Mechanism of Lmx1b-regulated Limb Dorsalization

Jennifer Feenstra
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Mechanism of Lmx1b-regulated Limb Dorsalization

by

Jennifer Feenstra

A Dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Anatomy

June 2012
Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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I would also like to thank my committee members for their advice, direction and editing skills. Especially to Dr. Fallon for always taking the time to pass along advice regardless of the topic, I thank you for that. To all of those with whom I have collaborated, argued, agreed, laughed, and cried, thank you for your time, support, and efforts. Special thanks go to Charmaine Pira my lab companion, friend, and confidant. Without your expert knowledge of grammar, life, and the -80 freezer I may never have graduated.

To my family and friends, your love and support through this long endeavor has been treasured and I could not have finished without you. I will always been fascinated by science and the wondrous secrets it still holds, I am happy to say that I can now look forward to a career exploring and hopefully discovering some of those wonders.
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<td>Electroporation</td>
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<tr>
<td>WMISH</td>
<td>Whole mount <em>in situ</em> hybridization</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Q-PCR</td>
<td>Quantitative or Real-time PCR</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse-transcriptase PCR</td>
</tr>
<tr>
<td>AER</td>
<td>Apical Ectodermal Ridge</td>
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<tr>
<td>ZPA</td>
<td>Zone of Polarizing Activity</td>
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<td>ACAN</td>
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<td>Radical fringe</td>
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<td>Lim homeobox transcription factors</td>
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<td>SOX – SRY</td>
<td>Sex-determining region Y</td>
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<td>HMG</td>
<td>High mobility group</td>
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<tr>
<td>GDF</td>
<td>Growth and differentiation factors</td>
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<td>PTHLH</td>
<td>Parathyroid hormone-like hormone</td>
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<td>IHH</td>
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<td>IGF</td>
<td>Insulin-like growth hormone</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<td>RUNX</td>
<td>Runt-related transcription factor</td>
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<td>Short stature homeobox transcription factor</td>
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<td>ROR2</td>
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<td>Leukocyte cell-derived chemotaxin</td>
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<td>Matrilin</td>
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<td>LOX</td>
<td>Lysyl oxidase</td>
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<tr>
<td>LIM</td>
<td>Lin11, Isl-1 &amp; Mec-3 domain</td>
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<td>IPA</td>
<td>Ingenuity Pathway Assistant</td>
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<td>HH</td>
<td>Hamburger and Hamilton</td>
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<td>KO</td>
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<td>DPC</td>
<td>Days post coitum</td>
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<tr>
<td>KB</td>
<td>Kilobase</td>
</tr>
<tr>
<td>BP</td>
<td>Base pairs</td>
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<tr>
<td>CNR</td>
<td>Conserved non coding region</td>
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<td>MEMFA</td>
<td>L 3-(N Morpholino) propanesulfonic acid, Ethylene glycol tetraacetic acid, MgSO4, 3.7% Formaldehyde</td>
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<td>CARMAweb</td>
<td>Comprehensive R based Microarray Analysis web</td>
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<tr>
<td>PGK</td>
<td>Phosphoglycerate kinase</td>
</tr>
<tr>
<td>MCS</td>
<td>Multiple Cloning Site</td>
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<tr>
<td>HBSS</td>
<td>Hanks buffered saline solution</td>
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<tr>
<td>TREP</td>
<td>Targeted Regional Electroporation</td>
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<tr>
<td>cDNA</td>
<td>Complimentary DNA</td>
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<tr>
<td>SLRP</td>
<td>Small Leucine Rich Protoeglycan</td>
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<tr>
<td>DUSP6</td>
<td>Dual specificity phosphatase 6</td>
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<tr>
<td>TFAP2C</td>
<td>Transcription factor AP-2 gamma</td>
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<td>Description</td>
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<td>--------------------------------------------------------</td>
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<tr>
<td>PET-1</td>
<td>Plasmacytoma expressed transcript 1</td>
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<td>MES</td>
<td>Mesencephalon</td>
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<tr>
<td>TEL</td>
<td>Telencephalon</td>
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<tr>
<td>DIE</td>
<td>Diencephalon</td>
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<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>Immunoglobulin G</td>
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<td>RNA polymerase II</td>
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<td>Ptk-EGFP</td>
<td>Thymidine kinase promoter - enhanced green fluorescent protein</td>
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<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>SEQ</td>
<td>Sequencing</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
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<td>Human Embryonic Kidney</td>
</tr>
<tr>
<td>μl</td>
<td>Microliter</td>
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<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>ML</td>
<td>Milliliter</td>
</tr>
<tr>
<td>NEO</td>
<td>Neomycin</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PAX</td>
<td>Paired box gene</td>
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<tr>
<td>LM</td>
<td>Lumbricals</td>
</tr>
<tr>
<td>IO</td>
<td>Dorsal interosseous</td>
</tr>
<tr>
<td>DH</td>
<td>Dorsal hood</td>
</tr>
<tr>
<td>PIP</td>
<td>Proximal interphalangeal joint</td>
</tr>
<tr>
<td>MP</td>
<td>Metacarpal phalangeal joint</td>
</tr>
<tr>
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<td>Full Name</td>
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<td>-----------</td>
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<tr>
<td>ECD</td>
<td>Extensor digitorum communis</td>
</tr>
<tr>
<td>FDS</td>
<td>Flexor digitorum sublimes</td>
</tr>
<tr>
<td>FDP</td>
<td>Flexor digitorum profundus</td>
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</tbody>
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ABSTRACT OF THE DISSERTATION

Mechanism of Lmx1b-regulated Limb Dorsalization

by

Jennifer Feenstra

Doctor of Philosophy, Graduate Program in Anatomy
Loma Linda University, June 2012
Dr. Kerby Oberg, Chairperson

Lmx1b, a LIM homeodomain transcription factor is necessary for limb, kidney, eye, and central nervous system development. Lmx1b knockout (KO) mice display; abnormal limb dorsalization with distal ventral-ventral limbs, nephropathy, ocular malformations, loss of dopaminergic/serotonergic neurons, and reduced cerebellum and tectum formation. The developmental mechanisms utilized by Lmx1b remain largely unknown; to investigate downstream targets, we have used the limb as a model because of its accessibility and striking Lmx1b KO phenotype.

To identify candidate genes, we compared gene arrays from Lmx1b KO and wildtype mouse limbs during limb dorsalization, i.e., 11.5, 12.5, and 13.5 days post coitum. We identified 54 target genes differentially expressed in all three stages. An abundance of extracellular matrix-related targets were found to be regulated by Lmx1b, including collagens and proteoglycans. Three Lmx1b-regulated proteoglycans, are clustered together at one genomic locus, Keratocan, Lumican, and Decorin (KLD). To determine whether Lmx1b’s regulation of these targets is direct, we used in silico comparative genomic analysis and identified a potential regulatory region with a known Lmx1b binding site (Peak 3). We hypothesized that Lmx1b directly regulates KLD via Peak 3. We isolated Peak 3 and linked it to a green fluorescent protein (GFP) reporter.
This construct was electroporated into chick embryos at the primitive streak stage (Hamburger-Hamilton (HH) 4) and during early neural tube formation (HH10). GFP expression was detected in neural tube and surrounding the developing midbrain and cerebellum. Expression of Lmx1b and KLD mRNA overlaps the activity of this regulatory region.  

Our data suggests Lmx1b regulates morphogenesis by altering the extracellular matrix composition such as KLD proteoglycans. Furthermore, the activity of KLD-associated Peak 3 localized to Lmx1b-dependant CNS structures during development supports a direct role for Lmx1b regulation.
CHAPTER ONE

INTRODUCTION

The Developing Limb

Around 1 in 600 human newborns present with some form of upper limb abnormality (Flatt AE 1977). Characterization of the molecular pathways that orchestrate limb development is essential to understanding the etiology, diagnosis and possible interventions for patients with limb malformations. Additionally, the limb is a powerful developmental model to examine how patterned structures arise. Therefore, the molecular mechanisms which orchestrate limb development have been the focus of intense study for several decades.

During early embryonic development, homeobox (Hox) transcription factors set up a segmental body plan along the cranial-caudal axis (Burke, Nelson, Morgan, & Tabin, 1995). Around the fourth week of human development, the presumptive limb fields are established, triggering the expression of Tbx5, Wnt and Fgf proteins that initiate limb outgrowth (Burke, et al., 1995). The limb bud emerges as a bulge of lateral plate mesoderm covered by a thin outer layer of ectoderm (Fig. 1.1). Following formation of the limb bud, development proceeds along three axes: proximal-distal, anterior-posterior (radial-ulnar), and dorsal-ventral (Fig. 1.1).
Figure 1.1) Limb Bud Coordinate Axes and Signaling Centers
The forelimb (boxed region, A) of a Carnegie stage 13 (Moore, Hutchins, & O'Rahilly, 1981) embryo is depicted with the three coordinate axes (B, C), each with their own signaling center. The apical ectodermal ridge (AER - orange) coordinates proximal-distal (Pr-Di) outgrowth and differentiation. The zone of polarizing activity (ZPA - purple) controls radial-ulnar asymmetry. The dorsal ectoderm (green) regulates dorsal-ventral (Do-Ve) asymmetry. Within the FGF responsive zone (FRZ - blue), the fate of mesodermal cells are determined by these signaling centers. The axes and signaling centers are shown in different orientations; (B) Dorsal view, and (C) Lateral, end on view (Modified from Oberg, Greer, & Naruse, 2004).

Formation of the Proximal-Distal and Anterior-Posterior Axes

Development and differentiation of each axis is controlled by a population of cells that signal patterning information to local cells and organize tissue along its axis, i.e., signaling centers. Mesodermal Fgf10 at the apical dorsal-ventral boundary induces ectodermal thickening to form the signaling center for the proximal-distal axis, the apical ectodermal ridge (AER) (Zakany, Zacchetti, & Duboule, 2007). The AER, in turn, secretes Wnt3 and several Fgf proteins (Fgf4, 8, 9 & 17), which maintain Fgf10 expression in the underlying mesoderm. Fgf10 sustains the proliferation of a sub-AER population of cells termed the Fgf responsive zone (FRZ) (Boehm et al., 2010). This
reciprocal loop of ectodermal and mesodermal Fgf/Wnt proteins maintains proximal-distal outgrowth (Barrow et al., 2003; Kawakami et al., 2001).

Development and differentiation along the anterior-posterior (radial-ulnar) axis is controlled by the zone of polarizing activity (ZPA) in the posterior (ulnar) limb mesoderm. The ZPA expands limb width and posteriorizes (ulnarizes) the developing limb through a secreted morphogen, sonic hedgehog (SHH) (Riddle, Johnson, Laufer, & Tabin, 1993). The AER and the ZPA are closely linked by a reciprocal feedback loop that maintains SHH expression adjacent to the distal posterior (ulnar) border of the AER during progressive outgrowth (Lee Niswander, Jeffrey, & Martin, 1994; Sun et al., 2000).

**Figure 1.2) Molecules Linked to Dorsal-Ventral Limb Development**

Graphic representation of the molecular interactions initiating dorsal-ventral axis formation. Wnt7a expression in the dorsal ectoderm is denoted by blue, the boundary between dorsal and ventral is demarcated by the green dot (Radical Fringe) and En-1 in the ventral ectoderm is orange. Lmx1b expression along the dorsal compartment is shown in red, arrow in dorsal compartment indicates Lmx1b induction via Wnt7a. (+ indicates the molecule is a dorsalizing factor)
Formation of the Dorsal-Ventral Limb Axis

Patterning of the dorsal-ventral axis begins with delineation of the dorsal and ventral limb compartments (Fig 1.2). Initially the secreted protein Wnt7A is expressed in the presumptive limb ectoderm. The transcription factor Engrailed 1 (En-1) is induced by Bone Morphogenic Proteins in the ventral ectoderm. En-1 restricts Wnt7A expression to the dorsal ectoderm, where Wnt7A dorsalizes the underlying limb mesoderm through the induction of the Lim homeodomain transcription factor Lmx1b (Riddle et al., 1995; Vogel, Rodriguez, & Warnken, 1995). The role of each of these molecules in dorsal-ventral patterning is described in further detail below.

Wnt7A

The Wnt family of secreted proteins is essential for normal limb formation and several Wnt proteins are differentially expressed during limb outgrowth. Secreted Wnt7a in the dorsal ectoderm upregulates the expression of Lmx1b in the dorsal mesoderm and also modulates posterior Shh expression in the ZPA, thereby influencing both anterior-posterior and dorsal-ventral axis formation. Functional absence of Wnt7a in knockout mice causes ventralization of dorsal limb structures, i.e., a ventral-ventral limb with footpads on the dorsal autopod surface (Chen et al., 1998; Cygan, Johnson, & McMahon, 1997). In contrast, misexpression of Wnt7a in the ventral ectoderm induces Lmx1b expression in the ventral limb compartment and a subsequent dorsal-dorsal limb phenotype (Loomis, Kimmel, Tong, Michaud, & Joyner, 1998). In humans, mutations of WNT7A are associated with Fuhrman syndrome or Al-Awadi/Raas-Rothschild/Schnizel phocomelia. Fuhrman syndrome may arise from one or more mutations found in the
third exon of WNT7A correlated to bowing of the femoral bone, absent fibula and/or ulna, polydactyly, syndactyly, oligodactyly, hypoplastic fingers and fingernails (Woods et al., 2006). Al-Awadi/Raas-Rothschild/Schnizel phocomelia is associated with a mutation in the fourth exon of WNT7A associated with a variation of symptoms including absence of the ulna, pelvis, uterus and/or fibula or the more pronounced anomaly phocomelia (Lonardo, Sabba, Luquetti, Monica, & Scarano, 2007).

The striking morphological changes in both the Wnt7a KO mouse model and in the human studies have established a role for ectodermally expressed Wnt7a as an organizer of the dorsal-ventral limb axis with contributions to the anterior-posterior axis. Wnt7a is essential for up-regulation of Lmx1b in the underlying dorsal mesoderm, which initiates the cascade necessary for limb asymmetry along the dorsal-ventral axis. Although Lmx1b conveys dorsal limb fates, the mechanism by which Wnt7a induces Lmx1b remains to be shown.

**Engrailed-1**

Engrailed-1 (En-1) is a homeobox transcription factor expressed in the ventral ectoderm, which restricts Wnt7a to the dorsal mesoderm. In the mouse, loss of En-1 allows Wnt7a to maintain its expression in the ventral ectoderm, thereby creating a dorsal-dorsal limb phenotype (Loomis, et al., 1998). In contrast loss of Wnt7a does not alter or expand En-1 expression and produces a ventral-ventral limb (Parr & McMahon, 1995). Since cells not under the influence of En-1 produce a ventral morphology, it has been suggested that the presumptive limb field is programmed with a ventral specification *i.e.* the default pathway of the limb is ventral (Chen & Johnson, 1999).
Therefore, the presumptive limb is specified as ventral until intervention by Wnt7a which induces Lmx1b, thereby effectively dorsalizing the limb.

Lmx1b

Lmx1b is a LIM-homeobox transcription factor comprised of eight exons that produce a 7kb mRNA (Dunston, Hamlington, et al., 2004). Exon 7 can be alternatively spliced to produce two predicted proteins of 372 and 379 amino acids. It is currently unknown if these isoforms have any functional difference. The second and third exon of Lmx1b contains two N-terminal LIM-domain regions, characterized by two tandem repeats of cysteine/histidine and zinc finger domains. LIM-domains represent potent protein-protein interaction motifs and have been studied in many fields including cytoskeletal organization, organ development and oncogenesis. Found in exon four, five and six is a DNA/RNA binding homeodomain region, which is highly conserved (99%) between mouse and human. Using electrophoretic mobility-shift assays it has been shown that the Lmx1b homeodomain binds to the FLAT sequences, i.e. FLAT-E (TAATTA) and FLAT-F (TAATAAT) (Rohr et al., 2002). Many well-known homeobox transcription factors have been characterized as selector genes, which are considered to be or act as master switches with the ability to transcriptionally enhance or repress other genes (Kuziora & McGinnis, 1988).

Due to its functionally interactive structure and classification as a selector gene, it is not surprising that Lmx1b is essential for the correct development of several organ systems. In humans, loss of one Lmx1b allele (haploinsufficiency) causes Nail Patella Syndrome (NPS) (Chen, et al., 1998). Although commonly known as NPS this syndrome
may also be referred to as Onychoosteodysplasia, Turner-Kieser Syndrome, or Fong Disease. Mutations of the Lmx1b gene cause NPS and occur mainly within the LIM domains (82%) and the homeodomain (18%) (E. M. Bongers, Gubler, & Knoers, 2002), enhancing the importance of these regions for Lmx1b function. NPS is an autosomal dominant disorder symptoms of which can include nephropathy, open angle glaucoma, inhibition of dopaminergic-serotonergic neurons and various skeletal abnormalities including absent patella, elbow dysplasia, iliac horns and reduced/malformed nails.

The Lmx1b gene is highly conserved between mouse and human. To delineate the role and mechanism of Lmx1b during development, a functional mouse knockout (C57BL/6 background) was generated by replacing the first LIM domain and the homeodomain with a neomycin cassette (Chen et al., 1998). Interestingly, the heterozygous mutants (\textit{Lmx1b} \textit{+/-}), analogous to the human condition, do not exhibit a phenotype. However, the complete lack of Lmx1b function in these mice (\textit{Lmx1b} \textit{-/-}) produces NPS-associated organs with a much more severe pathology than seen clinically. The examination of NPS patients and generation of the Lmx1b KO mouse model has helped to delineate the functional roles for Lmx1b during development. These roles are reviewed below.

**Functional Roles of Lmx1b during Development**

**Kidney Development**

Renal dysfunction is considered the most life threatening of all the NPS symptoms, the homozygous \textit{Lmx1b} KO mice display severe kidney defects including large glomerular leaks along the vasculature causing proteinuria and death (Chen, et al.,
The glomerulus and surrounding Bowmans capsule make up the basic filtration system of the kidney. The glomerulus has a unique basement membrane that forms the boundary between the blood and urinary spaces and is flanked by a layer of podocytes. The processes extending from podocytes determine the size-selectivity of the filtration barrier. Podocytes are responsible for secreting basement membrane components including collagens and proteoglycans. Lmx1b directs podocyte differentiation and maintenance, thereby enabling formation of the distinct collagen fibril composition of the glomerular membrane (Rohr, et al., 2002). Specifically, in the mouse at 13.5 days post coitum (dpc), Lmx1b is expressed in presumptive glomerulus and persists in the visceral glomerular epithelium into postnatal life. Loss of Lmx1b causes disorganization of the glomerular basement membrane and disruption and/or fusion of podocyte processes (Morello et al., 2001). The disruption of these layers causes the renal dysfunction seen in NPS patients and the Lmx1b KO mouse. In Lmx1b KO mice, there is a decrease in the expression of collagen IV types (isoforms) three and four in the glomerular basement membrane. Additionally, studies using a podocyte-specific Lmx1b KO mouse demonstrate the importance of Lmx1b in initial development and maintenance of podocytes in the perinatal mouse (Suleiman et al., 2007). Interestingly, patients with Alport Syndrome, which is caused by a collagen gene mutation, develop similar nephropathy to NPS patients, further supporting the concept of collagen regulation during glomerular basement membrane formation (E. M. Bongers, et al., 2002).
Eye Development

Abnormal ocular structures are not common with NPS patients; however, congenital cataract, microcornea and sclerocornea have been noted in the literature. Also approximately 50% of NPS patients will develop clinical symptoms of open angle glaucoma (Lichter et al., 1997; McIntosh et al., 1998; Mimiwati et al., 2006). In the mouse, *Lmx1b* expression begins around 10.5 dpc in the periocular mesenchyme followed by a localization to the anterior eye including corneal endothelium, iris ciliary body and trabecular meshwork (E. M. H. F. Bongers et al., 2005; Pressman, Chen, & Johnson, 2000). Loss of Lmx1b causes cornea stromal defects and hypoplasia of the iris and ciliary body possibly causing an increase in intraocular pressure responsible for the open angle glaucoma commonly seen in NPS patients and the *Lmx1b* KO mouse. Together the morphology of NPS patients and the *Lmx1b* KO mouse demonstrate the importance of Lmx1b for establishing of the anterior segments of the eye.

Central Nervous System Development

A role for Lmx1b during CNS development has also been suggested. During CNS development, the isthmus organizer is a signaling center responsible for midbrain and hindbrain formation. In the mouse, *Lmx1b* is expressed in the isthmus organizer beginning at 9 dpc (Guo et al., 2007). In the *Lmx1b* KO mouse, genes normally associated with development of the isthmus are not expressed (*Fgf8, Wnt1, En1, En2 and Pax2*). Subsequently loss of Lmx1b causes a severe reduction of not only the midbrain dopaminergic neurons but also the tectum and cerebellum (Guo, et al., 2007). The tectum is part of the midbrain that contains the superior and inferior colliculi responsible for
visual and auditory receptors respectively. The cerebellum in Lmx1b KO mice is smaller but appears normally patterned indicating that Lmx1b is responsible for growth but not patterning of the cerebellum.

Lmx1b is also responsible for determining serotonergic neurons of the hindbrain (Y. Ding et al., 2003). Loss of serotonergic signaling is thought to contribute to several mental disorders including anxiety, depression, addictions, and post-traumatic stress. Correspondingly, NPS patients seem to be predisposed to attention deficit hyperactivity disorder and major depressive disorder, although they have normal intelligence with no apparent learning disorders (Lopez'Arvizu et al., 2011).

Lastly, Lmx1b is expressed in the dorsal interneurons of the spinal cord where it contributes to assembly of pain circuitry and differentiation/migration of sensory neurons/afferent fibers (Y. Q. Ding et al., 2004). In the Lmx1b KO mouse, no synaptic contacts are formed between the dorsal horn neurons and nociceptive afferent neurons responsible for pain response (Y. Q. Ding, et al., 2004). Similarly, NPS patients experience neurological deficits such as diminished pinprick sensation and tingling or numbness in the limbs (Dunston, Reimschisel, et al., 2004).

