and not the canonical β-catenin pathway. This mutant version of Dvl is homologous to alleles from other species which have been successfully used in dominant negative studies to override the function of endogenous Dvl (reviewed in Axelrod and McNeill, 2002; Veeman et al., 2003; see also Gong et al., 2004; Wallingford and Harland 2000).

The transcription and translation of Dvl-ΔPDZ is linked to the fluorescent reporter Venus, an improved version of yellow fluorescent protein (YFP). Thus, when introduced into stem cells, both genes will be transcribed onto a single RNA transcript. In this configuration, only the mutant Dvl gene would normally be translated into protein; however, an internal ribosomal entry site (IRES) has been added that will allow translation of the downstream YFP. Cells taking up this construct will therefore express not only the mutant Dvl gene but YFP as well, allowing us to distinguish mutant cells (fluorescent) from normal cells (non-fluorescent) in developing limbs and tissues. These genes are flanked by two regions (5’ Rosa 26 and 3’ Rosa 26) that will serve to target the construct to the Rosa 26 locus in the mouse genome. This locus is known to drive constitutive expression in all mouse cells (embryonic to adult). Rosa 26 would therefore drive ubiquitous expression of Dvl-ΔPDZ and YFP. However, three transcriptional stop sequences (ts) prevent transcription. These sequences are flanked by loxP sites which are substrates for recombinase (enzyme) called Cre. Thus, when this construct is exposed to Cre, recombination between the loxP sites is catalyzed removing the transcriptional termination sequences. Once these sequences are removed, Dvl-ΔPDZ will be expressed in a constitutive
fashion. We are using a version of Cre, CreER (Cre fused to a modified estrogen receptor), that is retained in the cytoplasm unless bound with tamoxifen.

Control ES cells have been derived from a cross between a male Heterozygous for Cre-ER (Fig. 13a) and R26R (Fig. 13b), and a female Homozygous for R26YFP (Fig. 13c). Cells heterozygous for all three transgenes were selected.

When 4-OH Tamoxifen is introduced to YFP3 cells, CreER enters the nucleus where it catalyzes recombination between loxP sites, resulting in constitutive expression of YFP and β-Gal. Following induction, YFP3 cells are referred to as YFP3-1 (Fig. 14) can be used to receive gene constructs targeted into the pROSA26 locus (Srinivas et al., 2001). Using well-established protocols, both constructs (Dvl-ΔPDZ and mSpry2) will be introduced into YFP3 cells that have potential to differentiate into any tissue in the mouse. After electroporation into stem cells, homologous recombinants are selected based on the following characteristics: 1) neomycin resistance, 2) absence of Diphtheria Toxin (DTX) in homologous recombinants, 3) loss of YFP or β-gal, and 4) inducible expression of YFP. Those cells which have incorporated the construct properly at the Rosa26 locus will be expanded.
The modified ES cells will be introduced to host embryos that have not implanted into the uterus. At E2.5 days of development, host embryos are flushed out of the uteri of superovulated and fertilized females. The zona pellucidae of these embryos are removed, and each embryo is incubated overnight with a small clump (8-12 cells) of modified embryonic stem cells. The ES cell/host embryo aggregates called chimeras will then be reintroduced into the uteri of pseudo-pregnant mice (female mice that have been bred with vasectomized males so that the uterus will be receptive to the implantation of the chimeras). The embryos are expected to develop normally until the introduction of tamoxifen.

The dominant negative activities of the Dishevelled genes described above will be tested by misexpressing them in mouse embryos. Lineage traceability of mutant clones will be made possible via the IRES-YFP construct placed downstream of the dominant negative Dvl gene product in the ES cells.

