# The Role of the *Lim1* Gene in Vertebrate Kidney Development

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Lim1 has been reported to regulate the head, nerve system and kidney development in mouse, zebrafish and *Xenopus*. In the present study, we demonstrate that *Xlim1* plays a pivotal role in the pronephric specification and development in *Xenopus.*

In *Xenopus* embryos at 4-cell and 8-cell stage, loss of function experiments using DEED depletion showed that down-regulation of *Xlim1* expression severely impairs the formation of pronephros, assessed by *pax8* expression. Overexpressing *Xlim1* by injecting constitutively active Lim1 constructs in *Xenopus* embryos induce remarking enlargement of pronephros at various stages during development, assessed by both *in situ* hybridization and immunohistochemistry staining. Furthermore we studied the working window of *Xlim1* on pronephric specification in mesoderm and observed the peak time point is at stage 10.5 while the whole working window is between stage 9 and 12.5. To understand the mechanism of *Xlim1*'s regulation in kidney development, then we performed the microarray analysis on the induced pronephric RNA sample extracted from animal caps, targeting couple of genes that either are the members involved in the lim1 pathway or in the networking that induce the pronephric specification.

In summary, our data suggest that Lim1 acts as a potent factor that determines the early specification of pronephros. Its expression levels at the early stage critically influences the specification of the pronephric tubules. More knowledge about XLim1's pathway in kidney development will be studied via examining the gene targets screened from microarray analysis.

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## PREFACE

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# 1.0 GENERAL INTRODUCTION

## 1.1. KIDNEY

### 1.1.1 Structure and function

Kidney is an intricate and critical organ that maintains homeostatsis of living creatures, that is, the stable internal environment for organisms. To maintain the healthy, balanced internal environment organisms must timely and efficiently remove the daily toxic byproducts generated from metabolism. Kidney functions largely as a filter for the elimination of the waste products generated from metabolisms, mainly including creatinine and urea as byproducts from protein metabolism in liver and some lipids metabolites[1]. One important function of kidney for all terrestrial animals is its role in maintaining an optimal and constant osmotic pressure[2]. Other functions of kidney include but are not limited to regulating concentration of acid, reabsorption of lipids, creating hormones that regulate the formation of red blood cells and blood pressure[3, 4].

Nephron is the functional unit of vertebrate kidneys[5]. Each nephron contains two parts: a filtering component (renal corpuscle) and a tubule part (renal tube). A highly permeable vascular bed, known as glomerulus, acts as a filter through which allows for the small molecules passing .Mostly enclosed by the big mouth of Bowman capsule, the glomerulus with Bowman capsule together constitutes renal corpuscle. The convoluted tubules that connect to the neck of Bowman capsule are known as renal tubules, which are segmented into several parts for their distinct functions. The last part of renal tubule, or the collecting duct, leads the waste to the renal pelvis and to the ureter where via the renal bladder urine will discharge to the exterior[6].

The formation of the urine could be divided into two steps. 1) When the blood via the dorsal artery travels through the capillary network within the glomerulus, the hydrostatic pressure pushes the fluid and small solute molecules to penetrate the capillary wall into the space between glomerulus and the Bowman capsule. Meantime, it keeps the large molecules like proteins to remain in the blood, resulting in a sticky blood, or ultrafilter blood.2) In the next step, as the glomerulus filtrates pass through the collecting tubes, the cells of the renal tubes selectively reabsorb the solute molecules and ions. Accompanying the reabsorption, some particles like lipids are secreted from the blood to the collecting tube alternatively. The solution that emerges at the end of the collecting tube (urine) thus is very different in composition and concentration from the solution that enters the tubule (the glomerulus filtrate). The collecting duct from one nephron and from many others meet up with bladder, from where liquid wastes go to the urethra and then is excreted from the body[6].

All three vertebrate kidneys contain nephrons. The complexity of the kidney partially counts on the number of nephrons the kidney has and the spatial assembly of these nephrons[5](See Dressler's review Figure 1, 2006). The most anterior and primitive kidney, the pronephros, has only one nephron[7]. The adult kidney of fish and amphibians, the mesonephros, has multiple units similar to nephron[7, 8]. The most complex one, the kidney of adult mammals and birds is called metanephros, which has up to one million nephrons as it has in the medullar and cortex of human kidney[9,10].

Pronephros, mesonephros and metanephros are the three successive stages in mammalian kidney development[11]. While the pronephros and the mesonephros are transiently present in embryonic stages, metanephros is the full-fledged organ for mammals. Yet, the presence of pronephros and mesonephros are indispensable for several species. For fish and amphibia, they rely on the function of pronephros to survive during the larval period[10]. The later replacement of pronephros, the mesonephros form the adult kidney of lower vertebrates and is also functional in the fetus of higher vertebrates like elephants, reflecting the aquatic ancestry of this huge terrestrial animal[8, 11].

The focus of this thesis is pronephros. Pronephros is a very simple organ indeed. The basic structure of pronephric of the *Xenopus* is shown in Brandli's review paper in 1999. The pronephros is composed of three distinct parts: the pronephric corpuscle, the pronephric tubules and the pronephric duct. Similar to the structure of the nephron, *Xenopus* pronephros contains a single corpuscle, which consists of the pronephric corpuscle and the pronephric glomus. The pronephric capsule protrudes into the filtration chamber of the pronephros, the nephrocoel, which

is partly connecting to coelom. The filtrates diffuse into the coelom and are reabsorbed in the pronephric tubules. Likewise, pronephric tubules are morphologically segmented into three parts: ciliated nephrostomes, connecting tubules, and a common tubule. The common tubule joins the connecting tubules and the connecting duct together in a way of connecting a sieve and a funnel so that the wastes could be selectively and completely excreted to the end of this filtering system, the rectal diverticulum, an outgrowth of the cloaca, to maintain the balanced salt and water environment of the body[7, 12].

### 1.1.2 Early development of the kidney

The development of urinary system and reproductive organs are closely associated. The urinary organ is developed from the intermediate mesoderm[11]. The permanent organs of mammals and birds are preceded by a set of embryonic structure, which all atrophy but with the ducts remained. This evolutionary organ-developing progression occurs rapidly in the mouse embryos that from the mesonephros to the metanephros it only takes about 1-2 days out of 20-day gestation[13]. In humans, pronephros is quite rudimentary. After the disappearance of the pronephros, the embryonic pronephric duct, and the initiated Wolffian ducts with the induced Mullerian ducts still remain and become a part of sex organs[14]. Along the Wolffian duct epithelia, the mesonephron and mesoureter form, which contribute to the excretory and the development of the reproductive organs in both sex[8]. Mesonephros undergoes rapid degeneration whilst the metanephors begins formation as a budding forms from the caudal end of the mesonephric ducts and invades the loose metanephric mesenchyme[8]. The specification of various nephric cells and the spatial assembly of the kidney structure are initiated from the bidirectional

inductive interactions between the ductal epithelium and nephrogenic mesenchyme. Signals from the ureteric bud on one hand promote the survival of the metanephric mesenchyme and, on the other hand result in the condensement of surrounding metanephric mesenchyme and the differentiation of the nephron. Interestingly, the metanephric mesenchyme, sends signals back to the ureteric bud. These signals promote the ureteric epithelium to grow and branch into the metanephric mesenchyme for the formation of the ultimate metanephric ducts[5, 11]. When nephrogenesis is complete before birth, metanephros not only is the organ with the excretory function but is an important endocrine organ *in utero* as well[13].

The early development of kidney is about the early kidney patterning. As aforementioned, the intermediate mesoderm generates most of the urogenital tract. So to study the early development of kidney, many efforts should be made to explore the signaling process that give rise to this mesodermal derivative. The current knowledge in this field reveals a number of genes, which are expressed both in the lateral plate mesoderm and intermediate mesoderm., are likely to involve in the later morphology shaping process[5].

For pronephros, the signals that direct patterning of the mesoderm towards pronephric lineages are unknown. In amphibians the perspective pronephric region could be traced back as early as the gastrula stage[15]. The marginal zone ventrolateral to the blastopore is destined to be future intermediate mesoderm where pronephros will emerge upon the induction of complicated signaling interactions[16]. The originals of these signals are suspected from Spemann organizer and ventral side of the embryos[17]. However, among the mesoderm formation factors, such as BMP and activin-like TGF- β family members, none of them plays the determinant role for pronephric

tissue[15, 18]. The involved signals emerge from the evidence that using activin in combination of retinoic acid can promote pronephric differentiation in explant cultures[19, 20]. Additionally, in *Xenopus laevis* model, pronephros formation in *Xenopus* embryos is severely impaired when retinoic acid signaling is inhibited via different methods. In concert with these results, the ectopic RA signaling expands the size of the pronephros, indicating RA is crucial in the early steps of kidney formation. The pronephric defects in *Raldh2* knock-out mouse embryos, which are retinoic-acid deficient types, again suggests the requirement of RA signaling for kidney formation is evolutionarily conserved[21]. However, the role of activin *in vivo* in kidney development induction is repressive[22].

*Lim1*, as one of the earliest and confirmed marker gene for early kidney development, expresses in the visceral endoderm, the anterior mesendoderm, and the lateral mesoderm that comprises the lateral plate and intermediate mesoderm[23]. The subsequent expression of *Lim1* is found in the nephric duct, mesonephric duct, mesonephric tubules, and parts of the developing metanephros[24]. Studies have shown that kidneys and gonads are missing in the *Lim1* null mutants[25]. Concomitant with its expression pattern, it is suggested *Lim1* is required for the correct patterning of the kidney. As a known intermediate mesoderm marker, *Odd1*'s expression is also seen in lateral and intermediate mesoderm at about the same time the *Lim1* does[26]. Despite the smaller size of the ducts compared to control group, the mouse of mutant *Odd1* is able to generate mesonephric ducts that express *pax2* but severely lacks posterior tubules or evidence of metanephric mesenchyme. These data demonstrate the requirement of *Oddl* for metanephros not early ones[26]. Other important genes that express during early developmental stages include *pax2* and *pax8*, both of which have been elaborately studied in various aspects.

