MORPHOMETRIC ANALYSES OF EMBRYONIC MOUSE LIMBS DEFICIENT IN ECTODERMAL SMAD4 SIGNALING

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By
Kimberly Novak

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Thesis written by
Kimberly Novak
Bachelor of Biology, Kent State University, 2010
M.S., Kent State University, 2012

Approved by

________________________ Dr. Y. Chen, Advisor

________________________ James L. Blank, Chair, Department of Biological Sciences

________________________ Dr. J. Stalvey, Dean, College of Arts and Science
# Table of Contents

Table of Figures ...................................................................................................................... vi

List of Tables .......................................................................................................................... vii

Abbreviations Used ................................................................................................................ viii

Acknowledgements ................................................................................................................ x

Introduction ............................................................................................................................ 1

Limb Development Background ............................................................................................ 1

The Apical Ectodermal Ridge, Proximal/Distal Patterning, and FGF8 ......................... 4

The Zone of Polarizing Activity, Anterior/Posterior patterning, and SHH ................. 6

Dorsal/Ventral Patterning, En1, Wnt7a, and the AER .................................................. 7

Bone Formation in Limbs ..................................................................................................... 8

Smad4 and the TGF-β Signaling Pathway ........................................................................... 9

Materials and Methods ......................................................................................................... 12

Mice ....................................................................................................................................... 12

Methods ................................................................................................................................. 12

Genotyping ............................................................................................................................. 12

Materials ................................................................................................................................. 12

Methods ................................................................................................................................. 13

Alcian Blue and Alizarin Red staining of the adult mouse skeleton .................................. 13

Materials ................................................................................................................................. 13

Methods ................................................................................................................................. 13
Alcian Blue staining of mouse embryos

Materials ................................................................. 14
Methods ................................................................. 14

Alcian blue and alizarin red staining of P1 mice

Materials ................................................................. 15
Methods ................................................................. 15

Beta-galactosidase staining of mouse embryos

Materials ................................................................. 16
Methods ................................................................. 16

Image Acquisition

Materials ................................................................. 17
Methods ................................................................. 17

Morphometric analyses of mouse embryo limb buds

Methods ................................................................. 17

Morphometric analyses of mouse embryo skeletal elements and limb buds

Methods ................................................................. 18

Statistics

Methods ................................................................. 18

Histology

Materials ................................................................. 19
Methods ................................................................. 19

Results

Removing Smad4 from the Ectoderm results in a Range of Limb Defects
Characterization of Forelimb Defects in Ectodermal Smad4 Mutants .......................... 23

The Hindlimb Phenotype is Comparatively Milder in Mutants ..................................... 28

The Shape of Mutant Limb Buds is Altered throughout Development ......................... 29

Discussion ......................................................................................................................... 35

Limb Patterning Defects are associated with a Range of Molecular Interactions .... 35

Early Shape Changes Reflect Later Phenotypes ................................................................. 41

Clinical Relevance for Findings ........................................................................................ 42

Conclusion and Future Directions of Study ...................................................................... 43

References ......................................................................................................................... 46
Table of Figures

Figure 1: Basic limb structures........................................................................................................... 2
Figure 2: Anatomy and signaling centers of the limb bud viewed from the dorsal or ventral surface. ........................................................................................................................................... 3
Figure 3: Generation of mutant and wild type embryos................................................................. 11
Figure 4: Mosaic expression of Cyp19cre.......................................................................................... 21
Figure 5: Loss of ectodermal Smad4 results in a high percentage of skeletal element loss and posteriorly biased phenotype.................................................................................................................. 23
Figure 6: Autopod and zuegopod defects in mutant forelimbs....................................................... 24
Figure 7: Digit tips in mutants are flatter and broader........................................................................ 25
Figure 8: Limb element measurement comparisons........................................................................ 26
Figure 9: D/V patterning is unaffected in mutant limbs............................................................... 27
Figure 10: Hindlimb phenotype exhibiting syndactyly and ectopic nails...................................... 28
Figure 11: Generation of reporter cross and reporter embryos.................................................. 30
Figure 12: Early mutant limb shape change. .................................................................................. 32
Figure 13: Limb shape change across stages of development....................................................... 33
Figure 14: Signaling pathways in the developing limb bud......................................................... 40
List of Tables

Table 1: The mutant phenotype is both penetrant and varied. .................................. 22
Abbreviations Used

A/P – anterior/posterior
AER – apical ectodermal ridge
BMP – bone morphogenetic protein
D/V – dorsal/ventral
d1, d2, ect – digit number
E10.5, E11.5, ect – days post coitum
En1 – Engrailed1
Fgf10, 8, ect – fibroblast growth factor 10, 8, ect.
Grem1 – Gremlin1
Lacz – beta-galactosidase
Lmx1b – LIM homeodomain transcription factor
mmol – milimole
P/D – proximal/distal
P1 – first day after birth, postnatal day 1
PBS – Phospho buffered saline
PCR – polymerase chain reaction
PFA – paraformaldehyde
R-Smad – receptor regulated smad
RA – retinoic acid

Rosa – B6.129S4-Gt(ROSA)26Sortm1Sor/J

S4f – Smad4 flox (Smad4<sup>Flox</sup>/Smad4<sup>Flox</sup>)

SHH – sonic hedgehog

Spry4 – sprouty4

ss – somite stage

µm – micron

ZPA – zone of polarizing activity
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Introduction

Limb Development Background

The limb is an essential structure for most vertebrates; used in feeding, mating, locomotion, and various other daily activities. It has evolved to facilitate running, walking, climbing, flying, the use of tools, and hundreds of other daily functions. Traditionally the limb is used in the study of evolutionary biology to determine how these different forms (wings, legs, fins, etc) progressed and diverged throughout time (Zeller et al., 2009). The limb is also an excellent model system for studying organogenesis. This is because while a limb is necessary for daily life it is not required for embryonic development. Because of this, the limb can be manipulated and explored in various ways while still allowing the embryo to develop to term, which would not be possible with other essential organs or structures. The limb also provides an excellent readout of early signaling, mainly the final skeletal structure, which can be easily analyzed (Zeller et al., 2009).

There are three main segments of a complete limb: the autopod (hand/foot), zeugopod (radius and ulna/tibia and fibia), and stylopod (humerus/femur) (Figure 1). There are also three main axes of limb development: proximal/distal (from shoulder to
tips of fingers/thigh to tips of toes), dorsal/ventral (back of hand to palm/back of foot to sole), and anterior/posterior (from thumb to little finger/big toe to pinky toe) (Figure 1).

