UNDERSTANDING THE MOLECULAR MECHANISMS OF RENAL PROGENITOR PATTERNING DURING ZEBRAFISH NEPHEROGENESIS

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Abstract

by

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The molecular mechanisms underlying nephron patterning establishment are intriguing. The zebrafish embryonic pronephros serves as a simplified and conserved genetic model to study this nephrogenesis process. The zebrafish pronephros arises from renal progenitors that are segmented into distinct epithelial regions including proximal and distal tubule domains, which consist of transporting epithelial cells and intercalated multiciliated cells (MCCs). Using whole mount in situ hybridization, my project uncovered essential roles of the transcription factors mecom and tbx2a/b in patterning of the renal progenitors during zebrafish nephrogenesis. Apart from their functions in segment formation,
mecom coordinates with Notch signaling to regulate MCC differentiation, while tbx2b acts downstream of Notch signaling to mediate development of an associated endocrine organ, the corpuscle of Stannius. Meanwhile, I have also discovered Notch signaling as a conserved factor in both zebrafish and mammalian nephron patterning that promotes proximal fates and restricts distal domains. Taken together, my work has identified crucial renal progenitor patterning factors, and has revealed novel molecular mechanisms guiding pronephros development in the zebrafish.
CONTENTS

Figures..............................................................................................................iv

Acknowledgements...........................................................................................xiii

Chapter 1: Introduction.........................................................................................1
  1.1 Vertebrate kidney organogenesis and birth defects – an overview........1
    1.1.1 The stages of kidney development in vertebrates.......................2
    1.1.2 The nephron – the basic functional unit of the kidney.............4
    1.1.3 Patterning of the mammalian nephron tubule.............................5
  1.2 Zebrafish as a promising model to study nephrogenesis....................8
    1.2.1 Zebrafish embryonic pronephros – organogenesis and structure...9
    1.2.2 Important genetic factors identified in pronephros patterning....12
  1.3 Summary and rationale.............................................................................16

Chapter 2: Materials and methods...............................................................27
  2.1 Zebrafish husbandry and ethics statement........................................27
  2.2 Morpholino knockdown, cRNA synthesis, and heat shock
      experiments.................................................................................................27
  2.3 RT-PCR......................................................................................................28
  2.4 Dextran injection......................................................................................29
  2.5 Benzidene staining...................................................................................29
  2.6 Chemical treatments................................................................................29
  2.7 WISH.........................................................................................................30
  2.8 Cell counting and statistics.....................................................................31

Chapter 3: Zebrafish nephrogenesis is regulated by interactions between retinoic
          acid, mecom, and Notch signaling.........................................................34
  3.1 Abstract.....................................................................................................34
  3.2 Introduction................................................................................................36
  3.3 Results.........................................................................................................40
    3.3.1 mecom delineates a dynamic caudal subdomain in the renal
         progenitor field.......................................................................................40
    3.3.2 mecom morphants exhibited renal edema and dysfunction........43
3.3.3 *mecom* knockdown induced PST specific expansion in the zebrafish pronephros ...........................................46
3.3.4 *mecom* morphants display PST expansion at the expense of the DL segment ..................................................47
3.3.5 RA negatively regulates *mecom* during pronephros segmentation ...............................................................48
3.3.6 The activities of RA and *mecom* also regulate epithelial cell fate choice in tubule segments .........................50
3.3.7 *mecom* and Notch signaling coordinate MCC density in zebrafish pronephros ..............................................52
3.4 Discussion ..........................................................................................................................................................55
3.4.1 Mechanisms of *mecom* function during zebrafish nephrogenesis ...............................................................56
3.4.2 The elucidation of new roles that RA plays during zebrafish nephrogenesis ..................................................59

Chapter 4: *tbx2a/b* transcription factors direct pronephros segmentation and corpuscle of Stannius formation in zebrafish .................................................................76
4.1 Abstract .........................................................................................................................................................76
4.2 Introduction ...............................................................................................................................................77
4.3 Results .......................................................................................................................................................81
4.3.1 The *tbx2* transcripts were expressed in the distal territory of the pronephros during zebrafish nephron development .................................................................81
4.3.2 Proper function of *tbx2* is crucial for DL segmentation and PCT specification during zebrafish nephrogenesis .................................................................82
4.3.3 Notch signaling and *tbx2* genes cooperate to repress CS development of the zebrafish pronephros ................84
4.3.4 Notch signaling also involves in segmental patterning by promoting proximal fates of the pronephros ..........87
4.4 Discussion ...................................................................................................................................................88
4.4.1 *tbx2a/b* play critical roles in renal progenitor patterning .................................................................89
4.4.2 *tbx2b* and induction of the CS in teleosts .........................................................................................90
4.4.3 Notch signaling pathway is involved in nephron segmentation formation during zebrafish nephrogenesis .................91

Chapter 5: Conclusion and perspective .................................................................................................................104
5.1 From development to regeneration - understand the cellular basis of kidney regeneration ................................105
5.2 Using zebrafish as a model to study nephrogenesis ..................................................................................108
5.2.1 New genome-editing technology in zebrafish ..................................................................................109
5.2.2 Zebrafish – a conserved model of nephrogenesis ........................................................................110

Bibliography ...................................................................................................................................................114
FIGURES

Figure 1.1 The nephrogenesis process in mammalian models......................18
The developing mouse intermediate mesoderm at approximately E10.5. At the anterior pronephric duct, the pronephros quickly forms and degenerates. Posterior to the pronephros, the primary nephric duct connects to a series of well-developed mesonephric tubules, which function transiently as the embryonic kidney organ. The most posterior region of the pronephric duct exhibits a single ureteric bud (UB). The metanephric mesenchyme surrounding the UB will generate most of the adult metanephric kidney. Right panel: Nephrogenesis in mammals involves series of morphogenesis processes of the metanephros mesenchyme (MM). Mesenchymal cells surrounding the invading UB in the MM form pretubular aggregates known as renal vesicles (RVs). Each RV exhibits a series of morphological changes to form comma-shaped body and then S-shaped body sequentially. The distal end of the S-shaped body fuses with the UB, which will develop into part of the collecting duct system. (Adapted from: Dressler GR. 2006. The cellular basis of kidney development. Annu Rev Cell Dev Biol. 2006;22:509-29.)

Figure 1.2 Segmentation arrangement of the human nephron. ......................19
The fully developed human nephron displays unique patterning features. From the proximal region to distal end, the nephron tubule is subdivided into the following domains by their physiological functions and morphology: glomerulus (G), proximal convoluted tubule (PCT), proximal straight tubule (PST), descending thin limb (DTL), ascending thin limb (ATL), thick ascending limb (TAL), macula densa (MD), distal convoluted tubule (DCT), and connecting tubule (CNT). (Adapted from: Li Y and Wingert RA. 2013. Clinical and Translational Medicine 2:11 doi:10.1186/2001-1326-2-11)

Figure 1.3 Genetic factors regulating mammalian nephron patterning. ..........20
Proximal-distal patterning is established at as early as the RV stage. Polarization of the RV could be detected by the expression of several genes, including the Notch ligands Dll1, Lfng and Jag1. The Notch signaling pathway is crucial for proximal segmentation establishment,
including formation of the proximal tubule and podocyte. The homeodomain-containing transcription factor Hnf1b promotes proximal and intermediate-medial tubule specification via modulating Notch ligand expression. Intermediate and distal domains are regulated by Brn1, which establishes distal polarity starting from RV stage. At the S-shaped stage, the proximal domain of the MM aggregate establishes the future glomerulus and S1–S3 segments of the proximal tubule. Intermediate segments give rise to the loop of Henle, while the distal domain forms the distal tubule, which fuses to the collecting duct through a connecting segment. (Adapted from: O’Brien LL, McMahon AP. 2013. Progenitor programming in mammalian nephrogenesis. Nephrology (Carlton). (3):177-9. doi: 10.1111/nep.12027.)

Figure 1.4 Segmentation pattern of the nephron is highly conserved among vertebrates. (a) Segmental organization of mammalian metanephric nephron. (b) Dorsal view of the zebrafish embryonic pronephros. At 24 hpf, pronephric segmentation is fully accomplished with eight distinct regions: podocyte (P), neck (N), proximal convoluted tubule (PCT), proximal straight tubule (PST), distal early (DE), corpuscle of Stannius (CS), distal late (DL), pronephric duct (PD), and cloaca (C). (c) Xenopus embryonic pronephros shown in a lateral view. Notably, analysis of gene expressions in analogous segment identities argues conserved nephron patterning in different species, shown by color-coding in each panel. (Adapted from: Wingert RA, Davidson AJ. 2008. The zebrafish pronephros: a model to study nephron segmentation. Kidney Int. 73(10):1120-7. doi: 10.1038/ki.2008.37.)

Figure 1.5 The zebrafish nephrogenesis process and patterning of the pronephros. (a) At the tail-bud stage, the intermediate mesoderm (IM) is a U-shaped structure located between the paraxial mesoderm (PM) and lateral plate mesoderm (LPM). (b) At the 5 somite stage, the IM renal progenitor field is close to the scl/tal1-expressing cells that are developing into the angioblasts and the scl/tal1+/gata1a+ co-expressing progenitors giving rise to primitive blood cells. The renal progenitors could be labeled by pax2a, pax8, and lhx1a. (c) At 24 hpf, the zebrafish pronephros is comprised of two nephrons that consist of eight segments including the podocytes (P), neck (N), proximal convoluted tubule (PCT), proximal straight tubule (PST), distal early (DE), corpuscle of Stannius (CS), distal late (DL), and pronephric duct (PD) and the cloaca (C). (Adapted from: Gerlach GF, Wingert RA. 2012. Kidney organogenesis in the zebrafish: insights into vertebrate nephrogenesis and regeneration. Wiley Interdiscip Rev Dev Biol. 2(5):559-85. doi: 10.1002/wdev.92.)
Figure 1.6 Establishment of pronephric segmentation in the zebrafish embryo…26
(a) The intermediate mesoderm (IM) which develops into the renal progenitor field is located directly adjacent to the paraxial mesoderm (PM). During the time frame of nephrogenesis, the PM secretes retinoic acid (RA) and generates a RA gradient along the IM. RA production is highest in the anterior PM, leading to the hypothesis that the rostral renal progenitor cells are exposed to the highest amount of RA, while distal region of the renal progenitors receive lower dosage of RA. (b-d) At the 5 somite stage, the rostral domain of the renal progenitors express wt1a+, and these cells will develop into podocyte progenitors. Until the 8 somite stage, the rostral and caudal domains have overlapping gene expression field. Then, a central region co-expresses jag2 and mecom starts to emerge. Establishment of each nephron domain is accompanied by complex and dynamic gene expressions that fluctuate throughout the whole process of nephrogenesis. By the 15 somite stage, irx3b expression labels a central domain, while the podocyte progenitors could detected by expression of foxc1a, hey1, lhx1a, mafba, and wt1b. (e) By the 28 somite stage (i.e. 24 hpf), segmentation has been fully accomplished. Subdomains could be defined by the expression of specific solute transporters or transcription factors. (Adapted from: Gerlach GF, Wingert RA. 2012. Kidney organogenesis in the zebrafish: insights into vertebrate nephrogenesis and regeneration. Wiley Interdiscip Rev Dev Biol. 2(5):559-85. doi: 10.1002/wdev.92. )