To date, there are no reliable molecular readouts for Wnt/PCP signaling. However, mice possessing mutations in this pathway exhibit neural tube closure defects and severe shortening of all axes, including head-to-tail, limbs, craniofacial structures, etc. (reviewed in Ueno and Greene, 2003; Yamaguchi et al., 1999).
When the chimeras are at 8.5 days of development, the mothers will be injected with low doses of tamoxifen to sporadically induce cells to express Dvl-∆PDZ and YFP (see Fig. 15). Chimeric embryos will be taken and subjected to various examinations to determine the behavioral consequences of the inability to respond to Wnt/PCP and FGF signals relative to control clones. If these alleles disrupt Wnt/PCP, it is expected that they will exhibit the common array of Wnt/PCP defects. We expect that Dvl-∆PDZ clones in the limbs will be rounded like Figure 11B unlike the columnar clones depicted in Figure 6. Such results would confirm the hypothesis that Wnt/PCP signaling promotes cell outgrowth.

**DISCUSSION**

Members of the FGF family and Wnt5a are co-expressed in many tissues that extend along an axis. Therefore, these studies were aimed to further define the general mechanisms for outgrowth and morphogenesis in vertebrate embryos as a whole. In the limb, we found that Wnt5a expression was activated by FGFs, and Wnt5a was not sufficient to induce a strong migratory behavioral response. However, we were unable to determine if Wnt5a caused limb mesenchyme cells to orient in a specific manner. This does not rule out the possibility that mesenchyme cells are polarizing due to directional cues from Wnt5a. Analysis of single cells may not be sufficient to visualize what is truly happening in vitro. Videos created at a lower magnification with a higher percentage of transfected cells may very well reveal results similar to Wallingford, et al. clearly depicting stabile actin rich filopodia orienting along a specific axis.

Studies in Xenopus have shown that filopodia (composed of actin microfilaments) are polarized 90º relative to the axis of outgrowth and are critical for mediolateral convergence (Wallingford et al., 2000) whereas mitotic spindles are aligned parallel to the axis of outgrowth.
and are essential for anteroposterior extension of the embryonic axis in zebrafish (Gong et al., 2004). If limb outgrowth is driven by similar convergent extension movements, then we would expect limb mesenchyme cells to be oriented orthogonal to the anteroposterior axis and the mitotic spindle to be aligned with the proximodistal axis (toward the AER). In trial #1 (Appendix E), several cells around the Wnt5a-expressing bolus pointed toward the bolus. In trial #2 (Appendix G), cells around the bolus were primarily parallel to the bolus, almost forming a swirl pattern. Without positional information from closely neighboring cells, we cannot determine if the cells are undergoing randomized cell movements, orienting orthogonal to the bolus, or directly migrating toward the bolus.

It is possible that these in vitro studies are not truly recapitulating in vivo events. For example, as stated above, Wnt5b is required in addition to Wnt5a to mediate the effects of FGF signaling. Additionally, FGF may be activating a parallel pathway to Wnt/PCP that is required to orient cells for convergent extension. Therefore, an exogenous source of Wnt5b, FGF, and/or other unidentified molecules may be required to cause cells to polarize as they would in the embryonic limb.

It is important to note, that Wnt5a and/or Wnt5b outgrowth defects do not phenocopy mice lacking components of the core PCP machinery (i.e., mutants lacking Celsr1(Flamingo), Vangl2, Dvl1/ Dvl2 double knockout mice; see below). It has been suggested that Wnt5a may signal through Ror (a member of the receptor tyrosine kinase family), which is required for cell migration and cell polarity in the nematode Caenorhabditis elegans (Kim and Forrester, 2003). Additionally, Ror1/2 double mutants almost phenocopy Wnt5a mutants. Thus, Wnt5a may directly signal through Ror and mutations in the Wnt/PCP pathway may have no effect on cell behavior.