**Figure 1: Basic limb structures.**

Dorsal image of E15.5 alcian blue stained right forelimb.

The limb bud from which the final adult limb develops begins as an outgrowth of mesenchyme covered with a thin layer of ectoderm (Tickle and Eichele, 1994). Prior to limb bud formation the area where the limb will grow is specified by overlapping domains of hox gene expression and mesenchymal-ectodermal interactions that begin
before the limb bud is even visible (Capdevila and Izpisúa Belmonte, 2001). The interactions between these two tissues will go on to set up the patterning and development of the limb as a whole (Capdevila and Izpisúa Belmonte, 2001). The patterning and growth of the limb is controlled in part by two main signaling centers: the apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA) which control proximal/distal and anterior/posterior patterning respectively (Towers and Tickle, 2009) (Figure 2).

![Diagram of limb bud anatomy and signaling centers](image)

**Figure 2:** Anatomy and signaling centers of the limb bud viewed from the dorsal or ventral surface.
AER-apical ectodermal ridge, ZPA-zone of polarizing activity.

The Apical Ectodermal Ridge, Proximal/Distal Patterning, and FGF8

In the specified limb fields on the flank of the mouse embryo active cell division begins and the mesenchymal outgrowth of the limb bud begins to form at approximately embryonic day 9.5 (E9.5) in the forelimb and E10.5 in the hindlimb (Capdevila and Izpisúa Belmonte, 2001; Theiler, 1989). As cells proliferate in the pre-limb bud area, they are also expressing Fgf10 (a fibroblast growth factor family member) which signals to the overlying ectoderm (Capdevila and Izpisúa Belmonte, 2001). This Fgf10 expression is crucial to the formation of the proximal/distal signaling center known as the apical ectodermal ridge (AER) as it induces expression of the AER’s main signaling molecule, Fgf8 (Capdevila and Izpisúa Belmonte, 2001).

At E10.5 a thickening of ectodermal cells at the tip of the developing limb bud begins to form, this is called the Apical Ectodermal Ridge (AER) (Fernandez-Teran and Ros, 2008). This structure is crucial for the development of the limb bud as it supplies factors necessary for cell survival, proper outgrowth, and patterning. At 27 somite stage (ss, approximately E9.5) the AER begins producing Fgf8, a major signaling molecule of the AER (Lewandoski et al., 2000). Fgf8 is largely responsible for the outgrowth and patterning in the developing limb and is essential for cell survival within certain parts of the limb (Niswander, 2003). In Moon et al, RAR-cre was used to conditionally knock
$Fgf8$ out of the developing forelimb prior to E9.0 (before limb formation) (Moon and Capecchi, 2000). These mice were shown to have no $Fgf8$ present in the forelimb region, including the AER, and as a result were lacking digit 1 and the radius (anterior limb elements) and occasionally digit 2 and the humerus (Moon and Capecchi, 2000). These mutants also displayed a loss of anterior mesenchyme and a disorganized AER, showing that $Fgf8$ is required for the maintenance of cell survival in these areas (Moon and Capecchi, 2000).

However, $Fgf8$ is not the only $Fgf$ expressed by the AER, though it is the first (expressed even before the AER is completely formed) (Lu et al., 2006). In fact there are a group of $Fgfs$ (collectively known as the AER $Fgfs$) which are expressed by the structure: $Fgf4$, $Fgf8$, $Fgf9$, and $Fgf17$ (Lu et al., 2006). Of these only $Fgf8$ is expressed throughout the entire circumference of AER, the rest are expressed along its posterior region (Lu et al., 2006). $Fgf8$ is also unique in that it is the only AER $Fgf$ that when removed alone causes limb defects (Lewandoski et al., 2000). $Fgf4$ also plays an important role in AER function as it can partially compensate for $Fgf8$ loss; this is why only anterior structures are lost in the $Fgf8$ RARcre mutants previously described (Lu et al., 2006; Moon and Capecchi, 2000).

Regression of the AER is also an important step in the patterning of the limb as the outgrowth of the limb must be controlled. Regression occurs around E12.5 and is mediated by signaling via the bone morphogenetic proteins (BMPs), members of the transforming growth factor beta (TGF-β) superfamily. BMP2, 4, and 7 are expressed in the developing limb (Robert, 2007). When Noggin, a BMP inhibitor, is misexpressed in
chick embryos prior to limb bud formation the result is a persistent AER, longer Fgf8 signaling, and extra soft tissue growth (Pizette and Niswander, 1999). This shows that without BMP signaling the underlying mesenchymal cells of the limb will continue to proliferate via Fgf8 signaling and cause excessive outgrowth of the soft tissue of the limb (Niswander, 2003; Pizette and Niswander, 1999).

**The Zone of Polarizing Activity, Anterior/Posterior patterning, and SHH**

In the limb bud the control of anterior/posterior (A/P) patterning is done via the Zone of Polarizing Activity (ZPA). This is a region located in the posterior mesenchyme portion of the limb bud where the signaling molecule Sonic Hedgehog (SHH) is produced (Bénazet and Zeller, 2009). As the cells in the ZPA produce SHH, a gradient is created. This gradient is essential to the patterning of the developing digits.

When tissues from the ZPA region, a bead containing SHH, or a bead containing retinoic acid (RA, an inducer of Shh expression) was transplanted to the anterior side of a chick wing bud, a mirror image duplication of digits formed (Pearse and Tabin, 1998). This showed both that the ZPA was responsible for anterior/posterior patterning and that SHH was the signaling molecule responsible for this. In Chiang et al. a Shh complete knockout mouse was developed and studied (2001). This Shh mutant had severe limb defects, with only d1 forming and the zeugopod forming only one bone with no
anterior/posterior polarity (Chiang et al., 2001). This shows that Shh expression is critical for the anterior/posterior patterning of the developing limb.

