2008-08-14

Determining the Role of Wnt5a Signaling in Embryonic Limb Outgrowth via Clonal Analysis

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DETERMINING THE ROLE OF WNT5A IN EMBRYONIC LIMB OUTGROWTH VIA CLONAL ANALYSIS

by

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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 2008

BRIGHAM YOUNG UNIVERSITY

GRADUATE COMMITTEE APPROVAL

of a thesis submitted by

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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, Chair
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BRIGHAM YOUNG UNIVERSITY

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ABSTRACT

DETERMINING THE ROLE OF WNT5A IN EMBRYONIC LIMB OUTGROWTH VIA CLONAL ANALYSIS

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The exact mechanisms that regulate limb outgrowth the mouse embryo are unknown. Although there are several models, we favor a hypothesis where cells become polarized by signals secreted from the AER which orient their cell migration and/or divisions causing limb outgrowth. Clonal analysis has provided a mechanism to study cell behavior. We have generated a targeting construct containing the Fgf8 inhibitor, Sprouty2, in order to generate mutant clones for behavioral analyses in the limb.

In order to more effectively study clonal behavior we report the modification of a novel clonal analysis approach, exo-utero surgery. We have modified, enhanced and proven that this technique can be used successfully in mouse embryos in which we directly apply 4-OHtamoxifen to the limb to induce YFP or β-gal reporter genes in limb mesenchyme. Using this method, we can closely control the timing and location of the

induced clones and observe cell behavior during embryonic limb development.

Phenotypes of Wnt5a$^{-/-}$ and Ror2$^{-/-}$ exhibit shortened limbs suggesting they function in a similar pathway. Wnt5a and Ror have been found to "colocalize" in the growing limb bud and have also been shown to bind *in vitro*. Here we show preliminary results about Wnt5a and Ror2 *in vivo* association by immunoprecipitation of limb bud extracts.

ACKNOWLEDGEMENTS

I would like to thank my mentor and friend Dr. Jeffery Barrow. His excellent tutelage and guidance throughout this process has been a blessing. He is a great teacher who was there helping every step of the way. I appreciate his patience and understanding when I made mistakes. I would also like to thank my committee members Dr. Stark and Dr. Bridgewater for all their support and help with my project.

I would also like to thank all the students in the lab who have so generously devoted time and work to this project. I would like to thank Enos Heinzen, Jared Barrott, Jed Kendal, Jaime and Tyler Mayo, Mike Rice, Adam Hills, Melina Jones, Erin Cox Jubal Stewart, Kate Kmetzsch, Jeanette May, Rick Otto, Chris Tobler and Corey Tingey.

My family has been an interested and supporting force. They have shown a daily interest in unfamiliar studies, experiments and results. I especially appreciate my parents, both sets. Their advice to continue my education, their support and love gave me the strength to go forward.

Most importantly, I would like to thank my wonderful husband for all his support and love through this journey. He was always there to listen to my problems and offer comfort and advice and to continue to push me forward. I am thankful for his example, which has made me a better student. I could not have done this without him.

“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

("Be Ye Clean," Ensign, May 1996, 48).

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Introduction

Understanding how our bodies develop ultimately increases the knowledge and understanding of how our adult bodies function. Developmental biology is an increasingly important area of study with research having only scratched the surface of many complex systems in a developing organism. Many studies focus on cellular signals, cascades and cell behavior. Others study organogenesis and the development of entire systems (e.g. vasculogenesis). Of great interest to Dr. Jeffery Barrow's lab and myself is to determine the genetic basis whereby a structure develops in three dimensions. An excellent model to study 3D development is the vertebrate limb. Furthermore, one of the more common birth defects is limb anomalies occurring at ~0.7 to 1 per 1,000 births (McGuirk et al., 2001). Long-standing dogmas of the mechanisms of limb outgrowth have recently been challenged and many new hypotheses and ideas are surfacing.

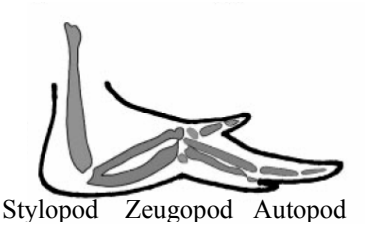

Fig.1. Segments of Limb (Capdevila et al., 2001)

To understand limb development, one must understand the components of the limb. The limb is divided into three different segments: the stylopod (the most proximal element), zeugopod and autopod (the most distal element) also known in the human as the humerus, radius and ulna, and digits, respectively (Fig. 1). The three dimensional axes of the limb are defined as the following: the humerus is the most proximal element and the digits are the most distal elements, the back of the hand is dorsal and the palm is ventral. Anterior designation is given to the first digit of the hand (the thumb) and posterior designation is given to digit 5 (the pinky). Research has shown that the limb grows in a proximal to distal fashion, the stylopod forming first and the

autopod forming last. The mechanisms whereby morphological changes are generated along these axes are called patterning. The limb begins as an outgrowth of the lateral plate mesoderm originating from the main trunk of an embryo. Limbs begin to appear around embryonic day nine (E9) in the mouse embryo. Although many signaling pathways have been identified that are required for patterning the limb in three dimensions the mechanisms whereby they do so remain poorly understood.

Previous Studies

John W. Saunders, Jr. began limb experiments in 1948 when he removed a ridge of ectodermal tissue called the apical ectodermal ridge (AER) from the distal margin of the limb bud in a chick embryo (Saunders, 1948). The AER is located at the distal end of the limb and divides the dorsal/ventral axis. Saunders found that early AER removal resulted in severe truncation of limb outgrowth. Later removal of the AER allowed progressively more distal outgrowth of the limb, providing evidence the limb grows in a proximal to distal fashion (Saunders, 1948). These beginning studies revealed that the AER is necessary for regular limb development. Exactly how the AER regulates the distal outgrowth of the limb is the focus of current research.

One early theory proposed that the AER secreted different signals at different times in development to generate the different segments of the limb. To test this idea, ectoderm recombination experiments were performed to see if the signals were changing, i.e. that an older AER would be producing different signals than a younger AER and would alter limb segments if transplanted. However, ectoderm recombination experiments had no effect on skeletal patterning (Zwilling, 1955). Fgf beads implants and rescue experiments by other groups help elucidate the role of the AER (Cohn and

Tickle, 1999; Fallon et al., 1994; Niswander et al., 1993). Experiments where one replaced AER with beads soaked in a single Fgf factor rescued and generated a fairly normal phenotype further demonstrating that a single factor is capable of generating all three elements. As the signals secreted by the AER do not change during limb development, there must be another means of creating proximal-distal patterning.

Mesenchyme recombination experiments were then conducted in chicks, where the distal most 300μm of early limb buds (both mesenchyme and ectoderm) was grafted onto older limb buds. These grafted limbs contained duplications of the stylopod and zeugopod (Fig. 2) (Summerbell and Lewis, 1975). Conversely grafting the distal-most 300μm of old limbs (ectoderm and mesoderm) onto young limb buds resulted in limb buds where the graft only gave rise to digits. This experiment led to the conclusion that the age of the mesenchyme dictates the patterning.

Fig.2. Replicated autopod and zeugopod in chick limb after young mesenchyme graft (Summerbell and Lewis, 1975).

All these experiments proved that the AER did not play an instructive role in establishing the PD axis of limb identity but rather, the mesenchyme contained the information needed for patterning and that the AER was sending permissive signals to the mesoderm, i.e. "the AER is not saying 'Become toe,' but rather 'Consult the mesoderm subjacent to you and become the next level (distally) to it!'"(Rubin and Saunders, 1972). Still the question remained as to exactly how the mesenchyme dictates positional fate and

how cells receive positional cues from its environment and correctly interpret and act upon these cues to create patterning.

The "Progress Zone Model" was developed to explain proximal-distal patterning of the limb (Summerbell et al., 1973; Summerbell, 1974a; Summerbell, 1974b; Tickle et al., 1975; Wolpert et al., 1975). It was proposed that the limb is specified in a proximal-distal fashion controlled by an autonomous timing mechanism operating in the 'progress zone' (the distal most 300μm of the limb) containing undifferentiated growing mesenchyme under the influence of the AER. Ultimately, cell fate is determined by internal clock that gives more distal fates the longer the cell resides in the progress zone. The cells that are forced out of the progress zone early, due to proliferation, become the more proximal components while those that remain in the progress zone longer become the more distal components of the limb (Summerbell and Lewis, 1975).

This model has been the prevailing hypothesis for the past 30 years, however there is a lack of evidence for an internal autonomous timing mechanism (Tabin & Wolpert, 2007). In response, the early specification model (Dudley et al., 2002) was developed and proposed that cells are specified early and then expand to generate outgrowth of the limb. However, no markers differentiating the different cells have been found. Many continue to develop models that better explain limb development.