*Pax2* and *pax8* have very intriguing relationship. *Pax2* mutants can generate a primary nephric duct but in the absence of renal tubule. The knock-out of *pax8* itself will not affect the formation of kidney, yet it is reported that the double mutant of *pax2* and *pax8* fail to generate any epithelial structure within the region of the intermediate mesoderm and do not exhibit Lim1 expression by E9.5 in mouse embryos[25, 27, 28].

Of all the genes mentioned before, *pax2* and retinoic acid signaling pathway appear to be able to specify renal tissue. The convincing role of pax2 in specification stems from the expansion of the region fated to become renal epithelia by its overexpression. However, in pronephros, the expression of *pax2* is a much later event after the expression of *Lim1* and *pax8*[5], arising the suspicion of the conserved role of *pax2* in renal early specification in vertebrates. RA is long known for its vital role in A-P regionalization and new evidence shows that it is also responsible for pronephros A-P segmentation by targeting cdx genes[29]. In addition to segmentation, as a diffusible morphogen in other tissues, the gradient of RA in intermediate mesoderm (IM) significantly induces distinct fates of pronephros tubules.[21] Moreover, the heterologous expression of RA enlarges the pronephros. Despite the unknown targets of RA in IM , its defined role in pronephros development is acknowledged.

### 1.1.3 Significance of the early development of the kidney

There are many reasons to study kidney development, but the most fascinating prospect of the kidney development studies is the contribution to clinic application beyond the basic research. Kidney diseases, no matter, acute or chronic, all have the potential to progress to end-stage kidney disease. People with end-stage-kidney diseases (ESKD) must undergo dialysis and

or transplantation to stay alive. Either dialysis or transplantation is a painful experience and costs a huge expense[30]. Given this situation, the emphasis on the research to treat kidney disease is repeatedly addressed.

In the research of genetics, researchers supported by the National Insititute of Diabetes and Digestive and kidney diseases have located two genes that cause the most common form of PKD, (Polycystic kidney disease) a genetic disorder and learned that owning a defective copy of either PKD1 gene or PKD2 is sufficient to initiate the phenotypic changes and results in the development of PKD[31]. Researchers have also found homologs of PKDs in *C.elegans*, introducing the role of cilia in the polycystin pathway[32]. This new knowledge will be used in the search for effective therapies to prevent or treat PKD. For other inherited kidney diseases, the search for genes that gives rise to the defective form of kidneys is underway and most of the disease-related genes play important role in developmental process. Thus the kidney development is the basis to understand the renal diseases[33].

In the area of transplantation, the mature surgical procedure and the newly developed drugs help the organs to be adapted by recipients easily and increase the rate of survival. To solve the gaps between the limited donor organs and the large number of ESKD patients, the expectation that in the future scientists may develop an artificial kidney for implantation or repairing is appealing[34]. This idea arises closely with the rapid advance of the stem cell research and biomedical engineering[34]. There are several different speculations in taking advantages of renal stem cells for kidney disease treatment. Kidney contains stem cells with regenerative capacity[35]. The renal papilla is considered niche of adult stem cells that are resistant to apoptosis

and that divides slowly under normal circumstance but accelerates the speed of division after the ischemia-reperfusion[36]. Other research alternatively underpins the idea to use bone-marrow-derived tubule progenitor cells to repair damage because there is evidence that damage caused by glomerulosclerosis in old mice can be repaired by transplantation of bone marrow from younger mice, that the speed of repair of renal ischemia–reperfusion injury increases when mesenchymal stem cells are injected into the suprarenal aorta, and that there is potential to switch the developmental program of cells[37, 38]. Inducing kidney cells *in vitro* via embryonic stem cells is another possibility. It is, however, difficult because it heavily depends on the correct differentiation orientation that could be induced, the purity and the net amount of cells that could be obtained, and the life cycles of the cells that could be maintained. Compared to pure cell therapy, currently a wearable dialysis system or a hybrid bioartifical kidney is more pratical[34]. But we expect that eventually we can use biomaterials to cure kidney diseases as the way we do to conquer bacteria.

## *1.2 XENOPUS*

### 1.2.1 *Xenopus* as a research model

*Xenopus* are a genus of carnivorous frog native to Africa[39]. It consists of 15 species and all have the enormous webbed, five-toed, three-clawed rear feet typical of the group[39]. *Xenopus laevis* (African clawed frog) has been known to science since 18$^{th}$ century. Besides *Xenopus laevis* was the first vertebrate to be successfully cloned[40].

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*Xenopus laevis* has benefited numerous vertebrate biological areas, distinguishably for early embryology, cell biology and electrophysiology. As a matter of fact, *Xenopus* is a major vertebrate model for the cellular and developmental biology research that is supported by most of the Institutes of the NIH[41].

It has several advantages over other model organisms like zebrafish and mouse in developmental studies. First, the large number of high quality embryos is easy to obtain in most time of a year. With proper management and culturing, two to three females can provide enough embryos for experimental needs on a daily basis. Second, the large size of the embryos makes them easily amenable to micro-surgery and micro-injections. The property that yolk is partitioned in *Xenopus* embryos during development and all cells have an autonomous supply of nutrients, make the embryos ideal for experimental embryological approaches[41].Third,the external development and the *in vitro* fertilization expose the whole development process making easy access to all development stages and tissues. All these advantages have helped to establish Xenopus as a leading model organism in embryology study[42].

Following the emergence of fate maps, many molecular approaches have been applied on *Xenopus* embryology. RNA generated *in vitro* or from other organism can be injected into the embryos or oocytes and the results could be studied using molecular methods and electrophysiological methods[43, 44]. Gene expression can be knocked down or splicing modified using morpholino antisense oligo or DEED injected into *Xenopus* oocytes or early embryos[45-47]. The development of whole-mount *in situ* hybridization and immunohistochemistry render the

correlation with fate maps and specification maps[48, 49]. Other approaches that have been frequently used for genome-scale gene analysis in early embryo development are RT-PCR and microarray, both of which provide a high resolution at molecular level[49, 50]. With all these techniques, many well-known signaling pathways are elucidated or validated in Xenopus embryos, such as TGF β s, Wnts, hedgehogs and receptor tyrosine kinases pathways[51-53]. Besides, new components are underway of discovery[54, 55 56].

Meanwhile *Xenopus* oocyte is a widely used system for studying cell biology, especially the electrophysiology. *Xenopus* oocytes have been used to study protein posttranslational modifications, channel ion fluxes with various biophysical approaches, changes in membrane capacitance, reconstituted transmitter release, channel gating currents, multiprotein subunit assembly, receptor pharmacology, subcellualr metabolic activity and so on[57](X.Johne Liu, *Xenopus protocol*,*2006*). Eukaryotic chromosomal DNA replication has been extensively studied in cytoplasmic egg extracts from the frog *Xenopus laevis* as it is a good cell-free system, depleted with antibodies[57]. Other studies show that the *Xenopus* egg extracts prove to be an ideal translation system in which to study the biosynthesis of proteins and present many advantages for the study of the cell cycle, including the availability of a large quantity of material synchronized at the particular phase of the cell cycle[58, 59]. As a conclusion, *Xenopus* is a model full of potential contributing to a variety of research aspects.

The drawback of the *Xenopus laevis* resides in their pseudotetraploid genome, which is complicated for genetic studies. So the diploid *Xenopus troplicalis* is considered as a complementary system which is amenable to the modern genetic approaches whilst possessing

the similar biological advantages of *Xenopus laevis.* The undergoing genome assembly project of *Xenopus troplicalis* carried out by the Department of Energy's joint Genome Institute acknowledge the importance of *Xenopus troplicalis* in the field of genetics. Furthermore, the knowledge revealed from *Xenopus* genome probably will enhance our understanding to human genome granted a number of regulation factors are extraordinarily conserved in vertebrates. Similarly, making transgenic frogs and obtaining stable lines with reporter gene may complement the transgenic mouse system considering it is easy, inexpensive and efficient[41, 60-63].

The sequencing of EST and cDNA library of both *Xenopus laevis* and *Xenopus tropicalis* would provide more information about the allelic variants, which will enhance the accuracy of allele-specific knock out and differential hybridization techniques in gene chip analysis. Despite the difficulty of sequencing EST from *Xenopus laevis,* it is still important to finish the sequencing project because the nature of the pesudotetraploid genome would provide key information about genome evolution that is divergent from mammals'[61].

While studying *Xenopus* embryonic early development, there are several convenient tools that could be taken advantages of. Apart from the aforementioned EST and cDNA library, *Xenopus* full length libraries from Kirschner lab at Harvard Medical School and *Xenopus* micro-array from the Brivanlou lab at the Rockefeller University are the two enriched *Xenopus* genetics resources. These resources provide a plateau for biologists and researchers to efficiently complete their projects by using these organized and useful information and tools [41].

### 1.2.2 *Xenopus* as a model for kidney studies

*Xenopus* have pronephros, the simplest kidney of all three kidneys in vertebrates. *Xenopus* has long been considered as a popular model to study the nephorgenesis because *Xenopus* embryos are fertilized *in vitro* and have a relatively short developmental cycle, which turn them into a unique subject for organogenesis analysis. Additionally their huge embryos are easy to manipulate at molecular level and the developmental stage is morphologically visible as described before. The embryonic explants of *Xenopus* can be induced to pronephric differentiation *in vitro* by adding activin and retinoic acid, providing another interesting system to study *in vitro* nephrogenesis[19]. This makes *Xenopus* a nice model to dissect molecular events relevant to nephrogenesis.