Limb Development

Lmx1b is robustly expressed in the dorsal mesodermal compartment of the developing limb. In the mouse, strong Lmx1b expression can be detected in the dorsal compartment at 10.5 dpc. With stage progression, Lmx1b expression fades in the proximal tissue but remains strong in the distal limb/autopod until 15.5 dpc (Riddle, et al., 1995; Vogel, et al., 1995). NPS patients demonstrate a disruption of dorsal limb
structures such as the patella and nails (Vollrath et al., 1998) while the Lmx1b KO mouse limb displays a complete loss of distal symmetry with ventral-ventral morphology of the joints, tendons, limb musculature and nerves (Chen, et al., 1998) (Fig 1.3).

Lmx1b is essential for normal dorsal-ventral limb axis formation. Loss of Lmx1b causes distal ventral-ventral limb symmetry while overexpression of Lmx1b forms dorsal-dorsal structures (Chen & Johnson, 2002; Cygan, et al., 1997; Loomis, et al.,
indicating Lmx1b is both necessary and sufficient for distal limb dorsalization. Additionally, recent fate mapping experiments in the chick and mouse (Arques, Doohan, Sharpe, & Torres, 2007; Pearse, Scherz, Campbell, & Tabin, 2007; Qiu, Chen, & Johnson, 2009) have shown Lmx1b-expressing cells create or define the dorsal limb compartment. Moreover, while Lmx1b does not initiate compartment formation it is essential for dorsal compartment maintenance and dorsal morphogenesis. Collectively, these data indicate that Lmx1b is responsible for limb dorsalization, however, the mechanism and downstream targets remain to be determined. Thus, the underlying objective of this study is to discover the mechanism of Lmx1b-regulated limb dorsalization.

**Dissertation Hypothesis**

Based on the evidence from literature, I hypothesized that Lmx1b dorsalizes the limb by modifying the expression of genes involved in the formation of joints, ligaments, tendons and bones. I investigated this hypothesis through the following specific aims.

**Project Specific Aims**

Specific Aim 1.) Identify Genes in the Dorsal Mesoderm Regulated by Lmx1b During Limb Development

**Rationale and Approach**

Haploinsufficiency of Lmx1b in Nail Patella Syndrome (NPS) patients and complete loss of Lmx1b in the KO mouse indicate the significance of Lmx1b to pattern the dorsal tissues of the limb. However, downstream Lmx1b targets in the limb are
currently not well characterized. Thus, my **working hypothesis** was that **Lmx1b altered downstream skeletal and soft tissue targets, causing asymmetrical limb patterning.**

To examine this hypothesis and discover potential Lmx1b gene targets, we used microarray analysis and compared *Lmx1b* KO mice to wildtype littermates. Specifically we targeted stages during limb dorsalization and limb tissues with robust *Lmx1b* expression (11.5-13.5 dpc). Genes differentially expressed in the microarray analysis were validates with Real-time PCR. Additionally, whole mount *in situ* hybridization (WMISH) was performed to determine the corresponding tissue pattern of expression for the validated Lmx1b target genes.

**Outcomes**

Targets discovered that were differentially expressed in the microarray, validated by Real-time PCR and asymmetrically expressed along the dorsal-ventral axis included a variety of genes ranging from angiogenesis to skeletal formation. Interestingly, a significant portion of genes discovered were extracellular matrix molecules such as Collagen11a2, Collagen9a3, Aggrecan, Lumican, Decorin, Keratocan. We also discovered several genes that are known to associate with or accentuate proteoglycan function including Matrilin1, Matrilin4 and Lysyl oxidase. From these findings I concluded that Lmx1b may function to regulate extracellular matrix composition, thereby patterning developing tissues. See chapter three for the detailed description and discussion of these experiments published in *Development, Growth, and Differentiation.*
Specific Aim 2.) Determine Direct Induction of Downstream Lmx1b Targets

Rationale and Approach

I consider genes discovered in Specific Aim 1 to be downstream Lmx1b targets, a preponderance of which are extracellular matrix molecules. The discovery of multiple extracellular matrix molecules suggests that Lmx1b dorsalizes the limb, at least in part, by changing extracellular matrix composition. Among these extracellular matrix molecules were four proteoglycans, three of which (Keratocan, Lumican, Decorin) were dorsally expressed in the mouse limb at 12.5 dpc. Thus, my working hypothesis was that Lmx1b directly regulates the proteoglycans: Keratocan, Lumican and Decorin.

To confirm this direct relationship, we chose to use the chick as a bioassay because of its accessibility and our ability to focally manipulate gene expression. We successfully developed an avian retroviral construct, which expresses human Lmx1b. In addition, we used an electroporation technique to focally express the construct in the ventral wing mesoderm of the chick and induce dorsalization of ventral tissues. (see Chapter 2).

However, when I performed WMISH using Keratocan, Decorin and Lumican in the chick, I discovered that these targets, which are dorsally-restricted in the mouse, are expressed in the dorsal and ventral wing mesoderm. Thus, native expression of Keratocan, Lumican, and Decorin in the chick wing was different from the mouse and would impair our ability to determine up-regulation via our human Lmx1b construct. Thus, we abandoned the chick as a bioassay to demonstrate direct Lmx1b regulation of these proteoglycans as originally planned. Instead we reported these unique differences...
in expression and refocused our energies on other techniques to determine direct Lmx1b regulation as outlined in Specific Aim 3.

Outcomes

Many developmental pathways are conserved across vertebrate species. Thus, this disparate expression between chick and mouse was not expected. To clarify this discrepancy, we performed a detailed temporal and spatial expression pattern analysis of these proteoglycans (Decorin, Lumican and Keratocan) during dorsalization, comparing both forelimbs and hindlimb in the chicken and mouse models. Our analysis demonstrated a marked species-specific difference in the expression patterns of these proteoglycans. We also included Aggrecan which is expressed in proliferating chondrocytes and is conserved between chick and mouse. Our publication describing this disparate proteoglycan expression pattern is detailed in chapter four.

Specific Aim 3.) Determine the Mechanism by which Lmx1b Regulates Direct Downstream Targets

Rationale and Approach

New sequencing technologies have allowed the field of genetics to flourish. Furthermore, new sequences from evolutionarily diverse species are being added to public databases regularly. This has created new opportunities to compare evolutionary gene changes across species which may correspond to function. In particular, we are interested in the discovery of noncoding sequences that participate in gene regulation called cis-regulatory regions. These regulatory regions can lie nearby or significantly up/downstream of their target genes (Gomez-Skarmeta et al., 2006; Pfeifer et al., 1999)
Regulatory functions that are conserved across species typically maintain these non-coding sequences and appear to evolve more slowly than associated coding sequences (Blanchette and Tompa, 2003). To identify these conserved noncoding regions (CNRs), we used VISTA Genome Browser (http://pipeline.lbl.gov/cgi-bin/GenomeVista, Kent et al., 2002; Frazer et al., 2004). VISTA compares the human genome to many diverse species and indicates areas of conservation in increments of 100bp intervals. For our analysis we used dog, mouse, opossum, and chicken. We initially examined Keratocan since it revealed the greatest fold-change in the microarray analysis and WMISH of Keratocan was undetectable in the Lmx1b KO mouse limb. When we examined the location of Keratocan on the genome, we recognized that Lumican and Decorin lie just upstream (5’) of Keratocan. We referred to this cluster of three proteoglycan genes as the KLD locus. Additionally, 5’ to the KLD locus is a 900kb gene desert, a region devoid of protein coding genes. When searching for conserved sequences, gene deserts have been shown to be regions rich in regulatory elements. VISTA analysis produced 12 CNRs surrounding the KLD locus. Using more detailed genomic alignment, we discovered two of the CNRs, Peak3 and Peak10, contain FLAT sequences (potential Lmx1b binding sites) which are conserved across the multiple species we examined. Thus, my working hypothesis for this specific aim was that Lmx1b binds to its specific binding site (FLAT sequence) within Peak3 and Peak10 to directly regulate Keratocan, Lumican and/or Decorin.
Outcomes

Peak3 and Peak10 constructs were electroporated into the primitive streak stage chick embryo and allowed to incubate to HH14 to determine activity. Peak10 showed no activity in the early embryo but Peak3 showed activity in the neural tube and developing brain vesicles beginning around HH10. Later CNS targeted expression of Peak3 reveals activity surrounding the developing brain vesicles at HH19/20. Isolation and overexpression of the FLAT binding site in the developing brain also shows activity indicating the FLAT site may be the region necessary for Peak3 activity. Together we believe this indicates that Lmx1b uses the FLAT site of Peak3 to direct KLD expression in the developing brain. Data acquired to support this aim is presented in chapter 5.

Peak3 and Peak10 were also electroporated into the presumptive limb at HH14 and monitored for GFP every 24 hours from HH19-HH25. However no activity was detected for Peak3 or Peak10 in the chick limb.

Specific Aim 3.) Alternate Strategy

Rationale and Approach

In addition to the identification of regulatory regions using software methods, newly developed next generation sequencing techniques have not only emerged but have now become affordable. The combination of next generation sequencing and chromatin immunoprecipitation (ChIP-seq) is a powerful tool to examine DNA-protein interactions. Our goal is to use ChIP to isolate Lmx1b-bound chromatin during limb development, when Lmx1b is expected to interact with DNA to actively regulate transcription responsible for dorsalization (12.5dpc). At 12.5 dpc limbs are harvested and fixed using
formaldehyde to reversibly cross-link DNA-protein complexes. The chromatin is then fragmented and combined with a high affinity Lmx1b antibody. The chromatin bound to Lmx1b is isolated and purified for sequencing. For our experiments, a high affinity Lmx1b antibody is available (Suleiman, et al., 2007). However to implement ChIP-seq a significant challenge remains, a direct downstream target that will act as a positive control is important for optimization. Currently the only reported direct downstream targets of Lmx1b are in the kidney.

In the kidney, podocytes secrete basement membrane components including collagens and proteoglycans. Lmx1b directs podocyte differentiation and maintenance, and loss of Lmx1b causes aberrant basement membrane formation, thereby preventing normal glomerular filtration (Rohr, et al., 2002). Two genes have been implicated as direct downstream Lmx1b targets in the kidney: Col4a4 and Nphs2 (Rascle et al., 2009).

The disruption of renal morphology in the Lmx1b KO mouse, suggests that kidney development is similar to limb development, with Lmx1b regulating extracellular matrix during morphogenesis. Therefore, we believe targets discovered in embryonic kidney cells using ChIP-seq may overlap with, and also expand, our understanding of Lmx1b-mediated limb patterning. We hypothesize that Lmx1b regulates Keratocan, Lumican and Decorin (KLD) by binding to predicted CNRs of the KLD locus during development.

Outcome from Alternate Strategy

Human Embryonic Kidney (HEK) cells have been grown in culture and transfected with an Lmx1b-hemagglutinin (HA) tagged construct. Lmx1b-HA cells were
harvested to extract DNA, RNA and nuclear protein. We successfully amplified \textit{Lmx1b}, \textit{Col4a4} and \textit{Nphs2} from HEK RNA and the FLAT site of \textit{Col4a4} and \textit{Nphs2} from HEK DNA by PCR. We also successfully detected enhanced \textit{Lmx1b} protein levels in \textit{Lmx1b-HA} transfected HEK cells by Western blot. Since we determined the HEK cells mimicked in vivo conditions, we continued with the ChIP-it Express Enzymatic kit from Active Motif. Using the HA antibody, we were able to isolate \textit{Lmx1b-HA}-bound chromatin and subsequently confirmed the presence of the FLAT sequence by PCR. However, we have had trouble immunoprecipitating enough chromatin for sequencing purposes. \textit{Lmx1b} may be a low abundance molecule so we will need to perform multiple ChIP runs with a high number of cells. The progress of this alternative approach is detailed in chapter 6.
CHAPTER TWO
MATERIALS AND METHODS

Specific Aim 1 Methods

Lmx1b Mouse Model

The Lmx1b KO mouse was generated by Randy Johnson (Chen, et al., 1998). The homeodomain and first LIM domain of the Lmx1b gene was conditionally knocked out by inserting a neomycin cassette using the C57BL/6J mouse background. Males and females are housed in separate cages with individual ventilation. Colony is maintained by mating Lmx1b (+/-) males with Lmx1b (+/+) females, additionally new C57BL/6J unrelated virgin females are ordered from Charles river every four months to maintain genetic diversity of the colony. Time dated matings use heterozygous (Lmx1b +/-) males mated with heterozygous females to obtain homozygous Lmx1b mutant or KO embryos (Lmx1b -/-) and wildtype littermates (Lmx1b+/+). For colony maintenance matings male (+/-) and female (+/+) mice were housed together for 14 days then separated to allow female to deliver pups. For time dated matings female mice were placed in the male cage for a 12 hour period, from 7pm to 7am with females checked in the morning for vaginal plugs to confirm copulation. Noon on the day the vaginal plug was identified was considered as 0.5 days post coitum (dpc). Pregnancy was determined by weighing females on day 7 to determine a baseline weight then repeated on days 10/11/12/13 depending on stage required. Pregnant females on average will gain 1 gram per day, older females with bladder problems can show sporadic weight gain, which does not increase
over time. Colony Pups were weaned at four weeks after which tail snips were taken to perform genotyping. Mice are anesthetized with Isoflurane in a closed chamber (200 μl/750 ml) for 30 seconds. Approximately 1cm of the tail tip is removed and diced with a sterile razor blade. Mouse tails are dipped in hemostatic powder to decrease bleeding then placed back in cages and observed to ensure proper recovery from anesthesia. Diced tail specimens are placed into 750 μl tube containing 250 μl of QuickExtract DNA extraction solution (EPICENTER). Tails are vortexed for 15 seconds and incubated 6 minutes at 65°C, vortexed again and incubated for 2 minutes at 98°C. 5 μl of this solution was used for PCR genotype analysis, primers used demonstrate the presence or absence of the Neomycin (Neo) cassette inserted into the Lmx1b gene. Mice containing the Neo cassette on both alleles are labeled mutants (-/-), one Neo copy are heterozygous (+/-), and normal mice with no Neo insert are wild type (+/+). PCR parameters are as follows: 95°C/5min (1cycle), 95°C/15sec, 60°C/30sec,72°C /45sec (30cycles), 72°C/7min (1cycle), 4°C ∞. Following timed matings pregnant females are anesthetized the day of harvesting in the closed container with isoflurane. Females are sacrificed using cervical dislocation after which embryos are rapidly removed under sterile conditions. Embryos are removed from their gestational sac and placed in 1% phosphate buffered saline (PBS) to continue cleaning. Heads were placed in QuickExtract DNA extraction solution (EPICENTER) and used for genotyping in the same manner as tail snips. The digestive tract, heart and urogenital tract are removed from embryos followed by overnight incubation in MEMFA (0.1 mol/L 3-(N Morpholino) propanesulfonic acid (MOPS), 2 mmol/L ethylene glycol tetraacetic acid (EGTA), 1 mmol/L MgSO4, 3.7%
formaldehyde) at 4°C. Following fixation embryos are rinsed in PBS and stored in 90% Methanol at -20°C.

Microarray Analysis

Lmx1b is a transcription factor utilized during the development of multiple organ systems. In 1998, Col4a4 was discovered as a direct downstream target of Lmx1b in the kidney. With this discovery it was thought that further determination of the Lmx1b cascade would be imminent. However since that discovery nearly 14 years ago only one additional direct downstream kidney target has been discovered (Nphs2). In order to elucidate previously undiscovered downstream Lmx1b targets in the limb we utilized a Lmx1b KO mouse model in conjunction with microarray analysis. Limbs were analyzed during stages of initial tissue differentiation and Lmx1b expression using the Affymetrix GeneChip® Mouse Genome 430 2.0 array. This array is a comprehensive analysis of the mouse genome utilizing a powerful probe set of eleven mismatched oligonucleotide probes per transcription sequence for over 39,000 transcripts and variant sequences. Mice were time-mated and embryos were harvested at 11.5, 12.5 and 13.5 days post coitum (dpc). Embryos were placed in 1% PBS, heads were used for genotyping and limbs were removed and stored in RNAlater (Ambion) at -80°C. We harvested RNA from Lmx1b +/+ and -/- littermate limbs using the RNeasy kit from Qiagen. We modified the protocol slightly using three freeze thaw reactions with liquid nitrogen to crush the tissue. Buffer RLT including 1ul/100ul B-Mercaptoethanol was added to tissue followed by a 10 min incubation at room temperature before spinning in QIAshredder column. Final elution was performed twice, 15ul each elution. RNA utilized was obtained from at least three
separate litters producing three unique forelimb sets pooled together to minimize biological variability. RNA was quantified with nanodrop spectrophotometer and around 12 μg was sent to University of California Irvine (UCI Genomics) where RNA and microarray analysis was completed.

![RNA Analysis](image)

**Figure 2.1) RNA Analysis done by UCI on 11.5 dpc Samples**

An example of the RNA analysis done by UCI shows RNA sent in by our lab in Lanes labeled 1-4 and RNA sent in from other labs in lanes 5-7. Lanes 1-2 are wildtype RNA Lanes 3-4 are Lmx1b Knockout RNA.

Microarray data were received and uploaded to Comprehensive R based Microarray Analysis web frontend (CARMAweb, https://carmaweb.genome.tugraz.at/carma/). CARMAweb simplifies the statistical programs R and Bioconductor to normalize and quantify fold-change and significance. Data for each gene chip was normalized using JustRMA. Fold-change and significance were determined using the Limma package which preforms a moderated t-test, since we
used two chips per group this was the most powerful statistical analysis, since it corrects for possible sampling error. Note that chips for each stage were treated as replicates, for example 11.5 dpc WT1 and WT2 data was normalized individually then treated as a replicate data set to determine fold change and significance. Output analysis from CARMAweb is annotated with Affymetrix probe data and can be easily exported to Excel, any genes which display a significance of less than 0.005 were removed from further analysis. The Excel document was searched to discover genes represented in more than one array. Many genes were discovered in multiple arrays including genes up-regulated at one stage and down-regulated in the next. However, for the purpose of this study, we focused on genes up-regulated or down-regulated in all three stages since these should represent Lmx1b specific targets rather than stage specific targets. The list of genes discovered at all three stages was cross-referenced with literature and genes that were sex specific were discarded.

Real-time PCR

Possible downstream target genes were analyzed by Real-time PCR to validate microarray gene expression. Primers were generated for each gene target using Sequencher 4.6, each primer set met several parameters including: an annealing temp near 60°C, 16-22bp length, GC clamp on 5’ and 3’ end, produces a amplicon of 50-150bp. Primers were tested using mouse limb +/- cDNA libraries, resulting PCR was purified and sent out for sequence analysis to determine primer specificity. Primers which produced more than one amplicon were discarded. Real-time PCR was performed using the Roche Lightcycler 2.0 and Roche recommended sybergreen mastermix reagents.
Runs were standard for all target genes beginning with denaturation (95°C for 5 minutes), amplification 40 cycles (95°C for 10 seconds, 60°C for 10 seconds, 72°C for 10 seconds-data acquisition), melting curve (95°C for 10 seconds ramping at 0.1 degrees/sec continuous acquisition). Melting curves were performed following RT runs to determine product specificity. Lightcycler software was used to determine WT and KO fold change using phosphoglycerate kinase (PGK) control samples to normalize gene expression (Crossing point data) for each run. Each run was performed a minimum of 3 times, standard deviation for each was calculated with Excel to create bar graph error bars found in figure 3.3.

Whole Mount in situ Hybridization

Primers were generated using the same parameters to pull down 700-1000bp probe sequences in the chick and mouse models. Sequences were isolated and cloned using the pCR™ II-TOPO (Invitrogen) vector. Constructs were screened for insert and grown up overnight to perform Qiagen Miniprep, constructs with insert were sent for sequencing to determine sequence fidelity. Sequences with few or no mutations are isolated with PCR, then T7 is linked to PCR fragment using the Block-it kit by Invitrogen. The linkage of T7 allows for a second PCR of both sense and antisense strands using T7 and either the 5’ or 3’ gene specific primers. PCR products are purified (Quiagen) and concentration is determined with the nanodrop spectrophotometer. Sense and Antisense strands (1 μg) are transcribed using T7 enzyme in conjunction with a Digoxigen labeled UTP to create the final Dig-labeled riboprobe (Block-it kit, Invitrogen). Probes are treated with DnaseI and purified using Pellet Paint (Novagen).
Probes were then heated to 75°C for 10 minutes placed on ice and diluted in cold hybridization buffer to 10 ug/ml.

To determine 3-dimensional gene expression we utilized whole mount *in situ* hybridization (WMISH) with digoxigenin-labeled riboprobes as previously described (Yamada et al., 1999). Sterile reagents were made aloquated and frozen to ensure cleanliness. Protienase K reactions were 30 min for 11.5dpc mice and HH 23 chick and 45min for 12.5-14.5 dpc mice and HH 23-29 chick. Hybridization of the transcribed RNA probes was carried out at 60°C with stringent post-hybridization washes at 65°C. Sense probes were also generated and tested to verify antisense probe specificity. Embryos were checked every 20 minutes during colorization to ensure optimal probe staining then washed with PBS, fixed in MEMFA and stored in 90% Methanol. We expect that genes with confirmed differential expression overlapping Lmx1b expression during limb development will be considered downstream targets. A minimum of two expression pattern WMISH runs was done in both the chick and mouse using both upper or lower limbs to establish a baseline expression pattern.

**Specific Aim 2 and 3 Methods**

**Chick Model**

Further studies of downstream Lmx1b targets were performed using the chick model system. The chick is a longstanding invaluable model for limb development research allowing researchers to perform in ovo experiments such as transplantations and AER removal. These types of historic experiments have producing data, which substantially increased our understanding of how the limb develops. The reasons for the chick model
systems popularity include the ability to harvest sufficient numbers of chick embryos at precise stages but most importantly the chick is readily accessible in the egg throughout development.