A New Model

We have developed our own model designated the Limb Mesenchyme Recruitment Model. We propose that Fgfs signaling from the AER establish a gradient of a secreted Wnt ligand, Wnt5a, in the limb mesenchyme. Wnt5a has been previously shown to signal in Wnt/PCP (Planar Cell Polarity) pathway, which has been shown to

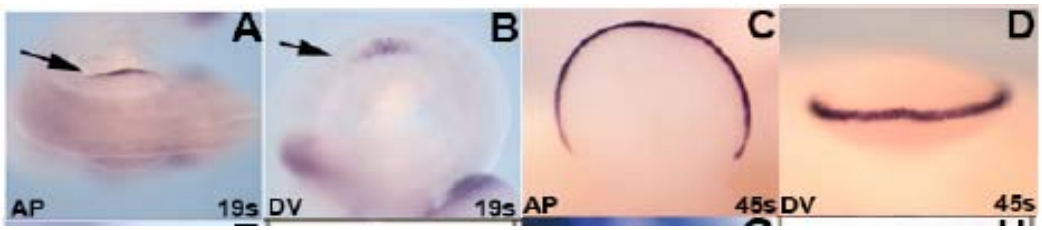

Figure 3. (A,B) The AER (visualized by Fgf8 expression) in 19 somite mouse embryos (arrows) is short along the AP (A) axis and thick along the DV axis (B). (C,D) Conversely at 45 somites, the AER is extended along the AP axis (C) and thin with respect to the DV axis (D) (Barrow unpublished data).

regulate cellular polarity (Barrow, 2006; Kilian et al., 2003; Qian et al. 2007). The Wnt5a expression gradient directs proximal to distal cell migration and/or cell divisions by polarizing the cell which results in limb outgrowth. The size and shape of the AER would then be responsible for patterning the limb. We will test the hypotheses that Fgf and Wnt5a/Ror signaling in the distal limb are necessary for directed migration and oriented cell divisions of limb mesenchymal cells

FGFs

As the bead experiments showed that the AER was secreting the same signal over time, many others have also shown that members of the secreted fibroblast growth factors (Fgf) family are expressed in the AER and are necessary for its growth (Crossley and Martin, 1995; Martin, 1998; Min et al., 1998; Sekine et al., 1999) which has led to experiments studying the molecular mechanism whereby Fgfs regulate limb growth. Many Fgfs function in the development of the limb and the early expression of Fgfs in the limb suggests an important role in directing the initial outgrowth of the forelimb and hindlimb buds (Crossley et al., 1996; Vogel et al., 1996). Fgf10 has been shown to be necessary for outgrowth of the mesoderm of both the fore and hindlimb (Min et al., 1998; Sekine et al., 1999). Other Fgf genes, Fgf4, Fgf8, Fgf9, Fgf17, are expressed in the AER,

however, only Fgf8 is expressed throughout the entire AER (Fig 3) (Lewandoski, et al., 2000; Niswander et al., 1993). Null mutations of Fgf4 (Moon et al., 2000; Sun et al., 2000), or Fgf9 (Colvin et al., 2001), or Fgf17 (Xu et al., 2000) do not affect limb growth while conditional $Fgf8^{-/-}$ knock out results in severe limb defects (Lewandoski et al., 2000; Moon and Capecchi 2000). An Fgf8 and Fgf4 double knockout mouse exhibits an even more severe phenotype with no hindlimbs forming, however, all elements of the three segments in the forelimb form to some extent (Boulet, 2004; Martin, 1998; Sun et al., 2000). These mutant studies indicate an important role of Fgfs in the developing limb. Studies determining the mechanism whereby Fgfs govern limb growth indicate the absence of limbs is not due to reduced proliferation or increased cell death of limb mesenchyme (Li and Muneoka 1999; Saxton et al. 2000; Sun et al. 2002) demonstrating that the AER drives outgrowth by other mechanisms, such as a chemoattractant, rather than simply acting as a mitogen or cell survival mechanism.

Wnt5a

One possible chemoattractant may be the secreted protein, Wnt5a, which is expressed in gradient fashion next to the AER (Fig. 4). Though Wnt5a can signal through the canonical Wnt pathway (Sumanas et al., 2000; Mikels and Nusse, 2006; Gordon and Nusse, 2006), there is accumulating evidence that it functions primarily through noncanonical pathways *in vivo* (Barrow, 2006; He et al., 1997; Ishitani et al., 2003; Krauss et al., 2004; Kühl, 2004; Moon et al., 1993; Sheldahl et al., 1999; Slusarski et al., 1997; Torres et al., 1996; Wallingford et al., 2001; Yamanaka

Fig.4. Wnt5a expression in a WT E11.5 mouse limb bud (Barrow unpublished data).

et al., 2002). The pathway(s) though which Wnt5a signals in the limb are unknown. Wnt5a has been shown to play roles in directed cell movement and polarized cell division, thus it is possible that Wnt5a may mediate cell migration and cell division during limb outgrowth (Gong et al., 2004; Kilian et al., 2003; Qian et al., 2007; Rauch et al., 1997; Westfall et al., 2003).

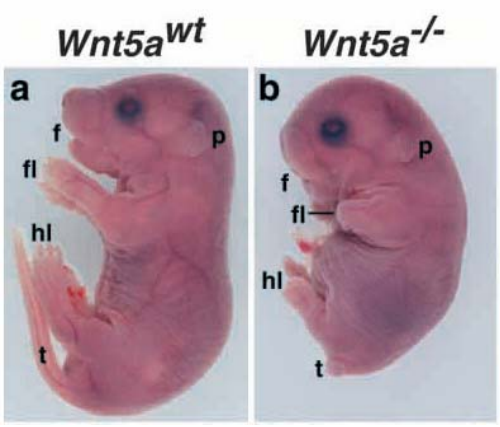

Fig.5. Phenotypic compassion between (a) wildtype and (b) $Wnt5a^{-/-}$ mutant (b) (Yamaguchi et al., 1999).

Analysis of Wnt5a expression reveals a proximal-distal gradient (Gavin et al., 1990; Parr et al., 1993; Barrow unpublished data). Along the dorsal-ventral axis, Wnt5a RNA is found primarily in the ventral half of the limb ectoderm (Gavin et al., 1990). Wnt5a transcripts are found in the limb from E9.5 to 14.5 days (Parr et al., 1993). The highest levels of Wnt5a are found in the apical ectoderm at the distal tip of the limb until E11.5 and then become associated with only the distal mesenchyme from E11.5 to E14.5 days.

The phenotype of a Wnt5a knockout exhibits morphological defects: an open neural tube and shortening in the snout, mandible, limb, genital primordium, and body axis (Fig. 5) (Qian et al., 2007; Yamaguchi et al., 1999). Despite the abnormal phenotype many genes (i.e., Fgf8, Hoxd11-13 and Hoxa13) known to play an important role in the development of these structures yielded normal patterns of expression compared to WT suggesting that Wnt5a acts downstream of these genes. Reduced mitotic activity was observed in the normal domain of Wnt5a and suggested that truncations in the limb may be due to a decreased proliferation of cells in the progress zone (Yamaguchi et al., 1999).

Ror

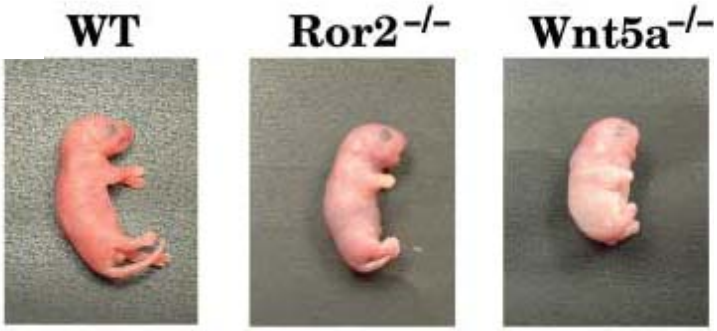

Fig.6. Comparison of developmental phenotypes in wild-type (WT), *Ror2*$^{-/-}$ and *Wnt5a*$^{-/-}$ newborns. Both *Ror2*$^{-/-}$ and *Wnt5*$^{a-/-}$ newborns exhibit dwarfism, facial abnormalities, and foreshortened limbs and tail (Oishi et al., 2003).

A general practice to deduce members of a pathway is to screen for mutants with similar phenotypes. Mice with homozygous loss of mROR, a member of the receptor orphan tyrosine kinase family (RTK), exhibit cardiac septal defects, dwarfism, facial abnormalities, dysplasia of lungs and genitals, and more importantly, short limbs and tail (Fig. 6) (DeChiara et al., 2000; Takeuchi et al., 2000).

Observations of the Ror homolog in C. elegans, cam-1, revealed that it is necessary for oriented cell division, directed cell migration and axon outgrowth (Forrester et al., 1999; Kim and Forrester, 2003). Others have shown that Xror2 in *Xenopus laevis* has roles in convergent extension. It is known that Xwnt5a and Xwnt11 can bind to Xror2 via a Frizzled-like domain in the extracellular region (Hikasa et al., 2002).

Ror receptors belong to the RTK family and consist of a single membrane-spanning region and an intracellular region that contains a conserved tyrosine kinase domain, two serine threonine rich regions separated by a proline rich region, and an extracellular region that contains Immunoglobulin (Ig), Cysteine-rich, and Kringle domains (Fig. 7). Ror proteins contain an extracellular cysteine-rich domain (CRD) that is defined by the presence of 10 conserved cysteines and by several additional conserved amino acids. A similar CRD is found in the seven-pass transmembrane protein Frizzled which is known

the bind Wnts (Forrester, 2002; Masiakowski and Yancopoulous, 1998; Rehn et al., 1998; Saldanha et al., 1998; Xu and Nusse, 1998). Ror receptors are different than the ROR family of nuclear receptors. The presence of the kringle domain is unique to Ror with only one exception and is used as identification (Forrester, 2002).