Although it is not clear whether Wnt5a signals through PCP or another pathway such as Ror, Wnt5a is still considered a likely candidate capable of polarizing limb mesenchyme, directing outgrowth of the limb.
METHODS

Preparation of FGF-4 Beads

Recombinant Human FGF-4 protein was obtained from R & D Systems and reconstituted to a final concentration of 1000 μg/ml in sterile D-PBS containing 0.1% BSA. Half of the protein was diluted to 250 μg/ml. To prepare FGF-4 beads, heparin beads (Sigma) were rinsed twice with PBS. An aliquot of rinsed beads was transferred to a separate tube and all PBS was removed by aspiration. Using a clean pipette tip, 30-40 beads were removed and placed into a two μl drop of FGF-4 protein in a 0.2 ml tube. Beads were incubated in protein overnight at 4ºC.

Implantation of Beads into Chick Limbs

White Leghorn chicken eggs were incubated for either 55, 70, or 85 hours to obtain stage 14-15, 18-20, and 21-23 eggs respectively (Hamburger and Hamilton). Eggs were windowed as described by Van Raay and Stark (2002). A small incision was made into the lateral plate mesoderm (coelom), proximal limb bud, or distal limb bud with a finely pulled glass pipette. After overnight incubation in protein or D-PBS with 0.1% BSA, beads were resuspended in 100 μl of sterile D-PBS. Beads were transferred to eggs using a micropipettor and manipulated into place using sterile forceps. After 7-24 hours of incubation in a humidified incubator at 37ºC, embryos were collected and fixed in 4% formaldehyde in PBS overnight. Wnt5a expression was examined using a standard in situ hybridization protocol.

Primary Limb Mesenchyme Cell Culture

White Leghorn chicken eggs were incubated for 85 hours and embryos were collected in sterile D-PBS. Wing and leg buds were removed with sterile forceps, collected into a conical vial, and rinsed twice in D-PBS. D-PBS was replaced with 2% Trypsin in D-PBS and limb buds
were incubated for 35 minutes at 4°C. After trypsinization, limb buds were rinsed twice in D-
PBS. Ectoderm was dissected from the limb bud and limb mesenchyme was collected into
DMEM supplemented with 15% serum, non-essential amino acids, nucleotides, β-mercapto-
and pen/strep glutamine. Limb mesenchyme was triturated vigorously to dissociate the cells. Once a
single cell suspension was created, cells were centrifuged for 15 minutes at 1500 rpm. The
resulting cell pellet was resuspended to a final concentration of two million cells per ml and
used as described below.

**Preparation of Limb Mesenchyme for Live Cell Imaging**

For live cell imaging, two six cm dishes each received seven million limb mesenchyme
cells and were incubated at 37°C in 5% CO₂. After overnight incubation, cells received fresh
media and were transfected with plasmids expressing tubulin-GFP or actin-GFP fusion proteins.
Lipofectamine 2000 (Invitrogen) and its suggested protocol were used over
Lipofectamine (Invitrogen) and Trans-it Jurkat (Mirus) after preliminary testing.
After overnight incubation with liposome complexes, actin-GFP and tubulin-GFP transfected cells were collected and
combined. Two million cells were plated onto a Delta-T dish (Bioptechs) and were incubated with or without a cell bolus (Fig. 16). This
incubation period proved necessary for cell survival during live imaging. Additionally, it
allowed cells to become confluent and spread out their cellular processes.

L-cell and Wnt5a expressing L-cell lines (ATCC) were used to create cell boluses. L-
cells were cultured to confluency in DMEM supplemented with 10% FBS, non-essential amino
acids, nucleotides, β-mercapto, and pen/strep glutamine. Cells were collected and concentrated to
seven million cells per ml. 30 μl drops were dispensed onto the lid of a cell culture dish, which was inverted over a dish containing 10 ml of D-PBS. Hanging drops were incubated 24 hours prior to simultaneous plating with limb mesenchyme as described above. A control L-cell line was also used in live imaging experiments.

**Live Cell Imaging**

After overnight incubation with cell boluses, cell culture media (described above) was replaced with DMEM containing 25mM HEPES buffer lacking sodium bicarbonate supplemented with 10% FBS, non-essential amino acids, nucleotides, β-mercaptoethanol, and pen/strep. In order to examine the movement of cells relative to Wnt5a cell boluses, cells were imaged live using Slidebook 4.1 (Olympus). Coordinates from this program were plotted in Microsoft Excel. The Delta-T dish was maintained at a constant temperature of 37°C during live imaging experiments lasting up to 12 hours.