Figure 3.1 mecom transcripts mark an early caudal domain of the renal progenitors, and the mecom expression domain is dynamic during nephrogenesis………………………………………………………………………63
(A) Schematic depictions of zebrafish pronephros at 24 hpf, shown in lateral view. Enlargement represents segmental organization of the nephron at 24 hpf. Abbreviations: G (glomerulus), N (neck), PCT (proximal convoluted tubule), PST (proximal straight tubule), DE (distal early), CS (corpuscle of Stannius), DL (distal late), PD (pronephric duct), C (cloaca). (B) At the 2 and 3 somite stages, the renal progenitor field was labeled by pax2a (purple) and forming somites were marked by dlc (red). Onset of mecom expression in renal progenitors could be detected at the 3 somite stage, in a caudal domain exclusive to the dlc-expressing rostral domain. Inset shows non-overlapping expression territory of dlc (red) and mecom (purple) at 3 somites in the renal progenitor field. (C) Expression of mecom (purple) and myod1 (red) at various time points between 6 to 14 somite stages in wild types. At 14 somites, the expression domains of solute transporters slc4a4a and slc12a3 indicate premature patterning of the pronephros proximal versus distal segment regions. (D) Upper panel:
genomic structure of zebrafish *mecom202* (dark purple, bottom) and *mecom201 (evi1)* (light purple, top). The *mecom202*'-5' and *mecom202*'-3' riboprobes (orange filled lines) were designed to distinguish *mecom202* and *mecom201* transcripts by targeting the 5' and 3' region exclusively present in *mecom202* transcripts. Lower panels: in 24 hpf wild type embryos, WISH using a full-length *mecom202* probe and *mecom202*-specific probes showed that *mecom* expression was restricted to the DL and PD regions of the pronephros.

Figure 3.2 *mecom* morphants exhibit pericardial edema and symptoms of renal failure. .................................................................65

(A) Schematic indicates targeting sites of *mecom* morpholinos (blue lines) blocking splice sites of *mecom* mRNA between exon 3 and 4. Primers (red arrowheads) were designed to amplify the 180 bp linkage region between properly spliced exon 3 and 4. Right panels: cDNA isolated from wild type embryos showed the 180 bp band indicating proper splicing to remove intron 3. In contrast, amplification of this product was abrogated in *mecom* morphants. *eef1a1l1* was used as internal control. (B) *mecom* morphants showed gross developmental defects when compared with mismatch controls. Pericardial edema could be visualized at 30 hpf in *mecom* morphants indicating fluid accumulation (left panels). At 50 hpf, *mecom* morphants displayed severe pericardial edema and body curvature (right panels). (C) Fluorescent 40 kDa dextran was injected into the somite of wild types or *mecom* morphants at 48 hpf. A failure of renal clearance, indicated by dextran accumulation, was observed in the yolk and edema of *mecom* morphants at 72 and 96 hpf.

Figure 3.3 *mecom* knockdown leads to an expanded PST segment. ...............66

(A) At 24 hpf, WISH indicates proximal segments marked by *slc4a4a* elaborated in *mecom* morphants. Within proximal domains, PCT labeled by *slc20a1a* was not affected comparing with wild type embryos. Knockdown of *mecom* induced a 3-somite expansion in *trpm7*-expressing PST in morphant pronephros at 24 hpf, which could be rescued by co-injecting *mecom* cRNA along with *mecom* morpholinos. (B) Schematic summary of proximal segment organization in wild type, *mecom* morphant, and *mecom* rescued embryos, with PST alterations highlighted in yellow. Abbreviations: P (podocytes), PCT (proximal convoluted tubule), PST (proximal straight tubule), DE (distal early), DL (distal late), PD (pronephric duct).

Figure 3.4 *mecom* knockdown leads to the formation of a reduced DL segment..67

(A) WISH using distal segment markers showed a 3-somite reduction in DL region by 24 hpf. The distal segments and pronephros ducts
were labeled by *clenk*. Expression of *slc12a1* and *slc12a3* marked DE and DL respectively. Pronephros segment boundaries were evaluated relative to the somites, which were shown by *smyhc1* expression (red). The reduced DL could be ameliorated by co-injection of *mecom* morpholino and *mecom* cRNA. (B) Schematic depiction of distal segment alterations, with DL domains highlighted in orchid. Abbreviations: P (podocytes), PCT (proximal convoluted tubule), PST (proximal straight tubule), DE (distal early), DL (distal late), PD (pronephric duct).

**Figure 3.5** *mecom* and RA have opposing roles in PST and DL formation during proximodistal segmentation of the pronephros. .............................69
(A) Wild type embryos or *mecom* morphants were incubated with 1x10⁻⁷M RA, 1.6x10⁻⁵M DEAB or DMSO. WISH using the PST marker *trpm7* and DL marker *slc12a3* showed that exogenous RA resulted in a more severe segmental phenotype in the *mecom* morphant pronephros, with further expanded PST and reduced DL. DEAB treatment partially rescued the segmentation phenotype in *mecom* morphants. (B) Schematic summary of segmentation changes in wild type embryos, *mecom* morphants, and wild type embryos or morphants treated with RA or DEAB. Yellow and blue boxes highlight the PST and DL segments, respectively.

**Figure 3.6** RA negatively regulates *mecom*, which enables MCC formation……71
(A) Wild type embryos treated with 1x10⁻⁷M RA, 1.6x10⁻⁵M DEAB or DMSO. Analysis of *mecom* transcripts using WISH shows that RA treatment diminishes the *mecom* domain while abrogation of RA signaling expands the *mecom* domain. (B) MCCs were labeled by *odf3b* in wild types and *mecom* morphants treated with RA or DEAB. *mecom* knockdown resulted in a caudal shift of MCC domain in the DL and PD regions, and this effect was partially rescued by treating morphant embryos with DEAB. Notably, wild type embryos treated with DEAB had abolished MCC formation, while RA induced ectopic MCC formation in the distal region of the pronephros.

**Figure 3.7** *mecom* acts upstream of Notch signaling to modulating MCC differentiation and regulates the MCC domain. ..............................73
(A) Wild type embryos treated with 100 µM DAPT showed a significant increase of MCC number without ectopic MCC formation. *mecom* knockdown led to a caudal expansion of MCC in the DL and PD, and exhibited increased MCC density compared to wild types. A similar condensed MCC arrangement could also be seen in *mecom* morphants treated with DAPT. The overexpression of Notch1a resulted in decreased MCCs in heatshock induced Tg(hsp70:gal4; uas:notch1a-intra) embryos. Ectopic MCC formation associated with...
mecom knockdown was abolished by Notch signaling activation, though the domain of MCCs was still expanded. (B) Differentiated MCCs at 24 hpf under 10× magnification in a single nephron from wild types, wild types treated with DAPT, mecom morphants and morphants treated with DAPT, and finally wild types and mecom morphants with NICD overexpression. Note MCCs displayed a condensed organization/cluster pattern in DAPT-treated wild type and mecom morphant pronephros, while DAPT treatment in mecom morphant failed to induce further MCC density. Arrows indicate large MCC aggregates observed in DAPT-treated wild types, mecom morphants, and DAPT-treated mecom morphants, which were absent from the wild type pronephros. For each experiment, at least 20 embryos were examined. (C) Quantification of MCC density in wild types, wild type embryos treated with DAPT, mecom morphants, and mecom morphants treated with DAPT. Student t test revealed significant increase of MCC density in DAPT treated wild types and mecom morphants relative to untreated wild types (***(p=0.0005). Alterations of MCC density between mecom morphants and morphants treated with DAPT did not reach statistical significance. For each experiment, at least 20 embryos were examined.

Figure 3.8 Model of mecom function during nephrogenesis. …………………75
(A) Role(s) of mecom during pronephros proximodistal segmentation. At early somitogenesis, forming somites generate a gradient of RA, which diffuses along the IM and modulates proximodistal patterning of the renal progenitors by promoting proximal segmentation and restricting distal fates. Initially expressed in the caudal domain of the renal progenitor field, mecom is negatively regulated by RA signaling and executes a contrary role to that of RA by favoring distal tubule formation and/or limiting proximal segmentation. Interplays between RA and mecom, as well as other transcription factors and signaling pathways precisely define the patterning of the renal progenitors during nephrogenesis, which develops into a pronephros with at least eight distinct segments by 24 hpf. mecom is specifically essential to promote the DL and possibly to restrict the PST. (B) RA, mecom, and Notch activities coordinate MCC formation. RA signaling is required for MCCs to develop, and one modality is that RA acts to negatively regulate mecom expression. RA likely has other targets that promote MCC formation, which remain unidentified. To further refine the balance between MCC and transporting epithelia fate choice, mecom represses ectopic MCC formation by promoting Notch signaling. Notch is the penultimate signal that inhibits MCC induction via lateral inhibition. By 24 hpf, MCCs are dispersed along the pronephric tubules in PST, DE, and DL regions adopting a proper ‘salt-and-pepper” fashion. Abbreviations: RA (retinoic acid), PCT (proximal convoluted tubule), PST (proximal straight tubule), MCC
Figure 4.1 tbx2 transcripts are specifically expressed in a caudal domain of the renal progenitor field during nephrogenesis, and knockdown of tbx2a and/or tbx2b led to disrupted function of the pronephros. .........95 (A) Schematic depiction of zebrafish pronephric segmentation at 24 hours post fertilization (hpf). Segment abbreviations: P (podocytes), N (neck), PCT (proximal convoluted tubule), PST (proximal straight tubule), DE (distal early tubule), CS (corpuscle of Stannius), and DL (distal late tubule). (B) Using whole mount in situ hybridization, expression of the tbx2b transcript could be detected in the distal region of the pronephros starting from the 15 somite stage (ss) (arrow head). At 24 hpf, tbx2b expression is restricted to the DL and the PD domains of the pronephros. At 24 hpf, expression of tbx2a in the pronephros were detected in the distal and duct region, though with lower expression level compared to tbx2b. (B, bottom panel) (C) Morpholino knockdown of tbx2a, tbx2b or tbx2a/b induced cardiac edema at 48 hpf (arrow head). Morphant embryos also displayed malformation in the brain and body curvature.

Figure 4.2 tbx2 knockdown leads to slight expansion in the PCT segment in morphant pronephros. .......................................................... 96 Upper panel: At 24 hours post fertilization (hpf), whole mount in situ hybridization was used to label segmental domains in wild types and tbx morphants. Proximal segments marked by slc4a4a elaborated in tbx2 morphants. Within proximal domains, morpholino knockdown of tbx2a, tbx2b, or tbx2a/b induced a consistent 1-somite expansion in the slc20a1a-expressing PCT segment in morphant pronephros at 24 hpf. The PST segment, labeled by transcripts encoding trpm7, was not affected compared with wild type embryos. Representative results are based on examination of at least 20 embryos per marker. Morphant pronephros structures showed consistent segmentation disruption based on somite counting of at least 5 embryos per marker. Lower panel: Schematic summary of proximal segment organization in wild type and tbx2 morphants, with PCT alterations highlighted in orange and PST in yellow. Abbreviations: PCT (proximal convoluted tubule), and PST (proximal straight tubule).