**Dorsal/Ventral Patterning, En1, Wnt7a, and the AER**

In order to for a limb to develop properly dorsal/ventral patterning must also take place. Bmps play a crucial role in D/V patterning as evidenced by the removal of Bmp2 and Bmp4 from the embryonic AER using Msx2-cre, a cre line expressed in the AER (Maatouk et al., 2009). In this mutant a bi-dorsal phenotype occurs with a nail plate (a dorsal structure) growing from both the dorsal and ventral side of the limb (Maatouk et al., 2009). Expression of Engrailed-1 (En1) was absent in the AER of these Bmp2 and 4 mutants (Maatouk et al., 2009). En1 is usually expressed in the ventral ectoderm where it inhibits expression of the dorsalizing transcription factor Limx1b (LIM homeodomain transcription factor) in the mesenchyme (Maatouk et al., 2009). En1 does this by restricting Wnt7a, a Wnt family signaling molecule, to the dorsal ectoderm (Niswander, 2003). Wnt7a induces Lmx1 expression in the underlying dorsal mesenchyme; Lmx1 is required for cells to adopt a dorsal fate (Niswander, 2003). With a loss of Wnt7a in the dorsal ectoderm a bi-ventral phenotype occurs (Niswander, 2003). Conversely, when En1 is absent from the ventral ectoderm a bi-dorsal phenotype develops (Niswander, 2003).

The role of BMPs in dorsal/ventral patterning is also supported by an earlier chick embryo experiment by Pizette et al. (2001). In this experiment they misexpressed the
Bmp antagonist Noggin prior to limb formation (Pizette et al., 2001). This misexpression led to dorsalization of the entire limb bud, as evidenced by reduced or absent En1 expression in the ventral ectoderm, ectopic expression of Wnt7a in the ventral ectoderm, and ectopic expression of Lmx1 in the ventral mesenchyme (Pizette et al., 2001). They also misexpressed constitutently activated Bmp receptors in the same manner with opposite results (Pizette et al., 2001). With the increase in BMP signaling the limb was ventralized; ectopic En1 signaling was found in the dorsal ectoderm where it reduced the dorsal factors Wnt7a in the ectoderm and Lmx1 in the mesenchyme (Pizette et al., 2001). This further illustrates the crucial role of Bmps in the dorsal/ventral patterning of the embryonic limb.

**Bone Formation in Limbs**

Bones in vertebrate limbs are formed via endochondrial ossification of the mesenchymal tissues based on the patterns set up and controlled by the signaling centers previously mentioned. The limb mesenchyme undergoes proliferation, a process controlled by the feedback loop between the ZPA and AER, and then forms condensations (Stricker and Mundlos, 2011). The recruitment of these cells into condensations as well as the control of their final size and shape is controlled in part by BMPs (Hall and Miyake, 1995; Horton, 2008). Condensations form in the limb bud around E10.5 (Horton, 2008). Sox9, a marker for limb cartilage and also a key
transcription regulator required for cartilage formation is visible at E10.5 (Bi et al., 1999; Shimizu et al., 2007). The forming of condensations is the first step in chondrogenesis, the second being differentiation of the cells into chondrocytes (Shimizu et al., 2007). Cartilage then elongates and segments to further set up the framework for the final bone and joint formation (Horton, 2008; Stricker and Mundlos, 2011). With the cartilage pattern fully set up, bone cells invade the limb framework as apoptosis of chondrocytes take place in the center of the condensations, setting up primary ossification centers that will complete development of the bones (Horton, 2008).

Smad4 and the TGF-β Signaling Pathway

The TGF-β signaling pathway regulates a dizzying number of cellular functions including apoptosis, cell differentiation, and cell growth among others (Schmierer and Hill, 2007). It is absolutely crucial for correct embryonic development where its ligands specify germ layers and set up various morphogen gradients (Schmierer and Hill, 2007). A TGF-β signaling ligand will bring together type I and II transmembrane serine/threonine kinase receptors, allowing the type II to phosphorylate type I receptor (Shi and Massagué, 2003). The type I receptor goes on to recruit and phosphorylate receptor-regulated SMADS (R-SMADS) (Schmierer and Hill, 2007). The R-SMADS can now complex with the mediator SMAD, called SMAD4, and translocate to the nucleus where they are able to regulate gene expression (Schmierer and Hill, 2007).
Smad4 knock out animals die around E7.5, before the onset of major organogenesis events, precluding the possibility of studying the late functions of Smad4. Therefore, a conditional knockout strategy was utilized to assess the requirement of Smad4 at embryonic stages beyond the lethal time point of the conventional Smad4 knockout animals. In this study, a Smad4 flox (S4F) mouse line, with loxp sites flanking a critical exon 8 of Smad4 (Yang et al., 2002) was crossed to a Cyp-19-Cre line (Wenzel and Leone, 2007) that expresses the recombinase, Cre, in the developing ectoderm prior to limb bud formation (Li, 2012, unpublished data). Utilizing this cre/loxp conditional knockout method, we removed Smad4 from the developing ectoderm, including the surface ectoderm covering the developing limb bud, allowing us to assess the requirement of Smad4-mediated signaling in the developing limb. Through a multigenerational mating scheme, we were able to obtain the genotype best suited for our research (Figure 3). Antibody staining against SMAD4 confirmed the ectoderm specific deletion of Smad4 in the developing limb (Li, 2012, unpublished data).
Figure 3: Generation of mutant and wild type embryos.

Figure indicates the multigenerational crosses utilized to obtain mice of the desired genotype. The bottom most cross indicates a timed mating cross which will yield both mutant and wild type control embryos.
Materials and Methods

Mice

Methods

Smad4 Flox (Smad4\textsuperscript{tm2.1Cxd}) mice on a mixed 129Sv and C57BL/6 background were from Dr. C. Deng's lab at NIH (Yang et al., 2002). Cyp-19 Cre mice on a mixed 129Sv and C57BL/6 background were made in Dr. G. Leone's lab (Ohio State) (Wenzel and Leone, 2007). ROSA26 reporter line obtained from JAX, stock #:003474; formal name: B6.129S4-Gt(ROSA)26Sortm1Sor/J. The animals are housed in the animal facility of the Department of Biological Sciences at Kent State University. All experimental procedures were approved by the Kent State University Institutional Animal Care and Use Committee.

Genotyping

Materials

Lysis buffer (25 mmol NaOH, 0.2 mmol EDTA), Primers used: Smad4flox (Smad4FloxB 5-GGGCAGCGTAGCATATAAGA-3, Smad4FloxA 5-GACCTTGGCTACCTTCAC-3, wt: 390bp, mut: 450bp), Cyp19cre (PW51-Cyp19Cre GACCTTGGCTAGATTAGATC, PW22-Cyp19Cre GAGAGAGAA-GCATGTTTAGCTGGCC, mut: 545bp) and ROSA (ROSA M GCG AAG AGT TTG
TCC TCA ACC, ROSA W GGA GCG GGA GAA ATG GAT ATG, ROSA WM AAA GTC GCT CTG AGT TGT TAT, wt:550bp, mut:300bp).