Moreover the available data show that the same bunch of transcription factors and signaling molecules play a role in *Xenopus* pronephros differentiation as in mammalian nephrogenesis. *Lim1* and *pax2*, for example, are essential for correct patterning of kidneys in many vertebrate model organisms[5].Thus, the study of *Xenopus* pronephros formation is significant for the general nephrogenesis understanding, and furthermore, the factors identified may provide insights in genetic renal diseases in humans. One of the genes identified in the diabetic nephropathy screen is the bone morphogenetic protein antagonist gremlin[64]. The expression pattern of gremlin was analyzed in *Xenopus* and suggested a requirement for gremlin activity in kidney development[65]. Another study particularly exploits the function of human HNF1ß. In that study, a frameshift mutation in HNF1ß gene associated with renal ageneses is shown to have the similar effect on pronephros development in the frog[66].

Additionally, with the development of transgenesis in *Xenopus,* it offers us a real prospect of characterizing and generating the pattern of interacting genes in pronephros[7].Transgenesis renders the spatial and temporal control of targets genes. More significantly, it displays a whole picture of its primary and secondary role in the studied organism. The network drawn from this transgene phenotype provides us with solid evidence of the real interaction *in vivo* which might be beneficial for medical field.

## 1.3 GENE LIM1

### 1.3.1 Structure, location, expression pattern and function of lim1 gene

Structure

*Lim1* (LHX in human and mouse, Xlim1 in Xenopus) encodes a protein with two LIM domains and one homeodomain[67]. It belongs to group 1 LIM protein defined by Dawid's group typically having 2 different classes of LIM domains in tandem in the protein[68]. As found in organisms like C-elegans, rat, and drosophila, LIM domain is a conserved motif. Its main structure is a specialized double-zinc finger motif. Physically they mainly interact with other domains from various proteins and thus endow proteins possessing LIM domain diverse functions[68, 69].

Xlim1 is found to show activity in the presence of XLdb1 in *Xenopus,* inducing the partial secondary axis formation in embryos[69]. The LDB binding domain-fused Xlim1 construct shows even higher activity in secondary axis induction[70]. Thus it is demonstrated that Xlim1's

activity requires the presence of adaptor, Lim domain binding proteins. The homeodomain implies the nuclear-acid binding ability of LIM1 family proteins. LIM domain-containing protein is a large and diversified family, Lim1 and its homologs in the known vertebrates and non-vertebrates only represent a small portion of this family[71]. Xlim1/Lhx1's multiple-domain structure convenes its existence in both cytoplasm and nuclear, or the ability to shuttle in between[68].

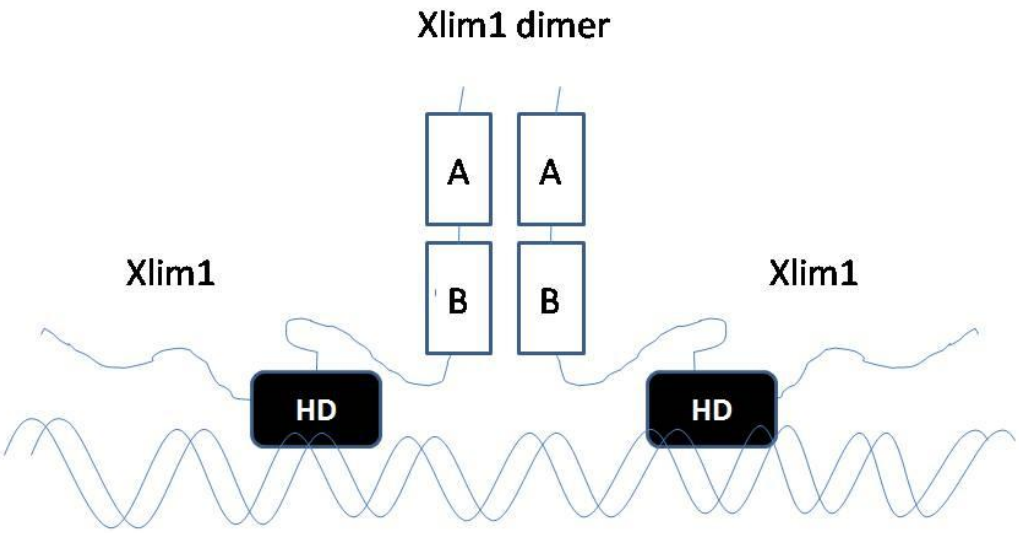


Figure1. Presumptive lim1 dimers bind to nucleic acid. A, B are the two distinct cystenine rich Lim domains and HD is the homeodomain that binds to DNA.

Location and expression patterns

At early developmental stage in *Xenopus*, *Xlim1* is expressed in the organizer region. At later time, its expression extends to the future head region, and finally locates in the kidney and central nervous system, namely, the midbrain, hindbrain and the spinal cord[72]. Likewise, *Lhx1*'s expression in mouse is detected underlying the region of epiblast for the future primitive streak and mesodermal wings at the very early stage of gastrulation, the region for the future central nervous system. With the developmental progression, it expresses from the mesodermal wing, to the anterior mesoderm, and in the prechordal mesoderm. Shortly afterwards, the area of presumptive mesonephros is condensed with *Lhx1*'s expression at day E8.5 in mouse embryos. Its expression is also detected at the transient pronephros region, but with a quite low level. Yet unlike in the adult mouse, at early stages, it does not localize in sex organs, such as gonad, ovary, testis and epididymis, nor in the derivatives of the Wollfian and Mullerian ducts. In the metanephric kidney, the expression of *Lim1* is regionalized in the ureteric bud and the proximal tissue. Also the distinguished expression could be seen in the renal vesicles and S-shaped body[23, 24, 69].

Function

As a summary of previous studies, *Lim1* plays multiple roles in developmental process. Shawlot and Behringer report that targeted mutation of the homeobox gene *Lim1* in the mouse results in embryos that lack anterior head structures but have normal trunk and tail development.[25]. This result indicates the functional analogy between *Lim1* and *Hox* gene family, both of which are required for organ organization[73]. Hence this knock-out evidently support the existence of an organizing center which is different from the center for tail and trunk proposed by Hans Spemann[74]. Again, injected Lim1 RNA into *Xenopus* explants induce the formation of

the secondary axes, which accounts for its role in the organizing center[70]. *Lim1*$^{-/-}$ mouse, on the other hand reveals its role in kidney and sex organ development from the evident loss of kidney and gonad[24].

Other than transgenesis, the function of *Lim1* was revealed via several other approaches. For example, mRNA microinjection experiments in *Xenopus* indicate that *Xlim1* and *Goosecoid* have synergistic effects on notochord formation, and that *Chordin*, but not *Noggin* can be activated by *Gsc* and *Xnot*[75, 76]. So we can expect to find both parallel and convergent pathways in the organizer. Loss of *Xlim1* via DEED injection in *Xenopus* severely affects the cell movements and cell behavior and it is found that the depletion of *Xlim1* mRNA results in a failure to activate the PAPC gene in early gastrulation of frog and zebrafish embryos. These results suggest that *Xlim1* affects cell behavior in the embryo through its regulation of PAPC and , possibly , other uncharacterized protocadherin genes[45].

Figure2. The cartoon of the postulated Xlim-1/Ldb complex and the Ldb1-Xlim1 fusion protein. LDB-Xlim1 fusion proteins are in a constitutively active form because the homedomains of the Xlim1 part are always free to bind to DNAs.

Lim1 is a presumed transcription factor but the genes under its regulation hardly have been reported. One of its targets is gene *goosecoid*, albeit the complicated regulation pattern with the aid of Ldb proteins[69, 77]. Intriguingly, the transcription ability of Lim1 is maneuvered by LIM domain. The lack of LIM domain completely abolishes the function of LIM-domain containing protein, taking example of Apterous, the Lim1 ortholog in Drosophila. These observations could be reconciled under the model of dimer Lim1s indispensable for functioning (Fig2). This model hypothesizes that via interacting with LIM domain binding proteins, Xlim1s are dimerized and stimulated into active form that is able to bind to specific DNAs to regulate gene expressions. Considering it is true, then the LDB-domain fused Lim1 protein should work as well as the natural counterparts, the Lim1 and LDB proteins[78].

Other than LIM domain and homeodomain, the function of the C-terminal of Xlim1 has drawn attention as well[78]. Researcher identified two independent activation domains and a negative regulatory domain involved in axis formation in the arbitrarily designated 5 portions of lim1 C-terminal. But none of the known coactivators tested interacted with the Xlim-1 transactivation domain while its own transactivation ability is quite weak. Thus it is anticipated that Xlim-1 requires a specific adaptor protein or transactivators attached to its C-terminal to execute transactivation[78]. It is speculated that the mechanism to switch the inactive state to active state is to displace some negative repressor and associate with transactivators.

### 1.3.2. Lim1's role in kidney development

Previously, I mentioned in *Lim1*$^{-/-}$ mouse heads, kidneys and gonads are found missing but all other gut structures, organs and tissues were present and appeared to be normal. As *Lim1* is expressed in the developing kidney, the absence of these structures is unlikely due to the secondary effects of the loss of lim1. In combination of *Lim1*'s expression pattern, it suggests that Lim1 is required in multiple tissues during urogenital development.