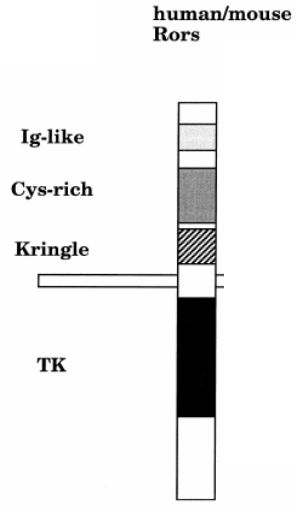

Fig.7. Depiction of Ror receptor domains (Oishi et al., 1999).

Strong expression of Ror2 is detected at E9.5 in the limb (Fig. 8) (Matsuda et al., 2001). Along with the similar mutant phenotypes, it has been recently demonstrated that Ror2 is involved in the non-canonical Wnt5a/JNK signaling pathway. Wnt5a was shown to interact both physically and functionally with Ror2 by binding to its CRD region, suggesting that Ror2 acts as a receptor for Wnt5a to activate the non-canonical Wnt5a/JNK pathway during developmental morphogenesis in mammals. It was also shown that Wnt3a, which appears to signal uniquely through the canonical pathway, lacks the ability bind to the extracellular Ror2-CRD demonstrating the selectivity of the Ror2/Wnt5a interaction (Oishi et al., 2003). Ror2 was shown to be required for Wnt5a-mediated inhibition of canonical signaling, and that the CRD and the intracellular, cytoplasmic domain are crucial for the receptor's inhibitory function (Mikels and Nusse, 2006).

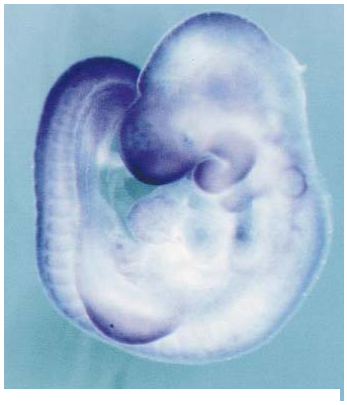
Fig.8. Whole-mount in situ hybridization of E9.5. WT embryos with mRor2 anti-sense probes (Takeuchi et al., 2000)

However, mRor mutants do not exhibit as severe a phenotype as Wnt5a mutants, suggesting that Wnt5a may be

acting through additional receptors. A potential candidate, the Ryk (receptor-like tyrosine kinase) receptor has been shown to mediate Wnt5 signals in Drosophila. Ryk mutant mice exhibit shortening of the face, body axis, and limbs similar to but not nearly as severe as Wnt5a and Ror2 mutants (Halford et al., 2000). It has also been reported that in *Drosophila*, Derailed (Drl), a member of the RYK family, can be a receptor for Wnt5, an ortholog of mammalian Wnt5a (Yoshikawa et al., 2003). RYK family members are atypical receptor tyrosine kinases that lack catalytic activity but have been suggested to transduce signals with other active tyrosine kinase receptors, perhaps in this case, Ror (Yoshikawa et al., 2001). Thus, it is interesting to consider the possibility that RYK and Ror2 also interact physically and functionally, and contribute to form a receptor complex for Wnt5a.

Upon examination of the above data, we hypothesize that the Fgf signals from the AER are inducing nearby cells to secrete Wnt5a, which then binds to the Ror receptor

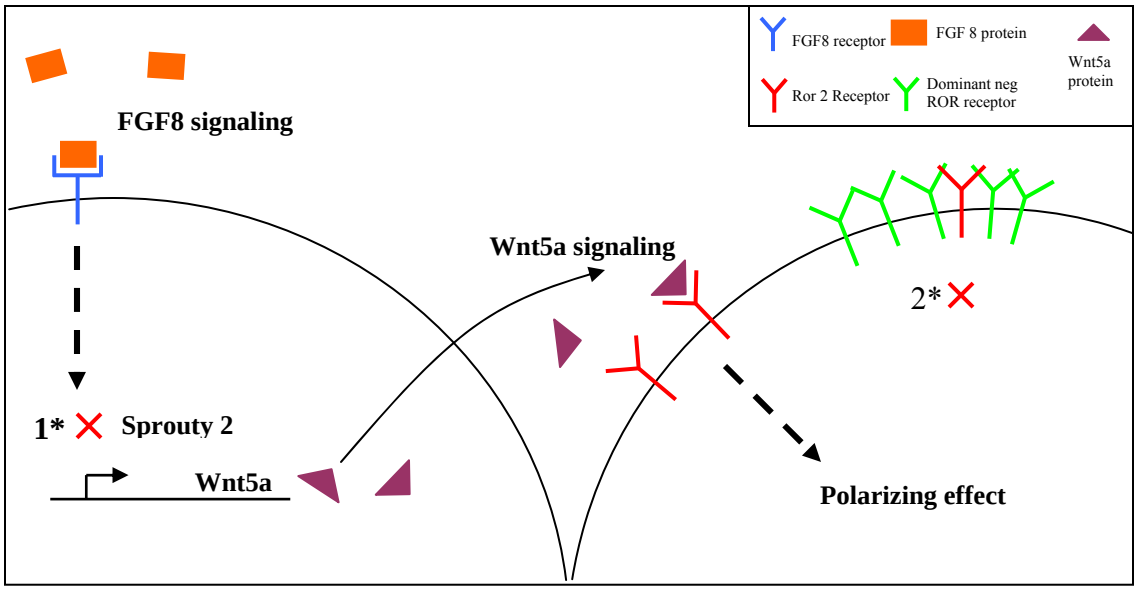

Fig. 9. A putative signaling pathway. Fgf8 induces Wnt5a expression that is secreted to create a proximodistal gradient, polarizing cells through asymmetric activation of the Ror receptor.
*(indicates proposed experiments to disrupt the polarization of cells)

resulting in polarization of the cell (Fig. 9). The polarized cell will now migrate towards the AER and/or undergo oriented cell division in a manner that causes outgrowth of the limb.

Clonal Analysis

Historically, clonal analysis has been a powerful tool in *Drosophila* to analyze the molecular and behavioral characteristics of cells with altered gene activity in a wild type milieu (McGuire, 2004).

Dr. Barrow and colleagues previously developed an approach to induce lineage traceable clones with altered gene activity in the stem cell lines YFP3, containing the inducible reporter genes YFP and β–gal, and the YFP3.1 stem cell line, constitutively active YFP and β–gal reporter genes (Mao et al., 2005). These two embryonic stem cell lines each contain two reporters, YFP (yellow fluorescing protein) and β-gal (β-galactosidase), and have already been homologously targeted to the Rosa26 loci (Fig. 10). The Rosa26 locus drives ubiquitous and constitutive expression of both reporters. The YFP3 line gene expression is dependent on Cre recombinase, a Type I topoisomerase that catalyzes site-specific recombination of DNA between loxP sites. The loxP recognition element is a 34 base pair (bp) sequence comprised of two 13 bp inverted repeats flanking an 8 bp spacer region which confers directionality. Cre recombinase is coupled to a modified estrogen receptor (Cre^{ER}) which is cytoplasmically bound. Cre^{ER} is released from the cytoplasm, or "induced," upon exposure to the drug 4-hydroxytamoxifen (4-OHtamoxifen) (Hayashi and McMahon, 2002; Mao et al., 2005). Injection of low levels of tamoxifen into surrogate mothers bearing the chimeric embryos induces widespread nuclear transport of Cre^{ER}, resulting in the expression of a

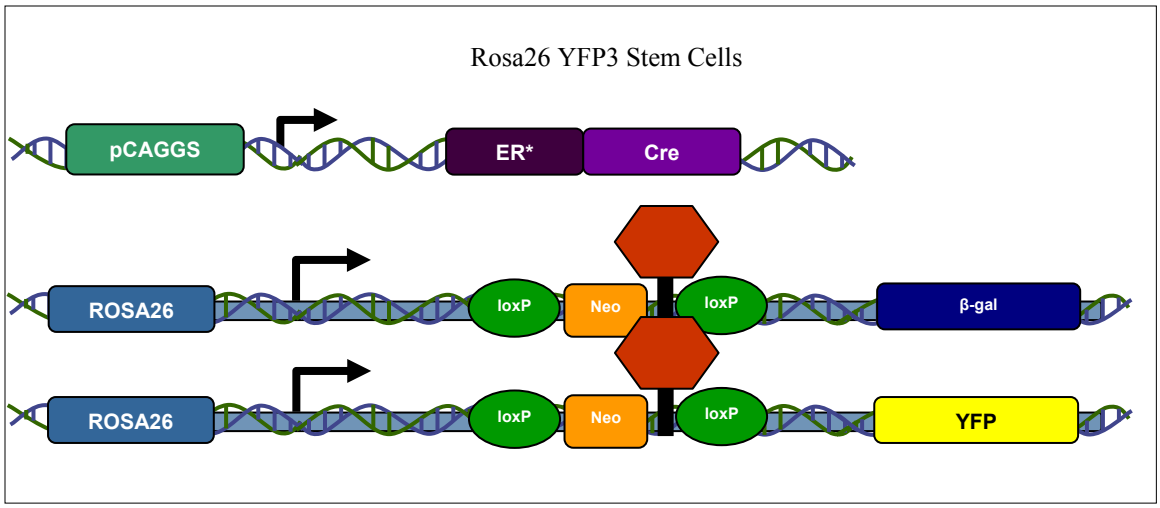

Fig.10. Cre^{ER} inducible recombinase and YFP and β–gal reporter genes targeted to the ROSA26 loci.

fluorescent or colored reporter. The ubiquitous and constitutively active Rosa26 promoter will drive reporter gene expression in induced cells (Srinivas, 2001). This allows one to precisely control the timing of gene/reporter activation. The YFP3 cell line will allow one to perform tamoxifen inducible, clonal analysis in wild type cells in chimeras produced by morula aggregation (Nagy et al., 2003). Reporter genes were induced in control YFP3 mice and cell behavior exhibited linearity in clones suggesting polarized cell migration or division toward the distal end of the limb (Fig. 11).