For all experiments using chick embryos fertilized white leghorn chick eggs (Chino Ranchers, CA) were incubated at 39°C in a standard humidified avian incubator. Depending on the type of electroporation or harvesting the eggs were windowed and staged according to Hamburger and Hamilton (HH,Hamburger & Hamilton, 1951).

WMISH of Lmx1b targets was also performed using comparable staged chick embryos. Similar to mouse the chick embryos were harvested in phosphate buffered saline, fixed in MEMFA overnight at 4°C and stored in 90% Methanol at -20°C.

Electroporation Studies

The accessibility of the chick limb means it can be surgically manipulated, electroporated and/or treated with chemicals/proteins. In particular we use several different types of electroporation in conjunction with two different vector delivery systems. The first vector used is the pTK-EGFP which contains the the f1 phage origin; an upstream SV40; a multiple cloning site (MCS); the Herpes simplex virus thymidine kinase minimal promoter; an enhanced GFP reporter gene; and the ampicillin resistance gene (Uchikawa et al., 2004). Plasmids were isolated from E. coli using the Qiagen EndoFree Plasmid Maxi Kit to minimize lipopolysaccharide-related cytotoxicity and improve transfection efficiency in chick embryos. The blank pTK-EGFP construct was used as a negative control. The second vector system is an avian specific retroviral
system RCASBP(A). This vector is replication competent, ampicillin resistant and contains a \textit{ClaI} insertion site (Hughes et al., 1987).

Whole embryo EP done at Hamburger-Hamilton (HH) stage 4, transfects the entire embryonic disc just as it is begins gastrulation. Embryos can be followed in culture for 48hrs up to HH14. Therefore electroporation of DNA at HH4 allows for screening of DNA activity in early chick development. First embryos are harvested from the egg by clearing albumin using the blunt end of forceps then a 2x2 cm Whatmans filter paper with a 1cm hole punched in the center is placed onto the Vitelline membrane with the embryo in the center of the punched hole. The filter paper is cut from the yolk and excess yolk is removed with Hanks Buffered Salt Solution. The embryo is placed on a silicone support with a 2mm deep well containing a 2 x 2 mm platinum cathodal electrode (see Fig 10). Using a glass capillary pulled-needle, a mixture of the construct plasmid (1μg) and Fas-green dye to visualize delivery is injected in the space between the vitelline membrane and the excised embryo. The anodal electrode is held above the embryo by a micromanipulator with a 3mm inter-electroodal space. HBSS is used between the electrode and the embryo to ensure electrical conductivity. The reporter plasmids are driven into the embryo by 5 pulses of 5 V for a duration of 50 ms at 100 ms intervals using a CUY21 electroporator (Protech International, Austin, Texas). Following electroporation embryos are incubated on a yolk agar plate in a humidified chamber at 37°C. Construct activity measured as fluorescent GFP is visualized with fluorescent microscopy and imaged at 24 and 48hrs.
Figure 2.2) Apparatus for Whole Embryo Electroporation at HH4
Upper panel, silicone support and micro-manipulators used for precise delivery of plasmid and stable upper electrode placement. Lower panel, embryo on silicone support showing needle with plasmid/phenol red marker at needle tip. The electrode is placed over the embryo in contact with buffer solution.
HH 10 EP was performed to determine construct efficacy in the brain. Embryos were staged and stained with neutral red to facilitate removal of the vitelline membrane surrounding the head and electroporation. DNA (1 µg) was injected into the neural tube lumen and pushed cranially toward the tip of the anterior neural pore. Electrodes are 0.5mm in diameter and are placed on either side of the head approximately 4mm apart delivering 3 pulses of 50 ms at 10 V at 100 ms intervals. Embryos are incubated and imaged with fluorescent microscopy at 24 and 48 hours to determine construct activity.

Targeted regional electroporation at HH14 was directed to transfect the lateral plate mesoderm thereby introducing DNA into the entire presumptive limb. Briefly, eggs are windowed and embryos stained with neutral red to determine somite number. The vitelline membrane overlying the limb is removed and a small slit cut on the yolk membrane near the heart. Two platinum electrodes (0.3 mm diameter, at 2.5 mm distance) are mounted on a micromanipulator and positioned parallel to the embryo. The cathode is placed through the slit and into the yolk. Using a glass capillary pulled-needle, a mixture of the regulatory-construct plasmid (2 µg/µL) and fas-green are injected into the embryonic coelom underlying the right lateral plate mesoderm. The anode is placed above the embryo and 3-5 drops of PBS are used to ensure conductivity. Electroporation (EP) was performed with 3 pulses of 50 ms at 8 V at 100 ms intervals. Following surgery embryos can be imaged for construct activity and/or incubated for 10 days allowing formation of the presumptive skeletal structures. We utilized TREP to test the limb dorsalization properties of an RCASBP(A) vector expressing hLmx1b. Overexpression of our RCASBP-hLmx1b construct successfully produced feather buds on the ventral limb surface.
Figure. 2.3) Dorsalization of the Chick Limb using the RCASBP-hLmx1b Construct
The chick wing was electroporated at HH14 along the lateral plate mesoderm to overexpress Lmx1b in the entire limb.

We also perform a focal limb electroporation technique using HH21/22 embryos. The Vitelline membrane is removed around the limb and a small amount of fluid is removed then limbs can be flipped to expose the ventral surface. A bolus of 1 μl containing 1 μg of DNA is injected into the limb mesoderm. Electrodes are placed on either side of the limb with fluid placed on top to ensure conductivity. Focal electroporation is performed with 3 pulses of 50 ms at 50 V at 100 ms. Below is an example of our focal electroporation technique using the RCASBP-hLmx1b expressed on
the ventral limb surface. Embryos were incubated for 48 hours and harvested to performed hLmx1b expression WMISH.

Figure 2.4) Focal Electroporation of RCASBP-hLmx1b into the Ventral Wing Mesoderm
Whole mount in situ hybridization probing for hLmx1b in the chick wing. Expression is absent from the control limb and can be seen in the ventral mesoderm of the experimental limb.
References


CHAPTER THREE
DETECTION OF GENES REGULATED BY LMX1B
DURING LIMB DORSALIZATION

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Running title: Lmx1b Regulated Genes in Limb Dorsalization

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Abstract

Lmx1b is a homeodomain transcription factor that regulates dorsal identity during limb development. Lmx1b knockout (KO) mice develop distal ventral-ventral limbs. Although induction of Lmx1b is linked to Wnt7a expression in the dorsal limb ectoderm, the downstream targets of Lmx1b that accomplish limb dorsalization are unknown. To identify genes targeted by Lmx1b, we compared gene arrays from Lmx1b KO and wildtype mouse limbs during limb dorsalization, i.e., 11.5, 12.5, and 13.5 days post coitum.

We identified 54 target genes differentially expressed in all three stages. Several skeletal targets, including Emx2, Matrilin1 and Matrilin4, demonstrated a loss of scapular expression in the Lmx1b KO mice, supporting a role for Lmx1b in scapula development. Furthermore, the relative abundance of extracellular matrix-related soft tissue targets regulated by Lmx1b, such as collagens and proteoglycans, suggests a mechanism which includes changes in the extracellular matrix composition to accomplish limb dorsalization.

Our study provides the most comprehensive characterization of genes regulated by Lmx1b during limb development to-date and provides targets for further investigation.

Keywords:
Limb development, mouse, patterning, Lmx1b, microarray
Introduction

The limb is a complex organ that requires unique multi-axis asymmetry for function. The limb emerges from the lateral plate mesoderm as a bulge of undifferentiated mesoderm covered by ectoderm. In the mouse model at 11.5 days post coitum (dpc) the limb bud mesoderm is diffuse and relatively uniform. Centrally, the mesoderm condenses to form cartilage anlagen and peripheral mesoderm condenses to form tendon precursors. Joint segmentation begins as a zone of high cell density (an interzone) within the mesodermal condensation (Craig, Bentley, & Archer, 1987; Dalgleish, 1964). The joint capsule and associated ligaments and tendon attachments develop from condensations surrounding the interzone regions (Mitrovic, 1978). This process proceeds in a proximal-to-distal fashion. By 13.5 dpc, the skeletal elements, joints, ligaments and tendon precursors of the stylopod (humerus/femur), zeugopod (radius-ulna/tibia fibula) and autopod (hand/foot) are largely defined.

Development of the limb bud can be described along three axes. The proximal-distal axis depicts the progressive outgrowth of the three limb segments: stylopod, zeugopod and autopod. The anterior-posterior axis defines the patterning of the digits (e.g., from thumb to little finger) and zeugopod (e.g., radius and ulna). The dorsal-ventral axis delineates the extensor and flexor compartments of the limb. The limb has marked asymmetry in structure and function along the dorsal-ventral axis despite being composed of similar elements. This limb asymmetry is under the control of Lmx1b, a dorsalizing homeodomain-containing transcription factor. Lmx1b knockout (KO) mice (Chen, et al., 1998) display distal ventral-ventral morphology in the bone, muscle and soft tissues. In humans, single allele mutation of LMX1B causes Nail Patella Syndrome (NPS) (Dreyer et al., 1998) with incomplete dorsalization of extremities, i.e., loss of the...
patella, deficient nails, and joint malformations. However, limb-specific downstream target genes of Lmx1b have yet to be clearly defined.

The molecular control of dorsalization is initiated by Wnt7a expression in the limb bud ectoderm (Riddle, et al., 1995). Progressive expression of the En-1 transcription factor in the ventral ectoderm restricts Wnt7a to the dorsal ectoderm (Cygan, et al., 1997; Loomis, et al., 1998). Secretion of dorsally-restricted Wnt7a into the underlying mesoderm induces Lmx1b expression limited to the dorsal mesoderm (Vogel, et al., 1995). During progressive joint and tendon formation (11.5dpc - 13.5dpc), the expression of Lmx1b proximally decreases and becomes restricted to differentiated tissues while retaining intense expression in the distal less-differentiated dorsal mesoderm (Dreyer et al., 2004).

The restriction of Lmx1b to dorsal tendons and joints during differentiation suggests a role in tendon/joint formation, but no direct link between Lmx1b and any tendon- or joint-related genes have been described. In this report, we used microarray analysis to identify differentially expressed genes during joint, ligament and tendon formation in the presence (wildtype mice) or absence (Lmx1b KO mice) of Lmx1b. Progressive stages were analyzed and compared in an effort to minimize stage-specific differences and accentuate Lmx1b-specific targets. Our analysis identified genes regulated by Lmx1b that are involved in asymmetric nerve, bone, joint, ligament, and tendon formation during dorsalization.
Materials and Methods

Lmx1b KO Mouse

*Lmx1b* knockout (KO) mice were a kind gift of Randy L. Johnson (Chen, et al., 1998). *Lmx1b* homozygous mouse embryos were obtained by mating heterozygous male mice with heterozygous female mice. We time-dated matings using noon on the day that the vaginal plug was found as 0.5 days post coitum (dpc). At 11.5 and 12.5 dpc, embryos were harvested and the limb buds with the limb girdles were isolated. Embryos at 13.5dpc were also harvested and their distal limb buds (zeugopods and autopods) were isolated. Embryos were genotyped to confirm *Lmx1b* homozygosity (-/- or +/-).

Gene Expression Array

RNA from embryonic forelimbs and hindlimbs of wild type (WT) and *Lmx1b* KO mice was harvested using the Rneasy Kit (Qiagen). RNA was pooled to decrease genetic variability, *i.e.*, six limbs at 11.5 dpc, three limbs at 12.5 dpc and six limbs at 13.5 dpc. Duplicate samples were generated using different embryos for each stage and then hybridized to the Affymetrix GeneChip® Mouse Genome 430 2.0 Array (UCI, Irvine, CA). Microarray data was submitted to Gene Expression Omnibus and can be located under series accession number GSE34732. The data was normalized using RMA and analyzed using the Comprehensive R and Bioconductor based web service for microarray data analysis (Rainer, Sanchez-Cabo, Stocker, Sturn, & Trajanoski, 2006). Genes differentially expressed between WT and *Lmx1b* KO mice with *p*-value >0.05 were not considered significant and were not further analyzed.
In order to enhance gene discovery, all genes with significant differential expression were examined to compare expression differences at all three stages. Genes which demonstrated a 2-fold change at any stage were considered for further analysis.

Validation by Real-time PCR

Complementary DNA (cDNA) was transcribed from limb mRNA at each stage using both Lmx1b KO and WT mice. PCR products were sequenced to determine specificity (Supplemental Data Table 2). Differential expression was determined by Real-time PCR (Quantitative-PCR (Q-PCR)) measuring SYBR Green I fluorescence with the Roche Lightcycler 2.0 and Roche-recommended reagents. Triplicate Lightcycler runs with duplicate samples were compared to simultaneous PGK runs to normalize fold changes between Lmx1b KO and WT expression. Gene targets were considered validated if Q-PCR data confirmed microarray data with at least a 2-fold change at any stage.

Whole Mount in situ Hybridization

Sense and antisense digoxigen-labeled RNA probes ranging from 600bp – 1000bp were generated for each of the genes validated by Real-time PCR. Probes were sequenced to ensure specificity (Supplemental Data Table 2). Aggrecan probe sequence was generously provided by the Nancy Schwartz lab (Cortes, Baria, & Schwartz, 2009). Lmx1b KO and WT mouse embryos were harvested at 12.5 dpc and fixed overnight in MEMFA (0.1M MOPS, 2mM EGTA, 1mM MgSO4, 3.7% formaldehyde). Whole mount in situ hybridization was performed as described (Yamada, Szendro, Prokscha, Schwartz,


& Eichele, 1999) with each probe hybridized at 60°C, and embryos were washed post hybridization at 65°C.

**Results**

**Determination of Candidate Genes by Comparative Microarray**

In an effort to identify targets of Lmx1b involved in limb dorsalization, we isolated limb bud tissue with robust *Lmx1b* expression between 11.5 days post coitum (dpc) and 13.5dpc. At 11.5 dpc, expression of *Lmx1b* is throughout the dorsal limb bud mesoderm (Fig 3.1B). Thus, we harvested the entire limb bud as depicted in Fig 3.1A. The tissue harvested includes presumptive limb girdle elements (e.g., scapula) and tissue that will become all three segments of the limb (stylopod, zeugopod and autopod). Expression of *Lmx1b* at 12.5 dpc begins to localize to the condensing tendons proximally, while in distal less-differentiated dorsal mesenchyme, expression remains diffuse (Dreyer, et al., 2004). At this stage, we also harvested the entire limb including limb girdle elements (Fig.3.1A). *Lmx1b* expression at 13.5 dpc wanes proximally but remains strong in the autopod (Fig.3.1B). Therefore, the autopod was harvested at 13.5 dpc to coincide with strong *Lmx1b* expression (Fig.3.1A). In order to diminish biologic variability and enhance Lmx1b-associated changes, RNA was pooled from several different embryos/litters. Genes with significant (p<0.05) differential expression were included in our data for this study.
Figure 3.1) Depiction of Methods

A.) Depiction of the three developmental stages analyzed by microarray. Presumptive structures are color-coded at each embryonic stage depicting the progressive differentiation of the limb. Dashed red lines are drawn to illustrate the limb tissue harvested for gene array experiments. (dpc- days post coitum)  

B) Section in situ hybridization for Lmx1b at 11.5 dpc (2) with expression pseudocolored red demonstrating restricted dorsal expression. By 13.5 dpc (4), proximal expression of Lmx1b is less intense and localized to condensing musculo-tendonous tissue; distal Lmx1b expression remains diffuse in the dorsal mesoderm. H&E of corresponding limbs show the amount of relative tissue differentiation at each stage(1,2). (musc- muscle) (cart- cartilage)
Figure 3.2) Analysis of Microarray Data
A.) The number of genes with significant (p < 0.05) differential expression between WT and Lmx1b KO microarray analyses for each stage. The last column identifies the subset of genes differentially expressed in all three stages.  

<table>
<thead>
<tr>
<th></th>
<th>11.5 dpc mice</th>
<th>12.5 dpc mice</th>
<th>13.5 dpc mice</th>
<th>Recurring Genes</th>
</tr>
</thead>
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<tr>
<td>Up-regulated</td>
<td>311</td>
<td>282</td>
<td>494</td>
<td>46</td>
</tr>
<tr>
<td>Down-regulated</td>
<td>347</td>
<td>997</td>
<td>398</td>
<td>11</td>
</tr>
</tbody>
</table>

B.) Graphical representation of ingenuity pathway assistant (IPA) analysis that identifies developmental processes and system networks containing the genes that are differentially expressed at each stage. The relative significance determined by IPA defined parameters is listed on the y-axis. The number of genes in each network is listed at the top of each bar. The P value < 0.05 is denoted by the orange threshold line.

At 11.5 dpc, we identified 331 genes that were up-regulated in the presence of Lmx1b, while 368 genes were down-regulated. Lmx1b up-regulated the expression of 303 genes and down-regulated 1051 genes at 12.5 dpc. By 13.5 dpc, Lmx1b up-regulated 942 genes, and down-regulated 423 genes (Fig 3.2A).

To identify functionally relevant developmental pathways or networks used by Lmx1b, we analyzed our dataset with the Ingenuity Pathway Assistant (IPA) software (Fig 3.2B). When a comparison analysis is performed across stages, the data cluster into
several networks affected by Lmx1b. These include skeletal, connective tissue and nervous system developmental networks which correlate with morphology disrupted in the Lmx1b knockout (KO) mouse. Many of the targets discovered at individual stages may represent stage specific changes. In order to enrich genes downstream of Lmx1b during limb dorsalization, we identified genes with similar changes in expression for all three stages. We discovered 45 up-regulated genes and 9 down-regulated genes across all three stages. These genes were considered Lmx1b targets. Similar studies by Kania et al., used a 1.4-fold change; we chose a more stringent criterion since we have a larger data set incorporating several embryonic stages. For further analysis, we required candidate genes to have at least a 2-fold change at one or more stages. In addition, we selected genes that we suspected to be involved with limb development pathways. Table 1 lists the 23 genes which met this criterion, along with the fold change demonstrated at each stage.
Table 3.1) Partial list of genes from microarray analysis regulated by Lmx1b during all three experimental stages

A partial list of up and down-regulated genes identified by gene array across all three stages. Gene symbols and descriptions are listed along with the fold change at each stage. All data points listed in the table have a p value of < 0.05. Genes included in this partial list had a two fold change at one or more stages by gene array analysis. The final column indicates Q-PCR validation of this inclusion criteria.

<table>
<thead>
<tr>
<th>Description</th>
<th>Symbol</th>
<th>11.5 FC</th>
<th>12.5 FC</th>
<th>13.5 FC</th>
<th>Validated</th>
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<tr>
<td><strong>UP-REGULATED</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Empty spiracles homolog 2 (Drosophila)</td>
<td>Emx2</td>
<td>2.57</td>
<td>5.30</td>
<td>2.21</td>
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<tr>
<td>Keratocan</td>
<td>Kera</td>
<td>3.56</td>
<td>13.40</td>
<td>7.82</td>
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<tr>
<td>Lumican</td>
<td>Lum</td>
<td>2.56</td>
<td>3.67</td>
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<tr>
<td>Decorin</td>
<td>Dcn</td>
<td>1.43</td>
<td>2.10</td>
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<tr>
<td>Aggrecan 1</td>
<td>Agc1</td>
<td>1.53</td>
<td>1.88</td>
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</tr>
<tr>
<td>Matrilin 1, cartilage matrix protein 1</td>
<td>Matn1</td>
<td>2.09</td>
<td>3.07</td>
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</tr>
<tr>
<td>Matrilin 4</td>
<td>Matn4</td>
<td>1.41</td>
<td>1.60</td>
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<td>Growth differentiation factor 5</td>
<td>Gdf5</td>
<td>1.61</td>
<td>2.10</td>
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<tr>
<td>Procollagen, type IX, alpha 3</td>
<td>Col9a3</td>
<td>1.51</td>
<td>1.53</td>
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</tr>
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<td>Procollagen, type XI, alpha 2</td>
<td>Col11a2</td>
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<td>Cerebellin 2 precursor protein</td>
<td>Cbln2</td>
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<td>2.45</td>
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<td>Lysyl oxidase</td>
<td>Lox</td>
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<td>Epidermal growth factor-containing fibulin-like extracellular matrix protein 1</td>
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<td>1.68</td>
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<tr>
<td>Noggin</td>
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<td>Leukocyte cell derived chemotaxin 1</td>
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<td>Apolipoprotein D</td>
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<tr>
<td>Fam</td>
<td>MGC99845</td>
<td>1.45</td>
<td>1.60</td>
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<td>Retinoic acid receptor, beta</td>
<td>Rarb</td>
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<td>2.04</td>
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</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regulatory factor X, 4</td>
<td>Rfx4</td>
<td>1.99</td>
<td>2.73</td>
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<td>RIKEN cDNA 1700109F18 gene</td>
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<td>1.31</td>
<td>2.49</td>
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<td>Myeloid ecotropic viral integration site 1</td>
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<td>1.41</td>
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<tr>
<td>AF4/FMR2 family, member 3</td>
<td>Aff3</td>
<td>1.40</td>
<td>2.02</td>
<td>1.43</td>
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</table>
Validation of Candidate Lmx1b Target Genes by Real-time PCR

Candidate genes derived by microarray analysis were confirmed by Real-time PCR (Q-PCR) using SYBR green fluorescence. Q-PCR data met the previously described 2-fold change criteria for 18 candidate genes (Fig 3.3). Four genes (denoted in red) fell below our threshold and were not examined further. The fold-change at 12.5 dpc was greater than at other stages for most of the Lmx1b related targets. This finding suggests that 12.5 dpc is a crucial time during dorsalization, with Lmx1b exerting its greatest influence on gene targets. Thus, we examined the expression patterns of candidate genes in the WT and Lmx1b mutant mice at 12.5 dpc.