To dissect *Lhx1*'s role in early organogenesis, *Lhx1*$^{-/lacz}$ mouse strain is generated and the reduced expression of *Pax2* and *Hoxb6-lacZ* transgenes were found in the intermediate mesoderm in *Lhx1* null line. The severe disorganized structure in intermediate mesoderm indicates that *Lhx1* regulates the differentiation of the intermediate mesoderm in mouse[23]. Another study using mouse chimeras found that *Lhx1*-deficient cells were unable to contribute to the Mullerian duct resulted a truncated Mullerian duct , drawing a conclusion that Lim1 is required cell-autonomously during Mullerian duct development and Lim1 acts by regulating and /or maintaining the differentiation of the nephric epithelium[79, 80].

# 2.0 STUDY OF THE REGULATORY ROLE OF *XLIM1* IN KIDNEY DEVELOPMENT AND IDENTIFICATION OF THE TARGETS AND COFACTORS OF XLIM1 IN REGULATING KIDNEY DEVELOPMENT

## 2.1 EXPERIMENTAL DESIGNS

### 2.1.1 Cell targeting injection and gene specific knock-down

Rationale:

Previous work has shown that depletion of *Xlim1* by early dorsal injection of DEED antisense oligonucleotides cause truncated embryos. These embryos make an axis, as demonstrated by the presence of notochord and somatic muscle but pronephric development is severely disrupted. In consistent with the phenotype shown in *Lhx1* knockout mouse, it is indicated Lim1 and its homologs have conservative role in kidney development. However, whether the loss of kidney tissue is a direct result of the depletion of *Lim1* or is a secondary effect needs elucidation. Since we know that the *Lim1/pax8* mRNA co-injections in *Xenopus* embryos induce enlarged and even ectopic nephric structures, it is likely that *Lim1/pax8* convert more cells in intermediate mesoderm into nephric material and may recruit additional cells from the paraxial mesoderm. So in experiment 1, our goal is to understand the role *Xlim1* and *pax8* play in kidney specification/morphogenesis. The specific questions we want to address are

1) Whether *Xlim1* affects the specification of kidney

2) How *Xlim1* temporally involves in the specification of kidney tissue.

3) Whether *Xlim1* contributes to the cell movement event

Strategy

We used constitutively active Lim1 construct to examine the *Xlim1*'s role in the specification of kidney. According to the known division fate of *Xenopus* embryos we inject Lim1-DEED into V1 at 4-cell stage and 8-cell stage to investigate the direct effect of *Xlim1* depletion on kidney formation. We injected the Dex-regulated Lim1 construct into embryos to investigate the temporal involvement of *Xlim1* in kidney specification. The immunohistology and fluorescein dextran staining are followed to confirm the kidney formation and cell migration path.

### 2.1.2 Microarray and IMAGE clones

Rationale

By utilizing *Xenopus* in vitro organ culture system, we can induce the ectoderm cells of *Xenopus* (animal caps) into a variety of tissues by culturing animal caps in media containing different sets of growth factors. When animal caps are treated with media containing activin in combination of retinoic acid, *Xlim1*, *pax8* and *wt1*, the kidney markers are present, indicating the differentiation trend towards kidney. This model provides a unique chance to analyze kidney development without interpreting the influence of other adjacent tissue development in whole embryos on this process. The previous work completed in our lab shows that over expression of

*Xlim1* can replace the need of activin presence in the *in vitro* culture system, that prompt animal caps to enter into kidney differentiation path. This suggested that Lim1 might be the downstream factor of endogenous activin. However, whether internal activin actually influences on the kidney formation still is an enigma. In order to understand *Xlim1*'s role and other protein pathways in *in vitro* kidney differentiation, we took the RNA from *Lim1* knock-out samples treated in activin/retinoic acid condition for microarray analysis and pair-wise comparison of each sample is conducted. From the microarray data, we expect to target several Lim1 downstream effectors.

Strategy

Based on the microarray data, we select 20-50 potential targets of lim1 based on the fold change in comparison to the control group. Purchase IMAGE clones to obtain the probes for expression pattern analyses of interesting targets. The expression pattern will be examined via *in situ* hybridization.

## 2.2 METHODS AND MATERIAL

### 2.2.1 *Xenopus laevis* embryo manipulation and microinjection

Embryos were cultured in 0.2 X MMR and staged according to Nieuwkoop and Faber. *Xlim1* DEED-AS synthesis, sequence and specificity have been previously described. 4-8 cell

*Xenopus* embryos were injected with 80-800pg DEED-AS. Synthetic mRNAs were made using SP6 mMessenger mMachine kit (Ambion, Austin.TX). For overexpression studies 300pg LLVP16 was injected into the 2 dorsal or ventral blastomeres at the 4-cell stage or 150pg[81]. For temporally controlled overexpression, the glucocorticoid receptor was fused to the C-terminus of the LLVP16 construct. 50pg of LLVP16-GR mRNA was injected into the left dorsal or ventral blastomere at the 4-cell stage thus allowing an internal control. Injected embryos were then treated with 10uM of dexamethasone at indicated stages. Targeted injections used 2-3% flurorescien dextran as a lineage tracer.

### 2.2.2 *In situ* hybridization

Whole-mount in situ hybridization was performed as previously described[82] . *Xpax8* probe construct (A kind gift from Dr. Tom Carroll from Harvard University) was linearized by Not I and was transcribed with T7 mMessenge mMachine kit (Ambion, Austin TX). All EST image clones were purchased from Open Biosystem, Huntsville, AL, verified by sequencing, blast search similarity and analogous to previously published expression pattern.

### 2.2.3→Expression specificity and ontological analysis

To evaluate pronephros expression specificity of identified genes, gene symbols and locus numbers were used to retrieve their relevant expression information in the NCBI and Open Biosystem. The gene chips for *Xenopus* genes expression were designed by Affymetrix and the

quality report and the primary analysis was conducted with the assistance of Dr. Zhao Hui from NIH.

#### 2.2.4→Immunohistochemistry and fluorescein dextran staining

Antibody staining was carried out following Vize's protocol outlined on the XMMR website. Fluorescein dextran lineage trace was detected post-fixation using an HRP-linked anti-fluorescein dextran antibody (mouse monocolonal antibody, Roche Indianapolis, IN,) and DAB substrate (Sigma, Saint Louis, MO). 3G8 and 4A6 antibodies (a kind gift from Dr. Elizabeth Jones at the University of Warwick)targeting proximal tubule and distal tubules respectively, were detected using a goat-anti-mouse AP-linked secondary antibody (Sigma, Saint Louis, MO) and BM Purple (Roche, Indianapolis, IN) or Fast Red (Sigma, Saint Louis, MO) respectively.

## 2.3 RESULTS

### 2.3.1 Constitutively active *Xlim1* expands kidney tissues and the precise working window is between stage 9 to 12, with the most effective time point at 10.5

It has been reported that using a constitutively active construct of Lim1 bearing a mutation in the negative LIM domain can cause expanded or ectopic tubule formation in *Xenopus* embryos[79]. However, this phenotype was only observed in a low percentage of the injected embryos, less than 30% in the injected pool. To test whether we can mimic this phenotype, we use a differently modified Lim1 construct, LLVP16, that has been described in Kodjabachian's paper for injection[70]. Briefly, this construct does not contain LIM domain and is

fused on to the C-terminus of the dimerization domain of Ldb1.To increase its activation ability, the transactivation domain of VP16 is fused to the C-terminus of this construct. We found that after injection of 300pg LLVP16 mRNA into 2 ventral blastomeres at the four-cell stage, the pronephros region, as assessed by *Xpax8 in situ* hybridization, at stage 15and 26, conspicuously enlarged in comparison to the uninjected side. Moreover, the final pronephros as assessed by immunohistochemistry using the monoclonal antibody 3G8 for tubule detection showed an increase in either tubule number or length and therefore more coiling at stage 42, while the staining by 4A6, the duct specific antibody, did not show much difference compared with control side at the same stage. 12/101 staining of the abdominal muscles was decreased in both sets of stage 42 embryos, which may due to a developmental delay caused by the injection.