While we have used this technique with much success, we have found that injection of surrogate mothers with tamoxifen frequently yields chimeras that exhibit a wide range of induction making it difficult to identify isolated clones (Fig. 12). This prevents behavioral analysis of a specific induced clone. Altering the dosage of tamoxifen does not help alleviate ubiquitous induction (Barrow unpublished data). Improved methods of clonal induction must be developed in order to achieve mosaic and sporadic clones.

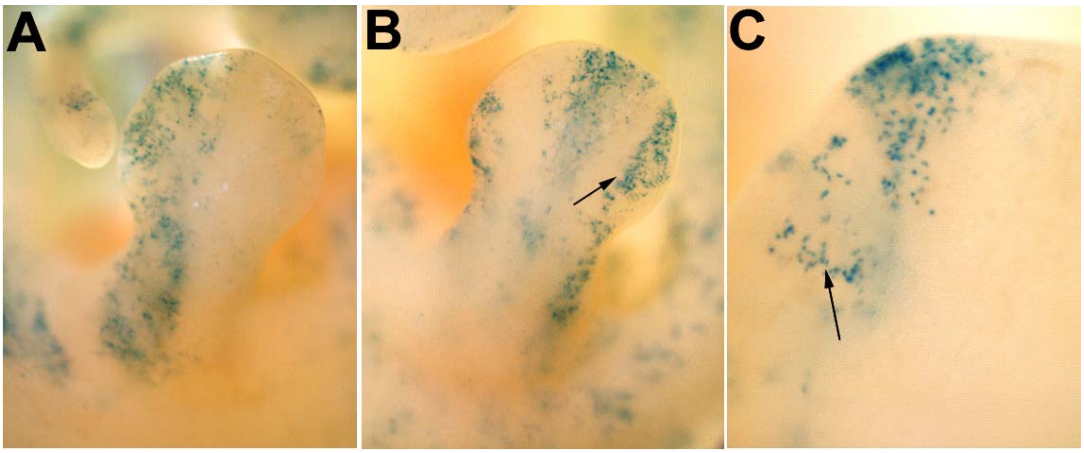

Figure 11. Clonal analysis in mouse embryos chimerized with the tamoxifen inducible reporter cell line, YFP3. Surrogate mothers that were carrying the embryos were injected with 2 mg of tamoxifen, when the embryos were at E8.75. Chimeras were harvested at E11.5 and stained with X-gal. Arrows indicate examples of linear columns of cells (Barrow unpublished data).

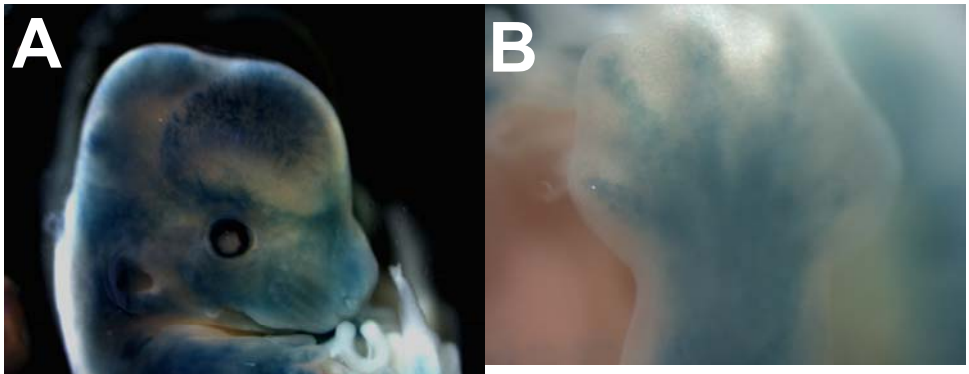

Fig. 12. (A) 24x magnification of a YFP3 tamoxifen induced embryo (B). 38.4x magnigication of a chimeric hand. Note in both pictures widespread induction of clones by tamoxifen.

Proposed Experiments

Specific Aim #1: In order to elucidate how Wnt5a affects cell behavior during limb outgrowth, we propose to block the mesenchyme's ability to produce/respond to Wnt5a. Overexpressing an Fgf inhibitor will prevent cells from responding to the Fgf signals and thus its ability to produce Wnt5a. Cell behavior will be analyzed in the absence of a Wnt5a gradient. By using an Fgf inhibitor instead of removing the AER, we specifically target the Fgf signaling pathway and avoid the possibility of confounding variables due to removing many other proteins secreted/induced by the AER.

Sprouty2 Construct

Sprouty2 has been shown to be an endogenous inhibitor of the FGF pathway through the Ras/MAP kinase pathway that functions by binding to and inhibiting activity of Drk and Gap1 (Casci et al., 1999; Chambers and Mason, 2000; Hacohen et al., 1998; Minowada et al., 1999). An FGF (Fgf 2 or 4 or 8) soaked bead can induce Sprouty2 transcription within minutes in some tissues (Chambers et al., 2000).

An inducible constitutively active construct will be designed to overexpress Sprouty2 with homologies to a locus in the mouse, as well as other factors, to facilitate homologous recombination following electroporation.

Another way to disrupt cell polarization signaling is to block the cell's ability to respond to the Wnt5a signal through its hypothetical receptor Ror2. Overexpression of WT or a ΔCRD mutant Ror2 has been shown to act as a dominant negative (DeChiara et al., 2000; Kim and Forrester, 2003; Mikels and Nusse, 2006; Nishita et al., 2006; Oldridge et al., 2000). Future studies will focus a creating a conditional targeting constructs similar to the Sprouty2, and also analyzed for clonal behavior.

***Specific Aim #2*:** Clonal analysis is the basis for our limb outgrowth studies. However, as shown above, clones are often induced ubiquitously and in a non-localized fashion preventing study of clones. With high induction, it is impossible to determine the correct progenitor/daughter lineage. A novel exo-utero approach will be tested and developed in order to provide greater control of location and amount of clonal induction in the mouse. A reduction in the amount of induced clones will provide clearer evidence as to the origin of clones and their behavior.

Exo-Utero Experiments

We will employ embryonic stem cells (ESC) technology to introduce conditionally regulated reporter genes into mice. Chimeric mice will be generated via morula aggregation. Heterozygous chimeric mice will then be crossed and resulting embryos will be exposed during development and clones will be induced by drug injection. The embryos will then be allowed to develop *in utero* for a period of 24 hours. The progeny of induced clones will retain the transcriptionally active locus causing the reporter genes to be constitutively expressed in all descendant cells, allowing visualization and study of clones after embryo harvesting.

Specific Aim #3: Clarify the Wnt5a/Ror2 interaction *in vivo* during limb development. Co-precipitation studies will be performed through Immunoprecipitation/Western Blot experiments to determine if Wnt5a and Ror2 are actively associated in the limb.

Immunoprecipitation and Western Blot

As Wnt5a has been shown to signal through many different pathways, the pathway through which it functions in the limb remains to be determined (Mikels and Nusse, 2006). The mutant phenotypes of Wnt5a and Ror2 are similar and both proteins have overlapping expression domains. A conclusive experiment to determine if Wnt5a actually binds to the Ror2 receptor in the limb is to perform co-immunoprecipitation studies. Many immunoprecipitation (IP) experiments have been performed on transfected cells but *in vivo* studies have yet to be conducted (Billiard et al., 2006; Hikasa et al., 2002; Mikels and Nusse, 2006; Nishita et al., 2006; Oishi et al., 1999/2003). We propose to conduct two IP experiments: the first using Wnt5a antibody and the second using a Ror2 antibody for immunoprecipitation. Each IP will be tested for co-precipitation. If Wnt5a binds to Ror2 receptors in the limb they should co-precipitate. This experiment will provide the foundation for future studies analyzing Wnt5a signaling via Ror receptors in the limb.

A better understanding of the mechanisms that regulate patterning and outgrowth of the limb are of high interest to the medical community. The developing limb is one of the most powerful research models for examining how organs develop in three dimensions. Hence elucidating general principles in the limb may have broad implications for the development of other structures as well.

Results

Specific Aim #1: Sprouty2 Construct

Sprouty2 DNA from the mouse has been obtained from Gail Martin, UCSF. We have generated a conditional targeting vector containing Sprouty2 gene (Fig 12).