Figure 3.3) Graphical representation of Real-time PCR (Q-PCR)
The fold change of each gene was measured at each stage; a 2-fold threshold is denoted by a blue line. Q-PCR validated microarray data for genes shown in black type. Genes denoted in red failed to meet validation criteria. The greatest fold changes are consistently seen at stage 12.5 dpc, therefore this stage was used for in situ hybridization analysis. * Col9a3 produced a 1.9-fold change at 12.5 dpc. Since several other collagen related targets were also validated, Col9a3 was analyzed further.
WMISH was utilized to determine differential expression for 19 genes. Their expression patterns are briefly described below and illustrated in figures 3.4, 3.5, and 3.6, along with the expression pattern of Lmx1b (Fig 3.4 A,a, & A’,a’).

Apolipoprotein D (ApoD) is a carrier glycoprotein that is part of the lipocalin superfamily. In humans, ApoD is a minor component of plasma high density lipoproteins. Apod has been reported as a potential downstream target of Lmx1b with dorsal ectodermal expression of ApoD demonstrated at 13.5 dpc (Gu & Kania, 2010). We found expression of ApoD proximally in the dorsal limb mesoderm (Figure 3.4 B,b, & B’,b’) and accentuated at the anterior presumptive wrist boundary in the presence of Lmx1b at 12.5 dpc (Fig. 3.4 B, white arrow). We also confirmed the previously reported expression within the dorsal ectoderm at 13.5 dpc (data not shown), indicating a stage-specific shift in ApoD expression.

We found Empty spiracles 2 (Emx2), a transcription factor encoding gene linked to the formation of the scapula (a dorsal limb girdle structure), to be expressed diffusely within the dorsal limb girdle at 12.5 dpc (Fig 3.4 C,c). Emx2 expression also extends from the limb girdle out into the dorsal autopod mesoderm. In Lmx1b KO mice, expression of Emx2 is absent in both the scapular and dorsal limb mesoderm (Fig 3.4 C’,c’).

Cerebellin 2 (Cbln2) is a cerebellum-specific, membrane-associated hexadecapeptide derived from Cerebellin 1. Leukocyte cell-derived chemotaxin 1 (Lect1) is a cartilage-specific glycoprotein also known as Chondromodulin I. Interestingly in our WMISH Cbln2 and Lect1 both exhibit focal points of intense dorsal expression (Fig 3.4
D,d, D’,d’, E,e, & E’,e’). Cbln2 displays focal anterior and posterior limb girdle expression in the dorsal mesoderm (Fig 3.4 D white arrows). There is also concentrated expression overlying the proximal zeugopod (black arrowhead) and two discrete zones of dorsal expression at the zeugopod-autopod boundary (white arrowheads). Lect1 has strong focal dorsal expression over the stylopod and a pattern similar to Cbln2 expression along the zeugopod-autopod boundary (Fig 3.4 E). The limbs of Lmx1b KO mice show near absence of Cbln2 and Lect1 expression at all of these sites (Fig 3.4 D’d’ and E’,e’ respectively).

Several genes up-regulated by Lmx1b localize to the developing skeleton. Growth differentiation factor 5 (Gdf5) is a member of the TGF-beta superfamily of secreted growth factors. Gdf5 expression in WT limbs localizes to developing joints, interdigital regions and dorsal mesoderm along the zeugopod (Fig 3.4 F,f), and is prominent in the developing elbow joint (Fig 3.4 F, white arrow). In the Lmx1b KO mouse limb, Gdf5 expression is absent from the area associated with the olecranon and reduced within interdigital regions (Fig 3.4 F’). Matrilin 1 (Matn1) and Matrilin 4 (Matn4) are cartilage matrix proteins. Aggrecan (Agc) is a chondroitin sulfate proteoglycan, Agc, Matn1, and Matn4 expression surrounds condensing cartilage in the limb and the scapula at 12.5 dpc (Fig 3.4 G,g,H,h,I,i respectively). Interestingly, the scapula lacks Agc, Matn1, and Matn4 in Lmx1b KO mice (Fig 3.4 G’g’,H’h’,I’,I’ respectively). Agc and Matn1 is also absent in the ulnar anlagen, while Matn4 in the ulnar anlagen is absent distally. Additional skeleton-related genes identified by our gene array show symmetrical expression along the dorsal-ventral axis and include Noggin, Scinderin, Collagen9a3 and Collagen11a2. (Fig 3.5). These genes display decreased expression in Lmx1b KO mice.
Lysyl oxidase 1 (Lox) encodes a connective tissue-related enzyme that is expressed strongly in the dorsal mesoderm from the limb girdle to the distal zeugopod (Fig 3.4 J,j). Lox in the Lmx1b KO mouse is greatly reduced (Fig 3.4 J’,j’). A cluster of connective tissue-related proteoglycans was also identified in our gene array as potential candidates: Keratocan (Kera), Lumican (Lum), and Decorin (Dec). Kera is expressed intensely in the dorsal zeugopod mesoderm (Fig 3.4 K,k) and is undetectable in Lmx1b KO limbs (Fig 3.4 K’,k’). Lum and Dec demonstrate strong dorsal mesodermal expression with very light staining of the proximal ventral mesoderm in WT limbs (Fig 3.4 L,l,M,m). Lmx1b KO limbs display greatly reduced dorsal expression of Lum and Dec; the residual staining is symmetrical and consistent with the light ventral expression seen in WT limbs (Fig 3.4 L’,l’,M’,m’).

The up-regulated target Family with sequence similarity 196, member B (Fam196b) is a predicted gene which was not detectable by WMISH.
**Figure 3.4** Whole Mount *in situ* Hybridization of Targets Up-regulated by Lmx1b

WMISH for Q-PCR validated up-regulated genes at 12.5 dpc. Cartoon provides orientation for pictures, line along AP axis depicts cut performed for dorsoventral pictures. Dorsal views along with cross sections are shown for each gene. Expression patterns for each gene are as follows: Capital letter (e.g., A) denotes expression in WT limbs along the dorsal surface, capital letter with apostrophe (e.g., A’) denotes expression in Lmx1b KO limbs along the dorsal surface. Small letter (e.g., a) marks WT limb sections while small letter with apostrophe (e.g., a’) indicates KO limb sections. B, white arrow indicates anterior *Apod* expression. D *Cbln* expression in the autopod (white arrowhead), zeugopod (black arrowhead), and proximal/stylopod (white arrows). F, *Gdf5* in the olecranon (white arrow). H and I, black arrows both indicate scapular stain for *Matn1* and *Matn4*, respectively.
The two additional transcription factors that were identified by our gene array were down regulated. *Regulatory factor X,4 (Rfx4)* is a winged helix transcription factor, which is expressed in the ventral limb mesoderm proximally and anteriorly (Fig 3.6 A,A’,A’’white arrowheads), and is duplicated in the dorsal mesoderm of the *Lmx1b* mutant (Fig 3.6 B,B’B’’white arrowheads). The nuclear transcription factor *AF4/FMR2 family, member 3 (Aff3)* shows broad ventral expression within the presumptive soft
tissue of the zeugopod and autopod, surrounding the condensing cartilage and extending to the ectoderm, while on the dorsal aspect, the subectodermal expression is lacking (Fig 3.6 C,C’,C’’,c’’red line). Lmx1b KO limbs reveal broad symmetrical Aff3 expression patterns along the dorsal and ventral mesoderm (Fig 3.6 D,D’,D’’,d’’).

**Figure 3.6** Whole Mount *in situ* Hybridization of Targets Down-regulated by Lmx1b

WMISH for Q-PCR validated down-regulated genes at 12.5 dpc, cut sections performed as illustrated by cartoon in Fig.4. Rfx4 is expressed dorsally (A) and ventrally (A’, white arrowhead; A”, cut section, white arrowhead) WT limb. In the Lmx1b KO limb, Rfx4 is expressed dorsally (B) and ventrally (B’). A cut section shows duplicated expression (B”, white arrowhead). C and C’ show dorsal and ventral Aff3 expression in WT limb, respectively. C” displays enhanced ventral Aff3 expression, magnification area denoted by the white box demonstrates absence of Aff3 in the dorsal subectodermal mesoderm (red line). D and D’ show dorsal and ventral expression of Aff3 in the Lmx1b KO limb, respectively. D” magnified in d”, Aff3 is enhanced both dorsally and ventrally limb.
Discussion

Lmx1b is a homeodomain transcription factor known to be necessary and sufficient for dorsalizing distal limb structures including skeletal elements, nerves, tendons and ligaments. Loss of Lmx1b expression in Lmx1b KO mice causes scapular hypoplasia, distal ulnar hypoplasia, and loss of soft tissue dorsalization, i.e., symmetrical ventral-ventral or flexor-flexor ligaments, tendons and muscles (Chen & Johnson, 2002). Retroviral mediated overexpression of LMX1B in limb mesoderm generates a dorsal-dorsal distal phenotype (Vogel, et al., 1995). Therefore, it is expected that Lmx1b regulates many diverse targets during limb dorsalization. Supporting this concept, our microarray analysis revealed clusters of target genes associated with bone, connective tissue, and nerve development.

Lmx1b Genes Associated With Skeletal Development

Our microarray data included several skeletal targets: Noggin, Growth differentiation factor 5 (Gdf5), Aggrecan (Agc), Scinderin, Collagen9a3, Collagen11a2, Matrilin1 (Matn1) and Matrilin4 (Matn4). Of these targets, Gdf5, Agc, Matn1 and Matn4 demonstrate asymmetric expression in normal mice that is disrupted in Lmx1b KO mice (Fig 3.4 F’,G’,H’,I’).

Gdf5 is a regulator of joint and cartilage formation (Buxton, Edwards, Archer, & Francis-West, 2001). In the 12.5 dpc limb, Gdf5 is expressed within the developing joints and interdigital spaces (Fig 3.4 F). Notably, the developing elbow joint is an asymmetric structure with a prominent dorsal joint interface. Correspondingly, Gdf5 expression includes a strip of dorsal mesoderm (Fig 3.4 F,white arrow). In Lmx1b KO embryos, this
expression is absent, consistent with our microarray and Real-time PCR analysis. In a previous report, no difference in Gdf5 expression was seen in autopod joints between normal and Lmx1b KO mice, however the elbow was not examined (Dreyer, et al., 2004). Although Lmx1b may regulate Gdf5 expression, Gdf5 KO mice do not present with obvious dorsoventral abnormalities; instead, loss of Gdf5 in mice and humans causes shortened limbs with abnormal joint formation and ossification of digits (Storm et al., 1994). Lmx1b plays a role in elbow development, since over 90% of patients with haploinsufficiency present with elbow dysplasia (E. M. Bongers, et al., 2002). Thus, the loss of Gdf5 expression in Lmx1b KO mice may signify a contribution to or reflection of deficient elbow dorsalization.

Agc is a highly expressed structural proteoglycan associated with cartilage development and maintenance. In the cartilage matrix deficiency mouse (CMD), wherein Agc is spontaneously/naturally deleted, there is marked limb hypoplasia with no apparent dorsal-ventral limb axis abnormalities (Watanabe & Yamada, 2002). However, in our review of the published data, Agc deficient mice do appear to have more pronounced ulnar hypoplasia with ulnar deviation at the wrist. Lmx1b KO mice show a loss of Agc expression within the presumptive ulna (Fig 3.4 G’). This deficiency in Agc expression in the posterior cartilage anlagen may contribute to the ulnar hypoplasia seen in the Lmx1b KO mouse.

Matrilin (Matn) genes exhibit overlapping expression patterns; in particular Matn1 and Matn4 are expressed in cartilaginous tissues where they bind collagen and proteoglycans to aid in skeletogenesis. Similar to Agc in the Lmx1b KO limb, Matn1
expression is lacking in the presumptive ulna (Fig 3.4 H’). In contrast, Matn4 expression is present within the proximal ulna anlagen, but is absent distally (Fig 3.4 I’).

Ulna formation is under the control of Sonic Hedgehog (Shh) (Ros et al., 2003). The differential expression patterns of Agc, Matn1 and Matn4 in response to Lmx1b suggest that posterior cartilage proteoglycan regulation may be a collective target of Lmx1b and Shh. Furthermore, these findings support the concept that Lmx1b may work in concert with other patterning factors along different axes, such as Shh, to generate asymmetrical structures.

Lmx1b Regulated Genes Associated With Connective Tissue Development

A large second group of target genes uncovered by our analysis express within the dorsal limb mesoderm and, therefore, may be regulated by Lmx1b to pattern dorsal soft tissues. These include: Empty spiracles homolog 2 (Emx2), Apolipoprotein D (ApoD), Lysyl oxidase (Lox), Keratocan (Kera), Lumican (Lum), and Decorin (Dec).

Emx2 expression extends along the dorsal mesoderm of the zeugopod, continuing distally into the autopod and digits (Fig 3.4 C). This expression is absent in Lmx1b KO limbs (Fig 3.4 C’). Overexpression of Emx2 in the chick model induced a single posterior or mirrored digit on the anterior border (Pröls et al., 2004). A role for Lmx1b in the integration of Shh signals has been suggested by Tzchori and co-workers (Tzchori et al., 2009), although no disruption of Shh signaling is evident in Lmx1b KO limbs (Chen, et al., 1998). As suggested for posterior proteoglycans, it is possible that Lmx1b and Shh work in combination to also regulate digit-specific Emx2 activity. Alternatively, Emx2
and Shh could work in concert to establish dorsal digit identity. Irrespectively, overexpression of Emx2 in the limb appears to mimic or induce ectopic Shh expression. ApoD is a multi-ligand, multi-functional transporter (Rassart et al., 2000). The expression of ApoD at 13.5 dpc has been reported in the dorsal limb ectoderm (Gu & Kania, 2010). We confirmed dorsal ectoderm expression at 13.5 dpc; however, at 12.5 dpc, we found ApoD expression in the anterior dorsal mesoderm (Fig 3.4 B). With its stage-specific shift from dorsal limb mesoderm to ectoderm and loss of expression in Lmx1b KO limbs, it is unclear what role ApoD might play in limb development since ApoD KO mice have no reported limb abnormalities (Ganfornina et al., 2008).

Lox is a soft tissue associated target involved in collagen formation/maturation. Lox expression is broad in the proximal dorsal mesoderm extending out into the zeugopod (Fig 3.4 J). Lox expression at 12.5dpc overlaps Lmx1b expression in the zeugopod; however this expression is absent in the Lmx1b KO limb (Fig 3.4 J’). The role of this differential expression in response to Lmx1b is unclear. Since Lox is a copper binding enzyme involved in crosslinking collagen and elastin (Kagan & Trackman, 1991), it is possible that dorsal tendons/ligaments in mice have a composition of elastin and collagen that differs from ventral tendons and/or ligaments.

Kera, Dec, and Lum are proteoglycans associated with connective tissue development. Dec and Lum are associated with collagen fibrillogenesis and degeneration of the eye, skin and tendons. The ability to slow, but not to stop, the process of collagen formation allows for the homogeneous spacing between collagen fibrils during development. Not surprisingly, Dec KO (Danielson et al., 1997) and Lum KO mice (Chakravarti et al., 1998) present with irregularly spaced collagen, which causes weak or
abnormal connective tissue *i.e.* weak tendons with irregular attachment sites, and skin fragility. *Lmx1b* KO mice display a marked reduction of *Dec* and *Lum* expression in the distal dorsal mesoderm with limited expression remaining in the proximal ventral and dorsal mesoderm (Fig 3.4 L,M). Kera has been studied in the embryonic eye and adult tendon where it is utilized for collagen fibril development and maintenance (Conrad & Conrad, 2003; Liu, Birk, Hassell, Kane, & Kao, 2003). *Kera* expression in the *Lmx1b* KO was undetectable (Fig 3.4 K’). In addition, expression of *Collagen9a3* and *Collagen11a2* (Fig 3.5 C,D) is reduced in *Lmx1b* KO limbs(Fig 3.5 C’,D’). The collective differential expressions suggest that *Lmx1b* plays an active role in collagen formation and maintenance, which may, in part, confer the dorsal character of these structures.

Lmx1b Regulated Genes Associated With Neuronal Development and Angiogenesis

*Cerebellin 2* (*Cbln2*) and *Leukocyte cell-derived chemotaxin 1* (*Lect*) demonstrate a strikingly similar focal pattern of expression of the dorsal zeugopod and autopod (Fig 3.4 D,E), which is greatly reduced in the *Lmx1b* KO (Fig 3.4 D’,E’). *Lmx1b* guides the initial branching and subsequent trajectory of dorsal motor axons (Kania, Johnson, & Jessell, 2000). Interestingly, Cbln2 is abundant in the embryonic brain (Miura, Iijima, Yuzaki, & Watanabe, 2006) and has been linked to connectivity in sensory neurons (Reiner, Yang, Cagle, & Honig). Therefore, *Lmx1b* may work in conjunction with localized dorsal *Cbln2* expression to guide motor axon trajectory and branching. Lect functions as an inhibitor of angiogenesis in the growth plate (Shukunami, Iyama, Inoue, & Hiraki, 1999). Lect in the dorsal limb may assist *Lmx1b* in patterning blood vessels by
restricting vasculogenesis of targeted locations associated with innervation and joint formation.

Lmx1b Regulated Genes Associated With the Developing Scapula

The scapula, a component of the limb girdle, also develops as a dorsal structure. Scapular development is a complex process. Signals from the lateral plate mesoderm pattern the head of the scapula while Hox genes from somites create a segmental pattern along the blade (Huang, Christ, & Patel, 2006; Huang, Zhi, Patel, Wilting, & Christ, 2000). Several knockout (KO) mouse models display varying degrees of scapular aplasia. Emx2 (Pellegrini, Pantano, Fumi, Lucchini, & Forabosco, 2001) and Wnt-βcatenin (Hill, Taketo, Birchmeier, & Hartmann, 2006) KO mice display a complete loss of the scapula, while Pax1 KO mice develop hypoplastic scapulae lacking the acromion (Wallin et al., 1994). Tbx15 KO mice develop a foramen in the blade (Singh et al., 2005), Alx3 KO and Cart1 KOs (Brouwer, ten Berge, Wiegerinck, & Meijlink, 2003) reduce the rostral blade. Despite these mechanistic insights, a comprehensive list of genes and how they interact during scapula formation remains unclear. Expression of several genes discovered in our study localize to the developing scapula in normal mice. Other than Emx2, the targets identified in our analyses have not previously been associated with scapular formation. Agc, Lum, Dec, and Lox show a reduction of scapular expression in Lmx1b KO mice (Fig 3.4 C’,G’,L’,M’). Emx2, Matn1, and Matn4 exhibit a striking loss of scapular expression (Fig 3.4 H’,I’). Since Lmx1b KO mice have hypoplastic scapulae, we suggest Lmx1b works in concert with these collective targets to augment scapular development.
Genes Down-Regulated by Lmx1b During Limb Development

We discovered two genes down-regulated by Lmx1b in the dorsal limb bud.

*Regulatory factor X, 4* (*Rfx4*) encodes for a transcription factor and has proximal anterior expression in the ventral mesoderm (Fig 3.6 A’). This localized expression is duplicated in the *Lmx1b* KO limb (Fig 3.6 B’’). Rfx transcription factors have been associated with CNS development and may direct proximal nerve migration in the limb. A recent report found that an intraflagellar transport protein, *ift172*, is a target of Rfx4 regulation, and in a spontaneous mouse mutation, disrupted Shh and Gli signaling in the brain and spinal cord (Ashique et al., 2009). *Rfx4* expression was also observed in the limb, but no skeletal abnormalities were reported (Ashique, et al., 2009). Soft tissue or nerve patterning, however, was not examined. Thus, it is unclear what role this gene and the regulation of cilia may have on dorsal-ventral patterning.

*AF4/FMR2 family, member 3* (*Aff3*) is expressed in both the dorsal and ventral mesoderm but has broader ventral expression during normal mouse limb development that includes the subectodermal mesoderm (Fig 3.6 C’’,c’’). In the *Lmx1b* KO mouse, *Aff3* expression is equally accentuated in both the dorsal and ventral mesoderm (Fig 3.6 D’’,d’’). *Aff3* was initially discovered in association with lymphoid tissue (Ma & Staudt, 1996), a more recent limb study detected expression at 11.5dpc and 13.5 dpc (Gyurján, Sonderegger, Naef, & Duboule, 2011). The presence of Aff3 within the developing limb and Q-PCR detected down-regulation by Lmx1b, suggests a role for Aff3 in dorsoventral patterning.
Summary

Previous studies have performed microarray analysis to discover downstream Lmx1b targets. Krawchuk and Kania (2008) used proximal WT and Lmx1b KO 11.5 dpc hindlimb tissue. Keratocan was the only target discovered coincident with our study; however, Keratocan was considered “not significant or inconclusive”. Our data and data from others (Randy Johnson, personal communication) find Keratocan significantly elevated in comparative WT and Lmx1b KO mouse limb gene arrays. We propose Keratocan is a significant Lmx1b target important to dorsal tendon formation. A second microarray analysis was also published using 13.5 dpc limbs (Gu & Kania, 2010; Krawchuk & Kania, 2008). This report revealed ApoD as a downstream target and Cbln2 as a “target of interest”. These two genes were also detected by our microarray study, supporting their role as Lmx1b targets.

The persistent asymmetrical elevation of a cluster of proteoglycans during limb dorsalization is a noteworthy discovery from our data and may provide clues to the mechanical mechanism of dorsal morphogenesis. Kurpios and co-workers recently demonstrated differential proteoglycan content within left and right dorsal mesentery that caused asymmetric cell density, and the initiation of directional gut rotation (Kurpios et al., 2008). Thus, one mechanism Lmx1b may use to orchestrate dorsalization is the regulation of proteoglycans that modulate cell shape, density and migration.