Methods
Crude lysates derived from mouse ear punches, toe clips, or embryo yolk sacs were used in genotyping.

Alcian Blue and Alizarin Red staining of the adult mouse skeleton

Materials
Alcian blue stain (0.015% alcian blue 8GX powder (Acros Organics, cat # 400460100) dissolved in a solution of 80% of 95% EtOH and 20% acetic acid), Alizarin red stain (0.005% alizarin red (Acros Organics, cat # 400480250) dissolved in 1% KOH solution), 100% EtOH, 1% KOH, and 100% glycerol.

Methods
Protocol adapted from Kingsley whole mouse skeletal staining protocol and optimized for limb only (Rountree, 2003). Adult animals were euthanized and limbs were dissected off. Using a scalpel, as much skin and muscle tissue was removed as possible from the entire limb while focusing on the autopod. Samples were fixed overnight in 95% EtOH then stained for 2-3 days in alcian blue solution. Then samples were returned to 95% EtOH overnight and put into 1% KOH on the following day to clear, for approximately a day and a half. Clearing time varied between samples and had to be
monitored carefully to prevent disintegration of sample. Next the alizarin red stain was added and left on the samples for approximately 3 days. Samples were then put into 100% glycerol to clear overnight and replaced with fresh 100% glycerol the next day for storage and imaging.

**Alcian Blue staining of mouse embryos**

**Materials**

Alcian Blue solution (.05% alcian blue 8GX (Acros Organics, cat # 400460100) dissolved in 5% acetic acid), ammonium hydroxide: ethanol solution (0.1% ammonium hydroxide in 70% EtOH), BABB (1 part benzyl alcohol with 2 parts benzyl benzoate), Bouin’s fixative (Ricca Chemical Co, cat # 1120-16), methanol, and 5% acetic acid.

**Methods**

Taken from the Cold Spring Harbor Protocol (Nagy and Gertsenstein…, 2009). Embryos from E12.5 to E16.5 were dissected out of extraembryonic membranes and put into Bouin’s fixative for 2 hours. Embryos were then washed in the ammonium hydroxide: ethanol solution for a period of about 24 hours. During this time the solution was changed regularly until no yellow color was apparent on the embryos. Next the embryos were equilibrated in 5% acetic acid, two changes for 1 hour each. The alcian blue stain was then added and left on the samples for 2 hours. Once 2 hours had passed, the stain was replaced with 5% acetic acid for two washes of one hour each. Finally the
embryos were cleared by washing them in methanol two times for 1 hour each and then stored in BABB. Embryos could then be imaged.

**Alcian blue and alizarin red staining of P1 mice**

**Materials**

Alcian blue stain (.015% alcian blue 8GX (Acros Organics, cat # 400460100) dissolved in a solution of 80% of 95% EtOH and 20% glacial acetic acid), alizarin red stain (0.005% (Acros Organics, cat # 400480250) dissolved in 1% KOH), 1% KOH in 20% glycerol, 1:1 glycerol/EtOH solution, 2% KOH, and 100% EtOH.

**Methods**

Protocol optimized from skeletal preparation protocol obtained from Siu-Pok (personal communication). Euthanized neonatal mice (P1) were eviscerated, put into 65°C water for 1 minute, and skinned. During skinning, as much muscle tissue as possible was also removed, taking care not to damage the limbs. Samples were then fixed in 100% EtOH overnight. Alcian blue stain was then added for 8 to 12 hours, during this period staining was monitored. Once stained, samples were washed in 100% EtOH overnight then cleared in 2% KOH for approximately 6 hours. Then samples were counterstained with alizarin red stain for 3 hours to overnight. Excess stain was removed with a rinse in 1% KOH in 20% glycerol and samples were then cleared for approximately 4 hours in the same solution. Finally skeletons were rinsed once with 1:1 glycerol/EtOH and put into the same solution for storage and imaging.
Beta-galactosidase staining of mouse embryos

Materials

Phosphate Buffer (0.027M monobasic sodium phosphate, 0.073M dibasic sodium phosphate), fix buffer (0.1M phosphate buffer, 2mM MgCl2, 5mM EGTA, 0.2% gluteraldehyde), Wash solution (2mM MgCl2, 0.1M phosphate buffer, 0.01% deoxycholate, 0.02% NP-40), X-gal stain (2mM MgCl2, 0.01% deoxycholate, 0.02% NP-40, 5mM K3Fe(CN)6, 5mM K4Fe(CN)6, 0.1M phosphate buffer, 1mg/ml X-gal (Gold Biotechnology, cat # X4281C)), 4% PFA (paraformaldehyde), and 70% EtOH.

Methods

Protocol adapted from Sanger/Wellcome protocol (Institute, 2004) and CSH protocol (Nagy et al., 2007c). Embryo was dissected out of extraembryonic membranes and fixed for 30 minutes in the fix buffer. Samples were then washed in the wash buffer three times for 5 minutes each on a rocker. X-gal stain was then added and samples were stained at 37C overnight, protected from light. Embryos were then washed with wash buffer two times for 5 minutes each. A post fix was then done with 4% PFA overnight at 4C. Samples were stored in 70% EtOH prior to imaging.
Image Acquisition

Materials

1X Phosphobuffered Saline (PBS), 1:1 EtOH/Glycerol, and 70% EtOH as needed.

Methods

Pregnant female mice with embryos from stage E10.5 to E16.5 were sacrificed according to protocol. The embryos were dissected out of the mother and placed in 1XPBS for further dissection. The outer uterine wall was removed, then the decidua and yolk sac. The yolk sac was kept for the genotyping of the embryo. The embryos were then processed by the appropriate protocol according to which experiment was being conducted. For imaging of beta-galactosidase stained embryos, careful attention was paid to ensure that the limb bud was parallel with the surface of the dish when imaging. For imaging of alcian blue stained embryos the limb was gently removed from the body wall and placed flat in a dish with 1:1 EtOH/Glycerol.

Morphometric analyses of mouse embryo limb buds

Methods

Images of limb buds were opened in Adobe Photoshop Elements 6 and the ‘magic wand’ tool was used to trace each limb bud. Careful attention was paid to where each limb bud contacted the body wall of the embryo. Another layer was added and the trace was filled with solid color. These individual traces were grouped together into one image.
containing all mutants and a representative wild type of the corresponding somite stage. The points where the limb contacted the body wall were used to determine the midpoint of each limb bud and orient the limbs to one another along the P/D axis for comparison.