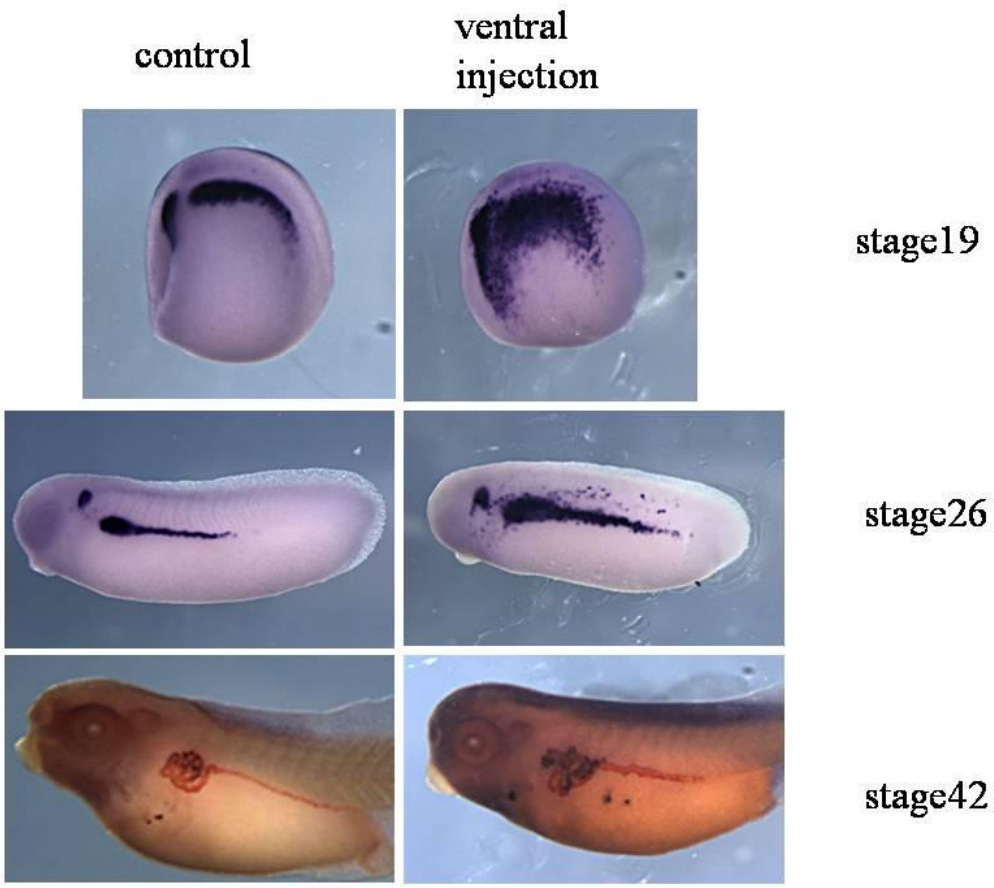


Figure 3. The constitutively active *Xlim1* expands the pronephros. 300pg LLVP16 was injected at 4-cell stage into two ventral blastomeres. *Xpax8 in situ* were carried out at stage 19 and stage 26 and 3G8/4A6 antibody staining at stage 42 ventral overexpression of *Xlim1* show expansion of kidney tissues with no gastrulation defects.12/102 antibody turquoise to mark somites.3G8 antibody was staining blue and lineage tracer was stained with b-gal red.(Data from Dr. Hukriede's lab)

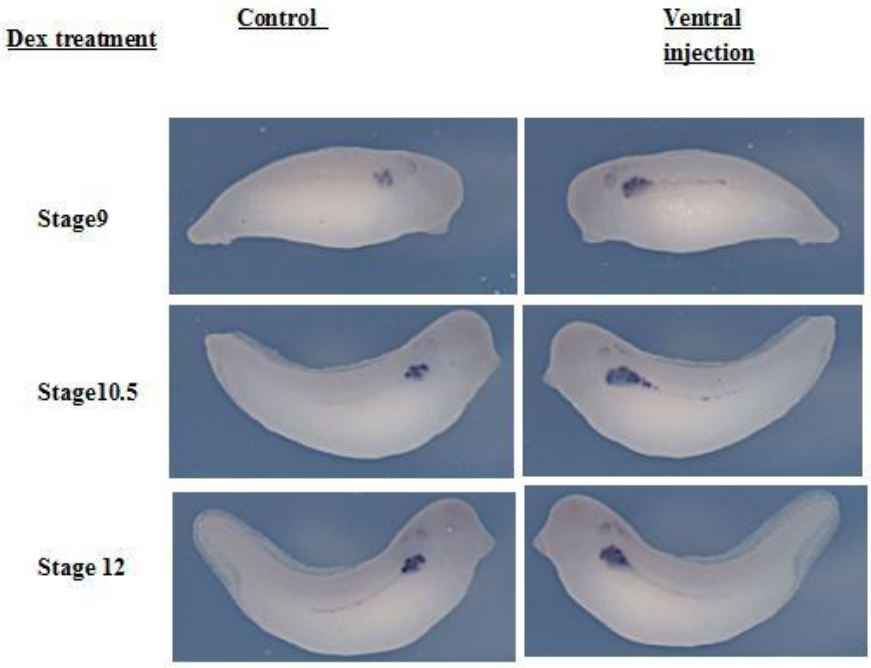


Figure 4. The working window of *Xlim1* is determined at an early stage. The precise working window of *Xlim1* in pronephric tubule specification was demonstrated by the injected LLVP-GR under dexamethsone regulation. (Data from Dr. Hukriede's lab)

We are clear that the early expression of *Xlim1* starts from stage 12.5. But the precise time window in which *Xlim1* functions in pronephric development remains elusive. In order to establish this time window, the glucocorticoid receptor was fused to the C-terminus of the LLVP16 construct (LLVP-GR). Addition of dexamethasone to the growth medium thus allows the active transcription of *Xlim1* at various points throughout development. 50pg of LLVP16-GR was injected at four-cell stage into the left ventral blastomere leaving right ventral blastomere as

internal control. Figure 6 shows the effects of activating *Xlim1* in either the ventral region of the embryos. Addition of DEX at stage 9 shows that *Xlim1* can function in pronephric development at this stage and overexpression causes formation of enlarged tubules in both ventral injection of LLVP16-GR. The greatest effect is seen when *Xlim1* is activated at stage 10/10.5. At stage 12.5, when the pronephros looks like a tear shape at the activation by DEX, entails little change in tubule size. Later treatment does not show obvious effect either. Hence, it suggests around stage 10-10.5, actually is the internal activating time for *Xlim1*, or the downstream factors are most effectively expressed at that time. Later than 12.5, it seems like that the networking to specify the pronephric tubule formation has halted.

### 2.3.2 **The potential downstream targets of lim1 retrieved from microarray analysis**

In previous research, our lab discovered that overexpressed *Xlim1* transcripts in *Xenopus* (animal caps) could replace the need for activin in the culture medium to differentiate explants into kidney pathway. To identify Lim1's downstream effectors and the genes involved in the *in vitro* kidney differentiation, we carried out microarray analysis to compare the gene expression profile difference between Xlim1-DEED injected animal caps and uninjected animal caps, between the retinoic acid/Activin (RA/Ac)treated animal caps and untreated control ones(Control). All the caps except the control group have been treated in the kidney inducing condition. We found 216 genes have been significantly upregulated and 576 genes were significantly downregulated in the DEED-injected vs. uninjected pair. We also found 438 upregulated genes and 266 downregulated genes in AcRA-treated vs. control pair. Among those upregulated and downregulated genes, we grouped them according to their expression patterns.

In the below four tables, list all the genes identified by microarray that we found to express in kidney via pubmed search. These genes cover several important fields in biological process. They are cell signaling/cell communication, transcription factors, gene/protein expression regulation, cell structure/motility, metabolism and unclassified/unknown. Most of the genes we found to be upregulated and downregulated in DEED vs. ACRA pair, which are expressed in kidney, belong to the unclassified/unknown group, suggesting unknown genes are critical to Lim1 involved process. In table 2, 43% of the 60 are classified as unknown, while factors in table 1 are too few to have statistic significance of grouping. Table 3 displays some genes with dramatic fold changes and low p-value, indicating positively they are pronephros-pro genes, in contrast to the genes in table 4 as the potential repressors in the differentiation process of pronephros.

Table1.Summary of upregulated genes expressed in kidney identified by microarray (DEEDAcRA-vs- AcRA)

| Uni Gene ID | lFold | Gene Description | Expression |
|---|---|---|---|
| XI.29876 | 21.33 | CCAAT-enhancer binding protein delta | tail; whole body; limb; fat body; spleen; heart; lung; brain; kidney; skin; ovary; bone; |
| Xl.41528 | 15.35 | Shisa | Whole body; ectoderm; endomesoderm; dorsal lip; kidney |
| XI.25992 | 8.20 | Transcribed Locus | whole body; kidney; ovary; digestive |
| XI.23471 | 6.45 | Transcribed locus, strongly similar to NP_001084654.1 hypothetical protein | Whole body; endomesoderm;ovary;kideny |
| XI.1085 | 5.54 | Zic2 protein (Zic2) | whole body; endomesoderm; ectoderm; dorsal lip; limb; ovary; kidney; head |
| Xl.1289 | 5.41 | Cripto-1 (cr1) | whole body; ectoderm; ovary; kidney |
| Xl.5107 | 5.31 | Transcribed locus, strongly similar to NP_001082000.1 beta-2 microglobulin [Xenopus laevis] | spleen; fat body; testis; lung; brain; kidney; head; thymus; whole body; digestive; skin; heart; bone; endomesoderm; limb; tail; ovary |

| | | | |
|---|---|---|---|
| Xl.23093 | 5.09 | Hypothetical protein MGC53111 (MGC53111) | whole body; kidney; brain; tail; endomesoderm; digestive |
| Xl.23425 | 4.15 | Transcribed locus | whole body; head; ectoderm; fat body; digestive; tail; kidney; endomesoderm |
| Xl.3201 | 9.10 | Hypothetical protein MGC83260 (MGC83260) | whole body; kidney; testis; ovary |
| Xl.6392 | 8.80 | Electrogenic sodium monocarboxylate cotransporter (smcte) | endomesoderm; whole body; kidney |