Our multi-stage microarray data expands previous investigations to include skeletal, connective tissue, neuronal, and angiogenic targets regulated by Lmx1b during limb dorsalization. Several of these genes exhibit the Lmx1b binding site as described by Morello and coworkers (Morello, et al., 2001), suggesting direct Lmx1b regulation.
Although Lmx1b is necessary and sufficient to accomplish distal limb dorsalization (Chen, et al., 1998), many of the genes in our data sets demonstrate a basal ventral expression. This finding supports the concept of Lmx1b functioning as a selector transcription factor in a multi-factor regulatory network as recently suggested by Qui and coworkers (Qiu, et al., 2009).

**Acknowledgements**

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Supplemental Tables

Table 3.2) Genes Common to all three arrays (11.5, 12.5, and 13.5 dpc). Positive fold change values indicate genes up-regulated by Lmx1b, negative fold change denotes genes down-regulated by Lmx1b. Genes shaded in grey were also analyzed with whole mount in situ hybridization.

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Table 3.3) Primer Sequences for qPCR and WMISH Probe Generation, primers are listed in the 5’ to 3’ orientation.

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CHAPTER FOUR
PROTEOGLYCAN GENE EXPRESSION DURING Lmx1b-DIRECTED LIMB DORSALIZATION REVEALS DISPARATE CONSERVATION BETWEEN CHICK AND MOUSE

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Loma Linda, CA  92350
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Abstract

Many of the molecular pathways used to pattern the developing limb are conserved across divergent species. The limb develops along three axes; proximal-distal, anterior-posterior, and dorsal-ventral. The molecular pathway by which dorsoventral limb patterning occurs is incompletely characterized. Lmx1b, a LIM homeodomain transcription factor is necessary and sufficient for limb dorsalization; however, downstream targets of Lmx1b are unknown. Identification of these downstream targets is essential for understanding patterning of the dorsal-ventral limb axis. Previous studies have identified several proteoglycans as potential downstream gene targets of Lmx1b. In 12.5 dpc mouse limbs, Keratocan, Lumican, and Decorin, were dorsally restricted, while Aggrecan was not. We hypothesized the expression of these target genes would be conserved across tetrapods. To test this hypothesis, we examined the expression of these proteoglycans in both mouse and chick embryonic forelimbs and hindlimbs at corresponding stages. (11.5 - 14.5 dpc and Hamburger-Hamilton stages 22 - 29, respectively) by whole mount in situ hybridization. The expression of Aggrecan was highly conserved, localizing to condensing cartilage during the stages examined in both mouse and chick fore/hindlimbs. In contrast the expression patterns of Keratocan, Lumican, and Decorin was not conserved between chick and mouse. In mouse, all three proteoglycans were initially (11.5 dpc) expressed within the dorsal mesoderm. By E14.5 Keratocan localized to the dorsal and ventral tendons, whereas Lumican and Decorin retained dorsal expression with additional focal proximal expression within ventral mesoderm of the hindlimb. In the chick, Keratocan and Decorin expression was initiated in both dorsally and ventrally mesoderm progressively localizing to ventral and dorsal
tendons. *Lumican* is expressed in the superficial dorsal and ventral mesoderm including the sub-AER region. Lumican also develops diffuse dorsal and ventral mesodermal expression in the hindlimb, beginning at HH25. The marked difference in expression between mice and chicks during limb dorsalization was unexpected. These contrasting expression patterns suggest that the Lmx1b-directed cascade of gene regulation during limb dorsalization is not conserved across species. The disparity may also reflect species-specific variations in dorsal limb evolution, structure, and function.

**Introduction**

Despite a variety of adult limb conformations, early vertebrate limb morphogenesis is remarkably similar across divergent species. From fish to humans, this observation was aptly recognized and described by 19th century embryologists such as Karl Ernst von Baer in 1828 (Russell, 1917). Early limb development begins as a bud of tissue emerging from the lateral plate mesoderm. Asymmetric development advances along three coordinate axes, proximal-distal, anterior-posterior, and dorsal-ventral.

In addition, to morphology, the molecular pathways that orchestrate limb development are largely retained. Development along the proximal-distal axis, utilizes the apical ectodermal ridge (AER), a thickened ridge of distal ectoderm. Fibroblast growth factors secreted from the AER stimulate growth in the underlying undifferentiated mesoderm and progressively distalizes the limb during outgrowth. This epithelial-mesenchymal interaction is conserved across vertebrate species, retaining the critical molecules in the pathway with some minor species-specific variations (Mercader, 2007). For example, in zebrafish Tbx5 in the presumptive fin mesoderm induces Fgf10 secretion
which up-regulates Fgf24, Fgf16, and Fgf8 in the AER through a Wnt intermediate. Similarly in mice, Tbx5 in the presumptive limb mesoderm up-regulates Fgf10, which in turn induces Fgf8, Fgf4, Fgf9 and Fgf17 in the AER via intermediary Wnt genes. Thus, the molecular pathway and downstream targets are conserved even though different species use distinctive Fgfs to accomplish limb outgrowth.

A cluster of cells known as the zone of polarizing activity (ZPA) directs development along the anterior-posterior axis. The ZPA secretes sonic hedgehog (Shh) inducing proliferative limb bud expansion and distal anterior-posterior patterning. The Shh pathway is also preserved in vertebrate appendages from fins (Dahn, Davis, Pappano, & Shubin, 2006) to tetrapod limbs (Mercader, 2007).

The dorsal ectoderm polarizes the dorsal-ventral axis. Initially, Wnt7a is expressed in the ectoderm overlying the presumptive limb. En-1 is up-regulated in the ventral ectoderm as the limb bud emerges and limits Wnt7a expression to the dorsal ectoderm (Cygan, et al., 1997). Wnt7a induces the LIM-homeodomain transcription factor Lmx1b, in the underlying mesoderm (Vogel, et al., 1995) and Lmx1b is responsible for limb dorsalization (Chen, et al., 1998; Riddle, et al., 1995; Vogel, et al., 1995). The molecular mechanisms initiating dorsal-ventral axis formation in the limb are also conserved across vertebrates (Mercader, 2007).

We wondered whether conservation of the molecular dorsal-ventral pathway in the limb extended to downstream targets, however, the molecular cascade beyond Lmx1b is not well characterized. Several microarray studies have been performed to identify limb related downstream Lmx1b targets (Feenstra et al., 2012; Gu & Kania, 2010; Krawchuk & Kania, 2008); all of these studies have discovered one or more proteoglycan
genes up-regulated by Lmx1b during limb dorsalization. Proteoglycans are extracellular matrix molecules that have been implicated in several developmental processes including CNS development (Bandtlow & Zimmermann, 2000) and skeletal formation (Waddington, Roberts, Sugars, & Schonherr, 2003).

In this report we examined the expression of proteoglycans discovered by differential gene array analysis during limb dorsal-ventral axis formation in the mouse; Keratocan, Lumican, Decorin, and Aggrecan. We further examined the expression in chickens during limb development to determine whether the dorsal-ventral molecular cascade was conserved. Mouse cells located in the dorsal limb mesoderm do not cross or migrate to the ventral compartment after 10.5 days post coitum (dpc) (Arques, et al., 2007; Pearse, et al., 2007; Qiu, et al., 2009), creating a distinct dorsal-ventral boundary which may be the first step in limb dorsalization. Therefore, we examined expression of proteoglycans beginning with compartment restriction (11.5 dpc and HH23) and ending with differentiation of skeletal elements when Lmx1b expression decreases. Despite the similarity of vertebrate limbs, chicks have a more derived forelimb with only three digits, whereas the hindlimb has four digits and more closely parallels rodent limbs. Thus, to compare target genes of the Lmx1b dorsalization pathway, we examined expression patterns in both the forelimb and hindlimb of mice and chickens. When comparing mouse and chick, we discovered an early disparate expression pattern for Keratocan, Lumican and, Decorin that terminally converged within tendons.
**Materials and Methods**

Brown Leghorn eggs were incubated at 39°C until Hamburger-Hamilton (HH) (Hamburger & Hamilton, 1951) stages 22-29. CB57/bl mouse embryos were harvested at stages 11.5, 12.5, 13.5 and 14.5 days post coitum. Embryos were harvested and fixed overnight in MEMFA (0.1M MOPS, 2mM EGTA, 1mM MgSO4, 3.7% formaldehyde). Digoxigenin labeled probes were used to detect Lmx1b, Keratocan, Lumican, Decorin, and Aggrecan. Table 1 contains previously unpublished chick probe sequences, mouse probe sequences were previously described (Feenstra, et al., 2012). Mouse and chick Aggrecan probe sequences were derived from plasmids which were a kind gift of the Nancy Schwartz lab (Cortes, et al., 2009). WMISH was performed as previously described (Yamada et al., 1999) with each probe hybridized at 60°C and embryos washed post hybridization at 65°C.

**Results and Discussion**

**Lmx1b**

As previously reported (Dreyer, et al., 2004; Vogel, et al., 1995), Lmx1b expression is evident throughout the dorsal mesoderm of the mouse 11.5/12.5 dpc and chick HH23/25 limb (Fig 4.1). Subsequent expression of mouse (13.5/14.5 dpc) and chick (HH 27/29) limbs is reduced in the proximal region but remains strong distally. Hindlimb expression is slightly delayed in both. Later stages of the mouse (13.5/14.5 dpc) display enhanced expression in the joints of the autopod. The expression and function (Chen, et al., 1998; Vogel, et al., 1995) of Lmx1b is similar in mouse and chicks, supporting the concept that Lmx1b is conserved across species as a mediator of limb dorsalization.
Figure 4.1) Lmx1b Expression Pattern Comparison
In the chick and mouse Lmx1b localizes to the dorsal limb mesoderm during limb development. Cartoons illustrate orientation of images, dorsal limb surface and a cut section to visualize the dorsal/ventral limb compartments. Line through the dorsal limb view (anterior/posterior) depicts where D/V cuts were made. Lmx1b expression is conserved between forelimb and hindlimb of the chick and mouse.
Aggrekan

In mice, Aggrekan (Acan) expression localizes to condensing limb cartilage with progressive development (Fig 4.2). Similarly, chick Aggrekan (ACAN) expression is apparent by HH25 and highlights the condensing cartilage of the stylopod, zeugopod, and distal posterior autopod. With stage progression, Aggrekan accentuates the developing digits in a posterior to anterior pattern. By HH29, intense ACAN expression delineates the majority of the limb skeleton. Thus, in both mice and chicks, Acan expression denotes where cartilage condensations will form during limb skeletogenesis.

Aggrekan is an important component of the cartilage extracellular matrix, where it forms charged aggregates within the collagen network. These charged aggregations alter water content and binding thereby providing cartilage elasticity and compression resistance. Loss of Acan function in the chick and mouse causes severe dwarfism (H. Li, Schwartz, & Vertel, 1993; Watanabe et al., 1994). Similarly in humans, a homozygous partial truncation of Acan causes severe dwarfism (Tompson et al., 2009). Our data further supports the notion that Acan is conserved across divergent species as a molecular component important for chondrogenesis.
Figure 4.2) Aggrecan Expression Pattern Comparison

Aggrecan localizes to condensing cartilage of the chick (HH25/27/29) and mouse limb (12.5/13.5/14.5 dpc). A dorsal view of the limb is shown along with a cut section to illustrate dorsal/ventral expression differences. Aggrecan is expressed in the digits in a posterior to anterior pattern best visualized in the chick (HH27).

Keratocan and Decorin

Keratocan, Decorin and Lumican are small leucine rich proteoglycans (SLRP) that are important for directing collagen fibril assembly during development. These three SLRP’s are genomically clustered together, which may suggest coordinated or possibly redundant function. Furthermore, Keratocan (Fig 4.3) and Decorin (Fig 4.4) have a similar expression pattern that localizes to developing tendons at HH29 and 14.5 dpc.

Keratocan expression in the mouse limb is first evident as a small region in the proximal dorsal mesoderm at 11.5 dpc. With development, expression spreads distally to involve the midline of the dorsal zeugopod and extends to the autopod but is absent from
the distal digits. By 14.5 dpc, Keratocan localizes to the dorsal tendons and a few ventral tendons of the murine limbs (Fig 4.3D, arrows).

In contrast, Keratocan within chicken limbs originates superficially in the distal dorsal and ventral mesoderm at HH23 (Fig 4.3A). Keratocan expression continues in the developing autopod region through HH25 (Fig 4.3B). By HH27 Keratocan expression has expanded proximally into dorsal and ventral zeugopod mesoderm with waning expression within the autopod and posterior clearing that excludes the region of the ZPA (Fig 4.3C). With further development (HH29), Keratocan expression localizes to forming dorsal and ventral tendons (Fig 4.3D).
Figure 4.3) Keratocan Expression Pattern Comparison
In the mouse Keratocan displays dorsally restricted proximal expression, which later localizes to tendons, arrows indicate ventral tendons. In the chick Keratocan displays distal mesodermal expression, which localizes to dorsal and ventral tendons. Dorsal, ventral and dorsal/ventral (DV) cut sections are shown; ventral images are flipped horizontally to facilitate expression pattern comparison.
Similar to Keratocan, Decorin is first expressed in the proximal dorsal aspect of murine limbs. At 11.5 dpc, Decorin is expressed in the forelimb as a small proximal region of dorsal mesoderm at the midline, while in the hindlimb Decorin expression localizes to the proximal posterior aspect of the dorsal mesoderm (Fig 4.4A). Decorin expression at 12.5 dpc broadens anteriorly within the dorsal mesoderm and also appears proximally within the ventral mesoderm (Fig 4.4B). In the forelimb and the hindlimb at 13.5 dpc Decorin expression extends toward the autopod to lie directly below carpals (Fig 4.4C). At 14.5 dpc Decorin expression remains intense within the dorsal zeugopod with expanded expression ventrally, condensing into presumptive dorsal and ventral myotendinous bundles (Fig 4.4D).

Within the chicken limb at HH23, Decorin expression, like Keratocan, begins distally within the central superficial dorsal and ventral mesoderm (Fig 4.4A). With continued limb outgrowth (HH25-HH27), Decorin expression extends proximally to include the zeugopod and autopod (Fig 4.4B). By HH29, Decorin expression within the autopod clears except for limited dorsal and ventral tendon expression; however, Decorin expression within the zeugopod mesoderm remains strong while highlighting condensing tendons.
Figure 4.4) Decorin Expression Pattern Comparison
Decorin displays dorsally restricted expression in the mouse beginning proximally, by 14.5dpc Decorin condenses to tendinous bundles (white arrow). In the chick Decorin begins distally and localizes to dorsal and ventral tendons.
Thus, in both mouse and chick limbs, Keratocan and Decorin localize to developing tendons in due course. This localization suggests a role in tendon formation or maturation. Supporting this concept, Keratocan and Decorin expression overlaps Scleraxis, a marker of tendon progenitors, in the superficial mesoderm (Schweitzer et al., 2001). Although little is known regarding the function of Keratocan in tendons, abnormal corneal collagen fibril formation has been reported with loss of functional Keratocan in humans and mice (Khan & Kambouris, 2004; Liu, et al., 2003). Decorin is thought to control the rate of collagen fibril assembly (Zhang et al., 2006) and Decorin KO mice display disrupted collagen fiber formation and skin fragility (Danielson, et al., 1997). Together these findings suggest Keratocan and Decorin have a complementary role in patterning the collagen composition of limb tendons.

However, there is a striking species-specific difference in the initial and evolving expression patterns during limb development. In the mouse, expression begins proximally and extends distally, while in the chick, expression begins distally and extends proximally. These disparate patterns of proteoglycan expression may indicate different mechanism of regulation to attain the same eventual localization. Alternatively, this species-specific discrepancy could indicate a fundamental difference in how mouse and chick tendons develop.

Lumican

In the mouse, Lumican expression is present at 11.5 dpc within the proximal dorsal mesoderm (Fig 4.5A) and subsequently expands distally across the zeugopod by 12.5 dpc (Fig 4.5B) similar to Keratocan and Decorin. Weak ventral staining is also
present proximally, at 12.5 dpc and increases in intensity through 14.5 dpc (Fig 4.5D). Dorsal expression expands to include the majority of dorsal mesoderm by 13.5 dpc, excluding the distal autopod. By 14.5 dpc, Lumican expression extends to the distal autopod accentuating dorsal and ventral digital tendons (Fig 4.5D).

Similar to Keratocan and Decorin, Lumican expression in chick limbs at HH23 involves the central dorsal and ventral mesoderm at the distal tip (Fig 4.5A). The expression extends along the anterior peripheral aspect of the limb. In addition, Lumican expression is present within the zone of polarizing activity (ZPA). Thus, a band of Lumican negative distal mesoderm separates the anterior and ZPA related areas of expression that involve both dorsal and ventral mesoderm. The pattern persists through HH25 with increasing expression intensity (Fig 4.5A/B). By HH25, weak Lumican expression is also evident superficially in the zeugopod mesoderm. The anterior and ZPA related expressions coalesce at HH27 generating a definitive sub-apical ectodermal ridge (AER) band of expression (Fig 4.5B/C). With further development (HH29) the superficial central limb expression in the forelimb becomes more restricted. Concurrently, there is an overall decrease in forelimb expression including the autopod, which highlights the sub-AER staining (Fig 4.5D). The hindlimb at this stage (HH29) retains sub AER staining but also displays diffuse mesodermal staining of the zeugopod and stylopod.
Figure 4.5) Lumican Expression Pattern Comparison
Lumican displays dorsally restricted expression in the mouse and an interesting sub AER pattern in the chick limb.

The unique sub-AER pattern of Lumican expression in chickens is similar to the pattern of downstream Fibroblast Growth Factor target genes such as DUSP6 (C. Li, Scott, Hatch, Tian, & Mansour, 2007) and TFAP2C (unpublished data). In the mouse
there are no detectable levels of Lumican expression in the same sub-AER region. This species specific difference in induction suggests that Lumican is regulated by AER related factors such as FGF in the chick and a different molecular network in the mouse.

**Conclusion**

In this report we examined the expression of proteoglycans that are associated with chondrogenesis and collagen formation during limb development in mice and chickens. In mice, these proteoglycans are elevated in the presence of Lmx1b and accentuated dorsally during limb development (Feenstra, et al., 2012). We confirmed similar expression of Lmx1b expression within the dorsal mesoderm of mouse and chicken limbs during the stages examined. We also found similar mouse and chick expression patterns for Aggrecan, a potential downstream target of Lmx1b.

However, the expression patterns of Keratocan, Decorin and Lumican were remarkably different in mice and chickens. In mice, the expression is first evident proximally in the dorsal mesoderm and progresses distally with limb outgrowth. In contrast, these proteoglycans are initially expressed distally in chick limbs and expand proximally. Furthermore, Lumican displays an additional sub-AER expression in chicks that is not present in mice. The initiation of expression in opposing limb regions followed by a convergence of expression and the unique sub-AER Lumican expression are interesting and unexpected differences between these species.

Although the expression of Lmx1b within dorsal limb mesoderm mediates limb dorsalization across vertebrate species (Mercader, 2007), our disparate patterns of proteoglycan expression suggest that the Lmx1b-directed cascade of gene regulation
during limb dorsalization is not conserved across species. The disparity may also reflect species-specific variations in dorsal limb evolution, structure, and function. Additional studies will be needed to clarify the role of these proteoglycans in limb development.
References


CHAPTER FIVE
DISCOVERY OF AN ENHANCER REGION FOR KERATOCAN LUMICAN AND DECORIN IN CONJUNCTION WITH A POSSIBLE LMX1B REGULATION MECHANISM

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Abstract

Lmx1b, a LIM homeodomain transcription factor, is necessary for limb, kidney, eye, and central nervous system development. Lmx1b knockout (KO) mice display reduced cerebellar and tectal formation, loss of dopaminergic/serotonergic neurons, ocular malformations, and ventral–ventral limb symmetry. The mechanism by which Lmx1b patterns so many diverse tissues remains unclear. Recent microarray studies in the limb have revealed multiple extracellular matrix molecules as possible downstream Lmx1b targets. In particular, the proteoglycans Keratocan, Lumican, and Decorin demonstrate a striking loss of expression in the Lmx1b KO mouse. Interestingly, these proteoglycans cluster together at one genomic locus (KLD) and 5’ to this KLD locus is a 900 kilobase region devoid of coding sequences, i.e., a gene desert. Using in silico analysis we identified 12 conserved noncoding regions (CNRs) surrounding the KLD locus and within the gene desert. Two of these CNR’s, Peak 3 and Peak 10, contain a known Lmx1b binding site (TAATTA or “FLAT” site). We hypothesize that Lmx1b directly regulates KLD via CNR Peak 3 and Peak 10. We isolated the CNR regions and linked them to a green fluorescent protein (GFP) reporter plasmid. These constructs were electroporated into chick embryos at the primitive streak stage (Hamburger-Hamilton (HH) 4), during early neural tube formation (HH10), and during early limb formation (HH14). GFP expression, and thus enhancer activity, was detected for Peak 3 in the neural tube beginning at HH10. As development progresses Peak 3 activity appears within the developing midbrain and cerebellum. At HH19, LMX1B and KLD mRNA expression overlap the region of Peak 3 enhancer activity. Additionally electroporation of a 40 base pair fragment, which contains the FLAT site and several potential surrounding
binding sites, displays localized activity similar to the complete Peak 3. This data indicates that this 40 base pair sequence containing an Lmx1b binding site is sufficient to produce Peak 3 activity. Our data further suggests that Lmx1b may regulate CNS morphogenesis by altering the extracellular matrix composition such as KLD proteoglycans. Furthermore, the activity of the minimal KLD-associated Peak 3 FLAT site localized to Lmx1b-dependant CNS structures during development supports a direct role for Lmx1b regulation.

Introduction

The establishment of techniques and software programs capable of genomic alignment and analytical bioinformatics has allowed researchers to compare diverse vertebrate genome sequences. Specifically, these methods can be used to detect cis-regulatory regions found in the noncoding areas of nearby gene targets. Cis-regulatory regions are thought to contain target sites for transcription factors thereby enhancing or silencing nearby gene function. Since it is likely that evolutionary conservation of cis-regulatory sequences occurs by fitness selection, areas of conservation across divergent species would be the most likely target regions to convey function. Genomic comparison studies have identified many regulatory domains, which are essential for development (Woolfe et al., 2004). The benefits and applications of genomic comparisons are considerable, in this report we will focus on Lmx1b, a transcription factor associated with pattern formation of multiple organ systems with very few identified downstream targets.