To make a targeting construct, we cloned the protein sequence for Sprouty2 into a series of vectors to generate a Cre-inducible, YFP expressing Sprouty2 allele designed to target to the Rosa26 locus in the mouse. The first attempt to ligate the mSprouty2 fragment from RCAS into pBC SK+/- vector was unsuccessful, thus Sprouty2 was ligated into another plasmid, pGem7z. We designed primers to add a new flanking restriction site, specifically NheI (5') or MluI (3'), to facilitate cloning into the next vector. These primers were used to PCR amplify a Sprouty2 fragment with new restriction sites which was then ligated into pGem-T easy obtained from Sen Wu in the lab of Mario Capecchi, University of Utah.

pBigT was designed with all the necessary elements to create a successful targeting vector including: loxP sites, IRES, neomycin resistance, transcriptional stops and the reporter genes YFP (yellow fluorescing protein) or β-gal (β- galactosidase). Flanking the cloning region, are homologous regions to the Rosa26 locus. These regions allow the construct and locus to undergo homologous recombination upon electroporation into mouse embryonic stem cells (ESC). The Rosa26 locus is constitutively and ubiquitously active and will overexpress the Sprouty2 gene upon induction. pBigT/Sprouty2 was digested to release a large fragment and then ligated into pBCR26. The pBCR26/Sprouty2 vector was linearized with a XhoI digest and prepped for electroporation.

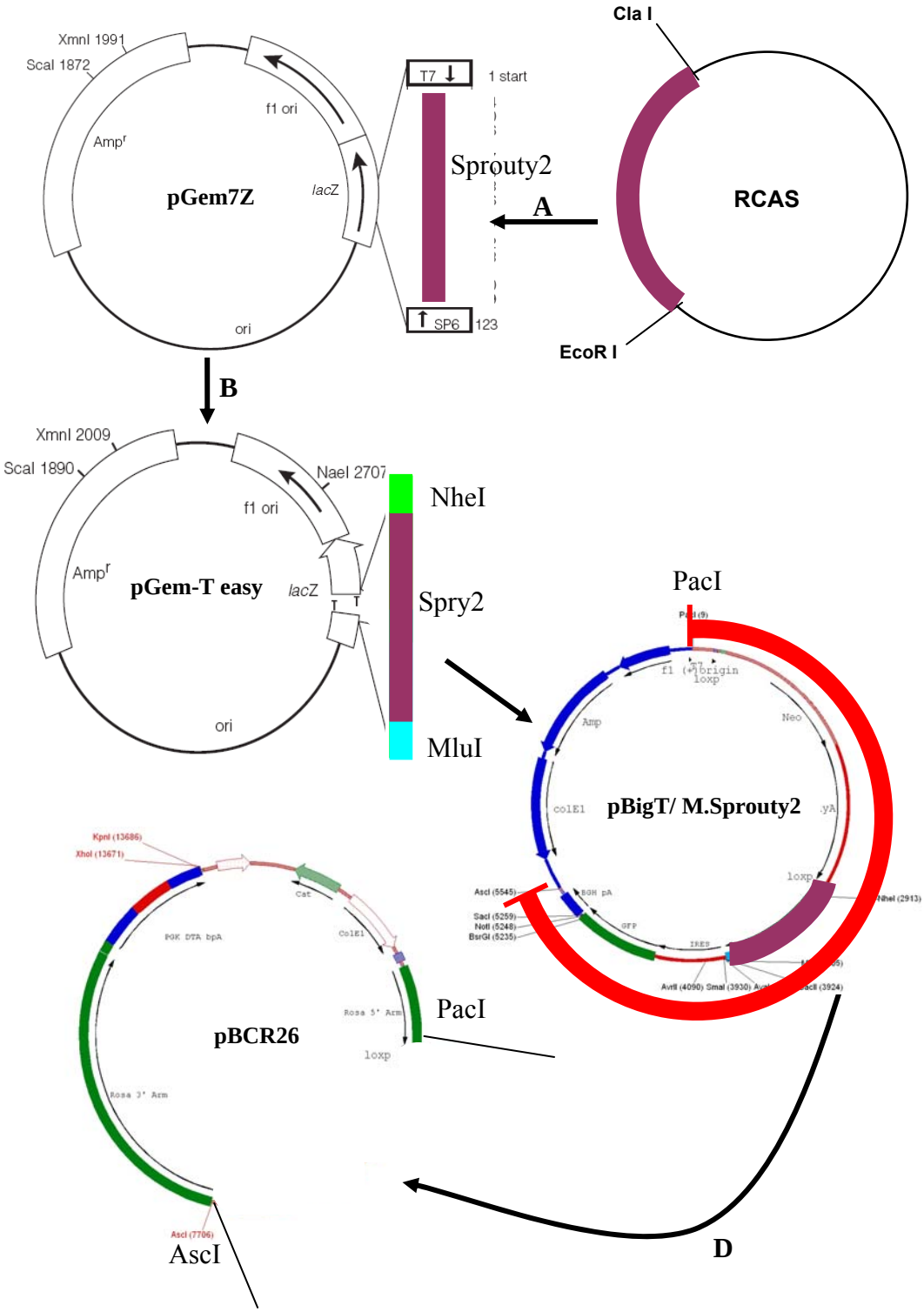

Fig.13. Sprouty2 targeting construct design. (A). Ligation from RCAS to pGem7z (B). PCR amplification with new restriction sites (NheI and MluI) and ligation into pGem-T easy (C). Targeting into pBigT and finally into (D). pBCR26 targeting vector.

Future experiments will electroporate the pBCR26/Sprouty2 construct or future pBCR26/Ror constructs into the YFP3.1 stem cell line. The construct will undergo homologous recombination into the R26 locus due to the homologies added from the pBCR26 vector. The YFP3.1 line expresses the two reporter genes constitutively. Cells that have taken up the new construct will contain new transcriptional stops and will lose reporter selection allowing one to quickly select targeted cells by visualization (Mao et al., 2005). YFP3.1 cells that have incorporated either Sprouty2 or Ror2 constructs will be used to generate chimeric mice via morula aggregation. These targeted cell lines can contribute to all tissues, including the germline (Mao et al, 2005).

We have successfully generated a conditional targeting vector that will allow manipulation and study of induced cells. Inhibiting the Fgf pathway will block any downstream effects it induces in cells. Clonal analysis will allow the study of mutant cells through visualization of YFP or β-gal reporters. Any behavioral changes or lack thereof will be directly related to Fgfs' ability to regulate limb outgrowth.

Specific Aim #2: Exo-Utero Surgery

One of the downfalls of ESC derived clonal analysis is that it is very difficult to control the extent of clonal induction. Regardless of tamoxifen concentration and dosage, often widespread induction results rendering clonal analysis impossible.

Here we report the generation of a novel clonal analysis approach where through exo-utero surgery we directly apply 4-OHtamoxifen to a developmental field of interest to induce YFP or β-gal positive clones. Using this method, we can closely control the timing and location of the induced clone. Further, the injected embryos can develop several hours to enable the analysis of the behavioral consequences of mutant clones. This approach will be especially useful in understanding the role of signaling pathways such as Wnt/Planar Cell Polarity that play critical roles in cell behavior during morphogenetic processes.

Chimera Production

In order to perform the exo-utero surgery, one must have inducible reporters derived from modified ESC. We have generated two YFP3 chimeras through morula aggregation which have been used to established two colonies of mice that contain either YFP or β-gal reporter genes. PCR was used to genotype the different reporter genes and to detect Cre recombinase. As these mice contain only the reporter genes and no Sprouty2 or Ror2 constructs, clonal analysis of these cells will provide the control data and will be used in future studies to compare with overexpression of Sprouty or dominant negative Ror chimeric mice.

We set up YFP/Cre^{ER} X YFP or β-gal/ Cre^{ER} X β-gal crosses for our tamoxifen inducible clonal analysis studies. At E11.5, the pregnant mouse was anesthetized and the

uterus was cut on the anti-placental side to reveal the yolk sacs of the embryos. Using a finely pulled pipet tip, a small amount of 4-OHtamoxifen was injected into the distal portion of the limb bud. After all embryos were injected, they were gently placed back into the abdominal cavity and the cavity was closed. Embryos were generally allowed to develop 24 hrs and then removed and evaluated for reporter gene expression.

We successfully achieved induced clones in the limbs (Fig. 14). The exo-utero surgeries performed on ESC modified chimeras replicate previous data obtained from regular tamoxifen injected mice further proving the efficacy of the exo-utero model.