Figure 4.3 tbx2 knockdown leads to the formation of a significantly reduced size of the DL tubule segment. ......................................................... 97 Upper panel: Whole mount in situ hybridization using distal segment markers showed a 2-somite reduction in the DL region by 24 hpf. The distal segments and pronephros ducts were labeled by clenk. Expression of slc12a1 and slc12a3 marked the DE and DL, respectively. Pronephros segment boundaries were evaluated relative
to the somites, which were shown by smyhc1 expression (red). Of note, an approximately one-somite gap region could be detected between the DE and DL in the morphant pronephros, which was not present in the pronephros of wild type embryos. Lower panel: Schematic depiction of distal segment alterations, with DL domains highlighted in orchid. Abbreviations: DE (distal early tubule), DL (distal late tubule), and PD (pronephric duct).

Figure 4.4 tbx2 genes regulate corpuscles of Stannius formation by inhibiting the number of stc1+ cells during zebrafish nephrogenesis. The corpuscles of Stannius were labeled by whole mount in situ hybridization to detect transcripts encoding stc1 at the 24 hours post fertilization (hpf) stage. In wild type embryos, each corpuscle is comprised of about 4-6 stc1+ cells per nephron (upper panels), while knocking down tbx2 transcripts induced a significant up-regulation of the stc1+ cell number in the corpuscles (lower 3 panels). Notably, knocking down tbx2b and double knockdown of tbx2a/b led to most severe phenotype while disruption of tbx2a only showed minor increase of the CS, suggesting prevailing role of tbx2b in regulation of the CS development by repressing stc1+ cell differentiation. Quantification of the corpuscle cell number is shown in the chart on bottom. For each experiment, at least 15 embryos were examined. (* P = 0.072, ** P = 0.000)

Figure 4.5 tbx2b acts downstream of Notch to inhibit CS differentiation. At 24 hpf, whole mount in situ hybridization identified expression of her9, a Notch signaling pathway factor, in the CS of zebrafish pronephros, suggesting a possible role for Notch signaling in CS development (upper left panel). Tg(hsp70::Gal4; UAS::NICD) embryos and siblings were heat-shocked at the 15 somite stage (ss) and then raised to 24 hours post fertilization (hpf). Post heat-shock induction, Tg(hsp70::Gal4; UAS::NICD) embryos exhibited a dramatic reduction of the CS, while siblings showed normal CS clusters. To block Notch signaling, wild type embryos were incubated in 100 µM DAPT/E3 solution from the 15 ss to the 24 hpf stage. DAPT treatment resulted in significant elevation in the corpuscle (right panels). (B) Heat-shocked Tg(hsp70::Gal4; UAS::NICD) tbx2b morphant exhibited significant expansion in the CS sized shown by stc1 expression. To quantify changes in the CS, stc1+ cells were counted for each experiment (left chart, P=0.000). Representative images were based on thorough examination of at least 20 embryos per treatment.

Figure 4.6 Notch promotes proximal tubule differentiation and represses distal fates prior to segmentation establishment.
(A) At 24 hpf, whole mount in situ hybridization identified expression of her9, a Notch signaling pathway factor, in the CS of zebrafish pronephros, suggesting a possible role for Notch signaling in CS development (upper left panel). Tg(hsp70::Gal4; UAS::NICD) embryos and siblings were heat-shocked at the 15 somite stage (ss) and then raised to 24 hours post fertilization (hpf). Post heat-shock induction, Tg(hsp70::Gal4; UAS::NICD) embryos exhibited a dramatic reduction of the CS, while siblings showed normal CS clusters. To block Notch signaling, wild type embryos were incubated in 100 µM DAPT/E3 solution from the 15 ss to the 24 hpf stage. DAPT treatment resulted in significant elevation in the corpuscle (right panels). (B) Heat-shocked Tg(hsp70::Gal4; UAS::NICD) tbx2b morphant exhibited significant expansion in the CS sized shown by stc1 expression. To quantify changes in the CS, stc1+ cells were counted for each experiment (left chart, P=0.000). Representative images were based on thorough examination of at least 20 embryos per treatment.

Figure 4.7 Working model. .................................................................103

(A) During zebrafish nephrogenesis, our data proposes a model where the tbx2 transcription factors regulate patterning of the pronephros by restricting PCT domain and promoting DL segment. Meanwhile, a new role of Notch signaling pathway is revealed in segmentation establishment of the zebrafish pronephros, where it promotes proximal fates and represses distal segmentation. (B) tbx2 transcription factors balance the CS size by limiting the number of stc1+ cells downstream of Notch signaling pathway. However, it remains unclear if there was direct epistatic relationship between these two factors. Notch signaling pathway also acts as a key modulator repressing CS differentiation shown by abolished CS formation in heat-shocked Tg(hsp70::Gal4; UAS::NICD) embryos. However, it remains unclear if other signaling factors were also involved downstream of Notch in regulating CS development.
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CHAPTER 1:
INTRODUCTION

1.1 Vertebrate kidney organogenesis and birth defects – an overview

One of the major causes of childhood morbidity involves the abnormal organogenesis of the kidney and urinary tract (Nakanishi and Yoshikawa, 2003). Termed congenital anomalies of the kidney and urinary tract (CAKUT), these disorders affect 1 in 500 births and account for ~31% of children with pediatric end-stage renal disease in the United States (North American Pediatric Renal Trials and Collaborative Studies, 2008, NAPRTCS Annual report; Daneman A and Alton DJ, 1991; Nakanishi and Yoshikawa, 2003). Further, patients with CAKUT are more inclined to develop conditions such as hypertension and cardiovascular disease later in life (Song and Yosypiv, 2011). CAKUT involves a broad spectrum of renal tract malformation including abnormalities in the kidney, collecting system, bladder and/or urethra (Nakanishi and Yoshikawa et al.). The leading causes of CAKUT remain poorly understood, but have been shown to include disruptions in the complex interplay between genetic and environmental factors (Nakanishi and Yoshikawa, 2003). Hence, integrated knowledge of kidney organogenesis on a molecular level is of great value to enable improved
understanding of the onset of CAKUT processes and the prevention of congenital renal system anomalies.

1.1.1 The stages of kidney development in vertebrates

Vertebrate kidney organogenesis proceeds through the formation and regression of as many as three successive structures known as the pronephros, mesonephros and metanephros (Dressler, 2006). These kidney structures all arise from the intermediate mesoderm (IM) located between the somatic mesoderm and the lateral plate mesoderm. During mammalian kidney ontogeny, the pronephric ducts, also known as the primary nephric ducts, emerge from the IM. These nephric ducts are bilateral epithelial tubular structures that grow progressively, ultimately extending caudally along the embryonic trunk until they reach the cloaca (Dressler, 2006). As organogenesis progresses, a series of epithelial tubes form from mesenchymal cells adjacent to the pronephric duct, with the most initial tubes that develop anterior to the nephric duct known as the pronephros (Figure 1.1, left panel) (Dressler, 2006). Whereas the pronephros is a vestigial organ in mammals, it serves as the embryonic excretory organ in lower vertebrates such as fishes and frogs (Dressler, 2006, described later). The mesonephros forms posteriorly to the pronephros, and contains complete nephron structures that include glomeruli and proximal tubule, for example. Depending on the vertebrate species, the mesonephros functions transiently in fetal life or serves as the permanent renal organ. If the mesonephros is transient, a third structure, the metanephros, is subsequently formed and functions instead as the definitive adult
kidney in many species including mammals (Dressler, 2006). The metanephros arises at the caudal end of the pronephric duct when an outgrowth of the pronephric duct, known as ureteric bud (UB) invades the surrounding metanephric mesenchyme (MM) (Figure 1.1, right panel). As the UB undergoes branching morphogenesis in the MM, it induces a mesenchymal-to-epithelial transition (MET) in cell aggregates adjacent to the ureteric bud tips (Little and McMahon, 2012). Once the mesenchymal cells in the MM aggregate undergo MET, they form a polarized structure named the renal vesicle (RV) (Dressler, 2006). The RV develops sequentially into a comma-shaped body, and then an S-shaped body (Dressler, 2006). At this stage, the S-shaped body exhibits a patterned proximal-distal axis that represents the future proximal and distal segments of mature nephron tubule (Mugford et al., 2009; Georgas et al., 2009; Brunskill et al., 2014). The distal end of the S-shaped body fuses with the UB to form a continuous tubule, while the proximal end of the S-shaped body develops into a glomerulus - a network of capillaries that performs the first step of filtering blood from the Bowman's capsule (Dressler, 2006). UB branching morphogenesis continues to induce new nephrons along the radial axis of the kidney as renal development ensues. In humans, this series of events leads to the formation of kidneys that contain between 800,000 and 1.5 million nephrons (Bertram et al., 2011).
1.1.2 The nephron – the basic functional unit of the kidney

The kidney has a number of essential physiological tasks: it filters waste metabolites from the circulation, maintains proper blood pressure, controls electrolyte homeostasis and balances pH (O’Brien and McMahon, 2013). The basic functional and structural unit of the kidney, known as the nephron, carries out most of these functions (Figure 1.2). As aforementioned, kidney organogenesis involves highly elaborated branching of the UB along the radial axis of the metanephric mesenchyme induces a complex network of the collecting duct system, with thousands of nephrons situated in an intricate, arborized three-dimensional organization (Dressler, 2006). Filtration proceeds from the podocyte located at the proximal end of the nephron. The filtrate solution subsequently passes through the nephron tubule, which consists of proximal and distal segments that are specialized to conduct discrete physiological roles. For example, the human nephron tubule consists of three major domains: the proximal tubule, the intermediate tubule (also known as the loop of Henle) and the distal tubule (Figure 1.2). These three major areas can be further subdivided into subdomains by gene expression profile and morphological appearance (O’Brien and McMahon, 2013). Specifically, the human nephron tubule is characterized by the following regions: glomerulus, proximal convoluted tubule, proximal straight tubule, thin limb, thick ascending limb, macula densa, distal convoluted tubule, connecting tubule, and collecting duct (Jacobson, 1981). Functionally, each segment region possesses specialized physiological roles in reabsorbing solutes and modifying filtrates, due to the expression of particular groups of solute
transporters and ion channels present on the membrane of epithelial cells of the nephron (Thiagarajan et al., 2011). At its terminus, each nephron tubule is then joined with the collecting duct system where the final urine solution is collected and discharged through the ureter (O’Brien and McMahon, 2013).