**Migration Assay and Flow Cytometry**

Quantitative cell migration assays were performed with ThinCert cell culture inserts (Griener), a disposable Boyden chamber assay. Limb mesenchyme cell cultures were prepared as discussed above. Supernatants were collected from Wnt5a producing and non-altered L-cells as described by ATCC. 600 μl of Wnt5a, L-cell, or fresh cell culture media was added to each well of a 24-well plate. In one data set, L-cells were not filtered from the media and had to be distinguished from the limb mesenchyme populations with gating during flow cytometry analysis (see Appendix B).

ThinCerts were placed into each well and 200,000 limb mesenchyme cells were added inside the insert. Cells were incubated overnight at 37°C in 5% CO₂. After incubation, any cells that had migrated through the filter were trypsinized from the bottom of the ThinCert and collected. Additionally, cells that had detached from the filter and plated onto the bottom of the well were trypsinized and collected. Just prior to flow cytometry analysis with FACSCanto, two
μl of propidium iodide (10 μl/1 x 10^6 cells, BD Pharmingen) was added to each sample tube. Cell counts of live and dead cells were obtained.

**Transfections, Viral Growth, Titrations, and Concentration**

The replication incompetent viral genome RISAP was a gift from the Kardon lab (University of Utah) as was the VSV-G (vesicular stomatitis virus G) packaging plasmid. RISAP is an abbreviation for Replication Incompetent avian retrovirus with a Splice acceptor site from the src gene and the immunohistochemical marker placental Alkaline Phosphatase. Therefore, PLAP (placental alkaline phosphatase) staining was used to detect transduced/infected cells.

The plasmid housing mSpry2, GenBank accession number AF176905, was obtained from the Martin Lab (Department of Anatomy and Program in Developmental Biology, School of Medicine, University of California, San Francisco, CA 94143-0452, USA). Spry2 was first cloned into SLIRES, an adaptor vector with the internal ribosomal entry site (IRES) of the encephalomyocarditis virus (Chen, et al. 1999). SLIRES–mSpry2 was then cloned into the pRISAP vector.

DF1 chicken fibroblast cells were co-transfected with 10 μg of VSV-G coat and 10 μg of RISAP or RISAP-mSpry2 as described by Chen et al. 1999 using Superfect reagent (Qiagen). Supernatants containing pseudotyped viral particles were harvested for three days (the third collection was obtained after a two day incubation) and concentrated for two and a half hours at 25,000 rpm using a Ti70 rotor in an ultracentrifuge. Viruses were titrated using standard protocols by staining for PLAP activity. However, viruses were concentrated to such a degree that a discrete titer was not determined. It is suggested that this experiment be performed with viral titers of 1 x 10^7 cfu/ml (colony forming units) when titered upon DF1 cells.
**PLAP Staining**

PLAP staining was used to detect RISAP infected cells. 24 hours after initial infection cell culture media was removed and cells were rinsed twice with PBS. Cells were fixed with 4% formaldehyde in PBS at room temperature for 15 minutes and then rinsed three times with PBS. Pre-warmed PBS was placed onto the cells and each multi-well plate was incubated at 65°C for 45 minutes to inactivate endogenous alkaline phosphatase. PBS was removed and replaced with a minimal volume of BM Purple. Cells were incubated at room temperature for 30 minutes and then moved to 4°C for overnight staining. BM Purple was removed and cells were rinsed with PBS twice. If needed, stained cells were stored in PBS at 4°C.