**Morphometric analyses of mouse embryo skeletal elements and limb buds**

**Methods**

Images of alcian blue stained limbs were opened in ImageJ (v10.2). The line drawing tool and measurement command were used to determine the length and thickness of various bones in the limb. Scaling factor was taken into account for images taken under different magnifications.

**Statistics**

**Methods**

Microsoft Excel 2008 was used for basic statistical calculations including averages, standard deviations, and t-tests. T-tests performed were one tailed, type 3 t-tests. If the P value was greater than 0.05 then the result was considered not significant. P values equal to or less than 0.05 were considered significant, with those less than 0.01 considered highly significant, and those less than 0.001 considered of very high significance.
Histology

Materials

4% PFA, 4% formic acid, 25% EtOH, 50% EtOH, 70% EtOH, 95% EtOH, 100% EtOH, ammonia, Citrisolv (Cat # 22-143975, Fisher), paraffin (Paraplast tissue embedding medium, Cat # 39501006, McCormick Scientific), Hematoxylin (Cat # C5401-1D, Fisher), eosin staining solution (0.33% eosin (Cat # E511-100, Fisher) and 0.2% acetic acid in 75% EtOH), and mounting medium (Cat # H157-475ML, Amresco).

Methods

E15.5 limbs were fixed in 4% PFA overnight then put into 4% formic acid for 2 days for decalcification. After decalcification, samples were rinsed in tap water for 10 minutes before proceeding. Limbs were then prepared for embedding and embedded in paraffin per protocol (Fischer et al., 2008b) (Nagy et al., 2007a) then sectioned on a Leica Supercut microtome into 10um sections (Nagy et al., 2007b). Slides were then stained based on standard protocol and mounted with cover slips (Fischer et al., 2008a).
Results

Removing Smad4 from the Ectoderm results in a Range of Limb Defects

Ectodermal deletion of Smad4 results in viable and fertile animals displaying a wide range of limb deformities. Defects associated with the autopod of the forelimb include carpal deformities, syndactyly, oligodactyly, digit curving, and broadening of digit tips. Our phenotype also affects the zeugopod of mutants where curving, shortening, or even loss of zeugopod element occurs. There is a posterior bias in the phenotype in that the majority of mutants missing a skeletal element (digits and/or zeugopod element) are missing the posterior one (99%, Table 1, Figure 5). The hindlimb phenotype of these mutants is far milder than that of the forelimbs. In the hindlimb we only see syndactyly and ectopic nail formation with varying degrees of severity. The penetrance of the mutant limb phenotypes is high, with nearly 95% of the Cre-transgene carrying limbs displaying a certain degree of deformity (Table 1). The few phenotypically normal “escapers” can be due to lower or mosaic Cre expression occasionally observed in some Cre-transgene carrying animals by reporter assays (Figure 4).
Figure 4: Mosaic expression of Cyp19cre.

Beta-galactosidase stained E10.5 embryo, arrows indicate examples of mosaic cre expression.
Overall (n=131) | Limb deformity penetrance (124/131) | 94.7%
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Zeugopod defects (21/131) | Posterior defects (ulna affected) (19/21) | 90.5%
Autopod defects (124/131) | Non-oligodactyly defects (19/124) (syncactyly ect) | 15.3%
 | Oligodactyly (digit loss) (105/124) | 84.7%
 | Oligodactyly with posterior deletion (104/105) | 99.0%

Table 1: The mutant phenotype is both penetrant and varied.

Percentages of various phenotypes are based on observations of Alcian blue stained embryos from E13.5-E16.5.
Characterization of Forelimb Defects in Ectodermal Smad4 Mutants

The forelimb of mutants is the most strongly affected structure in the limb with a range of deformities. In the autopod, oligodactyly (digit loss) was the most common defect (84.7%, n=124, Table 1). The severity of the oligodactyly ranges from autopods with 5 digits to autopods with only 2 digits (Figure 6). The most frequent phenotype lies
in between these two extremes. Digit tips were found to be blunted and broadened compared to wild type (Figure 7). Carpal defects are also observable though specific malformations are unable to be determined due to the severity of the phenotypes. In the more severe cases (16%, n=131, Table 1) the zeugopod is also affected. Of these cases 90.5% (n=21, Table 1,) are missing the posterior element, the ulna.

E15.5  Mut 5d  4d  3d  2d

|  WT  | (15.3%) | (37.1%) | (39.5%) | (8.1%) |

Figure 6: Autopod and zuegopod defects in mutant forelimbs.

All embryos are E15.5 stained with alcian blue for cartilage, preprocessed images are above each stained image. All images are dorsal view. WT- wild type, Mut- mutant, d-digits
Figure 7: Digit tips in mutants are flatter and broader.

P1 mouse limbs stained with alcian blue and alizarin red for cartilage (blue) and bone (red). Arrows indicate flattening and broadening of digit tips. Images A and B are forelimbs, images C and D are hindlimbs. RFL- right forelimb, LFL- left forelimb, RHL- right hindlimb, LHL- left hindlimb, P1- postnatal day 1, WT- wild type, MT-mutant.

Using digital image processing tools, detailed morphometric perimeters were measured on a large collection of stained skeletal preparations of wild type and mutant
limbs at E15.5 to gain an in depth understanding of the skeletal phenotypes associated with ectodermal Smad4 deletion. The perimeters measured include the length and average width of the zeugopod and stylopod elements. The zeugopod elements (radius and ulna) were both shorter (Figure 8, Panel A) and thinner (Figure 8, Panel B) than the wild type limbs with the ulna being the more severely affected of the two (Figure 8, Panel B). Although the length of the stylopod is not significantly affected in the mutants (Figure 8, Panel C), significant thinning of the humerus was observed (Figure 8, Panel D).

Figure 8: Limb element measurement comparisons.

Scale is in microns. WT- wild type, MT- mutant.
Dorsal/ventral patterning of a limb can be assessed via stereotypical tendon patterns, in particular by examining the presence of the ventral tendon (Maatouk et al., 2009). Presence of this tendon in the ventral side of the limb is often used to determine that dorsal/ventral patterning has occurred correctly (Maatouk et al., 2009). Detailed analyses were carried out on histological sections to assess the dorsal/ventral patterning of the forelimb based on patterns of the landmark tendons, specifically the ventral tendon (arrows in Figure 9). At this level of analysis, mutant forelimbs do not appear to have overt defects in dorsal/ventral patterning (Figure 9).