Lmx1b is a LIM homeodomain transcription factor known to contribute to patterning of the CNS, kidney, eye and limb in multiple species, however the mechanism
by which Lmx1b patterns these tissues is not fully understood. Lmx1b plays many
important roles in CNS formation including development of the midbrain, hindbrain,
serotonergic neurons and the differentiation/migration of spinal dorsal horn neurons.
Lmx1b is expressed in the neural tube at the isthmus organizing center at 9 days post
coitum (dpc) (Guo et al., 2007) where it acts to up-regulate Fgf8 and Wnt1 to ultimately
determine the boundary between the midbrain and hindbrain (Canning, Lee, Irving,
Mason, & Jones, 2007; Crossley, Martinez, & Martin, 1996; Wurst & Bally-Cuif, 2001).
Lmx1b KO mice do not express Fgf8 or Wnt1a causing a severe reduction of the tectum
and cerebellum (Guo, et al., 2007) conversely, overexpression of Lmx1b causes
overexpression of Fgf8 and Wnt1 creating an expansion of the tectum and cerebellum
(Matsunaga, Katahira, & Nakamura, 2002). Lmx1b also interacts with the transcription
factor Pet1 to direct terminal differentiation and maintenance of serotonergic neurons
(Cheng et al., 2003; Y. Ding et al., 2003). Additionally when Lmx1b is conditionally
knocked out of Pet-1 expressing neurons all serotonergic neurons in the mouse brain are
eliminated (Zhao et al., 2007; Zhao et al., 2006). It is thought that serotonergic
dysfunction is associated with depression, post traumatic stress disorder and addiction
(Naughton, Mulrooney, & Leonard, 2000). Interestingly the double Pet-1/Lmx1b KO
mice seem to exhibit increased anxiety, possibly due to the loss of serotonin transmission
(Dai, Hu, Shi, Guo, & Ding, 2008). Lmx1b KO mice do not form synaptic contacts
between dorsal horn neurons and primary nociceptive afferent neurons, thus indicting that
Lmx1b also plays a role in assembly of pain circuitry (Y. Q. Ding et al., 2004).

The molecular mechanisms and pathways utilized by Lmx1b to regulate CNS
development are still unclear. Since Lmx1b patterns several other organ systems studies
identifying downstream Lmx1b target genes in other systems may aid the discovery of CNS targets. Lmx1b has been well studied in the limb where microarray investigations using the Lmx1b KO mouse have implicated extracellular matrix molecules as downstream targets of Lmx1b during limb development (Feenstra et al., 2012; Gu & Kania, 2010; Krawchuk & Kania, 2008). In particular, the small leucine rich proteoglycan, Keratocan, was identified as a gene elevated in the presence of Lmx1b; and in Lmx1b KO embryos, Keratocan expression was not detected by whole mount in situ hybridization (Feenstra et al., 2012).

In an effort to identify and characterize cis-regulatory regions that Lmx1b might utilize, we examined the genome surrounding Keratocan, which is flanked by its family members Lumican and Decorin forming the KLD locus. Using VISTA analysis revealed 12 conserved noncoding regions (CNRs) surrounding the KLD locus. We screened several CNRs based on their location, the degree of conservation, and whether they contained an Lmx1b binding site. We hypothesized that Lmx1b uses one of these CNRs to regulate Keratocan, Lumican, and/or Decorin during its multisystem development.

**Materials and Methods**

We identified conserved cis-regulatory regions surrounding the KLD locus using VISTA genome browser (http://pipeline.lbl.gov/cgi-bin/GenomeVista) which uses the human genome as a base to perform pairwise analysis with dog, mouse, opossum and chicken (Frazer, Pachter, Poliakov, Rubin, & Dubchak, 2004). Pink peaks of conservation indicate greater than 70% conservation over a 100 base pair segment. KLD locus pairwise analysis was performed by entering the human chromosomal location of
the KLD locus (Chromosome12: 89,900,717-91,055,428), this 1.15kb range represents
the region of the KLD locus up to its nearest 5’ and 3’ gene. Peaks within this region that
are at least 70% conserved in dog, mouse, opossum and chicken were labeled 1-12. Peak
sequences for each species were examined for the FLAT Lmx1b binding site (TAATTA).
Of the 12 peaks only Peak 3 and Peak10 contain a FLAT site overlapping the same
region in human, dog, mouse, and chicken. Interestingly, the opossum shows a ~200 base
pair frame shift from the other species.

Forward and reverse primer sequences were generated which isolate CNR regions
including an additional ~50bp outside the region producing an 878bp fragment for Peak 3
and 894bp for Peak 10. Primer sets in the 5’ to 3’ orientation include;
Peak 10 Forward – GCAAAGGAAATTCTGGCTATC Peak 10 Reverse –
GTTTTGAACCACCATAGTTGTCC Peak 3 Forward -
CCTATTCATGACGCTGTTACATTGC, and Peak 3 Reverse -
GCAATCATTTCAGCTTGAGAACC Primers isolated the Peak 3 sequence by PCR
using chicken genomic DNA. Forward and reverse oligomers 40 bp in length were
annealed together to generate a 40 bp sequence that included the FLAT site in Peak 3, we
also generated a mutant FLAT sequence (FLATmut) that contained the same region with
the FLAT (TAATTA) site altered to form a HindIII cut site.

Primer sets in the 5’ to 3’ orientation, FLAT and HindIII sites are in bold;
FLAT Forward GCAGCTGCTAATTTGGTGATTAATTAATCCATATTACACATTGA
FLAT Reverse AGCAGCTGCTAATTTGGTGATTAAATTAATCCATATTACACATTG
FLATmut Forward GCTGCTAATTTGGTGATAAGCTTTCATATTACACATTGA
FLATmut Reverse AGCTGCTAATTTGGTGATAGCTTTCATATTACACATTGA
To generate FLAT and FLATmut fragments equimolar concentrations of forward and reverse primers were annealed using an annealing buffer (10 mM Tris, pH 7.5–8.0, 50 mM NaCl, 1 mM EDTA) primers were boiled for 5 minutes then cooled to room temperature over a 60 minute period. The resulting annealed primer sets were used for further construct generation. Peak 3, FLAT and FLATmut fragments were ligated into the pCRTM II-TOPO (Invitrogen) vector using manufacture recommended protocol. Constructs were screened for insert and sent out to be sequenced (Eton biosciences). Sequence confirmed pCRTM II-TOPO constructs were cut using KpnI and XhoI, inserts were then ligated into a pTK-EGFP vector, an enhanced GFP reporter driven by the Herpes simplex virus thymidine kinase promoter (Uchikawa, Takemoto, Kamachi, & Kondoh, 2004). Peak 3 FLATmut construct was created with the Peak 3 pTK-EGFP plasmid as a template along with the Forward FLATmut primer using QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) per the manufacturer’s protocol.

Whole embryo electroporation (HH4) is performed as described in (Chapman, Collignon, Schoenwolf, & Lumsden, 2001). 1μg of endotoxin free plasmid combined with Fas-green dye to visualize delivery is electroporated into the embryo using 5 pulses of 5 V for a duration of 50 ms at 100 ms intervals. Embryos are incubated on a yolk agar plate at 37°C and construct activity (GFP) is visualized with fluorescent microscopy at 24 and 48hrs.

HH10 electroporation in ovo was performed to determine construct efficacy in the brain. Embryos/eggs were windowed, staged and stained with neutral red to facilitate electroporation and removal of the vitelline membrane surrounding the head. 1μg of endotoxin free plasmid and Fas green was injected into the neural tube lumen and pushed
Since *in silico* analysis indicates Peak 3 and Peak 10 are likely targets of Lmx1b regulation we generated constructs for both CNR’s using a pTK-EGFP plasmid (Uchikawa, et al., 2004) which contains the the f1 phage origin; an upstream SV40; a multiple cloning site (MCS); the Herpes simplex virus thymidine kinase minimal promoter; an enhanced *GFP* reporter gene; and the ampicillin resistance gene (A). Electroporation of Peak 3 and Peak 10 into the whole embryo at the primitive streak stage allowed for detection of peak activity till HH14. Once the area of peak activity is determined, targeted regional electroporation (TREP) can be used to demonstrate activity in more developed organ systems such as the neural tube and developing brain (C).

cranially toward the tip of the anterior neural pore. Electrodes delivered 3 pulses of 50 ms at 10 V at 100 ms intervals. Embryos are incubated and imaged with fluorescent microscopy at 24 and 48 hours to determine construct activity.

Screening for activity in the limb was done by TREP of the lateral plate mesoderm between somites 14 and 21 in stage 14 chick embryos. Briefly, eggs are windowed and embryos stained with neutral red to determine somite number and assist removal of the vitelline membrane overlying the limb. Electroporation (EP) was performed with 3 pulses of 50 ms at 8 V at 100 ms intervals using 2 μg/μL and fas-green.

Whole mount *in situ* hybridization (WMISH) was performed using antisense digoxigen-labeled RNA probes for chick *LMX1B, KERATOCAN, LUMICAN* and *DECORIN*. Chick *LMX1B* probe sequences: Forward – GGATCGCTTTCTGATGAGG, and Reverse - CGAATGGAAGGAATGATGACATC. *KERATOCAN, LUMICAN*, and...
DECORIN probes were previously described (Dissertation Chapter 3, Feenstra et al 2012) Chicks embryos were harvested at HH19/20 and fixed overnight in MEMFA (0.1M MOPS, 2mM EGTA, 1mM MgSO4, 3.7% formaldehyde). WMISH was performed as described (Yamada, Szendro, Prokscha, Schwartz, & Eichele, 1999) with each probe hybridized at 60°C, and embryos were washed post hybridization at 65°C.

Results

Vista Analysis

VISTA analysis encompassed a 1.15 kilobase region which compares the human KLD locus to the dog, mouse, opossum and chicken (Fig 5.2). Upstream of the KLD locus lies an area devoid of other genes also known as a gene desert. Gene deserts are

![VISTA Analysis of KLD Locus](image)

**Figure 5.2) VISTA Analysis of KLD Locus**

VISTA output of the KLD locus comparing human to dog, mouse, opossum and chicken. Numbers below represent 12 areas of conservation surrounding the KLD locus. Keratocan (Kera), Lumican (Lum) and Decorin (Dec) are labeled above the human genome (black line), analysis extends to 5'/3' nearest neighbor genes. Pink peaks are conserved noncoding regions (CNR), dark blue regions are exons, light blue areas are untranslated regions. The horizontal line in each comparison represents 70% conservation. Peak 3 and Peak 10, highlighted in purple, contain conserved Lmx1b binding sites. Peak 10 is a small area of conservation between the 5’ untranslated region and first exon of Keratocan.
thought to be regions potentially rich with regulatory elements. Pink peaks denote areas of conservation; peaks with at least 70% conservation across species are labeled 1-12.

Further analysis of Peak 3 and Peak 10 revealed each had an Lmx1b FLAT binding site (TAATTA), which is conserved from human to chicken, suggesting that Peak 3 and Peak 10 are likely sites for Lmx1b mediated regulation. Consequently, we isolated Peak 3 and Peak 10 to screen for localized activity in the developing chick embryo.

Electroporation Studies

The conserved Peak 3 and Peak 10 sequences were incorporated into reporter constructs containing green fluorescent protein (GFP) driven by a basal herpes thymidine kinase promoter. Without an associated enhancer, GFP expression from this plasmid is minimal. After generating our reporter construct, we performed whole embryo electroporation at the primitive streak stage just prior to gastrulation. Following electroporation further development of the embryos was accomplished by incubation on a yolk agar plate for 48 hours. GFP expression and thus, activity of the Peak sequences was screened by fluorescent microscopy at 48 hours or approximately HH14. Results of whole embryo electroporation reveal Peak 3 activity in the HH10 developing brain (Fig 5.3A’). No activity of Peak 10 was identified after 48 hrs (Data not shown). To localize Peak 3 activity and correlate its activity with potential structure-related function in the brain, we performed electroporation during early brain development at HH10. Peak 3 localized to the mesencephalon and diencephalon within the developing brain at HH19 (Fig 5.3C’).
Figure 5.3) Peak 3 Activity in HH10 and HH19 Chick Brain
A) Light microscopy, dorsal view of HH10 brain electroporated with Peak 3 plasmid at HH4, A’) GFP picture of same embryo shows Peak 3 activity in developing brain vesicles. B/B’) HH4 embryo electroporated with base pTK plasmid as a negative control C) Light microscopy, lateral view of HH19 chick head with Peak 3 neural tube targeted electroporation performed at HH10, C’) GFP denotes Peak 3 activity in the mesencephalon (Mes) extending slightly forward into the diencephalon (Die) but is excluded from the telencephalon (Tel).
Using targeted regional electroporation we transfected the lateral plate mesoderm of the chick wing at HH14 with Peak 3 and Peak 10. Embryos were imaged with fluorescent microscopy at 24, 48, 60 and 72 hours. No activity was detected for either Peak 3 or Peak 10 in the developing limb.

Although Peak 3 activity was not detected in the limb, previously determined CNS activity (Fig 5.3) indicates Peak 3 may be a regulatory region for Keratocan (Kera), Lumican (Lum), and/or Decorin (Dec). To compare Peak 3 activity with LMX1B, KERA, LUM and DEC expression we performed whole mount in situ hybridization at HH19.

Expression Pattern Profiles

We confirmed the previously described expression of Lmx1b (Adams, Maida, Golden, & Riddle, 2000) in the mesencephalon and diencephalon. KERA, LUM and DEC are also expressed in the mesencephalon and diencephalon overlapping the areas of Peak 3 activity (Fig 5.4). This convergence of proteoglycan expression and enhancer activity supports a common regulatory mechanism. Since Peak 3 contains an Lmx1b binding site

![Image of expression patterns](image)

**Fig 5.4) WMISH of Lmx1b and Potential Downstream Target Genes**

WMISH detected LMX1B, KERATOCAN, LUMICAN and DECORIN in the mesencephalon (Mes), eye, telencephalon (Tel), and otic vesicle (OV). Mesencephalon expression overlaps Peak 3 activity.
we propose that Lmx1b binds the FLAT site to regulate the KLD proteoglycans. To test this possible mechanism of Lmx1b regulation we created a construct containing the Peak 3 sequence with the FLAT site mutated to a HindIII cut site. Additionally we generated a small (40 bp) region containing the FLAT site and a corresponding region with the mutated FLAT site.

**FLAT Sequence Electroporation**

If Lmx1b utilizes the FLAT site to regulate Peak 3 activity then mutation of the Lmx1b binding site should alter or ablate Peak 3 activity. Similarly, if the sequence surrounding the FLAT site is the minimal sequence necessary for Peak 3 activity

![Figure 5.5) Activity of the Lmx1b Binding Site (FLAT)](image)

Conserved noncoding region, Peak 3, identified with Vista contains a known Lmx1b binding site, pink box/1 (FLAT). Mutation of the FLAT site is represented by the black box/black 2. Minimal (40bp) constructs were also generated containing the FLAT site (3) and the HindIII mutated FLAT site (4). Embryos were electroporated at HH10 with the four constructs, followed by imaging with florescent microscopy to detect GFP at HH19. Peak 3 and FLAT constructs exhibit similar activity in the mesencephalon and diencephalon. Peak 3 FLATmut and Flatmut both display expansion of activity into the telencephalon (white arrows).
electroporation of this minimal sequence (40bp) should show similar activity to the entire Peak 3 sequence. If Lmx1b binding is essential to Peak 3 activity then mutation of the FLAT site in both the entire Peak 3 and 40bp fragment should abate activity. Interestingly, we discovered the 40bp FLAT does mimic Peak 3 activity (Fig 5.5 1/3) while Peak 3 FLATmut and the 40bp FLATmut fragment have increased activity not only surrounding the brain vesicles of the tectum but also extending into the telencephalon where Lmx1b is not normally expressed (Fig 5.3 2/4). A possible reason for this outcome is that by mutating the FLAT site to a HindIII site we may have created a new transcription factor binding site with enhanced activity.

To evaluate whether our mutation created a new binding site we used ALIBABA, a software program which uses TRANSFAC, a database of proven binding sites,

![Figure 5.6] ALIBABA Analysis of the FLAT and Mutated-FLAT Binding Sites

ALIBABA uses TRANSFAC analysis to determine possible transcription factor binding sites. Note Lmx1b is not listed in the TRANSFAC database and therefore does not appear in the results. Green region represents the FLAT site (TAATTA) while the red region below represents the FLAT site mutated to HindIII (mFLAT). In the native sequence we found five possible transcription factor binding sites in association with the FLAT binding site; our mutation of the FLAT binding site disrupted all five of these native sites, but generated 2 new binding sites.
consensus binding sites and regulated genes to discover predicted transcription factor binding sites. When we compare our FLAT and FLATmut sequences with ALIBABA, our mutation disrupted 5’ flanking binding sites; however, our mutation also generated new GATA and MCM1 binding sites (Fig 5.6). GATA transcription factors have been implicated in CNS development (Kornhauser et al., 1994), thus, creation of this new GATA site may have transformed the FLATmut sequence into an enhancer that is localizing to sites of GATA expression.

**Discussion**

Lmx1b is expressed in the isthmus organizer, a signaling center responsible for midbrain and hindbrain formation. Loss of Lmx1b causes a severe reduction of not only the midbrain dopaminergic neurons but also the tectum and cerebellum (Guo, et al., 2007). We have discovered a conserved noncoding region (CNR), Peak 3, which contains an Lmx1b binding site and is active in the mesencephalon and diencephalon, tissues that will form the midbrain and hindbrain region. Peak 3 lies 5’ to Keratocan, Lumican and Decorin (KLD), we have also determined that KLD expression overlaps Lmx1b expression and Peak 3 activity in the brain. Additionally expression of the small 40bp fragment including the Lmx1b binding site FLAT showed the same activity as the entire Peak 3 indicating this sequence is sufficient for Peak 3 activity. Together these results suggest Lmb1b binds to Peak 3 thereby regulating the proteoglycans Keratocan, Lumican and Decorin during mid/hind brain development.

No activity was detected for Peak 10 using whole embryo electroporation from HH4-HH14 and targeted regional electroporation of the limb from HH14-25. Although
Peak 10 may be active later in development our results suggest Peak 10 activity does not coincide with early stages of Lmx1b patterning.

For the completion of this study we will generate a different mutation in the FLAT site to determine activity in both the 40 bp fragment and the full Peak 3 sequence. A loss of activity with the newly mutated FLAT site would suggest that the initial mutation and the binding sites generated expanded enhancer activity. We are also concurrently running a chromatin immunoprecipitation which isolates Lmx1b targeted sequences. We will screen our Lmx1b-immunoprecipitated chromatin for Peak 3 to determine direct Lmx1b binding. Although the FLAT site is the only currently known Lmx1b binding site it is likely that other Lmx1b binding sites remain to be determined. It is also likely that Lmx1b uses a currently unidentified binding site to regulate any of the remaining 10 CNR’s surrounding the KLD locus. We will also use the ChIP-seq results to screen for possible KLD-associated Lmx1b regulatory regions in the limb.
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CHAPTER SIX
IDENTIFICATION OF GENOMIC LMX1B BINDING SITES IN HUMAN EMBRYONIC KIDNEY CELLS

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Abstract

Lmx1b is a homeodomain transcription factor critical to kidney development and glomerular filtration. In humans, Lmx1b haploinsufficiency causes a condition known as Nail-Patella syndrome (NPS). Individuals with NPS typically have under-developed nails, absent patellae, and impaired kidney function. In Lmx1b knockout mice, the absence of Lmx1b function disrupts glomerular filtration and abates urine production. The molecules targeted by Lmx1b that regulate glomerular development and function, however, are poorly characterized. Immortalized human embryonic kidney (HEK 293) cells provide a potential tool for high-throughput identification of Lmx1b targets and an in vitro system for dissecting the functional role of targets. Thus, we characterized the known Lmx1b pathway in HEK 293 cells and evaluated sites of Lmx1b binding. We demonstrate the expression of Lmx1b and its renal targets, collagen 4 alpha 4 (Col4α4) and podocin (Nphs2) in HEK 293 cells by RT-PCR. Additionally, protein expression of Lmx1b was verified by western blot analysis. In a preliminary Chromatin immunoprecipitation, we determined that Lmx1b binds to regulatory sequences associated with Col4α4 and Nphs2. Further characterization of the Lmx1b pathway in HEK 293 cells is underway and will likely enhance identification of relevant downstream Lmx1b targets.
Introduction

Throughout development, molecular pathways are routinely reused to pattern various organs or structures. Thus key molecules used in these pathways must be capable of patterning multiple organ systems. One such example is Lmx1b, a LIM-homeodomain transcription factor critical for central nervous system (CNS), eye, limb, and kidney development. Absence of Lmx1b function in \textit{Lmx1b} knockout (KO) mice disrupts CNS development, limbs exhibit a distal ventral-ventral symmetry and the kidneys are small, lack proximal tubules and fail to produce urine (Chen, et al., 1998). Lmx1b KO mice die shortly after birth due in part to the nephropathy. The filtering units of the kidney are convoluted vascular tufts of capillaries called glomeruli. Podocytes are a specialized type of epithelial cell that wrap around glomerular capillaries to regulate filtration. Podocytes secrete basement membrane components including collagens and proteoglycans. During kidney development Lmx1b directs podocyte differentiation and maintenance. Loss of Lmx1b causes aberrant basement membrane formation, thereby preventing normal glomerular filtration (Rohr, et al., 2002). Despite many years of study, only two direct downstream targets of Lmx1b have been discovered, Col4α4 (Morello, et al., 2001) and Nphs2 (Rascle, et al., 2009), while other targets in the kidney remain elusive. In other organs patterned by Lmx1b, no other direct downstream targets have been confirmed. Although, recent microarray studies in the limb have implicated the proteoglycans Keratocan, Lumican, and Decorin, it is unclear whether these molecules are direct or indirect targets (Feenstra, et al., 2012). Interestingly, disruption of Lmx1b patterning causes abnormal patterning of extracellular matrix proteins in the kidney and limb. So it may be that discovery of downstream Lmx1b targets in the kidney will enhance and/or
identify new limb targets.