1.1.3 Patterning of the mammalian nephron tubule

Recent studies using the mouse animal model have provided considerable insights into the molecular changes that accompany nephron development (Dressler, 2006). Patterning of the nephron tubule progenitors could firstly be observed when MM aggregates adjacent to the branched UB tips transition to become the epithelial RV (Dressler, 2006). RV progenitors undergo MET and morphogenesis to develop into comma- and S-shaped bodies sequentially (Dressler, 2006). At the S-shaped body stage, renal progenitors are organized in a polarized pattern where pre-nephron tubular domains are orientated along a proximal-distal axis representing the future proximal tubule and distal tubule (O’Brien and McMahon, 2013). This rapid remodeling of RV progenitors is promoted by signaling factors excreted from the UB tip, though the identity of these is an active area of investigation (Costantini, 2012). The current understanding of the nephrogenesis process argues that canonical Wnt signaling pathway is crucial for induction of RV formation and patterning of the pretubular structure (Carroll and Das, 2011; Kuure et al., 2007; Park et al., 2007; Park et al., 2012). Expression of the Wnt signaling factor in the distal RV suggests that Wnt could play essential roles in regulation of RV polarization, while evidence
suggests that proximal segmentation is induced by the Notch signaling pathway (O’Brien and McMahon, 2013).

Despite these insights, it remains unclear how the proximal-distal polarity is initiated within the RV and the pretubular aggregates. However, it is believed that intrinsic interactions within the polarized RV are possible playing the major role in the progress of nephron tubular patterning (O’Brien and McMahon, 2013). For example, the Wnt signaling component Wnt4 shows regionalized expression patterns in the distal domain of RV, suggesting that a Wnt signaling gradient acts as one contributory factor to the initiation of early RV polarity (Figure 1.3) (Mugford et al., 2009).

Evidence of the role for Notch signaling in promoting proximal fates during nephron segmentation came from a series of elegant loss-of-function experiments showing compromised proximal structure as Notch signaling is blocked with the gamma secretase inhibitor N-S-phenyl-glycine-t-butyl ester (DAPT) (Cheng et al. 2003; Wang et al., 2003). Specifically, Notch2 seems to play essential role on proximal specification, as Notch2 deficient kidneys only processed distal epithelial structures without proximal domains, demonstrating that Notch2 is indispensable for proximal segmentation (Cheng et al., 2003; Cheng et al. 2007). Apart from the Notch signaling pathway, the transcription factor Hnf1b is also involved in proximal segmentation of the nephron. Malfunction of Hnf1b led to nephrons missing the proximal tubule and loop of Henle, with glomeruli directly attached to the collecting duct by a short tubule (Heliot et al., 2013; Massa et al., 2013).
Opposing the role of Notch in specification of the proximal domain, distal segmentation is regulated by the transcription factors Brn1 and Lhx1 (Nakai et al., 2003; Georgas et al., 2008; Kobayashi et al., 2008). At the RV stage, Lhx1 is expressed in the distal region of RV (Georgas et al., 2008; Kobayashi et al., 2008). Lhx1-deficient RV structures showed abolished distal polarity, and the S-shaped body subsequently failed to form (Kobayashi et al., 2008). Abrogation of Brn1 expression induced nephrons with properly formed glomerulus and proximal tubule segments. However, Brn1-deficient nephrons had defects in the formation of distal tubule segments, in which the macula densa was compromised, and these phenotypes were accompanied with short and deformed loops of Henle (Georgas et al., 2008).

In summary, despite the signaling pathway and transcription factors described above, polarization and regionalization of the RV clearly involves complex gene networking and molecular mechanisms that are only beginning to be understood. Of note, it remains unclear about how terminal patterning of the proximal and distal identity is accomplished. Better understanding of the molecular mechanisms behind nephron segmentation establishment will facilitate knowledge of kidney organogenesis in the context of organ patterning and physiological functions.
1.2 Zebrafish as a promising model to study nephrogenesis

In recent years, the zebrafish *Danio rerio* has emerged as a promising model for developmental biology and vertebrate organogenesis (Dressler, 2006; Gerlach and Wingert, 2013). Approaches like large-scale forward and reverse genetic screens, genomics, and chemical genetics have been formulated for use with zebrafish, which has facilitated discoveries of a broad array of genetic factors that are essential for vertebrate organogenesis (Gerlach and Wingert, 2013). The *in vitro* fertilization and transparency of the zebrafish embryo also enables examination of organ development and function, facilitated by gene expression, transgenesis and various physiological methods (Gerlach and Wingert, 2013).

With respect to kidney ontogeny, the zebrafish embryonic pronephros has been established as a powerful system to study renal development (Drummond, 2000; Wingert and Davidson, 2008). Despite the fact that kidney types between vertebrates exhibit different macroscopic features as well as complexity, it has been shown that molecular mechanisms controlling kidney organogenesis are highly conserved among species (Wingert and Davidson, 2008). For example, studies of have demonstrated the importance of the transcription factors Wt1 and Pax2 for differentiation of renal progenitors in both pronephros and metanephros kidney types (Dressler, 2006). Both the amphibian pronephros and the metanephros in higher vertebrates like mammals are composed of nephrons as the basic functional unit, while segmental organization of the nephron is highly conserved among various vertebrate species (Figure 1.4). Therefore, using the
pronephros as a model to study kidney ontogeny provides an opportunity to identify essential factors for mammalian nephrogenesis. In particular, the anatomical simplicity of the zebrafish pronephros (discussed in the following section) provides an alternative to research using the more intricate kidney organs in other species. Historically, the complexity of mammalian nephrogenesis and kidney anatomy have led to gaps in understanding how nephron tubules are patterned into morphologically and physiologically distinct segments (Constantini and Kopan, 2010). To date, using zebrafish pronephros as a model system, a growing number of studies have discovered a host of novel factors that are crucial for renal development, especially for segmentation establishment of the nephron.

1.2.1 Zebrafish embryonic pronephros – organogenesis and structure

During embryonic life, zebrafish embryos form a pronephros that serves as the embryonic excretory organ over several weeks of larval life. Each pronephros is comprised of two nephrons that originate from bilateral stripes of IM, from which the renal progenitors are generated (Figure 1.5) (Drummond, 2003). During gastrulation, the IM renal progenitor field is organized as a U-shaped structure situated between neighboring stripes of the paraxial mesoderm (somatic mesoderm) and the lateral plate mesoderm, respectively (Krauss et al., 1991; Picker et al., 2002; Toyama et al., 1997; Swanhart et al., 2010). As development progresses, during early somitogenesis, the U-shaped renal progenitor territory further elongates through convergence-extension movements; next, by the 20-22 somite stage, the mesenchymal renal precursors undergo
mesenchymal-to-epithelial transition to form a pair of nephron accompanied by tubulogenesis and morphological rearrangements (Gerlach and Wingert, 2013; Drummond, 2003). Regional patterning of the nephron precursors specifies the renal progenitor field into spatially distinct segments (Gerlach and Wingert, 2013; Wingert et al., 2007). By the 28 somite stage, segmentation specification is fully established and eight segments can be distinguished. These segments are: the glomerulus (G), neck (N), proximal convoluted tubule (PCT), proximal straight tubule (PST), distal early (DE), corpuscle of Stannius (CS), distal late (DL), and pronephric duct (PD). Based on genes expression encoding solute transporter proteins that carry out exquisite functions of each segment (Figure 1.5) (Gerlach and Wingert, 2013; Wingert et al., 2007; Wingert and Davidson, 2008).

In addition to this nephron segment pattern, the zebrafish pronephros is comprised of two types of ciliated epithelial cells: single ciliated cells that exhibit the morphology of typical transporting epithelial cells, and multiciliated cells (MCCs) which function specifically for fluid propulsion in the pronephros to facilitate filtrate clearance (Liu et al., 2007; Ma and Jiang, 2007). Proper function of motile cilia in zebrafish pronephros is indispensable for a high rate fluid discharge, as deficiencies in cilia function could lead to cyst formation in the proximal pronephric tubule (Liu et al., 2007; Ma and Jiang, 2007; Kramer-Zucker et al., 2005). MCCs display distinct distribution within the pronephros epithelia – the majority of the tubule is comprised of transporting epithelial cells that possess a single cilium, while only a subset of the tubule cells is multiciliated (Liu et al., 2007; Ma and Jiang, 2007). MCCs could have up to 20 cilia per cell that are
extended from their apical surface into the lumen of the pronephros (Liu et al., 2007; Ma and Jiang, 2007). Examination of ciliogenesis genes shows that, by 34 hpf, MCCs are scattered in a spotted ‘salt-and-pepper’ pattern in the proximal and anterior-most distal region of the pronephros (Liu et al., 2007; Ma and Jiang, 2007). MCCs are surrounded by transporting epithelia expressing the cation transporter *trpm7* in the PST segment and the sodium-sulfate co-transporter *slc13a1* in the DE segment (Liu et al., 2007; Ma and Jiang, 2007). Previous research has demonstrated that the Notch signaling pathway is crucial for patterning of the MCCs via Jagged2-Notch interactions, proposing that the lateral inhibition mechanism plays a role in their differentiation (Liu et al., 2007; Ma and Jiang, 2007).

Another structure that is specific to the zebrafish pronephros is the corpuscles of Stannius (CS) (Garrett, 1942; Krishnamurthy, 1976; Kaneko et al., 1992). The CS is composed of a small group of cells that are initially situated between the DE and DL in the pronephros (Elizondo et al., 2005). In teleost fish, the CS functions as an endocrine organ that regulates calcium and phosphorus homeostasis (Elizondo, et al., 2005; Krishnamurthy, 1976; Kaneko et al., 1992). As nephrogenesis progresses, CS precursors emerge from the renal progenitor field and undergo morphogenesis rearrangements that situate them into bilateral lobes located immediately dorsal to the distal tubules of the pronephros (Camp, et al., 2003; Elizondo, et al., 2005; Wingert, et al., 2007). To date, it remains unclear what molecular mechanisms control development of the CS during nephrogenesis. However, a recent finding of our group has identified that the transcription factor
**single minded family bHLH transcription factor 1a (sim1a)** is crucial for determining the CS fate, as CS differentiation is totally abrogated in *sim1a* morphant embryos (Cheng and Wingert, 2015). However, little else is known about the identity or function of the other genetic factors that are required to regulate CS formation and patterning.

### 1.2.2 Important genetic factors identified in pronephros patterning

Proximal-distal patterning of the pronephros involves regional differentiation and specification of renal precursors during the process of nephrogenesis. Patterning of the pronephros requires dynamic and accurate regulation of signaling pathways and genetic networks. Using the zebrafish pronephros as a model to study nephron patterning, several key factors and pathways have been identified that act to direct the proximal-distal segmentation formation of the nephron (Gerlach and Wingert, 2013).

For example, the diffusible morphogen retinoic acid (RA) has been discovered to be essential for proximal-distal regionalization of the renal progenitor field (Wingert et al., 2007; Wingert and Davidson, 2008). RA has been well studied as a morphogen, which is a molecule that elicits dosage-specific effects on target tissues, during various organogenesis processes across metazoans (Duester, 2013). Proper effects of RA require elegant control of its accumulation and degradation in target tissues (Duester, 2013). Previous studies have shown that the rostral-caudal regionalization of the renal progenitor field is dependent on RA synthesis in the embryo, which has been proposed to affect the renal
progenitors due to a gradient distribution of RA along the proximal-distal axis of the embryonic trunk (Figure 1.6). Evidence suggested that, during the time frame of nephrogenesis, the anterior-most paraxial mesoderm (PM) secretes RA and generates a RA gradient along the RA-responsive IM that induces renal precursor identity specification (Wingert and Davidson, 2008). Zebrafish embryos deficient in RA biosynthesis, due to mutations in aldehyde dehydrogenase 1 family, member A2 (aldh1a2), fail to develop podocyte and proximal segments (Wingert et al., 2007). Supporting this, embryos treated with diethylaminobenzaldehyde (DEAB), which abrogates the activity of aldh RA synthesizing enzymes also induced the same distalized pronephros phenotype (Wingert et al., 2007). In contrast, exogenous exposure to RA caused the formation of a proximalized pronephros with expanded proximal segments. These results propose a model that the proper level of RA is necessary to induce proximal fates as well as to inhibit distal identities in renal progenitors during nephrogenesis process.