This technique circumvents the previous problem of wide spread clone induction. Once can successfully choose the developmental field of interest and induce genetically altered cells and study cell *in vivo* cell behavior. Previously, cell behavior was studied by *in vivo* analyses, by DiI labeling in the chick (Sang, 2004), or occasional mosaic clonal

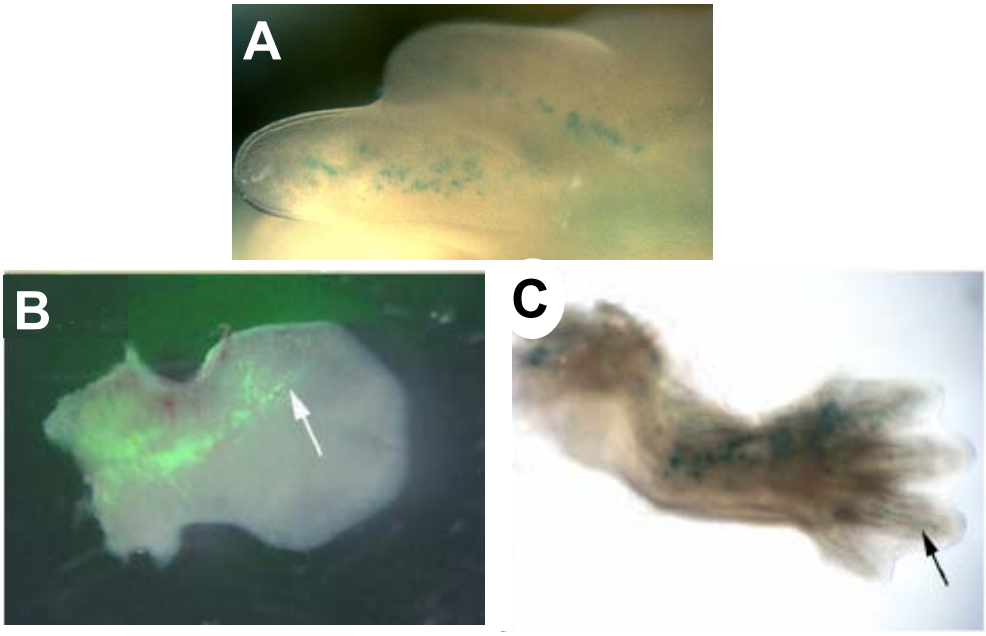

Fig. 14. Mosaic induction via exo-utero sugery of E11.5 YFP or β –gal reporter genes from YFP3 derived mice. Arrows depict linear clones. C 64x D 30x E 38.4X.

induction in the mouse but this method combines the ability to genetically and spatially manipulate cells in a living organism.

Specific Aim #3: Immunoprecipitation and Western Blot

In vitro immunoprecipitation experiments have shown that Ror2 and Wnt5a can physically associate (Hikasa et al., 2002; Oishi et al., 2003) but *in vivo* studies on Wnt5a/Ror2 association have yet to be conducted. However, the expression domains of both proteins overlap in the developing limb, suggesting that Wnt5a may participate in cell polarization by interacting with Ror2. In order to conclude that Wnt5a and Ror2 are interacting in the developing limb, preliminary immunoprecipitation experiments to detect co-precipitation were conducted.

Limb buds were extracted from E11.5 embryos from WT mice (Swiss Webster strain) and homogenized with a cell lysis solution and immunoprecipitated (IP) with a Ror antibody or with a Wnt5a antibody. A Ror antibody or a Wnt5a antibody was used on the blot to detect the IP and also for co-precipitation. Depending on the 1° antibody used, either an anti-rabbit or anti-goat 2° antibody was added along with HRP conjugate, and blots were analyzed by autoradiography.

The blots revealed that Wnt5a and Ror did not co-precipitate. Antibodies detected these proteins in the cell extract but failed to detect them from the IP. These preliminary results show that Wnt5a is not associated with Ror2 *in vivo*. However, these results are preliminary and previous *in vitro* studies highly suggest *in vivo* binding. Modification of the protocol and new antibodies will be used in future experiments to determine if an association does exist.

Discussion

The Sprouty2 construct created will provide a way to disable mesenchymal cell's ability to respond to the polarizing signal Fgf. The polarization and orientated migration/division of mesenchyme dictates limb growth. If Fgf is/or induces the polarizing factor, blocking the ability to respond to a polarizing signal will result in random non-linear cell behavior, opposite the linear clones seen in controls. The Sprouty2 construct, once targeted into ESC, will provide the mechanism to inhibit and identify unresponsive clones to determine if Fgf is initiating polarization of cells.

According to our model, Fgf is inducing a gradient of Wnt5a in the distal most portion of the limb. If Wnt5a is the downstream target of Fgf, blocking Fgf signaling will destroy the Wnt5a gradient preventing cell polarization. However, the Sprouty2 construct will only inhibit a cell's ability to produce Fgf, not its ability to respond to Wnt5a. Thus, if Wnt5a is secreted from other cells, the Sprouty2 expressing cells will still be able to respond to the Wnt5a signal and become polarized. Circumventing this problem, the future dominant negative Ror2 constructs, on the other hand, will render cells incapable of responding to Wnt5a. The Sprouty2 construct will therefore only provide evidence regarding Fgf signaling, not Wnt5a signaling while the dominant negative Ror2 will provide evidence for Wnt5a/Ror signaling. If Sprouty2 overexpression and Ror2 dominant negative expression are found to produce similar results in clonal cell analysis, it will greatly strengthen the evidence that Wnt5a and Ror2 interact to produce polarization of cells in the developing limb.

The modified and enhanced exo-utero technique provides a novel method to avoid the problems associated with ubiquitous tamoxifen induction. Other methods of clonal

analysis, such as those in *in ovo* experiments conducted in the chick, have used DiI labeling to observe cells but has been handicapped by the inability to modify the chick genome (Li and Muneoka, 1999; Sang, 2004) and also creating mouse-chick chimeras by injecting ESC modified mouse mesenchymal cells into the chick limb has shown that mouse mesenchymal cells cannot survive or contribute to the growing chick limb (unpublished data). *In vitro* cell studies present the possibility that they do not recapitulate *in vivo* events. The exo-utero technique is a far superior method than the previous tamoxifen injection for inducing small quantities of clones for behavioral analyses. This technique, in conjunction with modified ESC derived mice, provides and *in vivo* method for cell behavior analysis and will improve future studies involving mutant cells in chimeric mice

The preliminary results from the immunoprecipitation and western blot studies show that Wnt5a and Ror do not associate together in the developing limb bud. Although *in vivo* data has not replicated *in vitro* results, evidence strongly suggests that Wnt5a and Ror provide a major signaling pathway in the developing limb. Further analysis into IP protocols and antibody epitope binding will elucidate potential obstacles and may resolve the conflict between our preliminary results with previous *in vitro* experiments.

The pathway and mechanisms through which Wnt5a functions during limb outgrowth still remain to be elucidated. Future experiments will focus on embryonic manipulation of mutant mesenchyme with control mesenchyme using the Sprouty2 construct and exo-utero technique developed in this study. The studies performed therefore, have provided an essential foundation for future clonal analyses in the mouse.

Materials and Methods

Sprouty2

1a. Digest: Sprouty 2	1b. Digest: pGem7z*	2. Ligation
10µl mSprouty2	16µl pGem7z	0.5µl pGem7z
5µl BSA	5µl BSA	6µl msprouty gel purified fragment
5µl Buffer #4	5µl Buffer #4	2µl 10x ligase buffer
2µl Ecor1	2µl Ecor1	10.5µl H_2O
2µl Cla1	2µl Cla1	1µl T4 DNA ligase
26µl H_2O	20µl H_2O	20µl total
50µl total	50µl total	
2 hour digest in 37°C water bath	2 hour digest in 37°C water bath	Ligate at 16°C degrees overnight
Gel purified (mmBio UltraClean 15 DNA Purification Kit Cat# 12100-300)	Run gel to verify digest is complete *Promega Cat# P2251 or P2371	

3. Transformation
Thaw bacteria on ice. Pipet 5µl of ligation and triturate to mix. Incubate on ice for 30min. Heat shock at 42°C for *exactly* 45 seconds. Incubate on ice for 2 min. Add 500µl LB (IscBioexpress Cat# MBPE-1100) and shake for 30min for Ampicillin resistant vectors and 75 min for Chloramphenicol resistant colonies. Pick white colonies when MCR (Multiple Cloning Region) interrupts lacz sequence, the next day.

4. Grow picked colonies for a mini prep (Fastplasmid Mini Kit 250 prep ISCBioexpress Cat# G-3224-250). Extract DNA and run diagnostic restriction digests to determine size.

5. Sequencing
Spec mini/midi prep DNA. To submit to BYU Sequencing Center, dilute primers to 6ρmoles/µl. Add 2µl primer (12ρmole) and µg of DNA. Fill with ddH_2O up to µl. Submitted for sequence mSprouty-pGem7z using primers
T7 5'-TAATACGACTCACTATAGGG-3'
M13R 5'-CAGGAAACAGCTATGAC-3'

6. Use the program "Chromas v 2.31" (Technnelysium Pty Ltd chromas232.exe) to extract sequence. Blast sequence at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) under "Mouse Genomic Plus Transcript." Review results for matching sequences.