Keratocan, Lumican and Decorin are a unique set of small leucine rich proteoglycans tightly clustered together on the genome. Keratocan is best known for its role in maintaining transparency in the lens of the eye by aiding collagen fibril development and maintenance (Liu, et al., 2003). Lumican and Decorin are also associated with collagen fibrillogenesis; in particular affecting the rate of collagen fibril assembly, thereby giving tendons and skin their tensile strength (Rada, Cornuet, & Hassell, 1993). Lumican and Decorin are both expressed during kidney development and have been studied in the adult human diabetic kidney where overexpression of Lumican and Decorin is thought to have a protective effect (Schaefer et al., 2001). Upstream of the Keratocan, Lumican, and Decorin locus lies a 900 kb non-coding region, which contains developmentally conserved sequences. One region (800bp), which we named Peak3, contains an Lmx1b FLAT binding site (TAATTA) and is conserved between human, mouse, dog, opossum and chicken. These three proteoglycans clustered on the genome in conjunction with a conserved Lmx1b binding site and changes in proteoglycan distribution in the Lmx1b KO mouse makes Peak3 a compelling regulatory target in the Lmx1b cascade. We hypothesize that Lmx1b regulates Keratocan, Lumican and Decorin (KLD) by binding to Peak3 during development.

In an effort to characterize downstream targets of Lmx1b, including Peak3, we will use chromatin immunoprecipitation in conjunction with next generation sequencing (ChIP-seq). ChIP-seq will help us determine Lmx1b regulation by defining genomic sites of Lmx1b binding associated with its regulated targets. This is an important step in
beginning to understand the mechanisms utilized by the Lmx1b to regulate morphology and function.

**Methods**

**Cell Culture**

Human embryonic kidney cells (HEK) 293 cells were grown in 75 cm² culture flasks and maintained at 37°C in a humidified incubator with 5% CO₂. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM/ATTC) supplemented with 10% fetal bovine serum (Atlanta Biologicals). A subset of HEK cells were transfected with an pOZ-N-Lmx1b construct which contains both hemagglutinin (HA) and FLAG tags flanking the Lmx1b (pOZ-Lmx1b-HA/FLAG, generated in collaboration with Dr. Nathan Wall).

**HEK Cell Characterization**

HEK cell DNA was isolated using the DNeasy per manufacturers protocol (Qiagen.). Primers to flank the Lmx1b binding site of Col4α4 and Nphs2 were generated and resulting PCR amplicons were purified and sequenced (Eton Bioscience Inc.) to determine specificity. Primers were also generated to pull down RNA sequences for Col4α4 and Nphs2, which were also purified and sequenced.

**Western Blot**

HEK cells were homogenized via sonication in lysis buffer containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, and 50 mM Tris (pH 8). Protein (15-20 ul) was loaded into duplicate lanes of a 4-12 % Tris-glycine SDS-PAGE
gel (Invitrogen, Carlsbad, CA). Each electrophoresis and blotting was performed using nitrocellulose membranes at least twice. Membranes were stained with Ponceau S solution (0.1% Ponceau in 5% acetic acid) to check for variance in sample loading. The membranes were washed in 20 mM Tris-buffered saline solution with 0.1% Tween 20 (T-TBS). Membranes were blocked in 5% powdered milk in T-TBS for 1 h at room temperature and washed three times in T-TBS before being incubated overnight in primary antibody solution (Lmx1b AB, donated by the Ralph Witzgall lab, diluted 1:10/Hemagglutinin AB, Sigma, diluted 1:250). After incubating in the primary antibody, all membranes were washed three times in T-TBS and incubated in LI-COR® IRDye® 800CW Goat Anti-Mouse IgG or 680CW Goat Anti-Rabbit IgG (LI-COR®, Lincoln, NE; T-TBS, 1:15:000) for 1 h at room temperature. LI-COR® Odyssey® Fc Imaging System was used for detection of the protein bands.

Chromatin Immunoprecipitation (ChIP)

Cells were fixed with formaldehyde and chromatin extracted and sheared to produce chromatin fragments of 500-250 bp (optimized as per protocol, ChIP-it Express Enzymatic, Active Motif, Carlsbad, CA). The DNA concentration in the sheared chromatin was determined using the Nanodrop spectrophotometer. Approximately 50 ug of sheared chromatin was used for each immunoprecipitation. We isolated chromatin using the HA (Sigma) antibody from HEK cells and transfected Lmx1b-HA HEK cells. We also used the control kit (Active Motif) containing a Gapdh primer set, the RNAPolIII antibody for use as an in-run positive control and IGG as an in-run negative control. The ChIP-Express system uses protein G magnetic beads to precipitate antibody bound to
chromatic allowing for rapid purification of the protein-DNA complexes. The ChIP-isolated DNA was then purified using the minelute QIAquick PCR purification system (Qiagen) and DNA concentration determined using the Nanodrop spectrophotometer. Quality control check of all ChIP DNA (input chromatin, HEK, Ha-Lmx1b HEK) included PCR for 5’ region of Gapdh and known Lmx1b binding targets Col4α4 and Nphs2.

**ChIP Sequencing**

We will use the University of California at Irvine (UCI) Genomics High Throughput Facility for sequencing (http://ghtf.biochem.uci.edu/); they use an Illumina high throughput DNA sequencer (HiSeq 2000). We will submit 50 ng of ChIP-isolated DNA from our HEK and Lmx1b-HA HEK cell lines. HEK cell chromatin isolated with the HA antibody results will help identify nonspecifically bound chromatin. Results will be compared to previously identified conserved non-coding regions upstream of the KLD locus. In addition, results will be analyzed with the Genomic Regions Enrichment of Annotations Tool. This online software tool (http://great.stanford.edu/public/html/index.php) compares the location and association of potential regulatory elements with the annotations of the surrounding or nearby coding genes and assigns potential functional relevance to the site of binding.
Results

HEK Cell Characterization

Previous studies have shown that Lmx1b binds the FLAT site upstream of human Col4α4 (Morello, et al., 2001) and Nphs2 (Rascle, et al., 2009) using Cos7 and Hela cell lines. We suggest that the Human Embryonic Kidney cell line (HEK) would more accurately represent Lmx1b regulation in the developing human kidney. We began characterization of HEK cells by demonstrating that Lmx1b, Col4α4, and Nphs were all normally expressed (Fig 6.1B). We further isolated the previously described FLAT Lmx1b binding sites associated with Col4α4 and Nphs2 (Fig 6.1A).

![Figure 6.1](image)

**Figure 6.1) Characterization of Human Embryonic Kidney Cell Line**

A.) PCR of Lmx1b and the FLAT sequence of its renal targets, Collagen 4α4 and Podocin (Nphs2) using HEK 293 cell DNA. Gapdh was used as a positive control. B.) PCR of RNA from Collagen 4α4 and Nphs2 using HEK cell cDNA. C.) Demonstration of LMX1B in HEK 293 cells transduced with HA-LMX1B (293/L) by Western blot. HA-tagged LMX1B is demonstrated by both HA antibodies (αHA) and LMX1B antibodies (αLMX1B) (arrow ~56 kDa)
To enable optimization of the ChIP-seq protocol without depleting our limited supply of the ChIP proven Lmx1b polyclonal antibody, we transfected HEK cells with a pOZ-Lmx1b-HA construct. Generation of a stable HEK293 cell line with this construct (HEK293/L) allows us to probe for Lmx1b specific targets using commercially available ChIP proven monoclonal antibodies to either the HA epitope or the FLAG epitope. Using the Lmx1b antibody, Lmx1b protein was detected by western blot in both HEK293 and HEK293/L transfected cell lines (Fig 6.1C). Note, the construct generates greater than 10 times more Lmx1b that endogenously present. Thus the enhanced Lmx1b protein levels in the HEK293/L cells (Fig 6.1 +) by western analysis overshadows the level of Lmx1b protein visualized in control HEK cells (Fig -) although it is readily detected by Odyssey® Fc Imaging System (data not shown). While using the HA antibody, only the HEK293/L cells demonstrate Lmx1b protein (Fig 6.1 +). Importantly, the background HA recognized proteins in HEK293/L are also recognized in the control HEK293 cells (Fig 6.1-).

The HEK cell line mimics in vivo conditions making it an accurate reproducible model of Lmx1b mediated kidney development, which can be use for ChIP-seq. We were able to enhance Lmx1b protein levels in the HEK293/L cell line. Thus, using this model for Chip will accentuate Lmx1b targets. The unaltered HEK cell line will be used as a “in run” negative control since we were unable to detect Lmx1b protein with the HA antibody.
Preliminary ChIP Results

We confirmed the effectiveness of this approach by performing a chromatin immunoprecipitation to the known renal Lmx1b binding sites. Lmx1b bound chromatin was isolated, fragmented and then enriched from HEK293 and HEK293/L cells using an HA antibody. PCR confirmed the presence of Lmx1b binding sites (FLAT) to COL4 and Nphs2 from HEK293/L cells (Fig 6.2). FLAT sites were not found in control HEK293 cells isolated with the HA antibody.

Figure 6.2) ChIP Results from HEK and HA-LMX1B Transduced HEK Cells
PCR following HA-immunoprecipitation (HA Ab) of human embryonic kidney (HEK) 293 cells containing the HA-LMX1B construct. Lmx1b binding sites in kidney COL4α4 (C) and NPHS2 (N) loci are recovered by PCR. Sheared input DNA demonstrates the PCR fragment size of the predicted sites. The positive control uses an RNA polymerase II antibody (RP Ab) that binds to a GAPDH associated site (G) M=Molecular weight markers. Note absence of LMX1B COL4α4 and NPHS2 sites in the control HEK 293 cells using HA antibody (★).
**Discussion**

Characterization of the Lmx1b pathway in HEK293 cells will facilitate identification of downstream targets and the molecular cascade that regulates glomerular development and function. We have successfully isolated the FLAT sequences from Col4α4 and Nphs2 from HEK cell DNA. We have also shown Lmx1b, Col4α4 and Nphs2 RNA expression in the HEK cell line. Using western blot we detected Lmx1b protein in both the HEK and HEK transfected cell lines. Together these results indicate that the HEK cell line mimics in vivo conditions and is therefore a good model to perform ChIP-seq. PCR following ChIP confirmed Lmx1b binding to FLAT regulatory regions of both Col4α4 and Nphs2.

To complete this project we will perform multiple ChIP runs to pool the chromatin thereby allowing for sufficient chromatin to be isolated for sequencing. We expect ChIP-seq results to partially overlap microarray results discussed in Chapter 3. We also expect, since Peak3 is a very likely target of Lmx1b regulation, sequencing results from ChIP HA isolated chromatin will include Peak3 discussed in Chapter 5. Isolation of this region with ChIP will help determine direct Lmx1b binding.
References


Ma, C., & Staudt, L. (1996). LAF-4 encodes a lymphoid nuclear protein with transactivation potential that is homologous to AF-4, the gene fused to MLL in t(4;11) leukemias. *Blood, 87*(2), 734.


CHAPTER SEVEN
CONCLUSION AND FUTURE DIRECTIONS

Conclusion

Lmx1b is a homeodomain transcription factor necessary and sufficient for dorsalizing distal limb structures. This was established over a decade ago with the production of an Lmx1b knockout mouse (Chen et al., 1998). However, downstream targets of Lmx1b in the limb responsible for dorsalization remain uncharacterized. We began our search for Lmx1b target genes using the *Lmx1b* KO mouse model. In the mouse, loss of Lmx1b causes scapular hypoplasia, distal ulnar hypoplasia, and loss of soft tissue dorsalization, *i.e.*, symmetrical ventral-ventral or flexor-flexor ligaments, tendons and muscles (Chen & Johnson, 2002). Using microarray comparison of Lmx1b KO and wildtype limbs we discovered skeletal, connective tissue, neuronal, and angiogenic targets differentially expressed during Lmx1b mediated limb dorsalization. One of the most noteworthy findings of our microarray analysis was the discovery of several proteoglycans and proteoglycan related genes regulated by Lmx1b. The persistent asymmetrical elevation of a cluster of proteoglycans during limb dorsalization may provide clues to the mechanism of Lmx1b-regulated patterning.

We focused our efforts on the proteoglycans Keratocan, Lumican and Decorin for several reasons: the preponderance of proteoglycan and proteoglycan associated genes discovered in the microarray analysis, of all the downstream targets discovered, Keratocan showed the greatest fold change in the microarray, all three proteoglycans
have a dorsally restricted expression pattern in the limb while Keratocan was undetectable in the Lmx1b KO mouse limb, these three proteoglycans (Keratocan, Lumican and Decorin) are closely arranged on the genome (the KLD locus) suggesting a common or redundant mechanism of regulation and finally surrounding the KLD locus are two regions highly conserved across species which contain an Lmx1b binding site.

The role of Lmx1b mediating limb dorsalization is conserved across vertebrate species (Mercader, 2007). Since the developing chicken limb in ovo is accessible and provides a model to perform temporal and spatial experimentation we continued examination of the proteoglycans using the chick limb. We confirmed similar expression of Lmx1b expression within the dorsal mesoderm of mouse and chicken limbs. Unexpectedly however, the progressive patterns of Keratocan, Lumican and Decorin expression were not conserved between chick and mouse limbs although, in both species, these proteoglycans do eventually localize to tendons. The disparate patterns of proteoglycan expression suggest that mechanisms regulating these genes are not fully conserved.

Nevertheless, Keratocan demonstrated the greatest fold change in the microarray analysis, was undetectable in the Lmx1b KO mouse and is flanked by two conserved noncoding regions (CNRs), that contain Lmx1b binding sites. Peak 3, is upstream of the KLD locus and exhibits enhancer activity in the mesencephalon and diencephalon, tissues that will form the midbrain and hindbrain region. Correspondingly Lmx1b, Keratocan, Lumican and Decorin are expressed in the isthmus organizer, a signaling center responsible for midbrain and hindbrain formation. Loss of Lmx1b causes a severe reduction of not only the midbrain dopaminergic neurons but also the tectum and
The proteoglycans Keratocan, Lumican and Decorin lie very close along the genome suggesting a similar or redundant function. Although individual knockout mouse models exist for each of these genes, a compound KLD KO mouse would help determine the function of these molecules. It is unlikely that a triple KO of Keratocan, Lumican and Decorin KO could be accomplished by breeding individual KO mice, due to the close proximity of these genes within the KLD locus (within 60 kb of each other). I would propose that removal or disruption of the entire KLD locus would save resources and time. If KLD’s regulation by Lmx1b is critical for limb development, then the KLD KO mouse would present with aberrant dorsal limb morphology. It is likely that other Lmx1b...
patterned tissues including the eye, midbrain/hindbrain and kidney would also be disrupted.

In addition to the proteoglycans examined in this dissertation, many other interesting downstream Lmx1b targets were discovered in the microarray analysis (Chapter 3). An interesting group that remains to be examined are genes associated with scapula formation. *Agc, Lum, Dec, and Lox* show a reduction of scapular expression in *Lmx1b* KO mice while *Emx2, Matn1, and Matn4* exhibited a striking loss of scapular expression. Since *Lmx1b* KO mice have hypoplastic scapulae, we propose Lmx1b works in concert with these collective targets to augment scapular development. An interesting way to examine Lmx1b effect on scapula development would be to knockout Lmx1b expression from condensing cartilage. A recent study attempted this using a Sox9 promoter to induce Cre Lox removal of Lmx1b, the phenotype was very mild presenting with distal ventralized sesame bones (Li, Qiu, Watson, Schweitzer, & Johnson, 2010). Use of a similar method with an earlier marker to drive Lmx1b KO in the limb and scapula cartilage would be a very interesting model to study the effects of Lmx1b on skeletogenesis. Similarly a Scleraxis driven conditional Lmx1b KO would help to determine the role of Lmx1b and the downstream targets we discovered which localize to tendons.

Chip-seq data will be overlaid with the microarray data to confirm direct downstream targets. Initially ChIP-seq will be done using kidney cells, thus it is likely that not all relevant limb targets will overlap. However, with the confirmation of direct downstream Lmx1b targets in the limb we can proceed with ChIP-seq using mouse limb chromatin. Using our mouse colony we can harvest chromatin directly from mouse limbs
during Lmx1b mediated dorsalization. However since we have a limited amount of ChIP proven Lmx1b antibody available we may also use a transgenic mouse created by Dr. Randy Johnson with a Hemagglutinin tagged Lmx1b. Access to this breeding mouse colony would allow us to perform the ChIP on mouse limb chromatin using the Chip proven hemagglutinin antibody.
References


APPENDIX

DEVELOPMENTAL BIOLOGY AND CLASSIFICATION OF CONGENITAL ANOMALIES OF THE HAND AND UPPER EXTREMITY

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Abstract

Recent investigations into the mechanism of limb development have clarified the role of several molecules, their pathways and interactions. Characterization of the molecular pathways that orchestrate limb development has provided insight into the etiology of many limb malformations. In this review, we describe how the insights from developmental biology are related to clinically relevant anomalies and the current classification schemes used to define, categorize, and communicate patterns of upper limb malformations. We advocate an updated classification scheme for upper limb anomalies that incorporates our current molecular perspective of limb development and the pathogenetic basis for malformations using dysmorphology terminology. We anticipate that this scheme will improve the utility of a classification as a basis for diagnosis, treatment and research.

Introduction

Around 1 in 600 newborns present with some form of upper limb abnormality (1). The molecular mechanisms that orchestrate limb development have been the focus of intense study for several decades. Recent discoveries provide a more comprehensive understanding of the process and greater insight into how disruption of different molecular pathways correlates with specific presentations of upper limb anomalies.

Clinical diagnosis and treatment is enhanced by an accurate method to describe and categorize these anomalies. Furthermore, there is a benefit for communication between various clinicians and basic scientists if the classification scheme incorporates a contemporary understanding of the developmental process. The most commonly used
classification at present is based on a scheme proposed by Swanson (2) in 1964 and was subsequently accepted by the International Federation of Societies for Surgery of the Hand (IFSSH) in 1974 (3). This classification scheme merged the morphological understanding of limb development with a surgical perspective on treatment. Its simplicity promoted near universal adoption. However, its simplicity fails to group some developmentally related anomalies, thereby allowing for either misdiagnosis or multiple diagnoses for a related condition. Importantly, the scheme intermixes descriptive and presumed etiologic categories. Its descriptive terminology is unable to incorporate changes based on our increased understanding of developmental biology. Consequently, this method of classification has generated criticism (4-9).

Despite continued progress in limb development research, a comprehensive picture that explains a large spectrum of upper limb malformations has only recently emerged. Manske and Oberg recently proposed several modifications to the classification scheme based on clinical experience and developmental biology (6). The intent of this review is to present an overview of developmental biology highlighting current concepts of the pathogenetics of limb anomalies and to provide support for further modifying the classification scheme to make it more relevant and useful.

**Limb Development – Axis Formation and Differentiation**

During early embryonic development, homeobox (HOX) transcription factors set up a segmental body plan along the cranial-caudal axis (10). Around the fourth week of development, the presumptive upper limb fields are established, triggering the expression of TBX5, WNT and FGF proteins that initiate limb outgrowth (11). The upper limb bud
emerges as a bulge of lateral plate mesoderm covered by a thin outer layer of ectoderm (Fig A.1). Failure of limb induction, or tetramelia, is associated with WNT3 and FGF10 mutations (12;13). TBX4 and TBX5 provide clues for hindlimb\forelimb identity, respectively. Mutations in TBX5 (Holt-Oram syndrome) (14) produce a range of upper limb anomalies.

Following formation of the limb bud, development proceeds along three axes: proximal-distal, anterior-posterior (radial-ulnar), and dorsal-ventral (Fig A.1).

Figure A.1) Limb Bud Coordinate Axes and Signaling Centers
A) The forelimb (boxed region) of a Carnegie stage 13 embryo depicting the three coordinate axes – each with their own signaling center: the apical ectodermal ridge (AER - orange) coordinating proximal-distal (Pr-Di) outgrowth and differentiation; Radial-ulnar asymmetry is controlled by the zone of polarizing activity (ZPA - purple). Dorsal-ventral (Do-Ve) asymmetry is regulated by dorsal ectoderm (green); Within the progress zone (PZ - blue) the fate of mesodermal cells are determined by these signaling centers. The axes and signaling centers are shown in different orientations – B) Dorsal view, and C) Lateral, end on view. (Modified from Oberg et al., 2004, used with permission)
Development and differentiation of each axis is controlled by a population of cells that signal patterning information to local cells and organize tissue along its axis, i.e., signaling centers. Mesodermal FGF10, in conjunction with ectodermal radical fringe (R-FNG) at the apical dorsal-ventral boundary, induces ectodermal thickening to form the signaling center for the proximal-distal axis, the apical ectodermal ridge (AER) (15-17). The AER, in turn, secretes WNT3 and several FGF proteins (FGF4, 8, 9 & 17), which maintain FGF10 expression in the underlying mesoderm. FGF10 sustains the proliferation of a sub-AER population of cells termed the “progress zone” (PZ) (18). The mesodermal cells within the PZ are influenced by the signaling centers to determine their ultimate fate. This reciprocal loop of ectodermal and mesodermal FGF/WNT proteins maintains proximal-distal outgrowth (Fig A.2 A) (19;20).

Development and differentiation along the anterior-posterior (radial-ulnar) axis is controlled by the zone of polarizing activity (ZPA) in the posterior (ulnar) limb mesoderm. The ZPA expands limb width and posteriorizes (ulnarizes) the developing limb through a secreted morphogen, sonic hedgehog (SHH) (Fig. 2B & C) (21). The AER and the ZPA are closely linked by a reciprocal feedback loop that maintains SHH expression at the distal posterior (ulnar) border of the AER during progressive outgrowth (22-24).