While RA functions as key modulator of pronephros specification, patterning of each specific tubule and duct segment fate in the zebrafish pronephros requires additional dynamic and complex interactions of transcription factors and signaling pathways (Gerlach and Wingert, 2013). Descriptive studies using whole mount in situ hybridization (WISH) to map the expression domains of various renal genes have suggested the existence of a transcription factor code associated with the various segmental fates (Wingert, et al., 2007; Wingert and Davidson, 2008). Namely, the initial regionalization of the renal progenitor territory could be identified starting from as early as the 2-3 somite stage (Li et
al., 2014). Through the 6-8 somite stage, the renal progenitor field remains subdivided into two distinct and mutually exclusive territories: a rostral domain, characterized by expression of the Notch ligand genes delta-c (dlc) and jagged-2 (jag2), and a caudal domain marked by expression of the zinc finger transcription factor mecom (Wingert et al., 2007). Starting from the 8 somite stage, the rostral jag2 domain expands caudally and overlaps with the mecom – expressing domain (Wingert et al., 2007). At the 15 somite stage, a central domain marked by expression of irx3b transcripts emerges, while other transcription factors such as pou3f3a, pou3f3b, emx1, and gata3 are expressed in the caudal domain of the renal progenitor field (Wingert et al., 2007). As such, progressive refinement of segmental boundaries shown by active expression of nephrogenesis factors suggests that patterning establishment of the pronephros occurs at as early as 15 somite stage, while further definition of each segment follows at later stages (Figure 1.6).

Despite these observations of a ‘transcription factor code’ associated with pronephros development in the zebrafish, and the conservation of many of these domains during nephrogenesis in other species (Dressler, 2006), the functional assessment of each gene or combinations of these genes has not been completed. Thus to date, questions remain on the genetic network coordinating nephron segmentation and specifying each renal epithelial cell type.

There have been some loss-of-function experiments in zebrafish and Xenopus models which have revealed several molecular mechanisms underlying formation of individual pronephros domains. For instance, during Xenopus
nephrogenesis, the homeobox transcription factor *Ir3*, a member of the *Iroquois* (*Ir*) gene family, functions as a master modulator of intermediate tubule differentiation of the tadpole pronephros (Reggiani et al., 2007). Similarly in zebrafish, knocking down the corresponding orthologue *iroquois homeobox 3b* (*irx3b*) abolished DE segment formation in distal tubule cells, suggesting that *irx3b* is required for DE specification (Wingert et al., 2007). Supporting the idea that RA acts as a key upstream regulator of pronephros patterning, the *irx3*-expressing domain in *aldhla2* mutants (i.e. RA deficient embryos) was shifted, correlating with the altered proximal-distal patterning of the DE segment in these embryos (Wingert and Davidson, 2008).

More recently, work from our group has identified the transcription factor *sim1a* as a crucial factor negotiating the PCT/PST boundary within the proximal domain of the zebrafish pronephros (Cheng and Wingert, 2015). Morpholino knockdown of *sim1a* induced expanded the PCT and abrogated formation of both the PST and CS. Conversely, overexpression of *sim1a* led to a reduced PCT segment while modestly expanding the PST and CS (Cheng and Wingert, 2015). Moreover, *sim1a* expression seemed to be regulated downstream of RA: exogenous RA induced a caudal shift of the *sim1a* domain, while blocking RA synthesis led to a rostral expansion of the *sim1a*-expressing domain. Supporting a role of RA in modulating *sim1a* expression, a consensus retinoic acid response element (RARE) sequence (Bastien and Rochette-Egly, 2004) was identified in the putative promoter region of *sim1a* through sequence analysis.
1.3 Summary and rationale

Taken together, there have been many exciting advances in the identification of essential nephrogenesis patterning factors in the last several years. The zebrafish is an advantageous model for such work, due to its genetic tractability as well as the conserved but architecturally simple anatomy of the pronephros. Thus, this thesis was undertaken to ascertain the functional roles of several transcription factors expressed in the renal progenitors, namely *mecom* as well as the *tbx2a/2b* genes, and to establish their relationship with other signaling factors like RA and Notch. In addition to this research, future loss- and gain-of-function analysis for other transcription factors/signaling pathways is needed to unveil the molecular network mediating patterning of the nephron tubule.
Figure 1.1 The nephrogenesis process in mammalian models. **Left panel:** The developing mouse intermediate mesoderm at approximately E10.5. At the anterior pronephric duct, the pronephros quickly forms and degenerates. Posterior to the pronephros, the primary nephric duct connects to a series of well-developed mesonephric tubules, which function transiently as the embryonic kidney organ. The most posterior region of the pronephric duct exhibits a single ureteric bud (UB). The metanephric mesenchyme surrounding the UB will generate most of the adult metanephric kidney. **Right panel:** Nephrogenesis in mammals involves series of morphogenesis processes of the metanephros mesenchyme (MM). Mesenchymal cells surrounding the invading UB in the MM form pretubular aggregates known as renal vesicles (RVs). Each RV exhibits a series of morphological changes to form comma-shaped body and then S-shaped body sequentially. The distal end of the S-shaped body fuses with the UB, which will develop into part of the collecting duct system. (Adapted from: Dressler GR. 2006. The cellular basis of kidney development. Annu Rev Cell Dev Biol. 2006;22:509-29.)
Figure 1.2 Segmentation arrangement of the human nephron. The fully developed human nephron displays unique patterning features. From the proximal region to the distal end, the nephron tubule is subdivided into the following domains by their physiological functions and morphology: glomerulus (G), proximal convoluted tubule (PCT), proximal straight tubule (PST), descending thin limb (DTL), ascending thin limb (ATL), thick ascending limb (TAL), macula densa (MD), distal convoluted tubule (DCT), and connecting tubule (CNT). (Adapted from: Li Y and Wingert RA. 2013. Clinical and Translational Medicine 2:11 doi:10.1186/2001-1326-2-11)
Figure 1.3 Genetic factors regulating mammalian nephron patterning. Proximal-distal patterning is established at as early as the RV stage. Polarization of the RV could be detected by the expression of several genes, including the Notch ligands Dll1, Lfng and Jag1. The Notch signaling pathway is crucial for proximal segmentation establishment, including formation of the proximal tubule and podocytes. At the glomerulus, the homeodomain-containing transcription factor Hnf1b promotes proximal and intermediate-medial tubule specification via modulating Notch ligand expression. Intermediate and distal domains are regulated by Brn1, which establishes distal polarity starting from RV stage. At the S-shaped stage, the proximal domain of the MM aggregate establishes the future glomerulus and S1–S3 segments of the proximal tubule. Intermediate segments give rise to the loop of Henle, while the distal domain forms the distal tubule, which fuses to the collecting duct through a connecting segment. (Adapted from: O'Brien LL, McMahon AP. 2013. Progenitor programming in mammalian nephrogenesis. Nephrology (Carlton). (3):177-9. doi: 10.1111/nep.12027.)
Figure 1.4 Segmentation pattern of the nephron is highly conserved among vertebrates. (a) Segmental organization of mammalian metanephric nephron. (b) Dorsal view of the zebrafish embryonic pronephros. At 24 hpf, pronephric segmentation is fully accomplished with eight distinct regions: podocyte (P), neck (N), proximal convoluted tubule (PCT), proximal straight tubule (PST), distal early (DE), corpuscle of Stannius (CS), distal late (DL), pronephric duct (PD), and cloaca (C). (c) *Xenopus* embryonic pronephros shown in a lateral view. Notably, analysis of gene expressions in analogous segment identities argues conserved nephron patterning in different species, shown by color-coding in each panel. (Adapted from: Wingert RA, Davidson AJ. 2008. The zebrafish pronephros: a model to study nephron segmentation. Kidney Int. 73(10):1120-7. doi: 10.1038/ki.2008.37.)
Figure 1.5 The zebrafish nephrogenesis process and patterning of the pronephros. (a) At the tail-bud stage, the intermediate mesoderm (IM) is a U-shaped structure located between the paraxial mesoderm (PM) and lateral plate mesoderm (LPM). (b) At the 5 somite stage, the IM renal progenitor field is close to the scl/tall-expressing cells that are developing into the angioblasts and the scl/tall+/gata1a+ co-expressing progenitors giving rise to primitive blood cells. The renal progenitors could be labeled by pax2a, pax8, and lhx1a. (c) At 24 hpf, the zebrafish pronephros is comprised of two nephrons that consist of eight segments including the podocytes (P), neck (N), proximal convoluted tubule (PCT), proximal straight tubule (PST), distal early (DE), corpuscle of Stannius (CS), distal late (DL), and pronephric duct (PD) and the cloaca (C). (Adapted from: Gerlach GF, Wingert RA. 2012. Kidney organogenesis in the zebrafish: insights into vertebrate nephrogenesis and regeneration. Wiley Interdiscip Rev Dev Biol. 2(5):559-85. doi: 10.1002/wdev.92.)
Figure 1.6 Establishment of pronephric segmentation in the zebrafish embryo. (a) The intermediate mesoderm (IM) which develops into the renal progenitor field is located directly adjacent to the paraxial mesoderm (PM). During the time frame of nephrogenesis, the PM secretes retinoic acid (RA) and generates an RA gradient along the IM. RA production is highest in the anterior PM, leading to the hypothesis that the rostral renal progenitor cells are exposed to the highest amount of RA, while the distal region of the renal progenitors receives lower dosages of RA. (b-d) At the 5 somite stage, the rostral domain of the renal progenitors express wt1a+, and these cells will develop into podocyte progenitors. Until the 8 somite stage, the rostral and caudal domains have overlapping gene expression field. Then, a central region co-expresses jag2 and mecom starts to emerge. Establishment of each nephron domain is accompanied by complex and dynamic gene expressions that fluctuate throughout the whole process of nephrogenesis. By the 15 somite stage, irx3b expression labels a central domain, while the podocyte progenitors could detected by expression of foxc1a, hey1, lhx1a, mafba, and wt1b. (e) By the 28 somite stage (i.e. 24 hpf), segmentation has been fully accomplished. Subdomains could be defined by the expression of specific solute transporters or transcription factors. (Adapted from: Gerlach GF, Wingert RA. 2012. Kidney organogenesis in the zebrafish: insights into vertebrate nephrogenesis and regeneration. Wiley Interdiscip Rev Dev Biol. 2(5):559-85. doi: 10.1002/wdev.92.)
CHAPTER 2:
MATERIALS AND METHODS

2.1 Zebrafish husbandry and ethics statement

Zebrafish were maintained in the Center for Zebrafish Research at the University of Notre Dame Freimann Life Science Center. Wild type embryos were raised and staged as described (Kimmel, et al., 1995). The Institutional Animal Care and Use Committee at the University of Notre Dame supervised experimental procedures under protocols 13-021 and 16-025.