**In Vivo Injections and Staining**

White Leghorn chicken eggs were incubated for 55, 70, or 85 hours to obtain stage 14-15, 18-20, and 21-23 eggs respectively (Hamburger and Hamilton). Eggs were windowed as described by Van Raay and Stark (2002). 30 μl of virus was mixed with two μl of FastGreen and back-loaded into a finely pulled glass capillary needle. Using a picospritzer, the viral solution was injected into either the lateral plate mesoderm (coelom) or limb bud. Eggs were incubated for 48 hours in a humidified incubator at 37°C, embryos were collected and fixed in 4% formaldehyde in PBS. Embryos were then washed three times and PLAP staining was performed as described above.
APPENDIX A

Flow cytometry analysis following Boyden chamber assay. A & B Number of living cells in a 60 μl sample. C & D Percentage of cell death in a 60 μl sample. *p < 0.05.
APPENDIX B

A & C Scatter plots of flow cytometry analysis indicating cell count and granularity. A = Wnt5a and L-cell medias were filtered prior to Boyden chamber assay, and only limb mesenchyme cells are depicted (Trial # 2). C = Trial #1, Wnt5a and L-cell medias were NOT filtered prior to Boyden chamber assay and limb mesenchyme and L-cell populations are depicted. Only limb mesenchyme was gated for propidium iodide analysis. B & D Propidium iodide histograms used to gate living and dead cells.
Limb Mesenchyme (L-cell bolus)

Orientation & direction of cells relative to an L-cell bolus. Cells visualized for 12 hours. Arrows indicate direction of cell migration. Lines represent cell orientation when direction of migration was obscure. See Appendix D for explanation of dashed circle.
Individual pictures of cells encompassed in dashed circle in APPENDIX C. Each image representative of cell at final time point of capture near bolus (prior to any cell death).
Orientation & direction of limb mesenchyme cells relative to an L-cell bolus expressing Wnt5a. A culture of two million cells was visualized for 12 hours. Arrows indicate direction of cell migration. Lines represent cell orientation when direction of migration was obscure.
APPENDIX F

Individual pictures of cells encompassed in dashed circle in APPENDIX E. Each image representative of cell at final time point of capture near bolus (prior to any cell death).
APPENDIX F
APPENDIX G

Limb Mesenchyme (Wnt5a L-cell bolus)
Trial #2

Orientation & direction of cells relative to an L-cell bolus expressing Wnt5a. A culture of three million cells was visualized for 12 hours. Arrows indicate direction of cell migration. Lines represent cell orientation when direction of migration was obscure.
Individual pictures of cells encompassed in dashed circle in APPENDIX G. Each image representative of cell at final time point of capture near bolus (prior to any cell death).
APPENDIX I

List of Abbreviations

**AER** = apical ectodermal ridge

**Cre<sup>ER</sup>** = Cre fused to a modified estrogen receptor

**Dvl** = Dishevelled

**E11.5, etc.** = embryonic day 11.5

**FGF** = fibroblast growth factor

**Frizzled/LRP** = LDL-receptor-related protein

**GFP** = green fluorescent protein

**IRES** = internal ribosomal entry site

**JNK** = Jun N-terminal kinase

**Lef/TCF** = lymphoid enhancer factor-1 transcription factor

**mSpry2** = murine Sprouty-2, an FGF receptor antagonist

**PLAP** = placental alkaline phosphatase

**Rho kinase** = Rhodopsin kinase

**RISAP** = Replication Incompetent avian retrovirus with a Splice acceptor site from the src gene and the immunohistochemical marker placental Alkaline Phosphatase

**YFP** = yellow fluorescent protein


EDUCATION

M.S. in Physiology and Developmental Biology, GPA 3.96  
*Brigham Young University, Provo, UT*  
Thesis: “Fgf4 and Wnt5a/PCP signaling promote limb outgrowth by polarizing limb mesenchyme”  
*August 2006*

B.S. in Zoology, GPA 3.85  
*Brigham Young University, Provo, UT*  
Emphasis: Human Biology  
*April 2004*