Table2 Summary of the downregulated genes expressed in kidney identified by microarray (DEEDAcRA-vs-AcRA)

| Uni Gene ID | Fold change | Description | Expression |
|---|---|---|---|
| Xl.12858 | 4.11 | CDNAclone MGC:181766 IMAGE:8550943 | whole body; head; fat body; thymus; kidney |
| Xl.4083 | 4.13 | MGC83576protein (MGC83576) | whole body; spleen; kidney; testis; endomesoderm |
| Xl.51341 | 4.32 | Hypothetical LOC495150 (LOC495150) | whole body; head; kidney; ovary |
| Xl.3820 | 4.43 | Hypothetical protein MGC68533 (MGC68533) | whole body; kidney; ectoderm; testis; fat body; ovary; endomesoderm; spleen; head; heart; brain |
| Xl.3258 | 4.50 | Transcribed locus | spleen; heart; kidney; fat body; tail; bone; whole body |
| Xl.14521 | 4.60 | Hypothetical protein LOC398688 (LOC398688) | whole body; kidney; ectoderm; lung; fat body; tail; heart; skin |
| Xl.11114 | 4.63 | Transcribed locus, moderately similar to NP_001088258.1 hypothetical protein LOC495089 [Xenopus laevis] | whole body; kidney; head |
| Xl.1204 | 4.79 | Xlgv7 gene product (LOC397899) | whole body; kidney; ovary; |

| | | | |
|---|---|---|---|
| | | | ectoderm; brain; spleen; skin; endomesoderm; limb |
| Xl.13880 | 4.82 | MGC82677 protein (MGC82677) | whole body; animal cap; ovary; ectoderm; kidney; dorsal lip |
| Xl.14515 | 4.92 | Hypothetical protein MGC53361 (MGC53361) | whole body; ectoderm; brain; tail; skin; kidney; endomesoderm |
| Xl.5082 | 5.00 | Hypothetical protein MGC68521 (MGC68521) | whole body; kidney; digestive; brain |
| Xl.46026 | 5.32 | Similar to intergral membrane protein 1 (Itm1) | whole body; ectoderm; kidney; testis; thymus; skin; endomesoderm; spleen; lung |
| Xl.5583 | 5.37 | Hypotheticalprotein LOC100127280 (LOC100127280) | lung; kidney |
| Xl.4796 | 5.39 | Transcribed locus, strongly similar to XP_686412.2 PREDICTED: hypothetical protein, partial [Danio rerio] | whole body; ovary; digestive; kidney; ectoderm; spleen; fat body; endomesoderm |
| Xl.47404 | 5.62 | MGC80589 protein (MGC80589) | lung; ovary; whole body; heart; spleen; kidney |
| Xl.28895 | 5.82 | Hypothetical protein MGC82293 (MGC82293) | kidney; testis; ectoderm; whole body |
| Xl.12498 | 5.93 | Hypothetical protein MGC68533 (MGC68533) | whole body; brain; kidney; thymus |
| Xl.3820 | 4.43 | Hypothetical LOC494683 (LOC494683) | whole body; kidney; ectoderm; testis; fat body; ovary; endomesoderm; spleen; head; heart; brain |
| Xl.14452 | 5.96 | Transcribed locus | whole body; brain; head; kidney; fat body; limb |

| | | | |
|---|---|---|---|
| Xl.14232 | 6.05 | Transcribed locus, moderately similar to XP_922909.1 PREDICTED: similar to myosin IXA isoform 2 [Mus musculus] | whole body; ovary; kidney; lung; testis; fat body |
| Xl.5611 | 6.07 | Hypothetical protein LOC398440 (LOC398440) | whole body; ectoderm; ovary; dorsal lip; animal cap; kidney; testis; tail; endomesoderm |
| Xl.7178 | 6.14 | Transcribed locus | whole body; brain; kidney; thymus |
| Xl.965 | 6.26 | Epithelial sodium channel, gamma subunit (gammaxENaC-A) | kidney |
| Xl.6837 | 6.41 | Hypothetical protein MGC64330 (MGC64330) | whole body; endomesoderm; skin; ectoderm; ovary; kidney; fat body |
| Xl.5500 | 6.53 | Hypothetical protein LOC446948 (LOC446948) | whole body; head; skin; endomesoderm; fat body; spleen; kidney; testis; lung |
| Xl.12327 | 6.60 | MGC80468 protein (MGC80468) | whole body; lung; spleen; brain; kidney; ovary |
| Xl.25327 | 6.64 | Transcribed locus, moderately similar to NP_003612.1 PTPRF interacting protein, binding protein 2 (liprin beta 2) [Homo sapiens] | whole body; ovary; kidney; fat body |
| Xl.19291 | 6.80 | Hypothetical protein LOC398673 (LOC398673) | whole body; dorsal lip; ovary; kidney; ectoderm; brain |
| Xl.16481 | 6.86 | Hypothetical protein MGC82240 (MGC82240) | whole body; kidney; head; brain; testis; spleen; ovary |
| Xl.24700 | 7.36 | Transcribed locus | whole body; kidney |
| Xl.25179 | 7.39 | Hypothetical protein MGC68635 (MGC68635) | spleen; whole body; thymus; kidney; brain; limb; testis; skin; fat body; |

| | | | |
|---|---|---|---|
| | | | head; digestive |
| Xl.6133 | 7.47 | XK70 (LOC397756) | whole body; ectoderm; animal cap; head; skin; limb; dorsal lip; digestive; kidney |
| Xl.9576 | 7.55 | Carbonic anhydrase II (ca2) | whole body; head; brain; kidney; digestive |
| Xl.24116 | 7.56 | MGC86445 protein (MGC86445) | whole body; testis; ovary; kidney; spleen; digestive |
| Xl.2034 | 7.59 | Hypothetical protein LOC443724 (LOC443724) | whole body; spleen; kidney; testis; endomesoderm; ectoderm |
| Xl.34077 | 7.60 | Transcribed locus, strongly similar to NP_001011158.1 SEC31-like 1 [Xenopus tropicalis] | whole body; endomesoderm; kidney |
| Xl.14521 | 8.60 | Hypothetical protein LOC398688 (LOC398688) | whole body; kidney; ectoderm; lung; fat body; tail; heart; skin |
| Xl.695 | 9.61 | Polyprotein, serine proteases and ovochymase regions (LOC398190) | ovary; whole body; kidney |
| Xl.16095 | 9.80 | Transcribed locus | whole body; kidney |
| Xl.16255 | 10.02 | Transcribed locus | whole body; ectoderm; kidney; dorsal lip; ovary |
| Xl.19308 | 10.82 | Hypothetical protein MGC114839 (MGC114839) | kidney; whole body; ovary; dorsal lip; endomesoderm |
| Xl.47097 | 10.96 | Hypothetical protein MGC80262 (MGC80262) | whole body; ectoderm; testis; skin; endomesoderm; ovary; animal cap; kidney |
| Xl.206 | 11.2 | Nedd4 protein (nedd4) | whole body; dorsal lip; fat body; kidneymj |
| Xl.1605 | 11.32 | Transcribed locus, weakly similar to | whole body; tail; brain; skin; limb; |

| | | | |
|---|---|---|---|
| | | XP_425376.2 PREDICTED: similar to envoplakin [Gallus gallus] | spleen; lung; kidney; head |
| Xl.813 | 11.96 | Hypothetical protein MGC69066 (MGC69066) | spleen; whole body; brain; thymus; kidney; bone; digestive; lung; head |
| Xl.21860 | 14.17 | SPARC protein (MGC64258) | whole body; brain; limb; fat body; lung; testis; head; kidney; tail; spleen; skin; endomesoderm |
| Xl.15599 | 15.35 | Hypothetical protein MGC68953 (MGC68953) | whole body; kidney; testis; head; fat body; spleen; skin; limb; brain |
| Xl.5930 | 19.83 | MGC81015 protein (MGC81015) | kidney; whole body; limb |
| Xl.15997 | 24.75 | Synaptotagmin-like 2 (sytl2) | whole body; kidney; endomesoderm; head |
| Xl.16272 | 72.23 | Transcribed locus, weakly similar to XP_221384.4 PREDICTED: similar to mucin 4 [Rattus norvegicus] | whole body; kidney |
| Xl.18602 | 80.05 | Hypothetical protein MGC68485 (MGC68485) | whole body; kidney; ectoderm; ovary; endomesoderm; dorsal lip |
| Xl.8949 | 8.53 | Ornithine decarboxylase-2 (MGC52527) | whole body; testis; ectoderm; fat body; skin; animal cap; kidney; head; endoderm; endomesoderm |
| Xl.8939 | 8.73 | MGC81907 protein (MGC81907) | whole body; kidney; fat body; thymus |
| Xl.15221 | 8.78 | Similar to solute carrier family 27 (fatty acid transporter), member 2 (LOC398483) | whole body; kidney; brain |
| Xl.8759 | 10.65 | Hypothetical protein MGC68473 (MGC68473) | whole body; brain; head; kidney; testis; spleen; ectoderm |
| Xl.1500 | 15.25 | Similar to villin (LOC398504) | whole body; ectoderm; kidney; limb; |

| | | | |
|---|---|---|---|
| | | | skin; brain; testis |
| Xl.23708 | 22.25 | MGC81939 protein (MGC81939) | whole body; brain; ectoderm; kidney; skin |

Shown above are some genes that significantly decreased after the depletion of Lim1 in explants. Genes that are displayed in these four tables are the genes known to express in kidney in unigene database. For genes that are expressed in kidneys, their expression time windows and locations may not overlap pronephric development time window or reside in pronephric region in reality. Thus it is not wise to only depend on the quantitative evidence of the difference in expression levels, the actual expression level and location of these genes need to be validated by *in situ* hybridization. The same principle applies to the genes that have not been reported to associate with kidney development or express in kidney region, but reveal to show a difference in the supplemental materials.