7. Design primers: Oligo Calc: Oligonucleotide Properties Calculator
http://www.basic.northwestern.edu/biotools/oligocalc.html#helpadjusted

Diluted primers to 5µM
NheI '5-CTAGCTAGCTCTAGACCACTGTGGCCAGG-3'
MluI '5-CGACGCGTTATGTCGGCTTTTCAAAGTT-3'

8. PCR amplification

10µl 10x Buffer
10µl 10x dNTPs (Nucs)
15µl Mlu primer
15µl Nhe primer
48.6µl H_2O
0.4µl Taq Polymerase
1µl pGem-7z Sprouty
100µl total

Cycle Conditions:
94°C - 3min
94°C - 1 min
35 cycles:
    55°C -1 min
    72°C - 1.5 min
72°C - 10 min
4.0°C - hold

9. Ligation: PCR amplified Sprouty fragment into pGem-T easy

1µl PCR amp Sprouty2 fragment
1µl pGem-T easy
5µl 2x Ligation buffer
1µl T4 DNA Ligase
2µl H_2O
10µl total

Incubate at room temperature for 1 hour or overnight at 16°C

10. Transformation: 5µl and incubated 30min and plated onto Amp plates.

11. Picked colonies for next day mini prep

12. Ran gel to select possible transformed colonies

13. Digest: Mini prep pGem-T easy/Spry2

20µl Miniprep (pGem-T easy/Sprouty2)
5µl BSA
5µl Buffer#2
4µl Mlu
2µl Nhe
14µl H_2O
50µl total

Gel purified

14. Digest: pBigT

13.5µl pBigT (10ug)
5µl BSA
5µl Buffer#3
2µl MluI
24.5µl H_2O
50µl total

15. Phenol extracted and ethanol precipitate:

Add 50µl H20 and 100µl Phenol. Invert to mix. Spin at max (14 rpm) for 5min. Remove 100µl aqueous phase (top) and place in new tube. Add 1/10th (10µl) of 3M Sodium Acetate ph 5.2. Mix by flicking. Add 275µl ice-cold ethanol. Mix and spin for 10min at max in at 4°C. Remove and discard supernatant. Resuspend in H_20 (20µl).

16. Part II Digest pBigT

20µl pBigT
5µl BSA
5µl Buffer #2
2µl NheI
18µl H_2O
50µl total

Gel purified pBigT Nhe/Mlu digest

17. Ligation

1µl pBigT
6µl Nhe/Mlu digested pGemT easy/Sprouty gel purified frag
2µl ligase buffer
1µl ligase
10µl H_2O
20µl total

Incubate at 16°C overnight

18. Tranformation: Added 5µl of ligation to Mini preps

19. Picked colonies and performed mini prep with diagnostic digests

20. Performed a Midi prep of pBigT/ Sprouty

21a. Digest: pBigT/Sprouty2

34µl pBigT/Spry2
5µl BSA
5µl Buffer #4
2µl Pac I
2µl AscI
2µl H_2O
50µl total

Ran a gel

21.b Only partial digest Phenol extracted/ ethanol preciptation

Digest again:
20µl pBigT/Sprouty from phenol extract
5µl BSA
5µl Buffer# 4
2µl PacI
2µl AscI
26µl H_2O
50µl total

Phenol extracted ethanol precipitated

22. Sequence: pBigT/Sprouty2
T3 primer
5'- AATTAACCCTCACTAAAGGG -3'
T7 primer
5'-TAATACGACTCACTATAGGG -3'

Morula Aggregations/ Chimera Production

Stem cells are prepared before the day of morula aggregations. Stem cell preparation begins with plating MEFs onto 10cm or 15cm cell culture dishes (Cellstar Cat#) the Friday before aggregations. A vial of frozen MEFs is gently spun down to remove the freezing media and plated onto the dishes with fresh MEF media consisting of: DMEM (Invitrogen Cat# 11965126), 10% Fetal Bovine Serum (FBS; Millipore # ES-009-B), 1% NEAA (Invitrogen Cat# 11140050), 1% Nucleotides (Sigma: Cytidine C4654, Adenosine A4036, Uridine U-3003, Thymidine T1895, Guanosine G6264), 1% PSG (Invitrogen #10378-016), and a 1% β-mercaptoethanol-PBS solution (7µl β-merc and10ml PBS filter sterilized through a .2µm filter (IscBioexpress F-2754-2)). The MEFs are mito'd Monday by adding 150µl Mitomycin C (Fisher # BP25312) to 15ml of media and incubating at 37°C, 5% CO_2, for 2 ½ to 3 hours. The media is removed, rinsed three times with PBS (Invitrogen #14190-136), and trypsinzed with Trypsin-EDTA 0.05%; (Invitrogen #25300062) and incubated at 37°C for 5min shaking the dish at 2 ½ min. Media is used to deactivate the trypsin, the cells are gently centrifuged, counted on a hemacytometer and plated at a final concentration of 330,000/35mm dish. A vial of YFP3.1 frozen stem cells is thawed quickly, spun down to remove freezing media, and plated at a concentration of [3/4] onto one 35mm dish and [1/4] onto the second 35mm dish and given fresh ES media. ES media consists of 15% FBS, 1% Nucleotides, 1% NEAA, 1% PSF, 1% β-merc, and 0.1% LIF (Millipore #ESG1107). The [3/4] YFP3.1 are then split onto the remaining 2 35mm dishes on Wednesday at [1/2] or [3/4] depending on their confluency.

The Saturday before aggregations, 5-week-old CD-1 females are injected with PMS (.1ml containing 10units, EMD Biosciences # 367222) at 4pm. They are injected with HCG (.1ml containing 10 units, EMD Biosciences Cat# 869031) on Monday at 1pm. Matings are set up Monday afternoon and plugs are checked Tuesday morning. Tuesday afternoon mating as set up between vasectomized Swiss Webster (SW) males and SW females in estrus.

A divot plate is made on Wednesday, the day before aggregations. 30µl drops of M16 are placed in rows in a 10cm Nunclon tissue culture plate (ISC Bioexpress #T-2880-6). Mineral oil (Millipore#ES-005-C**)** is placed to cover the M16 droplets. A darning needle is then used to make 5 divots in each droplet. The divot plate is then incubated at 37ºC, 5%CO_2 overnight. The acclimation of the divot plate prior to morula aggregations is critical to the survivability of the morulae.

Thursday morning the CD-1 plugged females are sacrificed and their oviducts flushed with 30 Gauge ½" needles (Fisher #14-826F) with M2 media (Sigma-Aldrich #M7167). Morulae are collected and dezoned by rinsing through four drops of Acidic Tyrodes (Millipore #MR-004-D). Once the zona pellucida is disappearing, the morulae are immediately placed in M2 drops to inactivate the acidic tyrodes. They are rinsed through 4 drops of M16 (Sigma-Aldrich #M7292) and placed into the pre-made divots. The optimum stem cell dish is then trypsinized with 1ml media until the colonies begin to dislocate from the plate (~1min). 2ml of ES media is added to deactivate the trypsin. Small stem cell clumps containing 4-8 cells are selected. They are rinsed in M16 and added to the divot plate. The morulae/stem cells are incubated at 37ºC, 5% CO_2 overnight.

Friday morning the morulae/stem cell now blastocysts or blastocoels, are rinsed in M2 and placed in a mouth pipet. SW-vas-plugged mice and anesthetized with 2% Isoflurane and cleaned with isopropyl alcohol. A small longitudinal incision is made on the dorsal side and another small incision is made through the peritoneal cavity. The fat pad of the ovary is gripped to expose the uterus. 6-8 blastocysts/blastocoels are injected into each horn of the uterus. The uterus is placed back into the cavity and the epidermis is stapled shut (Stoelting Co. #59027). The mouse is placed on a warming pad until it awakes. Weight gain is recorded a week later to evaluate the success of implantation. Also a week later .1ml of tamoxifen (Sigma Aldrich # T5648) is injected IP into the mother. Embryos are allowed to develop until E10.5 or E11.5 or until birth.

Exo-Utero Surgery

Our YFP3 chimera was crossed and pups were genotyped for β-gal (F 5'-TTTAACGCCGTGCGCTGTTCG-3' and R 5'-GATCCAGCGATACAGCGCGTC-3') or GFP (5'- GCG AAG AGT TTG TCC TCA ACC-3' and 5'- AAA GTC GCT CTG AGT TGT TAT-3') expression as well as for Cre (F5'-CGAACGCACTGATTTCGA-3' R 5'-GGCAACACCATTTTTTCTGAC-3') expression in order to extablish YFP or β–gal positive colonies. YFP/Cre^{ER} X YFP or β-gal/ Cre^{ER} X β-gal crosses were conducted and surgery was scheduled for E11.5 (plug date E.5).

Surgery is performed as previously described (Muneoka et al., 1986, Ngo-Muller1999, Saito, 2006). A pregnant mother carrying YFP3 or β-gal reporter gene positive embryos at various stages (E10.5 and later) is anesthetized with 2% Isoflurane inhalant. The mouse is injected with 1ml of Ringer's Solution and is shaved and prepped for surgery. A lateral incision is made in the abdomen and the uterus is exposed. Frequent administration of 0.9% sterile saline is necessary to maintain proper hydration of exposed tissues. The embryos were exposed by making an incision on the anti-placental side of the uterus. The uterine muscles contract toward the placenta and expose the embryos still protected in their yolk sacs and amnion. A finely pulled injection pipette filled with 1μM 4-hydroxytamoxifen is inserted through the yolk sac and amnion and injected into the distal limb bud. The embryos are carefully placed back into the peritoneal cavity and the abdomen is sutured. Embryos are allowed to develop 48+ hours and then analyzed for either GFP or β-gal expression (Muneoka et al., 1986; Saito, 2006). This technique allows greater control of clone size, location and timing of clonal induction. 1mM 4-hydroxy tamoxifen (4-OHtamoxifen) is made by dissolving 3.87g into

10 ml 190% Ethanol and further diluting to 1μM for injection into limb buds by pipeting 1μl of 1mM solution into 999μl PBS (with fast green 20μl).