AWARDS, FELLOWSHIPS, SCHOLARSHIPS

- BYU Graduate Studies Fellowship  
  *2005 – 2006*
- Graduate Student Teaching Assistantship, Brigham Young University  
  *2004 – 2006*
- BYU Cancer Research Fellowship  
  *2005*
- Graduate School Scholarship, Brigham Young University  
  *2004 – 2005*
- Brigham Young Scholarship, Brigham Young University  
  *2001 – 2004*
- Brigham Young Scholarship, Brigham Young University  
  *2000 – 2001*
- Robert C. Byrd Honors Scholarship, Utah State Office of Education  
  *2000 – 2004*

RELATED RESEARCH & CLINICAL EXPERIENCE

*University of Pittsburgh Cancer Institute, Pittsburgh, PA*

**Research Specialist and Lab Manager** for Dr. Hideho Okada  
*2006*

I recently accepted a position at this accredited research pavilion and will be investigating effective immuno-gene therapy strategies for malignant brain tumors. These tumors possess dismal prognosis in humans, and therefore, there is a strong demand by society for the development of effective therapeutic modalities based on timely translation of rapidly advancing basic molecular immunology.

*Brigham Young University, Provo, UT*

**Master’s Student** in the lab of Professor Jeffery R. Barrow  
*2004 – 2006*

My master’s project required that I complete the following specific aims:

- **Specific aim #1:** I will test the hypothesis that FGF signaling activates the expression of Wnt5a in gradient fashion by transplanting beads soaked in increasing concentrations of Fgf4 into the flank or the proximal limb bud of chick limbs. The expression of cWnt5a and Wnt11 will be examined using in situ hybridization.
- **Specific aim #2:** I will test the hypothesis that Fgf and Wnt/PCP signaling are both necessary and sufficient to polarize limb mesenchyme cells by examining the movement of cells and expression of polarity markers relative to beads soaked in Fgf4 or Wnt5a in a two dimensional in vitro limb mesenchyme model. I will also generate clones of cells in chick limbs that lack the ability to respond to Wnt/PCP and Fgf signals and examine their behavior relative to control clones.

**Research Assistant** at BYU’s Center for Instructional Design (CID)  
*2003 – 2005*

Under the direction of Professor Robert E. Seegmiller and project managers at the CID, I helped develop a product to be used in embryology and anatomy courses concerning embryonic brain development. My primary responsibility at the CID was to gather and study research materials concerning the human embryonic brain. These materials were used to create an interactive CD containing a 4D model of embryonic brain development. Once I familiarized myself with these intricate developmental processes and the particular shape of the brain at various stages, I was able to critique three-dimensional images.
created by graphic designers. I ensured correct sequencing and appearance of structures associated with the embryonic brain. In addition to my work on the developmental movies, I wrote narratives to accompany two instructional videos, constructed testing questions, and provided brief descriptions of brain and spinal defects to be included in the interface. I also helped design interactive features of the CD (cited below under publications).

Undergraduate Researcher in the lab of Professor Michael Stark 2004
As part of a directed research course, I electroporated green fluorescent protein (GFP) expressing vectors into the neural tube and limb bud of developing chick embryos. After 24 hour incubation, I photographed any cells expressing GFP and then performed whole mount in situ hybridization to visualize Fgf4 expression.

Stanford Hopkins Marine Station, Pacific Grove, CA

Undergraduate Researcher for Professor Lee F. Braithwaite 2003
At the Hopkins Marine Station, I dissected the microscopic venom apparatus from the rocky shoreline sea snail Conus californius. I digitally photographed specimen as well as the general dissection process. I also performed experiments with C. californicus to determine if the snail when the snail used its venom apparatus and which endogenous prey it preferred.

Carbon High School, Price, UT

Health Professions Student Assistant/Shadow 1999-2000
While in high school, I participated in a community outreach program. The goal of this program was to allow students interested in the medical field to assist and shadow various professionals including a hospital dietician, several radiologist technicians, two physical therapists, multiple dentists, an LPN, an RN, a nursing care manager, a phlebotomist, and a hospital lab technician. During this time I was able speak with patients during their treatments, help plan patient menus, develop x-rays for the radiologist technicians, learn how to draw blood, and observe all of these careers in detail as I visited each for two weeks (two hours a day).