Table3. Summary of the upregulated genes identified by microarray (Activin/RA vs. control) that have been documented to express in kidney

| Unigene ID | Fold Change | Gene Description | Expression |
|---|---|---|---|
| Xl.12667 | 70.07 | transcription factor 2 /// hypothetical protein MGC68543 | whole body; kidney |
| Xl.15054 | 48.71 | Cnpr03 mRNA, complete sequence | whole body; ectoderm; fat body; tail; limb; endomesoderm; brain; kidney; spleen; lung |
| Xl.34 | 40.24 | Hypothetical protein MGC68907 | whole body; endomesoderm; limb; thymus; spleen; dorsal lip; lung; kidney |
| Xl.23957 | 38.09 | Ami | whole body; kidney; testis; spleen; fat |

| | | | |
|---|---|---|---|
| | | | body; digestive |
| Xl.6392 | 31.17 | electrogenic sodium monocarboxylate cotransporter | endomesoderm; whole body; kidney |
| Xl.1042 | 7.41 | eural specific DNA binding protein | heart; whole body; endomesoderm; kidney; fat body |
| Xl.899 | 7.37 | pleiotrophic factor-alpha2 | endomesoderm; whole body; fat body; ectoderm; head; kidney; brain; ovary |
| Xl.29610 | 6.79 | Transcribed locus, weakly similar to XP_237286.4 PREDICTED: similar to tensin [Rattus norvegicus] | lung; kidney; spleen |
| Xl.34749 | 6.69 | hypothetical protein MGC68579 | whole body; ectoderm; kidney; lung; dorsal lip; brain; tail; limb; endomesoderm; testis |
| Xl.23641 | 6.17 | enzymatic glycosylation-regulating gene | kidney |
| Xl.216 | 5.86 | Similar to macrophage stimulating1 (hepatocyte growth factor-like) | whole body; ectoderm; endomesoderm; kidney; dorsal lip; bone |
| Xl.25327 | 5.52 | Transcribed locus, moderately similar to XP_426399.1 PREDICTED: similar to coiled-coil like protein 1 [Gallus gallus] | whole body; ovary; kidney; fat body |
| Xl.15670 | 5.52 | Transcribed locus, weakly similar to XP_314118.2 ENSANGP00000003760 [Anopheles gambiae str. PEST] | whole body; kidney |
| Xl.24700 | 5.45 | Transcribed locus | whole body; kidney |
| Xl.1008 | 5.30 | FGF receptor 4a | whole body; endomesoderm; ectoderm; head; dorsal lip; kidney |
| Xl.50622 | 5.03 | similar to UDP-glucose dehydrogenase | whole body; endomesoderm; ectoderm; ovary; testis; dorsal lip; kidney |
| Xl.45046 | 5.00 | MRNA sequence | whole body; ectoderm; kidney; thymus; digestive; endomesoderm |
| Xl.16655 | 19.67 | solute carrier family 16 (monocarboxylic acid | whole body; kidney; ectoderm; |

| | | | |
|---|---|---|---|
| | | transporters), member 6 | endomesoderm |
| Xl.48471 | 16.38 | hypothetical LOC495093 | whole body; ectoderm; kidney; limb; endomesoderm |
| Xl.21562 | 13.46 | ras-related protein | whole body; ectoderm; dorsal lip; kidney |
| Xl.31935 | 8.31 | MAP kinase phosphatase X17C | whole body; spleen; kidney; ectoderm; endomesoderm |
| Xl.1210 | 7.66 | Hoxb7 protein | whole body; brain; kidney; fat body |
| Xl.1042 | 7.41 | neural specific DNA binding protein /// zinc finger protein Gli2 | heart; whole body; endomesoderm; kidney; fat body |
| Xl.2751 | 4.75 | fibroblast growth factor receptor | whole body; endomesoderm; testis; dorsal lip; ectoderm; kidney |
| Xl.29876 | 4.73 | CCAAT-enhancer binding protein delta | tail; whole body; limb; fat body; spleen; heart; lung; brain; kidney; skin; ovary; bone; |
| Xl.2243 | 4.33 | Fibronectin protein | whole body; endomesoderm; lung; bone; limb; tail; brain; fat body; testis; ectoderm; digestive; head; spleen; kidney |
| Xl.1130 | 4.80 | Xenopus laevis, Similar to paired box gene 2, clone IMAGE:5543325, mRNA /// Paired box protein | whole body; ectoderm; kidney |
| Xl.13563 | 4.07 | MGC80391 protein | whole body; kidney; digestive |
| Xl.49562 | 33.19 | hypothetical LOC495088 | endomesoderm; whole body; kidney |
| Xl.15593 | 13.90 | MGC82107 protein | whole body; kidney |
| Xl.1085 | 10.03 | Zic2 protein | whole body; endomesoderm; ectoderm; dorsal lip; limb; ovary; kidney; head |
| Xl.6155 | 12.59 | MGC83414 protein | whole body; brain; kidney |
| Xl.24776 | 10.57 | Transcribed locus, weakly similar to XP_517667.1 PREDICTED: similar to versican V2 core protein precursor [Pan troglodytes] | whole body; ectoderm; ovary; kidney; brain; spleen; endomesoderm |

| | | | |
|---|---|---|---|
| Xl.21860 | 10.43 | SPARC protein | whole body; brain; limb; fat body; lung; testis; head; kidney; tail; spleen; skin; endomesoderm |
| Xl.1299 | 12.58 | alkaline phosphatase, liver/bone/kidney | whole body; endomesoderm; tail; limb; fat body; kidney; dorsal lip; brain |
| Xl.3150 | 11.34 | follistatin-related protein | endomesoderm; whole body; bone; tail; limb; fat body; brain; head; testis; digestive; lung; kidney |

Table4. Summary of the downregulated genes identified by microarray (AcRA vs. Control) that have been documented to express in kidney

| Unigene ID | Fold Change | Gene Description | Expression |
|---|---|---|---|
| Xl.3209 | 3.46 | elongation factor 1-alpha O | ectoderm; whole body; ovary; kidney; endomesoderm; testis |
| Xl.53426 | 4.77 | Transcribed locus | whole body; kidney |
| Xl.6837 | 4.87 | hypothetical protein MGC64330 | whole body; endomesoderm; skin; ectoderm; ovary; kidney; fat body |
| Xl.16776 | 4.99 | Hypothetical protein MGC68565 | whole body; brain; kidney |
| Xl.48740 | 5.07 | MGC81884 protein | spleen; whole body; heart; brain; kidney |
| Xl.12327 | 5.08 | MGC80468 protein | whole body; lung; spleen; brain; kidney; ovary |
| Xl.931 | 5.29 | T-box transcription factor Tbx2 | whole body; kidney; digestive |
| Xl.5614 | 5.83 | hypothetical protein MGC68646 | whole body; kidney |
| Xl.814 | 6.38 | immunoglobulin light chain | spleen; fat body; kidney; brain; whole body; digestive |
| Xl.8193 | 6.90 | MGC86499 protein | whole body; kidney; brain; testis |
| Xl.1201 | 7.91 | glucocorticoid receptor protein | whole body; lung; kidney; fat body |
| Xl.2458 | 8.52 | hypothetical LOC496017 | whole body; spleen; ovary; brain; ectoderm; fat body; head; heart; limb; |

| | | | |
|---|---|---|---|
| | | | thymus; kidney; skin; digestive |
| Xl.21648 | 11.74 | Kit receptor tyrosine kinase homolog | whole body; spleen; kidney |
| Xl.14126 | 11.94 | Transcribed locus | whole body; kidney; endomesoderm |
| Xl.12819 | 12.64 | MGC81876 protein | thymus; kidney |
| Xl.5158 | 13.82 | Epor mRNA for erythropoietin receptor homologue | digestive; spleen; lung; kidney; head |

Thus, we carried out *in situ* hybridization for 35 potential targets on *Xenopus* embryos. The aim was to investigate whether these targets express in pronephric region and whether any of them express at the early specification period (data not shown). Our results demonstrated that some of the targets only expressed at the stage 12.5, the time tubule was specified. Some of them expressed at stage 19 the time early before the pronephric anlagen is visible. But, for most of the targets we investigated, they do not display an expression pattern early before the appearing of the pronephric anlagen. Additionally, among the 7 targets that could be detected at the right time frame for pronephric specification, none of which showed up in the pronephric region. We also did *in situ* hybridization using genes newly identified in microarray analysis on embryos. But their expression were not clearly associated with the pronephros development process(Data not shown).

### 2.3.3 Cell-targeted *Xlim1* knockdown affects the formation of the kidney. The expression of the early kidney marker pax8 is disrupted specifically in kidney region in Xlim1 knockdown embryos

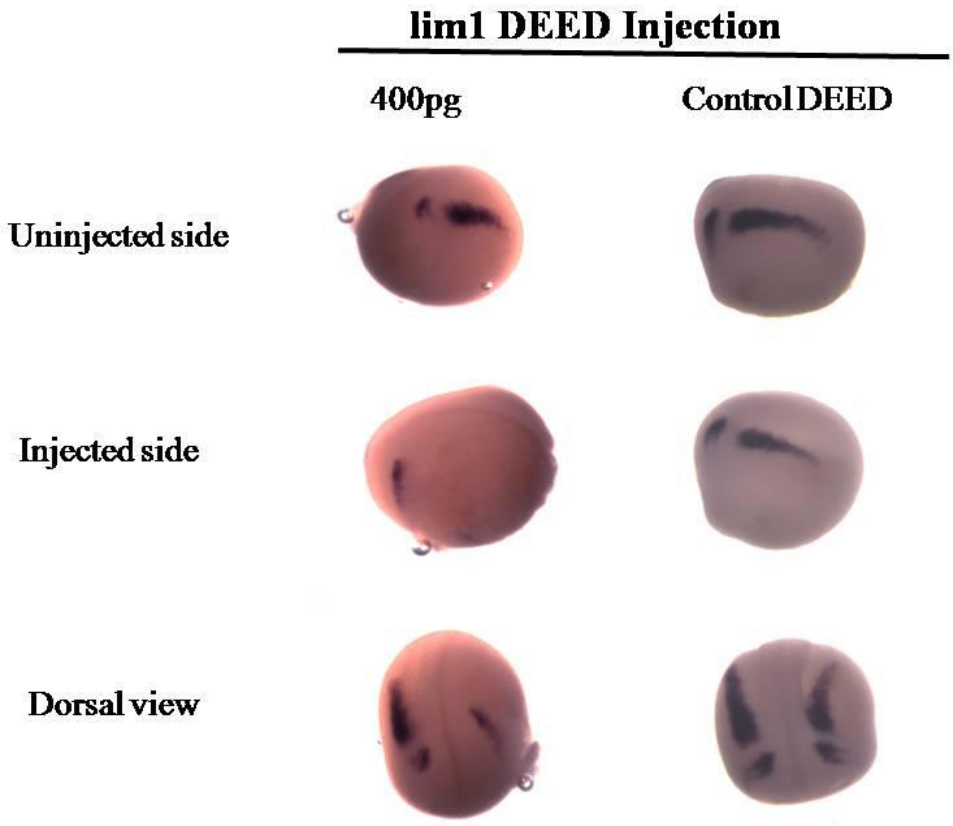


Figure 5 The depletion of *Xlim1* via DEED injection severely disrupts the formation of pronephros