2.2 Morpholino knockdown, cRNA synthesis, and heat shock experiments

All morpholinos were purchased from Gene Tools, LLC (Philomath, OR), and were solubulized as recommended and stored at a 4 mM concentration. The mecom morpholino e1SD was designed to target the splicing donor site of zebrafish mecom (XM_001920912) exon 3, and eISA targets the splicing acceptor of exon 4 (Table 2.1). The mecom mismatch morpholinos were used as controls (Table 2.1). A combination of mecom e1SD and eISA morpholinos produced fully penetrant effects, and was used for all mecom knockdown experiments. All tbx2 morpholinos were previously published (Table 2.1).
One-cell stage wild type embryos were injected with 1-5 nl 0.2 mM mecom or tbx2 morpholinos and raised to the desired stages at 28°C. For gene expression analysis, embryos were fixed in 4% paraformaldehyde/1×PBST and stored in methanol at -20°C. Synthetic mecom cRNA was synthesized from a mecom.pCS2 plasmid using the mMessage mMachine sp6 kit (Ambion). For rescue experiments, one-cell stage embryos were co-injected with 1-5 nl 5 ng/µl mecom cRNA and 0.2 mM mecom morpholinos. For NICD heat shock experiments, embryos were incubated at 37°C for 1 hour beginning at 90% epiboly, allowed to develop to the desired stage, fixed and processed for WISH, and then ultimately genotyped as described with primers listed in table 2.2 (Scheer and Campos-Ortega, 1999).

2.3 RT-PCR

RNA was extracted from mecom morphant and wild type embryos at 24 hpf with Trizol reagent (Invitrogen) according to the manufacturer’s instructions. RT-PCR was performed using the SuperScript First-Strand Synthesis System (Invitrogen). To confirm mis-splicing of mecom in morphant embryos, PCR primers were designed to specifically amplify the mecom mRNA region corresponding to 180 bp of sequence spanning exon 3 and 4 (exon 3 forward primer and exon 4 reverse primer). Primers located in intron 3-4 were used to amplify a 190 bp intronic fragment (intron 3-4 forward primer and intron 3-4 reverse, Table 2.2). To assess the exon 3-intron 3-4 fusion in mecom morphants,
the exon 3 forward primer and intron 3-4 reverse primer (Table 2.2) were used in combination to amplify a ~1.3 kb cDNA fragment.

2.4 Dextran injection

To assay kidney function, 40 kDa fluorescent dextran-FITC (Invitrogen) was injected into an axial somite of 1-phenyl-2-thiourea (PTU) treated wild type embryos or mecom morphants that were anesthetized with 0.02% tricaine at 48 hpf. Embryos were revived and incubated in PTU in the dark until later time points for observation. Dextran clearance was observed with a fluorescent microscope at 72 hpf and 98 hpf.

2.5 Benzidene staining

o-dianisidine stock was made by dissolving 0.07 g of o-dianisidine (Sigma D9134) in 50 mls of 100% ethanol. Live embryos were incubated in the dark for 15 minutes in a working solution comprised of 2 ml o-dianisidine stock, 500 ul of 0.1M sodium acetate pH 4.5, 2 ml of distilled water, and 100 ul of hydrogen peroxide. Embryos were rinsed three times in E3 and then fixed in 4% PFA for scoring and imaging.

2.6 Chemical treatments

RA and DEAB (Sigma-Aldrich) were dissolved in 100% DMSO to make 1M stocks and the aliquots were stored at -80°C (Wingert, et al., 2007). For RA treatments, tailbud stage embryos were incubated in 1×10⁻⁷M RA/DMSO made
with E3 embryo media in the dark until 24 hpf, washed 3 times with E3, then
fixed. For DEAB treatments, the embryos were incubated in 1.6×10⁻⁵M
DEAB/DMSO diluted in E3 media from 75% epiboly to 24 hpf. Control embryos
were allowed to develop in 1×10⁻⁷M or 1.6×10⁻⁵M DMSO over corresponding
developmental intervals. DAPT was dissolved in DMSO to make a 10mM stock
and stored at -80°C. Bud stage embryos were incubated in 100 µM DAPT/DMSO
or DMSO alone in E3 media to 24 hpf at 28°C in the dark. These chemical
treatments were fully penetrant and produced consistent results.

2.7 WISH

For our reported WISH expression studies and images, representative
results are provided based on analysis of at least 20 embryos, and gene expression
domains as reported by somite boundaries were based on counts of at least 5
separate embryos for accuracy. Zebrafish WISH was performed as previously
described (Wingert, et al., 2007). To generate antisense probes for pax2a, dlc,
egr2a, mecom, myod1, smyhc1, slc4a4a, slc20a1a, trpm7, clcnk, slc12a1,
slc12a3, wt1b, and nr5a1a, we used IMAGE clone template plasmids for in vitro
transcription, as previously reported (Wingert, et al., 2007; Wingert and
Davidson, 2011). Probes for odf3b were transcribed from PCR templates
amplified with primers (Table 2.2). To distinguish mecom from other transcript
variants, we generated antisense probes mecom829 and mecom555 targeting the
first 829 bp and the last 555 bp fragments specifically present in mecom using
PCR templates obtained with mecom829 primers and mecom555 primers
respectively (Table 2.2). To make riboprobes for detecting *tbx2a* and *tbx2b* expression, DNA templates were amplified by performing PCR with primers shown in Table 2.2.

### 2.8 Cell counting and statistics

Quantification of MCC density in the pronephros was conducted by counting the number of MCCs in a length ranging from 50 µm to 150 µm per nephron. MCC density was determined by calculating the number of MCCs per 10 µm. Quantification of the corpuscles was performed by counting the number of *stc1* cells in the CS per nephron. At least 20 embryos were examined for each experiment. Student’s *t*-test was applied for statistical analysis.
### TABLE 2.1

**MORPHOLINOS USED**

<table>
<thead>
<tr>
<th>Morpholino</th>
<th>Sequence</th>
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<tr>
<td>mecom e1SD mo</td>
<td>5’-CTGAGTGACCTACATATGAAGGGCT-3’</td>
</tr>
<tr>
<td>mecom e1SA mo</td>
<td>5’-TTGTGGCGAGAC-CTCAGGACGTTGTT-3’</td>
</tr>
<tr>
<td>mecom mismatch mo</td>
<td>5’-CTGATTGACGTACAAATGATGGGCA-3’</td>
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<tr>
<td>mecom mismatch mo</td>
<td>5’-TTGTAGCGAGCGCTCGC-GACTGTTGTA-3’</td>
</tr>
<tr>
<td>tbx2a mo</td>
<td>5’-ATCGGTGCATCCAAAAAGCCAGAT-3’ (Ribeiro et al., 2007)</td>
</tr>
<tr>
<td>tbx2b mo</td>
<td>5’-ATCGGTGCATCCAAAAAGCCAGAT-3’</td>
</tr>
<tr>
<td></td>
<td>5’-AAAATATGGGTACATACCTTGTGCT-3’ (Gross and Dowling, 2005)</td>
</tr>
<tr>
<td>tbx2a/b mo</td>
<td>5’-AAAACTGGATCTCTCATCGTGTCAT-3’ (Sedletcaia and Evans, 2011)</td>
</tr>
<tr>
<td>Primer</td>
<td>Sequence</td>
</tr>
<tr>
<td>------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td><em>mecom</em> exon 3 forward</td>
<td>5’-AACTTGCAGAGGCATATCCG-3’</td>
</tr>
<tr>
<td><em>mecom</em> exon 4 reverse</td>
<td>5’-GGCAGAGATTGG-AGAACTGC-3’</td>
</tr>
<tr>
<td><em>mecom</em> intron 3-4</td>
<td>5’-CCCTAGCTAGGGACACCTTG-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GCCTCGAATAACGTGATGATG-3’</td>
</tr>
<tr>
<td><em>odf3b</em> primers</td>
<td>5’-ATGATGGGCTCTGATG-3’</td>
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<td></td>
<td>5’-AATTAACCTCACTAAAGGGTGATC-3’</td>
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<tr>
<td><em>mecom</em>829 primers</td>
<td>5’-ATGATGGCTCTGAGGACAGG-3’</td>
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<td>5’-AATTAACCTCACTAAAGGGCATTTG-3’</td>
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<tr>
<td><em>mecom</em>555 primers</td>
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<td>5’-CATCATTGCGTCGAG-3’</td>
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<td><em>UAS: NICD</em> primers</td>
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<td>5’-CGGATCTTATGTTGGATC-3’</td>
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CHAPTER 3:

ZEBRAFISH NEPHROGENESIS IS REGULATED BY INTERACTIONS BETWEEN RETINOIC ACID, MECOM, AND NOTCH SIGNALING

This chapter appears in part in the published manuscript “Zebrafish nephrogenesis is regulated by interactions between retinoic acid, mecom, and Notch signaling” by Yue Li, Christina N. Cheng, Valerie A. Verdun, and Rebecca A. Wingert with minimal changes including removal of materials and methods section.

3.1 Abstract

The zebrafish pronephros provides a conserved model to study kidney development, in particular to delineate the poorly understood processes of how nephron segment pattern and cell type choice are established. Zebrafish nephrons are divided into distinct epithelial regions that include a series of proximal and distal tubule segments, which are comprised of intercalated transporting epithelial cells and multiciliated cells (MCC). Previous studies have shown that retinoic acid (RA) regionalizes the renal progenitor field into proximal and distal domains and that Notch signaling later represses MCC differentiation, but further understanding of these pathways has remained unknown. The transcription factor
mecom (mds1/evil complex) is broadly expressed in renal progenitors, and then subsequently marks the distal tubule. Here, we show that mecom is necessary to form the distal tubule and to restrict both proximal tubule formation and MCC fate choice. We found that mecom and RA have opposing roles in patterning discrete proximal and distal segments. Further, we discovered that RA is required for MCC formation, and that one mechanism by which RA promotes MCC fate choice is to inhibit mecom. Next, we determined the epistatic relationship between mecom and Notch signaling, which limits MCC fate choice by lateral inhibition. Abrogation of Notch signaling with the γ-secretase inhibitor DAPT revealed that Notch and mecom did not have additive effects in blocking MCC formation, suggesting that they function in the same pathway. Ectopic expression of the Notch signaling effector, Notch intracellular domain (NICD), rescued the expansion of MCCs in mecom morphants, indicating that mecom acts upstream to induce Notch signaling. These findings suggest a model in which mecom and RA arbitrate proximodistal segment domains, while MCC fate is modulated by a complex interplay in which RA inhibition of mecom, and mecom promotion of Notch, titrates MCC number. Taken together, our studies have revealed several essential and novel mechanisms that control pronephros development in the zebrafish.
3.2 Introduction

Vertebrate kidney organogenesis proceeds through the formation and regression of several successive structures, each comprised of excretory units known as nephrons (Dressler, 2006). The first structure is the pronephros, composed of rudimentary nephrons formed next to the nephric cord, a bilateral epithelial tubule derived from the intermediate mesoderm (IM). Whereas the pronephros is a vestigial organ in mammals, it serves as the embryonic excretory organ in lower vertebrates such as fishes and frogs (Dressler, 2006). During mammalian development, a mesonephros forms posteriorly to the pronephros and functions transiently in fetal life, then subsequently a third structure, the metanephros, is formed that functions as the definitive adult kidney (Dressler, 2006). The metanephros arises when the ureteric bud grows out of the caudal end of the nephric duct, invades the surrounding metanephric mesenchyme, and induces a mesenchyme-to-epithelial transition (MET) in cell aggregates adjacent to the ureteric bud tips (Little and McMahon, 2012). Mesenchymal cells undergoing MET form a polarized renal vesicle, which develops sequentially into a comma-shaped body, S-shaped body, and eventually into a segmented nephron tubule (Little and McMahon, 2012). Highly elaborated branching of the ureteric bud along the radial axis of the metanephric mesenchyme generates a complicated network within the collecting duct system, with thousands of nephrons situated in an intricate, arborized three-dimensional arrangement.