Immunoprecipitation and Western Blot Protocol

Limb buds are extracted from E9-12 mouse embryos, kept on ice and rinsed with PBS (it is imperative to keep chilled). Homogenizing Buffer from kit (Roche Applied Sciences #11719394001): 5ml Core Buffer, 3.75 ml NaCl, 2.5 detergent mix, 1 tablet. The limb buds are gently centrifuged to remove supernatant. The calculated amount of homogenizing buffer* is added to the limb buds, triturated and iced for 30 min with occasional mixing. The Protein A beads are precleared by gently centrifuging 50μl of given solution (50:50) and the supernatant is removed and discarded. 250-500μl of homogenizing buffer is added and the beads are incubated on ice for at least an hour. The lysed limb buds are centrifuged in a 4°C fridge for 10 min at full speed (14rpm) and the supernatant is removed and divided into two tubes (the pellet may be saved for WB)†. 5μl of Wnt5a antibody (Santa Cruz Biotechnology Wnt-5a (H-58): sc-30224) or Ror antibody (Cell Signaling #4105) is added to the supernatant and is incubated on a rocking platform at 4°C for 1 hour. Protein A beads are gently centrifuged, added (pure bead) to each tube and the Protein A/Supernatant solution is rocked at 4°C for 1 hour. It is again gently centrifuged (1000g for 30 sec) and the supernatant (A) is saved. The pelleted Protein A beads are washed three times by adding 1 ml of homogenizing buffer, resuspend by flicking, centrifuged and the supernatant discarded. An equal amount of homogenizing buffer as Sample Buffer (SDS-Page Buffer + BME 2μl/ml) is added to Protein A bead solution as well as an equal volume to supernatant(A). The solutions are heat blocked at 100°C for 5-10 min.

*(Example: 20 limbs / 50µl= 86 limb/x µl= 215 µl total. Thus add 107.5µl sample buffer to 107.5 lysis buffer (detergent mix) of supernatant. Add 107.5µl of 1% SDS and 107.5µl sample buffer to the pellet.)

†(When only running a western blot, resuspend the remaining limb bud pellet in 1% SDS (use ½ of total supernatant volume). Be very careful upon removal! Handle tube with caution as it may explode open. Add sample buffer up to volume (2^{nd} ½ of volume).

The supernatant(A) and limb bud pellets are run on a polyacrylamide gel. Running Buffer: 900 ml dH_2O and 100ml 10x Tris-Glycine-SDS Buffer (add last) (must be made fresh). The gel is placed into then gel apparatus, locked into place and filled ½ way with Running Buffer. The solutions (supernatant (A), Protein A bead solution and marker (5µl)) are run in separate lanes at 200V for 45 min. Transfer Buffer: 28.8g glycine, 6.06g Tris Base (Trizma), dH_2O up to 1600 ml, 400 ml Methanol (must be cold). The fiber pads are placed in the buffer to soak (at least 10 min) and rolled to remove air bubbles. The filter pads are soaked just before (~2min before otherwise it gets soggy) the gel is ready for transfer and also rolled to remove air bubbles.

Immobilon-P (Fisher Scientific #IPVH08100) paper is placed in methanol to equilibrate and rinsed 2-3x with dH_2O. It is then placed in Transfer buffer for at least 15 min to equilibrate. The transfer "sandwich" is set up as following: (white end) fiber pad, filter pad, Immobilon-P (membrane), gel, filter pad, fiber pad (black end) and the black side of the sandwich is placed next to the ice. Since it is imperative to keep the solution cold, a stir bad is placed under the gel apparatus to keep the cold water circulating. The

transfer is run at 100 Volts for 1 hour. The membrane is then carefully transferred to dH_2O and stained with Ponceau Red for 1-2 min. The membrane can be stored overnight in H_2O at 4°C.

Blocking Buffer (pBST): 50 ml 10x PBS, 450ml ddH_2O, 500µl Tween-20, 5 g powdered milk and mix with a stir bar. The membrane is cut into desired halves/segments (Ror will be in the upper portion and Wnt5a will be in the lower portion). The membranes are placed in blocking buffer on a rocker to equilibrate for 45 min.

Preparation of 1° antibody solutions: Ror has a 1:1000 dilution thus 3µl of antibody is added to 3ml of blocking buffer. Wnt5a (Santa Cruz Biotechnology Wnt-5a (C-16): sc-23690) has a 1:200 dilution thus 15µl of antibody is added to 3ml of blocking buffer and vortexed. The membranes are placed into a pouch with their respective antibody solutions, sealed and incubated at room temp for 1 hr.

The membranes are removed and separately rinsed 3 times in blocking buffer (each time rocking slowly for 15 min). Preparation of 2° antibody solutions: for the Wnt5a blot 1µl of anti-goat into 5ml of blocking buffer and for Ror2 blot 1µl of anti-rabbit into 5ml blocking buffer. The membranes are again placed into pouches with the correct 2° antibody solution and sealed. They are incubated at room temp for 1 hr and wash 3 times at 15 min intervals in blocking buffer. The membranes are exposed with ECL reagent (Pierce) or SuperSignal West Dura Extended Duration Substrate (Thermo Scientific Cat# 37071) at a 50:50 concentration (2.5ml and 2.5ml). The membranes are placed on a flat surface and the ECL reagent is added directly onto the membrane and

incubated at room temperature for 1 min. The blots are then placed and wrapped in saran wrap. Radiograph film is exposed (5sec up to 24 hours) is developed.

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Curriculum Vitae

Education

High School

High School Diploma 2002 — Buena High School
GPA 4.256 — Sierra Vista, Arizona
Class Ranking: 11/494

University

B.S. in Biology — Brigham Young University
September 2002 - April 2006 — Provo, Utah
GPA 3.58

Graduate School

M.S. in Physiology and Developmental Biology — Brigham Young University
September 2006 - August 2008 — Provo, Utah
GPA 3.91

Master's Thesis

M.S. Clonal Analysis of Wnt5a and Ror2 in Mediating Limb Outgrowth in Mouse Embryos

September 2006-Current — Brigham Young University

- Gene targeting: targeting constructs, DNA ligations and digests, gel purification, agarose gel electrophoresis
- Maintained stem cell culture and feeder cells and performed electroporation with my designed vectors (homologous recombination)
- Proficient in chimera production: morula aggregation,
- Modified novel exo-utero surgery technique
- Designed primers and performed PCR amplification of DNA
- Chemical and electrical bacterial transformation with mini, midi/maxi preps
- Prepared samples for DNA sequencing
- Performed Immunoprecipitation and Western Blot analyses
- Train and supervise over thirty undergraduates in the lab
- Managed lab ordering and keep detailed research records

Research and Training

Mentored Research

May 2005 - April 2006 — Brigham Young University

- Used the Elemental Analyzer and the Mass Spectrometer to analyze stable isotopes: carbon, nitrogen and hydrogen
- Labeled and recorded sample data

Academic Positions

Physiology 305 Lab Teaching Assistant
Sept. 2006 - April 2006 & Sept. 2007 - Dec. 2007 Brigham Young University

- Lectured 20 minutes for each two hour lab
- Taught four sections a week with class sizes of 24 students
- Managed nine lectures
    - Scientific Calculations, Osmosis, pH and Buffers, Action Potentials in the Mammalian Axon, Skeletal Muscle, Circulatory System, ECG and Blood Pressure, Respiratory System, Glucose/Insulin Regulation
- Assisted students with protocols, solutions, safety instruction
- Managed online quizzes, grades, posted announcements and documents through Blackboard program
- Calculated and submitted students' final grades

Employment History

Veterinary Assistant Animal Clinic May 2006 - August 2006

- Office management: improved client relations, scheduled appointments and billing, collections
- Blood work: assisted in blood draw, injections and vaccinations and developed radiographs
- Animal management: daily care, surgical prep, administered medicine

Veterinary Assistant Sierra Animal Hospital
Summers 2003, 2004, 2005

- Animal management: daily care, surgical prep, administered medicine
- Blood work and Radiology: assisted in blood draw, blood panels, injections and vaccinations and developed radiographs
- Office management: improved client relations, scheduled appointments and billing

Kid's W.O.R.L.D. Recreational Aid Parks and Leisure Services, City of Sierra Vista
May 2001 - July 2001

- Managed day camps for children kindergarten-5th grade
- Responsible for welfare of children

- Set up and instructed recreational, educational and social activities in a safe and supervised environment
- Assisted kids with arts and crafts, games, outside play, and group activities
- The programs focus was the total well-being of each and every child

Professional Qualifications

- Computer Skills: Knowledgeable in Microsoft Word, Excel, Outlook and Power Point
- Very familiar with Blackboard
- Relevant Coursework:
    - Chemistry 351, 352, 353: Organic Chemistry & Lab
    - Chemistry 105, 106, 107: Inorganic Chemistry and Lab
    - Developmental Genetics 582
    - Advanced Physiology 362 and Lab 363
    - English 316: Technical Writing
    - Cellular and Molecular Physiology 601
    - Molecular Biology and Lab 240, 241
    - Human Embryology 484
    - Developmental Biology 482
    - Statistics 221 and 510
    - Cell Biology 360
    - Biochemistry 481

Interests

- Talented pianist: studied over 10^{+} years
- Studied abroad for one semester in Rome, Italy
    - Learned Italian
    - Studied Art and European History