**Figure 9: D/V patterning is unaffected in mutant limbs.**

Images of E15.5 wild type and mutant limb cross sections, sectioned at 10 microns. Arrows indicate the ventral tendon, which is present in both wild type (WT) and mutants (MT). D- Dorsal, V- Ventral.
The Hindlimb Phenotype is Comparatively Milder in Mutants

The mutant hindlimb phenotype is characterized by syndactyly and ectopic nail formation (Figure 10, arrows). Overall it is much milder than the forelimb and does not exhibit oligodactyly or loss of zeugopod element as seen in the forelimb (Figure 7, panels C and D). When observing the skeletal elements of the hindlimb the tips of the digits appear to be broadened and blunted, (Figure 7, Panels C and D, arrows) this same phenotype is exhibited in the forelimb as previously mentioned (Figure 7, Panels A and B, arrows).

Figure 10: Hindlimb phenotype exhibiting syndactyly and ectopic nails.

Arrows indicate ectopic nail formation. WT-wild type, MT- mutant, RHL- right hindlimb.
The Shape of Mutant Limb Buds is Altered throughout Development

The architecture of a fully developed limb is a final product of earlier molecular signaling, in particular those signaling events that set up the shape and size of the primordium limb bud. As the limb grows, its shape changes in response to changes in gene expressions that affect cell proliferation and survival. Thus the shape of the limb bud across development becomes an excellent readout of the molecular regulations occurring at various developmental time points. To correlate the terminal limb deformities seen in Smad4 ectodermal mutants with potential morphological defects in early limb buds, we carefully examined the shape and size of limb buds across a 16 somite stage range (31-46ss; E10.5 to E11.5), over a developmental period of 32 hours. This allowed visualization of the phenotypic changes as they happened across the earliest stages of limb development.

To minimize the confounding effects of limbs with low or mosaic ectodermal Cre expression (Figure 4) a Lac-Z reporter transgene was bred into the Smad4 conditional allele background to allow for simultaneous visualization of Cre expression and assessment of the mutant phenotypes. Of the Cre-transgene positive embryos, only those with uniform lacZ reporter expression were used in subsequent analysis. This strategy effectively eliminated the inclusion of “escapers” in our analyses.

To increase the percentage of informative embryos in each cross, efforts were made to homozygous both the Smad4 conditional allele and the Rosa-lacZ reporter transgene (Figure 11, panel A). When crossed with S4F/S4F; Cyp19cre/+ mice, the
resulting embryos were stained for beta-galactosidase (lacZ) expression (Figure 11, panel B) to assess the level and distribution of the Cre transgene. PCR was done in parallel to confirm the genotypes of all embryos.

**Figure 11: Generation of reporter cross and reporter embryos.**

Panel A indicates the generation of the reporter line. Note that there is only a 6% chance of obtaining the desired genotype. Panel B indicates the cross performed to obtain mutant reporter embryos.

Over 225 mutant embryo limbs from E10.5 to E11.5 (31ss-46ss) were examined and compared to wild types of the same somite stage. Individual limb buds were carefully dissected and imaged. To minimize shape distortion due to variable imaging planes, careful attention was paid to each limb to assure it was parallel with the dish.
surface when imaged. These images were then imported, traced, and overlaid in Photoshop to allow for comparison between stage matched wild types and mutants. The change in shape is first clearly evident in 34ss mutant forelimbs lacking ectodermal Smad4. The lack of significant limb bud shape changes prior to this stage indicates that limb bud early development (approximately 21ss-33ss, approximately E9.5-E10.0) is not affected. At 34ss these mutant limb buds are noticeably narrower along the anterior-posterior axis. The smooth curved contour of the wild type limb is lacking, the sides instead have a much sharper slope (Figure 12, brackets). As development proceeds, the mutant limb shape continues to progress, further differentiating itself from the wild type. By 41ss there are three distinct categories of mutant limb shape present: thinned/narrowed limbs, broadened limbs, and limbs that contain a gapped distal region (Figure 13, Panels E-H). Fgf8 expression in these early shapes also follows a characteristic pattern (Figure 13, Panels A-D). Fgf8’s expression is shorter along the anterior/posterior axis in the narrowed limbs (Figure 13, Panel B), expression is broader in the broadened limb buds (Figure 13, Panel D), and there is a gap in Fgf8 expression in gapped limb buds (Figure 13, Panel C). These early shapes (41ss to 46ss) can be correlated with later shapes by tracing the limb outline of alcian blue stained embryos (E13.5-E16.5) (Figure 13, Panels I-P). The length in the proximal/distal axis is more difficult to assess, due to the lack of a definitive landmark that marks the exact position at which the limb bud joins the body flank. However, part of the proximal/distal developmental defects can be inferred from the measurement of the skeletal elements (Figure 8) which shows the shortening of both the zeugopod and stylopod elements.
Figure 12: Early mutant limb shape change.

The mutant (MT, blue) limb bud shape deviates from that of the wild type (WT) shape beginning at 34ss. At this stage the mutant becomes noticeably narrower, indicated by orange brackets. All views are dorsal.
**Figure 13: Limb shape change across stages of development.**

Images A-D are *Fgf8* in situ hybridization images, showing *Fgf8* expression in the AER (adapted from the research of Jibiao Li (Li, 2012, unpublished data). Used with permission.). Arrow in panel D indicates broadened expression of *Fgf8*. Images E and H are limb traces from 45ss embryos. Images F and G are limb traces from 46ss embryos.
Images I-P are stained with alcian blue for cartilage. Limb autopod shape has been traced with a black line. All views are dorsal. WT-wild type, Mut- mutant.
Discussion

By conditionally deleting \textit{Smad4} in the embryonic ectoderm, including the surface ectoderm of the early limb bud, we have uncovered a novel requirement for \textit{Smad4}-dependent signaling in limb development. The limb deformity associated with ectodermal \textit{Smad4} removal is multi-faceted, including preferential loss of posterior structures, retention of interdigital tissues, and excess ectodermal tissues. Detailed morphometric analyses of a temporal sequence of embryonic limbs revealed that the initial defects of these mutant limbs could be correlated with earlier changes in limb bud size and shape. Results presented in this study combined with ongoing molecular analyses of the mutant limbs in our lab has started to provide a deeper understanding of the role of \textit{Smad4}-dependent signaling in vertebrate limb morphogenesis.