To further examine the role of *Xlim1* in pronephros development, we used different amount of N, N-diethlethlenediamine antisense oligos to block the transcription of *Xlim1* in *Xenopus* embryos. 400pg, 200pg, 100pg, 80pg LIM1-DEED and 400 pg control DEED were injected into either V1 or V2, ventral vegetal cell at 8-cell stage. Concomitant with the previous data obtained

in the lab at 4-cell stage injection, 200pg DEED disrupts the normal condensement of pronephros severely. *Xpax8* expression is seen around the circumference of the embryos trailing down from where the tail of the teardrop of typical expression begins. These embryos never show a regular tear-shape in the correct place in addition to the ventral stain. More critically, when the dose of DEED was increased to 400pg per cell, *Xpax8* was not visible in the pronephros region. The normal tear-shape staining disappears, which indicates the loss of pronephros tissue in regards of that Pax8 is the marker of early pronephros. 100pg and 80pg injection, in the contrast, do not show a phenotype of kidney disruption. All those embryos were fixed at stage 19, the time the pronephros specification has been established. *Xpax8* staining, however is still visible in otic vesicle, which means cell targeting is correct and the loss of pax8 results from lim1-DEED injection simply .To rule out the possibility that the ventral expression *Xpax8* was in cells that had been fate-transformed from other tissues, several ventral differentiation markers were analyzed. *XSzl*, *XVent1*, *XmyoD* and *Xalpha T4globin* were unaffected.(date not shown).This result suggests it might be a failure in migration of cells in which Lim1 has been depleted and the failure of migration will give rise to possible apoptosis of the designated pronephric cells. Other possibility to result in such a pattern is the secondary effect of gastrulation problem (Discussed with Dr.Hukriede).

# 3.0 CONCLUSION AND PROSPECTUS

## 3.1 OBJECTIVES

My work was undertaken with the goal to elucidate the mechanism of *Xlim1* in *Xenopus* kidney development and it contains two parts. One part is some leftover work of Caroline's project which is to explore the regulatory role of *Xlim1* in pronephros and the other is the TAP work with intention to identify the components of Xlim1 complex, which I just started preliminary experiment and is not included in this thesis. For the mechanism of *Xlim1* in kidney development, this thesis tried to characterize *Xlim1*'s role in early kidney specification as well as to identify the downstream factors of *Xlim1* pathways

## 3.2 INTERPRETATION OF THE RESULTS

The early study of Lim1 dated back to 1992 when Taira's group which was fancied by a newly identified homeobox class that contains *mec-3* and *lin-11*, started to examine whether the members of this particular class are involved in the vertebrate embryogenesis. *Xlim1* was isolated and sequenced in *Xenopus* embryos in that study[67]. Soon after another paper came out reporting a homolog of *Xlim1*, *Lim1* (*Lhx1*), was identified from the mouse. Since then the role of *Lim1* has been very well investigated. In consistence with several papers published by Behringer's group

describing the *Lim1*$^{-/-}$ mouse phenotype, we found that in *Xenopus*, the specific depletion of Xlim1 at 8-cell stage in ventral vegetal cell results in the loss of pronephros, assessed by *Pax8*, one of the early pronephros markers in *Xenopus*. But the forming of this phenotype depends on the dose of Lim1-DEED used in the experiment. A relatively lower dose of Lim1-DEED injection (200pg/cell at 8-cell stage) gives rise to a tailing down pattern of *Xpax8*. 100pg or lower, interestingly, gives a normal phenotype. This indicates that different level of *Lim1* transcripts differentially regulates the gene networking that associates with pronephros specification. Actually, several genes that play critical roles in development show expression level-dependent effects on cellular differentiation or development. For instance, Oct3/4, an embryonic transcription factor, was found to be a master regulator of pluripotency that controls lineage commitment in embryonic stem cells. Three distinct fates of ES cells are governed by the precise level of *Oct-3/4* present in ES cells revealed by the tetracycline–induced system[83]. However, other suggests that the loss of visible pronephros anlagen may be due to secondary effects of gastrulation problem (Discussed with Dr. Hukriede). So the question remains to be whether the depletion of *Xlim1* gives rise to the loss of pronephros or the depletion of *Xlim1* gives rise to the gastrulation defects that consequently disrupts the formation of the pronephros. Additionally, it raises another question that whether it is the loss of tissue or it is the loss of inductive signal for pronephros.

To further understand and answer these questions, we took microarray analysis. Our microarray data indicates the depletion of *Xlim1* in animal caps induce couple of genes' expression going down under the kidney differentiation conditions. Many of these genes play a role in cell movement, proliferation and differentiation, such as N-cadherin, Marginal Coil, and

Annexin et al[84-86]. So it implies Lim1 does have close relationship with the molecular networking involving in cell movement. Consistent with the previous result that PAPC is a downstream factor of the Xlim1, we would speculate that perhaps more cell-movement factors are regulated by Xlim1 at some time point and on certain location in embryos[45, 87]. Yet, no interesting hypothesis has been raised to elucidate the mechanism how Xlim1 specifies pronephros fate because most of these gene expression patterns do not coincide with the pattern of Xlim1 or Pax8. However, microarray data provide us with a more restricted pool to probe the unknown genes that may affect the pronephric development.

The precious work completed by Behringer's group carefully dissected lim1's role in the later development of kidney and female reproductive tract[50, 88]. But nobody has ever attempted to regulate the level of *Lim1* at a very early developmental stage in mouse by utilizing regulatory systems like tet-inducible system. If such a system could be established in mouse, the analysis from that model may provide a better comparison with our results than the current data obtained from the mouse model of late kidney development and thus might provides more useful information.

Our results again confirm Carroll and Vize's results in 1999, that we found the overexpression of *Xlim1* successfully induces an enlarged tubules and glomerulus. The depletion of *Xlim1* in explants does not cause the significant change in *Xpax8*'s expression level, admittedly that *Xpax8* itself maintains a low expression level below the stringent threshold. So whether *pax8* is regulated by *Xlim1* is still elusive.

According to our results, the functionally active time window of *Xlim1* in manipulating kidney formation starts as early as stage 9 and ends at stage 12.5, with a peak time at stage 10 to 10.5. Stage 12.5 is the time point that tubule is specified and stage 14 is the time the duct is specified. In terms of specification, we mean a portion is capable of forming a given tissue when cultured in a neural environment (Sack, 1987). Clearly we can see that the overexpression of *Xlim1* at early time before stage 12.5 only induced enlarged pronephroi, which means that the specification of ducts requires other inducers rather than *Xlim1* only, while glomerulous and tubules mostly require Xlim1 involved networking. At the same time, the networking that has been activated at the stage 10.5 remains to be investigated. It would be helpful if we can screen EST library finding out all the ESTs expressed during 10.5. However the expression of *Xlim1* at 10.5 is not even close to the future pronephros region[67]. So there are no practical standards we can apply to predict where the cofactors should be expressed at an early stage like 10.5 that could efficiently affect the specification of pronephros. Hence, the molecular work that probes the factors directly or indirectly via complexes associated with Xlim1 turns to be critical.

Recently compelling evidence from both *Xenopus* and chicken supports the hypothesis that pronephros forms by means of secondary induction signals emitted from anterior somites[89, 90]. In addition, pronephros never forms in the absence of anterior somites. Thus we cannot rule out the possibility contributed by anterior somites. Possibly Xlim1 also plays some role in anterior somites formation, at least supports the formation of this tissue but it is not required for the formation. It is known that *Chordin*, a factor critical for somitogenesis, induces ectopic expression of myf5 at 10.5, a coincidence with the peak of *Xlim1*'s activity. *Myf5* expression at stage 10.5 is an indicator of whether a cell has the potential to form anterior somites[47]. This

interestingly suggests that Xlim1 and somitogenesis signaling pathway reciprocally induce each other's differentiation. In contrast, Seufert's paper in 1999 reported that reduced somites compensate for the enlarged pronephros. Whether it is true or not need experimental validation.

I use Unigene database as the main resource to retrieve the cellular location in different tissues and organs of the interested genes. However, when comparing with the targeted genes picked by hand by others, some of the genes that are related to kidney were not screened. After careful examining, those genes that are not listed as expressed in kidney in Unigene database, generally are annotated as expressed in endomesoderm. Thus, the pool for the potential targets of Xlim1 may expand by adding the genes that are expressed in endomesoderm too.

## 3.3 SIGNIFICANCE AND FUTURE WORK

The future direction of this work is to find specific targets of Lim1 as well as to answer the question that whether lack of lim1 entails the loss of kidney tissue or the loss of signals that induce kidney development.

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