WNT7A secretion in the dorsal ectoderm regulates limb development along the dorsal-ventral axis. WNT7A dorsalizes the underlying limb mesoderm through the induction of the Lim homeodomain transcription factor LMX1B (Fig A.2 D) (17;25;26). Disruption of WNT7A also disrupts ulnarization indicating an important role for WNT7A in maintaining ZPA-related secretion of SHH (27). Thus, SHH plays a pivotal role
during limb development, linking proximal-distal, anterior-posterior (radial-ulnar), and dorsal-ventral axes during outgrowth (28).

The signaling centers are involved in the coordinate control of downstream targets involved in skeletogenesis, vasculogenesis, myogenesis, and innervation, which may involve both common, and unique, asymmetric molecular pathways. For example, skeletogenesis requires appropriate temporal and spatial regulation of multiple factors including SOX9 for condensation of skeletal anlagen; WNTs and GDF5 for joint formation; and PTHLH, IHH, IGFs, BMPs, WNTs, FGFs, and RUNX2 for anlagen growth and subsequent endochondral ossification (29). In addition, SHOX2 is up-regulated in the proximal perichondrium and promotes humeral elongation, while SHOX is induced in the forearm perichondrium and regulates radial-ulnar elongation (30;31). Thus, appropriate induction of downstream pathways is critical to complete differentiation along the appropriate limb axis.

Disruption of the Proximal-Distal Axis (Transverse Deficiencies)

FGFs emanating from the AER promote PZ and ZPA related limb outgrowth. Complete removal of the AER (32) or blockade of FGF-receptor signaling (33;34) truncates the limb along the proximal-distal axis coincident with the timing of disruption (Fig A.2 E). These truncated limbs are akin to those described clinically for transverse arrest and the data in animal models suggest that the level of truncation/transverse arrest correlates with the timing of disruption (35).

Recently, Winkel and colleagues (36) demonstrated a link between WNT signaling and ROR2, the receptor tyrosine kinase frequently mutated in brachydactyly
type B1 (BDB1). WNTs, under the control of AER-related FGFs, contribute to elongation of the entire limb via ROR2 receptors; thus linking abnormal function of the AER outgrowth pathway to this form of brachydactyly and providing impetus for inclusion as a disruption of proximal-distal development.

Intersegmental defects or phocomelias have been difficult to align with developmental pathways and most of these fall under severe longitudinal or radial-ulnar axis defects as described below. However, recent insights into the mechanisms and potential roles of SHOX2 and SHOX in proximal-distal outgrowth may support some rare intersegmental defects (30;31;37).

**Disruption of the Radial-Ulnar Axis (Longitudinal Deficiencies)**

Whereas complete elimination of AER-related FGF function leads to transverse deficiencies, reduction in FGF function produces longitudinal deficiencies (Fig. 2F). Diminished FGF function leads to a reduction in limb outgrowth, volume and width, although proliferation and ulnarization promoted by the ZPA persists. The resulting phenotypes are remarkably similar to the classification spectrum of radial longitudinal deficiencies (RLD) (Fig A.2 F) (38-40). Malformation syndromes with mutations in FGF receptor-2, such as Apert, Pfeiffer or Saethre-Chotzen, also link radial/anterior joint abnormalities and synostoses of the arm to regulation of the FGF pathway (41).

SHH induces ulna formation in the forelimb and 4 ulnar-sided digits in the hand. In addition, SHH is responsible for posterior (ulnar-sided) limb growth and expansion. Progressive loss of SHH expression or targeted temporal interruption of SHH signaling during limb development reduces limb outgrowth, volume and width (42;43). The
phenotypes generated with SHH loss mimic those described in the spectrum of ulnar longitudinal deficiencies (ULD) (Fig A.2 G). The clinical spectrum of ULDs probably reflects variation in the timing, degree and duration of SHH disruption. Furthermore, the loss of SHH can also reduce FGF expression via interruption of the SHH-FGF loop. Thus, with reduced limb width, limb volume and FGF expression, formation of radial structures, particularly the thumb, can also be undermined. This correlates with the common clinical presentation of thumb deficiencies associated with ULD.

In the past, severe forms of longitudinal deficiencies that involve proximal losses have been classified as intercalary or intersegmental defects. Careful reexamination of many of these cases demonstrates distal defects consistent with RLD or ULD suggesting that the proximal abnormalities represent a continuation of the longitudinal deficiency (44;45).

Disruption of the Dorsal-Ventral Axis (Dorsalization Deficiencies)

Reduction or loss of either WNT7A in the dorsal ectoderm or LMX1B in the dorsal mesoderm impairs dorsalization (Fig A.2 H) (46;47). In the mouse model, haploinsufficiency does not generate a phenotype; however, in humans, single allele disruption of LMX1B has been shown to cause Nail-Patella Syndrome, with reduced limb dorsalization, i.e., abnormal elbow and fingernail formation (47). *(dorsalization is the embryonic term used prior to limb rotation and subsequently refers to the posterior or extensor aspect of the upper limb and the anterior-extensor aspect of the lower limb)*
Figure A.2) Disruption of Axis Formation/Differentiation
A-D) Impact of signaling centers on normal development. A) Fibroblast growth factors (FGFs) from the AER (orange) initiate and maintain limb outgrowth. Formation of skeletal elements progresses from proximal to distal shown in three progressive stages that depict arm, forearm and hand formation. B) During limb outgrowth sonic hedgehog (SHH) emanating from the ZPA (purple) establishes an “ulnarizing” gradient (purple arrows) and maintains posterior (ulnar) proliferation. In addition, FGFs (orange arrows) secreted from the AER promote proliferation in the underlying progress zone contributing to limb volume and width. C) Relative contributions of SHH (purple) and FGFs (orange) to skeletal elements. D) Dorsal-ventral section of an embryo through the forelimb illustrating the regulation of LMX1B (light green) by WNT7A (green). E-H) Failure of axis formation/differentiation. E) Abrogation of AER/FGF function as depicted in the top panel leads to transverse defects that correspond to the time of disruption. F) Progressive reduction of FGF function leads to loss of radial structures. G) Loss of SHH function leads to loss of ulnar-associated structures. H) Disruption of LMX1B is associated with loss of dorsalization.
Formation and Differentiation of the Handplate

The handplate becomes visible around the 5th week of development. HOX transcription factors (particularly HOXD9-13 and HOXA9-13) and SHH interact to establish digit number and identity (Fig A.3 A) (48-51). SHH also induces a posterior-anterior (ulnar to radial) BMP gradient that has at least two roles in the formation and differentiation of digits. First, BMPs induce apoptosis, or programmed cell death, within interdigital space. This is accomplished, in part, by repressing FGF expression in the overlying AER (52-54). Second, BMPs participate in completing digital identity via the phalanx forming region, a region overlying the distal digital anlagen that regulates chondrogenesis via SOX9 up-regulation and maintains the digit associated FGF in the overlying AER for continued digital outgrowth (55). However, it is still unclear how the various members of the BMP family (e.g., BMP2/4/5/6/7 and GDG5/6), which are expressed in the digital and interdigital mesenchyme, establish periodic thresholds that alternate between apoptosis and chondrogenesis.

Disruption of Interdigit Formation and Differentiation (Soft Tissue Deficiencies)

Several natural models of persistent interdigital webbing exist, such as ducks and bats (53;56). In both animal models, the BMP inhibitor Gremlin (GREM) is expressed within the interdigital region, limiting apoptosis (53). Ectopic overexpression of BMP inhibitors in the limb mesenchyme (57) and persistent FGF function in the AER or mesoderm (54;58) inhibit interdigital apoptosis and result in syndactyly. Mutations in Noggin (NOG), a BMP inhibitor, are clinically associated with joint synostoses, syndactyly and polydactyly reflecting the spectrum of BMP functions.
In Apert Syndrome, the acrosyndactyly is caused by mutations in FGF receptor 2 (FGFR2) that increase its activation affinity for FGF ligands and glycosaminoglycans that are diffusely present in the limb mesenchyme (41;59). The continuous stimulation of the receptor generates constitutively active signaling, i.e. always on. This constant FGF signaling disavows interdigital BMP signals and results in complex syndactyly.

The mechanisms that convey dorsal-ventral and digit specific ligament/tendon formation and attachment are less well-characterized. Candidate genes responsible for flexion-contraction disorders, such as camptodactyly, may be involved in neuromuscular interaction and/or extracellular matrix formation (e.g. PRG4). Further investigation is needed to characterize the pathogenetic basis of these hand-related soft tissue abnormalities.

Disruption of Digit Formation and Differentiation (Skeletal Deficiencies)

As described above, abrogation of SHH results in the loss of ulnar digits, a disruption of the radial-ulnar axis. However, abnormalities in the SHH signaling cascade can also yield polydactylies. GLI3, a transcription factor involved in SHH signaling, accentuates the functional gradient of SHH across the radial-ulnar axis (Fig A.3 B). In the radial aspect of the limb bud, GLI3 undergoes post-transcriptional modification into a shortened repressor form that counters SHH action on target cells. In the region of the ZPA, SHH blocks GLI3 modification to yield the full length activator form (60-62). Mutation of this bi-functional transcription factor can cause ulnar polydactyly (also called postaxial polydactyly types A & B) (63;64) and radial polydactyly (preaxial polydactyly type IV) with syndactyly (65). In mice, the complete absence of GLI3 causes a loss in
digit identity (Fig A.3 B) (60), which is morphologically similar to the human condition of a five-fingered hand or a non-opposable triphalangeal thumb (66). Additionally, mutations in the limb-specific SHH regulatory region cause abnormal anterior (or radial) expression of SHH and yield triphalangeal thumbs (67;68).

Disruption of HOXD13 (69) or deletion of the entire HOXD9-13 region (70) results in variant forms of synpolydactyly, a disorder characterized by disorganized digit/interdigit formation and identity (Fig A.3 C). Loss of HOXA13 function is associated with Hand-foot-genital Syndrome which exhibits hypoplastic middle phalanges and digit deviation (71;72).

Digit formation, segmentation and cartilage maturation involve multiple factors and pathways. Thus, mutation or disruption of many of these may result in shortened digits or brachydactyly. Mutations in Hoxd13 can cause brachydactyly type D and E1. Disruption of factors involved in cartilage maturation also cause brachydactyly, including IHH, (type A1), BMPR1B (type A1 and A2), Gdf5 (type A2 and C), ROR2 (Type B1), NOG (Type B2) and PTHLH (type E2).

During digit formation and differentiation, BMPs regulate the transition between digital SOX9 activation and FGF maintenance in the overlying AER. Complete absence of SOX9 results in regression of the limbs since cartilage formation is not induced (73). Haploinsufficiency of SOX9 results in camptomelic dysplasia with reduced cartilage formation, bowing of long bones and brachydactyly.

Ogino generated a model of central deficiency (cleft or split hand) (74) using the alkylating chemotherapeutic agent Busulfán. For a brief window during handplate development, the teratogen caused increased apoptosis in the distal progress zone and
AER leading to central clefting, central syndactyly, and central digit duplication (Fig A.3 D). The increase in handplate apoptosis also resulted in disruption of AER-related FGF expression, reduced and irregular BMP4 expression from the AER and underlying mesoderm, and reduced ZPA-related SHH expression (75). Thus, these data link the clefting, syndactyly, and polydactyly of central deficiency to a common, albeit molecularly complicated, mechanism during handplate formation.
Figure A.3) Disruption of Handplate Formation/Differentiation

A) From left to right: during early handplate formation SHH and HOX transcription factors create a posterior to anterior gradient (ulnar-radial; purple to peach) that establishes interdigital (ID) signaling centers, digital anlagen and a BMP gradient (brown to yellow). The BMP gradient participates in conveying digital and ID fates via the phalanx forming region (PFR – fuchsia region capping digits) and repression of the AER, respectively (arrows). Apoptosis in the ID region ensues with regression of the associated AER (stippled region). Overlying the digit, the AER persists and outgrowth continues as the PFR regulates digit construction.  

B) Expression pattern of SHH-related GLI3 expression: GLI3 repressor (GLI3r) predominates in the radial aspect of the developing limb, while GLI3 activator (GLI3a) surrounds the ZPA. In the complete absence of GLI3 (rather than targeted mutations as in the human), the digit number is expanded and the unique ulnar-radial digit identity is lost generating identical “default” digits.  

C) The nested expression pattern of HOXD9-13 is represented by HOXD9, HOXD11 and HOXD13. In the absence of HOXD9-13, the hand exhibits synpolydactyly with excessive carpals, irregular digital elements and loss of alternating ID regions.  

D) From left to right: The central distal region of the handplate appears to be particularly sensitive to disruption (cross hatched). Teratogenic exposure can induce cell death in the AER (1), the ID region (2) or both (3) resulting in partial duplication (1), syndactyly (2) or cleft hand (3), respectively. In all images the thumb is up. *Note - typically the central or third digit is the primary target for disruption with clefting and duplication. Digit two (D, center and right) is used to permit a collective illustration.*
Relationship to Classification

The insights gained from our increasing knowledge of developmental biology do not align with the IFSSH/Swanson classification scheme. During development, the molecular pathways that organize and define the unique asymmetries of the upper limb are established, i.e. the proximal-distal, anterior-posterior (or radial-ulnar) and dorsal-ventral axes. Disruption of these developmental pathways causes consistent abnormalities affecting the formation of the entire limb along these axes. Under the IFSSH/Swanson classification, the conditions classified in IFSSH Group I - “Failure of Formation” tend to emphasize axis-related abnormalities that affect the proximal limb (i.e., arm and forearm) which may extend to the hand. Consequently, it would be reasonable to group these conditions together into a category reflecting the etiology and global impact, i.e. Failure of Axis Formation/Differentiation – Entire Upper Limb, and to include sub-categories which indicate which axis is primarily involved.

The conditions listed under IFSSH Group 2 “Failure of Differentiation” tend to emphasize abnormalities of handplate formation and differentiation, rather than abnormalities of differentiation alone. Polydactyly, currently within IFSSH Group III – “Duplication” also involves failures of formation and differentiation affecting the anterior-posterior (radial-ulnar) axis. Exclusion of polydactyly from Groups I or II appears illogical. These handplate-limited malformations can be sub-categorized according to whether they disrupt axis formation and/or differentiation, e.g., polydactylies which by common definition are axial (radial-ulnar) defects, or whether they disrupt generalized handplate formation and/or differentiation (unspecified axis) e.g. syndactyly. Moreover, none of these IFSSH Groups adequately address the variety of
malformations evident in cleft hands, which prompted the Japanese Society of Hand Surgery (JSSH) to propose the addition of another group within the IFSSH/Swanson classification - “Abnormal Induction of Digital Rays” (76)

IFSSH Group IV - “Overgrowth” is a descriptive term that does not convey a mechanism of etiology. Similarly, IFSSH Group V - “Undergrowth” is nonspecific, uninformative regarding etiology, and the inclusion of disorders within the category appears arbitrary. Undergrowths such as brachymelia with brachydactyly or symbrachydactyly involve molecular pathways consistent with transverse deficiencies, while undergrowths such as longitudinal deficiencies are excluded from this category.

Although IFSSH Group VII - “Generalized Skeletal Disorder” noted syndromic entities, as the number of defined genetic syndromes with upper limb malformations and diverse skeletal involvement has accumulated, it has become less meaningful to separate these into a separate category rather than the presenting limb morphologies.

Our increased understanding of limb morphogenesis and dysmorphogenesis has accentuated problems of the IFSSH/Swanson classification and prompted surgeons, pathologists and geneticists working in the field of congenital limb anomalies to question the adequacy of this classification (4-6;8;9).

The terminology of dysmorphology offers a framework for conveying the etiology of congenital limb anomalies (77). A Malformation is an abnormal formation of a body part or complex tissue. Deformation differs from a malformation as the insult occurs after normal formation. A Dysplasia is an abnormality in the size, shape, and organization of cells within a tissue. Dysmorphologists describe a fourth term, Disruption. As this process involves alteration of tissue which is already formed, for the
purposes of classification, it is reasonable to include those conditions which are considered to be Disruptions alongside those considered to be Deformations.

Although the pathogenesis of some specific anomalies remains obscure, the concept of separating malformations, deformations and dysplasias provides a sound structure for categorizing congenital upper limb anomalies. Therefore, to align the classification scheme with our current understanding of limb development at a molecular level using dysmorphology terminology, we recommend three broad categories – Malformations, Deformations and Dysplasias. Malformations are further subdivided according to the axis of development which is predominantly affected and whether the defect involves the entire limb or restricted primarily to the hand. Incorporation of the above principles results in a classification scheme as follows:

I. Malformations

I A. Failure of Axis Formation/Differentiation – Entire Upper Limb

To more accurately reflect the pathogenesis of many of the malformations currently listed under IFSSH Group 1 - “Failure of Formation” we recommend a subcategory entitled “Failure of Axis Formation/Differentiation – Entire Upper Limb” with three divisions reflecting the three axes that can be disrupted (see table A.I). We include brachydactylies that impact the entire limb (Symbrachydactyly, and brachydactyly type B1) under defects of the proximal-distal axis together with transverse deficiencies and intersegmental deficiencies. Defects of the radial-ulnar axis include not only radial and ulnar longitudinal deficiencies, but also radial and ulnar duplications such as ulnar dimelia, and radial-ulnar synostosis (previously listed under IFSSH Group III -
“Duplications” and IFSSH Group II - “Failure of Differentiation”, respectively. We further add defects of the dorsal-ventral axis such as Nail-Patella Syndrome. This would bring together malformations that alter the unique upper limb asymmetries and tend to have a more comprehensive effect on limb development (which may also include the hand).

I B. Failure of Axis Formation/Differentiation – Handplate

Axial defects limited to the handplate are separated into the second subcategory. Previously, the polydactylies were considered duplication defects; however, these typically can be attributed to disruption of an axial signaling pathway. For example, disruption of GLI3 function, a transcription factor involved in the definition of the radial-ulnar axis, can cause preaxial polydactyly (type IV – or polysyndactyly) (78) and ulnar (postaxial) polydactyly (type A1) (65).

We also include triphalangeal thumbs in this group. Recent genetic studies of radial polydactyly (preaxial polydactyly type II) found point mutations in or duplications of the limb specific SHH regulatory region (79-81). In animal models similar point mutations induce ectopic radial SHH expression and preaxial polydactyly (82-84).

Disruption of the dorsal-ventral axis can also be limited to the handplate such as in dorsal dimelia; therefore such malformations are also included within this subsection.

I C. Failure of Hand Plate Formation and Differentiation – Unspecified Axis

The malformations listed under IFSSH Group II - “Failure of Differentiation” involve primarily, but not exclusively, handplate development. We have combined
defects limited the handplate that are not related to axis disruption under “I C”. For example, abnormalities that involve the distinctly hand-associated pathways that regulate interdigit formation and digit/phalanx designation are placed in this category. We also include malformations that probably involve multiple pathways such as synpolydactyly and cleft hand. Ogino and coworkers demonstrated that a single insult to the developing handplate can lead to syndactyly, central polydactyly or cleft hand (74;75;85). This demonstrates a link between these conditions, but not necessarily that the presence of one is obligatorily associated with that of the others.

II. Deformations

According to the terminology of dysmorphology, we have established a second category for deformation or disruption of any portion of a limb that has already been formed. Within this category “Constriction Ring Sequence” is listed which may be syndromic or related to amniotic banding. Arthrogryposis or congenital contracture can be isolated or involve multiple joints and have several etiologies including neurological, muscular or connective tissue (86;87). Arthrogryposis is also included in this category because the formation of contractures, typically evident during mid-gestation, occurs after joint and skeletal formation (88). Trigger digits are also included as deformations since they typically present in childhood rather than at birth. Deformations or disruptions from viral infection, vascular insults or mechanical damage do not typically follow a consistent pattern and, for the sake of classification, would be listed under subcategory D – “Not Otherwise Specified”.

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III. Dysplasias

Dysplasias include conditions which have previously been listed according to their appearance and are a unique group associated with cellular atypia or tumor formation. Limb hypertrophy which occurs in conjunction with tumor formation or macrodactyly which appears to be cellular dysplasia in response to some as of yet unknown factor are examples. These were previously classified within IFSSH Group II - “Failure of Differentiation” (tumorous conditions) or purely as a descriptive term within IFSSH Group IV - “Overgrowth”. There may be dissent as to whether these conditions are in fact malformations or deformations and further insights from developmental biology may prompt transfer of a specific entity to either our Group I or Group II.
Table A.1) Comparison of the current classification scheme and proposed modifications.

<table>
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<td>I MALFORMATIONS</td>
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<td>A. Failure of Axis Formation/Differentiation – Entire Upper Limb</td>
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<td>1. Proximal-distal outgrowth</td>
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<td>- Ulnar longitudinal def</td>
<td>Brachymelia with brachydactyly</td>
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<td>• Soft tissue deficiency</td>
<td>2. Radial-ulnar (A-P) axis</td>
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<tr>
<td>• Skeletal deficiency</td>
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<td>III Duplication</td>
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<td>• Radial polydactyly</td>
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<td>• Ulnar polydactyly</td>
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<td>Dorsal dimelia (Palmar nail)</td>
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<tr>
<td>1. Macrodactyly</td>
<td>Hypoplastic/aplastic nail</td>
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<tr>
<td>2. Upper limb</td>
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<td>3. Upper limb and macrodactyly</td>
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<td>B. Tumorous Conditions</td>
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Conclusion

Congenital anomalies in the hand and upper limb demand a reproducible and consistent terminology, a universal language which allows discussion of complex clinical entities, indications for treatment and comparisons of results. As our understanding of the molecular basis of morphogenesis and dysmorphogenesis continues to progress, the way that we categorize these disorders must also continue to be refined. This classification may also need revisions in the future; however, we believe that the use of dysmorphology terminology will provide an appropriate framework to easily accommodate further refinements.

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