Limb Patterning Defects are associated with a Range of Molecular Interactions

Loss of \textit{Smad4} from the surface ectoderm of the developing limb affects the skeletal development of the limb autopod and zeugopod across the different limb axes. Along the A/P axis there are digit-development malformations in our mutant. The A/P axis is set up by \textit{Shh} from the ZPA and when \textit{Shh} is removed completely from the
developing limb only one digit forms (Kraus et al., 2001). Both the amount and length of exposure to SHH are important for digit development. When Shh is removed in a time dependent manner fewer posterior digits are present, showing that these posterior digits require high concentrations of Shh for a certain amount of time to form correctly (Scherz et al., 2007). In our mutant Shh is reduced and occasionally absent in the posterior mesenchyme of our mutant forelimbs at E10.5 (Li, 2012, unpublished data). This lack of expression could also account for the occasional missing posterior zeugopod element as well as posterior digit loss. Shh is also important in mesenchyme proliferation, a decrease in the size of the skeletal elements will result if this proliferation does not take place (Stricker and Mundlos, 2011). This could account for both the loss of zeugopod elements as well as the thinning of these elements.

However, Shh is not the only factor affecting digit development; BMPs are also crucial to digit and bone development. While Bmp2, 4, and 7 are not needed for digit identity specification (either alone or together); Bmp2/4 are needed in the posterior digits to promote the condensation of digit anlagen and complete bone formation (Bandyopadhyay et al., 2006; Shimizu et al., 2007). In our mutant it has been shown that Bmp2/4/7 have truncated expression in the mesenchyme at E10.5 (Li, 2012, unpublished data). BMP signaling together with SHH signaling are likely the causes of the loss of posterior digits in our mutant limb.

Another important aspect of limb development is limb outgrowth. Our mutant limbs are significantly shorter with relation to the zeugopod element as compared to wild type limbs. The AER is responsible for outgrowth of the developing limb. When the AER
is removed at progressively earlier stages of limb development, fewer distal structures develop (Niswander et al., 1993). The AER is the site of FGF signaling, the first of which to appear is Fgf8 (MacKem, 2008). When Fgf8 is removed from the AER using an AER specific cre (RARcre), anterior limb structures do not develop (d1 and radius) in the forelimb (Moon and Capecchi, 2000). The anteriorly biased structure loss is due to compensation by other AER Fgfs, particularly Fgf4 (Boulet et al., 2004). In our mutant Fgf4 expression is upregulated in forelimb buds, likely to compensate for the loss of Fgf8 signaling in the AER. (Li et al., unpublished data). This could account for the occasional loss of the anterior most digit (d1) in our mutants (when Fgf8 loss is particularly severe). In our mutants the Fgf8 expression domain is shortened from E9.5 to E10.5 (Li et al., unpublished data). It should also be noted that when Fgf8 is removed from the AER, the expression of Shh and Bmp2 are also affected, details of which will be discussed later (Moon and Capecchi, 2000).

Fgf8 is also responsible for the proliferation and survival of cells in the developing mesenchyme (Niswander, 2003). In our mutants the expression of Fgf8 is truncated along the anterior/posterior axis and in particular Sprouty4 (Spry4, a downstream readout of Fgf8 signaling) expression is lost in the posterior mesenchyme (Li, 2012, unpublished data). Overall this shows that FGF8 signaling is no longer present in these areas and thus is not maintaining those cell populations in the mesenchyme. The loss of this posterior tissue likely also contributes to the posterior digit loss and zeugopod defects seen in our mutant phenotype.
Interdigital cell death is required for the correct separation of digits, without which syndactyly (fusion of soft tissue) occurs. In our mutants, syndactyly is present in various degrees. It has been shown that late Bmp activity is needed for AER regression by inhibiting FGF8 signaling, thus promoting limb cell apoptosis (particularly in interdigital tissue) (Guha, 2002; Pajni-Underwood et al., 2007; Sears, 2008). Our Smad4 ectodermal mutants show reduced Bmp expression at E10.5 (Li et al., unpublished data). It is likely that the reduced Bmp expression is causing the late AER regression in some places as shown by sustained Fgf8 signaling at late stage of limb development (Li, 2012, unpublished data). In particular this ectopic/extended Fgf8 expression usually occurs interdigitally and is likely the cause of syndactyly and excess tissue growth in both forelimb and hindlimb of the Smad4 ectodermal mutants (Li, 2012, unpublished data). Reduced interdigital cell death shown at E13.5 supports this hypothesis (Li, 2012, unpublished data). Extended FGF8 signaling could account for the ectopic growths often evident on limb autopods as studies done with FGF8 beads implanted in the chick limb showed formation of ectopic limb structures (Crossley et al., 1996). Ectopic and extended Fgf8 expression could also account for the ectopic nail phenotype found in the hindlimb of mutants (Li, 2012, unpublished data). Without BMP signaling to regulate it, Fgf8 persists longer than necessary, as the AER does not regress fully without correct BMP signaling (Robert, 2007).

It is critical to remember that these various signaling centers mentioned also interact with one another and regulate expression throughout the development of the limb (Figure 14). The AER and ZPA form a positive feedback loop with one another, the AER
*Fgfs* maintain expression of *Shh* in the ZPA just as *Shh* maintains the AER *Fgfs* (Duboc and Logan, 2009). Without this feedback loop between the limb epithelium and mesenchyme, digits are lost in the developing limb along with other defects (Benazet et al., 2009). However, *Bmp4* also plays a role in this feedback loop, it is responsible for regulating the AER length via signaling to the limb ectoderm (Benazet et al., 2009). Both of these signaling paths meet back at *Grem1*. *Bmp4* initiates *Grem1* early on (around E9.0) and *Grem1* expression is used in a self-regulatory feedback loop with *Bmp4* (Benazet et al., 2009). Later on in limb development (a few hours after it has been initiated by *Bmp4*) *Shh* also induces *Grem1* expression (Benazet et al., 2009). This causes a greater decrease in BMP signaling allowing *Shh* to pattern the digits correctly (Benazet et al., 2009). In our mutant the relative position of the *Grem1* domain appears closer to the posterior side of the bud, this is due to the preferential loss of posterior tissues (Li, 2012, unpublished data). The feedback loops within the developing limb bud are complex and intricate and are currently a growing area of study within this field.
Figure 14: Signaling pathways in the developing limb bud.

A) Green arrows indicate stimulation of expression, red lines indicate inhibition. B) The red X indicates the signaling that is disrupted by the loss of Smad4 from the developing ectoderm and can account for loss of posterior limb structures. C) Later on in development decreased BMP signaling caused by lack of ectodermal Smad4 (down arrow) lessens BMPs inhibition on the AER FGFs (dashed X) and allows them to persist in some areas, causing syndactyly.