There is currently a limited understanding of how nephron tubules are patterned into segments, due in part to the complexity of mammalian
nephrogenesis and kidney anatomy (Constantini and Kopan, 2010). However, in recent years the zebrafish has emerged as a useful vertebrate model to study nephron segmentation (Gerlach and Wingert, 2012). Zebrafish embryos form a pronephros with two nephrons that originate from bilateral stripes of IM, from which renal progenitors are generated (Drummond, et al., 1998). The rostral renal progenitors give rise to a single glomerulus that the nephrons share, while the remaining renal progenitors undergo a MET to generate tubules that fuse at the cloaca (Drummond, et al., 1998; Serluca and Fishman, 2001). Gene expression profiling, largely based on genes encoding solute transporter proteins that account for the exquisite functions of each segment, revealed that the zebrafish pronephros segment composition is analogous to other vertebrates (Wingert, et al., 2007; Wingert and Davidson, 2011). By 24 hours post fertilization (hpf), the zebrafish pronephros exhibits eight segments: the glomerulus (G), neck (N), proximal convoluted tubule (PCT), proximal straight tubule (PST), distal early (DE), corpuscle of Stannius (CS), distal late (DL), and pronephric duct (PD) (Figure 3.1A) (Wingert, et al., 2007).

Studies of zebrafish nephrogenesis have identified several transcription factors and signaling pathways that are crucial for renal progenitor patterning (Gerlach and Wingert, 2012). Among them, the diffusible morphogen retinoic acid (RA) is essential for proximal-distal regionalization of the renal progenitor field (Wingert, et al., 2007; Wingert and Davidson, 2011). In target tissues, RA regulates gene expression by entering the nucleus and binding to its nuclear receptors, which upon RA interaction directly bind to retinoic acid response
elements (RARE) to modulate transcription (Duester, 2008). Zebrafish genetic mutants lacking key RA synthesizing enzymes or wild types treated with diethylaminobenzaldehyde (DEAB), a chemical that blocks RA biosynthesis, develop a pronephros with reduced proximal segments and expanded distal segments (Wingert, et al., 2007; Wingert and Davidson, 2011). These studies established that RA induces proximal segment identities during the early somite stages, and may directly repress distal segments. Downstream of RA, the terminal boundaries of each segment are defined by the expression of domain-specific genes and appear to be controlled by the activity of multiple downstream transcription factors, presently known to include irx3b and hnf1β (Wingert and Davidson, 2011; Naylor, et al., 2013). Further, Notch signaling restricts MCC number by modulating the fate choice between transporting epithelium and MCC during mid-to-late somitogenesis (Ma and Jiang, 2007; Liu, et al., 2006). Despite these findings, many questions remain concerning how each nephron segment is precisely established during nephrogenesis—including the identity of other key factors involved in segmentation.

In searching for additional factors that may control nephron segmentation, we identified the zinc finger transcription factor Mecom as an intriguing candidate gene. Mecom is a splicing variant of the Evi1 (ecotropic virus integration site 1) and Mds1 (myelodysplastic syndrome 1) genes, which results in a N-terminal extension of the Evi1 protein (Wieser, 2007). Targeted disruptions that result in the loss of both transcripts causes embryonic lethality in mice associated with defects in neural, heart, and blood development—which suggests this locus has
multiple essential roles during ontogeny (Wieser, 2007; Hoyt, et al., 1997). More recent work has demonstrated that Mecom is required for long-term hematopoietic stem cell maintenance (Zhang, et al., 2011).

With respect to vertebrate kidney development, transcripts encoding Mecom have been detected in the pronephros distal tubule and duct in Xenopus (Mead, et al., 2005) and zebrafish mecom has also been reported in the pronephros (Wingert, et al., 2007; Wingert and Davidson, 2011). In the zebrafish, mecom is initially expressed in the renal progenitor field, but its domain changes dynamically during nephrogenesis (Wingert, et al., 2007; Wingert and Davidson, 2011). mecom marks a broad caudal domain in early stages, then later is restricted to the DL and PD at 24 hpf. A genome-scale in situ analysis of mammalian transcriptional regulatory factors reported expression of murine Mecom in nascent nephron S-shaped bodies in the developing metanephric kidney (Yu, et al., 2012), thus further suggesting it could be involved in nephron patterning across vertebrates. However, the mechanism of how mecom modulates vertebrate nephron segmentation and the signaling pathways that may interact with mecom in renal progenitors remain unclear.

Through the present study, we found that interactions between RA, mecom, and Notch signaling are essential for zebrafish pronephros development. We show that mecom expression is extremely dynamic in zebrafish renal progenitors and is negatively regulated by RA. Using both loss and gain-of-function approaches, we found that mecom is necessary for proper DL segment formation, and that the absence of mecom activity expands the PST segment and
MCC numbers. Moreover, *mecom* and RA have opposing roles in PST and DL formation, as *mecom* morphants treated with exogenous RA had a more expanded PST and an abrogated DL, while DEAB treatment rescued segmentation in *mecom* morphants. Consequently, we discovered a previously unappreciated role for RA in MCC development, since DEAB treatment prevented MCC formation while *mecom* knockdown in DEAB-treated embryos rescued MCCs. These data indicate that RA regulates MCC fate choice by inhibiting *mecom*. Furthermore, we established the epistatic relationship between Notch signaling and *mecom* during MCC differentiation, where *mecom* acts upstream to promote Notch activity. Taken together, our data suggest a model in which *mecom* and RA function during early nephrogenesis stages to arbitrate proximodistal segment pattern, and reveal that MCC fate choice is modulated by a complex interplay between RA, *mecom*, and Notch signaling to precisely define the MCC domain and density in the nephron.

### 3.3 Results

#### 3.3.1 *mecom* delineates a dynamic caudal subdomain in the renal progenitor field

Based on the gene expression patterns of specific solute transporters, the molecular anatomy of the zebrafish pronephros consists of proximal and distal segments that are analogous to mammalian nephrons (Wingert, et al., 2007). Eight similar regions have been identified, including two proximal tubule domains (PCT, PST) and two distal tubule domains (DE, DL) (Figure 3.1A).
Prior studies have documented the appearance of *mecom* transcripts as occurring between the 6-8 somite stages during pronephros development (Wingert, et al., 2007; Wingert and Davidson, 2011). To further examine the onset of *mecom* expression in the renal progenitor field, we assessed the domain of *mecom* transcripts by performing double whole-mount *in situ* hybridization (WISH) with several combinations of riboprobes consisting of both segmentation and somitogenesis gene probes that would enable labeling of renal progenitor domains compared to the somite boundaries. To confirm the embryonic stage and define somite boundaries, emerging somites were labeled in embryos between 1-6 somites using *deltaC (dlc)*, while embryos between 6-18 somites were labeled with *myogenic differentiation 1 (myod1).*

The domain of renal progenitors was visualized based on the expression of *pax2a* (Figure 3.1B) (Pfeffer, et al., 1998). At the 2 somite stage, *mecom* expression was absent in the renal progenitor field, whereas transcripts were present in the developing brain (Figure 3.1B). The onset of *mecom* expression in the renal progenitor domain occurred at the 3 somite stage, adjacent to where somite 4 was forming (Figure 3.1B). In addition to marking the somites, the Notch ligand *dlc* is known to mark the rostral territory of the renal progenitors, which will eventually develop into proximal segments in the pronephros (Wingert, et al., 2007; Wingert and Davidson, 2011). During the onset of *mecom* expression, there was a clear delineation between the *dlc*-expressing rostral domain and the *mecom*-expressing caudal domain at the 3 somite stage (Figure 3.1B, inset). Notably, the detection of non-overlapping domains of *dlc* and
mecom at the 3 somite stage is the earliest proximodistal distinction that has been observed within the renal progenitor field during zebrafish pronephros development.

Between the 6 and 15 somite stages, dynamic mecom expression was observed in the developing pronephros. The domain of mecom transcripts gradually shifted toward the caudal end of the renal progenitor field (Figure 3.1C). By 14 somites, pre-mature pronephric segments were established: the proximal domain was labeled by the solute transporter solute carrier family 4, member 4a (slc4a4a), while the distal domain was marked by solute carrier family 12 (sodium/chloride transporters), member 3 (slc12a3) (Figure 3.1C, lower two panels). At this time point, mecom transcripts were detected throughout the distal domain and partially overlapped with the proximal domain (Figure 3.1C). The progressive shifting of the mecom expression domain continued from 18-28 somite stages, until its expression became restricted to the DL and PD (Figure 3.1D). The presence of mecom transcripts in early renal progenitors, and its ongoing dynamic expression domain, suggested to us that mecom could be functioning at multiple times in developing renal progenitors.

In zebrafish, two mecom splice variants have been identified. The 3129 basepair (bp) mecom202 (mecom, also known as prdm3) (Sun, et al., 2008) possesses additional extensions in both the 5’ and 3’ ends of the 1807 bp mecom201 (evil). To distinguish expression of mecom202 from mecom201, we utilized the 5’ and 3’ end fragments present exclusively in mecom202 transcripts and designed riboprobes mecom202-5’ and mecom202-3’ (Figure 3.1D). WISH
using a full-length *mecom* riboprobe indicated *mecom* was restricted in the DL and PD regions of the pronephros at 24 hpf, while *mecom*202-5’ and *mecom*202-3’ probes further demonstrated the *mecom*202 transcripts were expressed in the pronephros and restricted to the DL and PD at 24 hpf as well (Figure 3.1D).