LABORATORY SKILLS

Basic Cloning Techniques
• Site-directed mutagenesis
• Restriction enzyme digests and ligations
• Chemical and electrical bacterial transformations
• Mini/midi/maxi preps
• Agarose and polyacrylamide electrophoresis

Cell Culture
• MEF and ES cell culture maintenance
• ES cell derivation from harvested blastocysts
• ES cell electroporation
• Primary limb mesenchyme cell culture derivation and maintenance
• Cell transfection using liposome reagents
• Live cell imagery using Slidebook 4.1

Manipulation of the Mouse Embryo
• Mouse colony maintenance (e.g. genotyping, breeding, weaning, hormone injections, etc.)
• Dissection of mouse embryos at various embryonic stages
• Chimeric mouse/embryo derivation (i.e. flush morula from oviducts of superovulated mice, de-zone morula, aggregate de-zoned morula with ES cells, allow for overnight incubation, implant chimeric blastocyst into pseudoepregnant mice using sterile surgical technique)
**Manipulation of the Chick Embryo**
- Chick egg windowing and India Ink staining
- In ovo electroporation and diI labeling
- Viral infections using replication competent and replication incompetent viruses

**Other Techniques**
- In situ hybridization and immunohistochemistry (sectioned tissue or whole-mount embryo)
- Confocal microscopy
- Some flow cytometry

### TEACHING EXPERIENCE AT BRIGHAM YOUNG UNIVERSITY

**Teaching Assistant** to Professor Jeffery R. Barrow in “Developmental Biology”  
*Fall 2005*  
Organized course information on Blackboard website; Lectured in bi-weekly review sessions; Graded midterm and final exams

**Student Teacher** for Professor Sterling Sudweeks in “Advanced Physiology Laboratory”  
*Winter 2005*  
Overviewed pertinent conceptual material previous to ‘wet’ or computer-simulated lab using PowerPoint or chalkboard demonstrations; Trained students in lab protocols and computer programs; Graded weekly lab reports

**Teaching Assistant** to Professor Robert E. Seegmiller in “Human Embryology”  
*Fall 2004*  
Collaborated on curriculum development; Prepared and revised PowerPoint presentations; Lectured in bi-weekly review sessions; Graded all written work (i.e. midterms, final, and course project); Organized course information on Blackboard website

**Teaching Assistant** to Professor Richard Neitzel Holzapfel in Doctrine & Covenants  
*2002 – 2003*  
Collaborated on curriculum, syllabus, and exam development; Researched photographic archives and text for course lectures; Prepared all PowerPoint presentations; Met with students upon request to discuss course material

**Teaching/Research Assistant** to Professor Richard C. Holzapfel in “World Civ I”  
*2001 – 2002*  
Collaborated on curriculum, syllabus, and exam development; Prepared all PowerPoint presentations; Graded ESL student projects; Met with students upon request to discuss course material; Maintained circulation and organization of prospective articles for the Religious Educator journal; Wrote and sent memos to those who provided submissions as well as the peer review committee; Organized office contact list in Microsoft Outlook

### PRESENTATIONS, PUBLICATIONS, AND PAPERS

Presentation: BYU Department Seminar, “Creating Multimedia for the Classroom: 3D animations, interactive CD’s, etc.” September 2005.


Poster: Annual Meeting of the Society of Developmental Biology, “Inducible Clonal Analysis,” Keri L. Low¹, Jason R. Mayberry¹, Michael Rule², Andrew P. McMahon², Jeffery R. Barrow¹² (¹Department of Physiology and Developmental Biology, Brigham Young University, Provo, UT 84602; ²Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138). July 2005.

## PROFESSIONAL AND LOCAL SOCIETIES

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## SCIENCE COMMUNITY SERVICE

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