AER- Apical ectodermal ridge, ZPA- zone of polarizing activity.
Early Shape Changes Reflect Later Phenotypes

Careful study of the limb shape change over development can give insight into the developmental defects previously described. Limb bud shape change correlates greatly with final limb patterning and shape. Across many species of mammals the limb bud shape is well conserved during very early development (Sears, 2011). The final limb shape is formed through the alteration of various pathways, which are then reflected in the overall structure of the limb. One can find many such examples throughout the animal kingdom. The pig limb contains four primary digits with d5 and d2 reduced in size (only d3 and d4 are weight-bearing), compared with the mouse which has a total of 5 digits (Sears et al., 2011). However, the pig limb bud is initially similar in shape to that of the mouse and even forms cartilage condensations for all five digits (Sears et al., 2011). Later in development (comparable to E11 stage in mouse) the pig limb bud is seen to be narrower than the mouse limb bud, and this trend continues to become more pronounced throughout development (Sears et al., 2011). This narrowness of the limb bud can be correlated with the loss/reduction of digits in the fully developed limb. This naturally occurring example bears resemblance to the early limb shape changes seen in the ectodermal Smad4 mutant limbs. In these mutant limbs, at early stages the limb bud is almost indistinguishable from that of the wild type. Starting at 34ss, the mutant limb becomes noticeably narrower however, a reflection of the final digit pattern in the adult animal. In the pig the cartilage condensations are proportionately sized when they are
formed; the condensations for the reduced digits are smaller than those for the non-reduced digits (Sears et al., 2011). This could be the case in our mutant, though further analyses of cell proliferation and cell death are needed.

**Clinical Relevance for Findings**

The study of *Smad4* in the limb field has numerous applications from understanding human limb malformations to the evolution of the limb. Mouse and chick limb models have already been shown to be very effective in understanding human limb malformations (Stricker and Mundlos, 2011). Human limb malformations are relatively commonplace, occurring in about 1 of every 600 births; syndactyly is one of the most common of these malformations (Chong, 2010; Oberg et al., 2010). Other limb defects such as ectopic nail growth are also seen in humans (Kikuchi, 1985). It is possible that *Smad4* has yet unappreciated roles in these limb defects, likely through BMP signaling, and this mouse model could help to elucidate these roles.

Mutational limb studies are often useful as they can help elucidate the mechanisms through which limbs evolved and changed their forms to better suit their environmental niches. The alteration of various limb developmental pathways can be used to determine how different species altered those same pathways to develop specialized limbs. For example the bat limb has a wider expression of *Fgf8* along the dorsal/ventral axis, which could contribute to a longer limb as *Fgf8* is important for
proximal/distal growth (Cretekos et al., 2007; Sears, 2008; Sears, 2011). There is also expression of \textit{Fgf8} in the mesenchyme between the digits and reduced BMP signaling in the limb which together lead to the growth of the interdigital tissue that allows the bat to achieve powered flight (Sears, 2008; Weatherbee et al., 2006). In one experiment the mouse limb elongation enhancer was replaced with the bat enhancer, resulting in mice with elongated forelimbs that were correctly patterned (Cretekos et al., 2008). The use of the bat enhancer in mice showed a way that organisms may modify transcription to produce different organ sizes across species (Cretekos et al., 2008). It is clear that the \textit{Smad4} ectodermal mutant could potentially provide insight into evolutionary changes across species, especially in the areas of digit loss, interdigital webbing, and reduced limb length.

\textbf{Conclusion and Future Directions of Study}

In conclusion, we have shown that ectodermal \textit{Smad4} is essential for proper limb development. Utilizing an ectodermal cre system it has been shown that \textit{Smad4} plays a crucial role in posterior digit formation, zeugopod formation, and digit separation. It has also been shown that the limb deformity can be traced back to changes in early limb bud shape, which can in turn be correlated with misexpressed molecular markers. The molecular signaling behind such malformations is beginning to uncover the nuances of \textit{Smad4}'s role in limb development, there is much yet to be understood.
With the mechanisms behind our mutant phenotype just becoming uncovered I would have liked to further explore the tendon patterning. Tendons can act as a readout for dorsal/ventral patterning and molecular analysis done by my lab mate Jibiao showed that the dorsal genes *Wnt7a* and *Lmx1* are invading the ventral anterior and posterior domains but our mutants show no overt dorsal/ventral defects (Li, 2012, unpublished data). More careful examination of tendon patterning could lead to an explanation of this molecular observation; dorsal/ventral patterning defects may exist only in a subtle form.

As the somite compartment which produces cartilage (sclerotome) develops the dorsolateral portion of this section forms the tendon lineage (Brent, 2005). Detailed study of tendon patterning in the mutant limb would allow for the dual examination of dorsal/ventral patterning as well as examination of the tissue lineage which shares a common somatic origin with the final bone progenitors. Tendon development is also closely linked to muscle development, as tendons will not differentiate properly without them (Eloy-Trinquet et al., 2009). Thus studying tendon may give insight into the muscular structure of the mutant limb as well.

To further study the digit phenotype a broader range of cell proliferation and cell death studies could be done across a wider range of time points. In this way cell death and proliferation could be tracked in a somite by somite stage manner and linked with the limb shape change studies. This could give insight into how and when digits are lost in the developing mutant limb.

The *Bmp* pathway of the TGF-β signaling family goes through the R-SMADS1/5/8 which then complex with SMAD4 before being translocated to the nucleus
to alter transcription. Utilizing the same cyp19cre a Smad1 single ectodermal knockout and Smad1/4 double ectodermal knockout have been created. The Smad1 knockout shows no limb phenotype, likely due to the compensation of SMADS 5 and 8 (Li, 2012, unpublished data). However, the Smad1/4 double knockout has shown more severe limb defects than that of the Smad4 single ectodermal knockout (Li, 2012, unpublished data). This suggests a Smad4 independent as well as dependent signaling pathway in limb development. More combinations of Smads1/5/8 and Smad4 ectodermal knockouts should be generated to further explore the molecular pathways affecting limb development.

There are also aspects of the phenotype that lie outside of the limb. These include malformations of the genital region (especially severe in males). The mechanisms behind this aspect would further uncover Smad4’s role in the ectoderm of the developing embryo. We have only just begun to understand the mechanisms behind Smad4’s important ectodermal role in development; there are numerous areas of further research to which this model can be applied.
References


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