### 3.3.2 *mecom* morphants exhibited renal edema and dysfunction

To assess the role of *mecom* in zebrafish pronephros segmentation, we performed morpholino knockdown studies. One-cell stage wild type embryos were injected with a *mecom* morpholino targeting the splice donor site of exon 3, the splice acceptor of exon 4, or a combination of these two morpholinos (Figure 3.2A). We found that the morpholino combination induced stronger phenotypes than either morpholino alone (Figure 3.2A). Based on these results, we co-injected the *mecom* splice morpholinos for the remainder of our studies on *mecom* loss of function. Next, we performed a series of RT-PCR experiments to assess changes in *mecom* mRNA splicing in co-injected morphant embryos compared to uninjected wild type controls. Primers were designed to amplify the *mecom* sequence spanning exon 3 and exon 4 to identify the properly spliced *mecom* mRNA fragment (Figure 3.2A). We found that cDNA isolated from wild-type embryos contained *mecom* transcripts with the predicted exon 3-4 band size of 180 base pairs (bp), which indicated appropriate splicing between these exons (Figure 3.2A). However, *mecom* morphant embryos injected with the combination of splice morpholinos showed low, if any, correctly processed *mecom* mRNA across exons 3-4 (Figure 3.2A). This suggested that there was a significant
reduction in normal *mecom* mRNA as a consequence of the morpholino injection. In *mecom* morphants, these primers failed to amplify a *mecom* cDNA product containing the entire intron 3-4 (data not shown), but this could be due to the size of that intron, which is predicted to be 4.4 kilobases (kb) in length.

To further address how the *mecom* transcript was processed in the morphants, we designed primers that would amplify smaller portions of the intronic interval. In *mecom* morphants, but not in wild type uninjected embryos, we detected the presence of transcripts corresponding to *mecom* intron 3-4 sequences (data not shown). Further, in *mecom* morphants we were able to amplify a 1.3 kb *mecom* cDNA sequence spanning the 3’ end of exon 3 and the 5’ end of intron 3-4; importantly, this intronic fragment contains a series of in-frame stop codons (data not shown). Thus, we hypothesize that the *mecom* morpholinos generate a mis-spliced mRNA that in turn results in a truncated Mecom protein. While we were unable to determine if *mecom* morphants retain the entire intron 3-4 after *mecom* transcript processing, our data nevertheless confirm that these splice junction *mecom* morpholinos lead to mis-spliced *mecom* mRNA.

Next, to broadly evaluate the role of *mecom* during zebrafish development, we followed the development of *mecom* morphants compared to control embryos injected with the mismatch morpholinos. At 30 hpf, *mecom* morphants showed pericardial edema, mild tail-axis curvature, and brain and eye defects (Figure 3.2B). In contrast, wild type embryos injected with the mismatch morpholinos had no gross developmental abnormalities (Figure 3.2B). At 50 hpf, *mecom* morphants displayed severe pericardial edema, suggesting possible pronephros
dysfunction and fluid imbalance compared to wild types (Figure 3.2B). Fluid accumulation could also result from cardiac defects and impaired circulation. However, the heart rate was normal in morphants compared with wild type embryos, circulation ensued normally at 24 hpf, and circulation continued to appear normal through 48 hpf (data not shown). To further scrutinize the circulation, we used benzidine staining, which labels hemoglobin in differentiated erythrocytes starting just after 32 hpf, and can more precisely assay if blood pooling transpired in mecom morphants (Wingert, et al., 2004). We found that 8.6% (4/46) of mecom morphants had cranial blood pooling at 36 hpf, and that the incidence of this phenotype increased to 54.5% (12/22) of mecom morphants by 48 hpf, while no wild type embryos displayed a pooling phenotype (out of 51 animals assayed at these time points). Taken together, these data suggest the mecom knockdown is associated with circulation and/or vascular integrity defects that worsen over developmental time. We hypothesize that this contributed to the pericardial effusion phenotype in mecom morphants. To assess renal function in mecom knockdowns, we explored renal absorption and clearance properties with dextran tracing experiments. We injected 40 kDa dextran-FITC into the somites of wild type embryos or mecom morphants at 48 hpf. From 72 hpf to 98 hpf, wild types exhibited clearance of the fluorescent molecule, whereas abundant dextran-FITC accumulation was visualized in mecom morphants (Figure 3.2C). These results indicate that mecom is necessary for proper development and function of the pronephros.
3.3.3 *mecom* knockdown induced PST specific expansion in the zebrafish pronephros

To further characterize nephron development in *mecom* morphants, we used double WISH with domain-specific markers and the somite marker *smyhc1* to precisely define each segment boundary relative to each somite. Interestingly, at 24 hpf, *mecom* morphants had expanded proximal tubules (Figure 3.3). The pan-proximal marker *slc4a4a*, encoding an electrogenic Na\(^+\) bicarbonate cotransporter, was expressed in the PCT and PST segments from somite 4 to 11 in wild-type embryos, whereas in *mecom* morphants, the *slc4a4a* domain was expanded from somite 4 to 14, and was rescued by *mecom* overexpression (62.5%, 20/32) (Figure 3.3). Scrutiny of each segment domain revealed that expansion of the proximal tubule was attributed to an enlarged PST. The PCT expresses the sodium-dependent phosphate transporter *slc20a1a*, a domain that was situated adjacent to somites 4 to 8 in both wild type embryos and *mecom* morphants (Figure 3.3). However, the PST labeled by the transient receptor potential cation channel gene *trpm7* was expanded from somite 9 to 14 in *mecom* morphants, while its expression in wild types was located next to somites 9 to 11 (Figure 3.3). The PST expansion was consistent in more than 90% (49/52) of *mecom* morphants. In addition, more than 60% (23/36) of *mecom* morphants could be successfully rescued by co-injecting the *mecom* morpholinos along with full-length zebrafish *mecom* cRNA (Figure 3.3). This result supports the conclusion that the segmentation defects observed after *mecom* knockdown are specific to the abrogation of mecom activity during nephrogenesis. The
development of other proximal nephron cell types like the podocytes, or the interrenal lineage that emerges next to the podocytes, was not affected in mecom morphants.

3.3.4 mecom morphants display PST expansion at the expense of the DL segment

To determine how the abnormal PST in mecom morphants affected distal segment patterning, we used double WISH with distal markers and smyhc1. Indeed, the increased PST in the morphant pronephros was accompanied by a robust reduction in the distal pronephros domain (Figure 3.4). In wild type embryos at 24 hpf, the expression pattern of the chloride conductance channel gene clcnk could be seen in the DE, DL and PD regions from somite 12 to 18 (Figure 3.4). In contrast, the clcnk expression domain was reduced in the mecom morphants, being located from somite 15 to 18 starting adjacent to somite 15; however, this aberrant phenotype was rescued by mecom transcript overexpression (66.7%, 22/33) (Figure 3.4). The DE domain marked by slc12a1, which encodes a Na⁺/K⁺/Cl⁻ cotransporter, had a similar length in wild types and mecom morphants (Figure 3.4). Surprisingly, the distal pronephros region was reduced specifically at the cost of the DL segment. The DL domain indicated by slc12a3, a Na⁺/Cl⁻ transporter gene, showed a significant reduction in mecom morphants, and was restricted to somites 17 to 18, whereas wild type embryos showed slc12a3 expression from somite 14 to 17 (Figure 3.4). The abrogation of the DL domain was not associated with heightened cell death in this domain at 24
hpf based on acridine orange staining (data not shown). Finally, we found that DL segment formation was rescued in nearly 70% (26/37) of embryos that were co-injected with mecom morpholinos and mecom cRNA (Figure 3.4A), further supporting the specificity of mecom morpholinos. We conclude from these findings that mecom is essential for normal nephron segmentation, such that mecom activity promotes the DL and restricts the PST.

3.3.5 RA negatively regulates mecom during pronephros segmentation

Next, we sought to identify possible developmental pathways relative to mecom in establishing the pronephric segmentation pattern. RA signaling plays important roles during segmentation: RA is required to induce proximal segments and to prevent distal segment expansion at early somitogenesis stages (Wingert, et al., 2007; Wingert and Davidson, 2011). To further explore the relationship between mecom and RA signaling, we treated wild type embryos and mecom morphants with exogenous all-trans RA at the concentration of $1 \times 10^{-7}$ M from bud stage to 24 hpf, and examined segmentation changes using WISH (Figure 3.5). At this RA dosage and time window, wild type embryos developed a minor ‘proximalized’ pronephros phenotype with an expanded PST, indicated by trpm7 expression, and a reduced DL segment marked by slc12a3 (Figure 3.5). However, at the same treatment condition, mecom morphants displayed a severe ‘proximalized’ pronephros indicated by the expansion of the trpm7 expressing PST domain throughout the entire tubule and an almost complete loss of the slc12a3 expressing DL segment (Figure 3.5). Furthermore, as shown previously
(Wingert, et al., 2007), treating wild types with DEAB, an inhibitor of RA aldehyde dehydrogenase (aldh) synthesizing enzymes, resulted in a ‘distalized’ pronephros with an expanded DL and reduced PST (Figure 3.5). When compared to mecom morphants or mecom morphants treated with RA, mecom morphants incubated with DEAB showed a partially reduced PST, as shown by trpm7 expression from somite 9 to 13, and partially expanded slc12a3 expression from somite 15 to 17 (Figure 3.5). Altogether, these results show that mecom and RA have antagonistic activities in PST and DL formation: mecom inhibits the PST while RA promotes the PST, and mecom promotes the DL while RA inhibits the DL.

Based on these findings, we hypothesized that RA could negatively regulate mecom, which would provide a mechanism to account for the patterning change in the PST and DL segments. Interestingly, RA treatment beginning at 60% epiboly has been shown to reduce the mecom expression domain at the 6-8 somite stage, while DEAB exposure beginning at this time leads to an expansion of the mecom domain at the 6-8 somite stage—changes that correlate with reduced and expanded distal segments, respectively (Wingert, et al., 2007). To further assess the relationship between RA signaling and mecom, wild type embryos were treated with RA or DEAB and mecom expression was evaluated using WISH (Figure 3.6A). Wild type embryos treated with RA showed a dramatically reduced mecom expression domain at 24 hpf, while embryos treated with DEAB had a dramatically expanded mecom domain, consistent with the
notion that RA negatively regulates *mecom* expression during nephrogenesis (Figure 3.6A).

### 3.3.6 The activities of RA and *mecom* also regulate epithelial cell fate choice in tubule segments

In the zebrafish pronephros, intercalated along the PCT, PST, DE, and the anterior-most DL transporting epithelia are distinct multiciliated cells (MCCs), which can be evaluated based on the expression of various ciliogenesis genes, such as *odf3b*, via WISH (Ma and Jiang, 2007; Liu, et al., 2006). How the MCC domain is defined within the renal progenitor field has yet to be understood, but MCC cell fate choice is limited through Notch signaling (Ma and Jiang, 2007; Liu, et al., 2006). Interestingly, we found that *mecom* morphants exhibited an expanded MCC domain and a noticeable overall increase of MCC density at 24 hpf (Figure 3.6B). These observations indicate that *mecom* negatively regulates MCC development.

While RA is vital for patterning pronephros segmentation (Wingert, et al., 2007; Wingert and Davidson, 2011), prior studies have not addressed how RA might affect MCC formation. Given the relationship between RA and *mecom*, we assessed whether RA is also involved in MCC patterning. Wild type embryos were treated with $1 \times 10^{-7}$M all-trans RA from tailbud stage to 24 hpf. The RA-treated embryos had a caudal expansion of the MCC domain (Figure 3.6B). When *mecom* morphants were treated with the same dosage of RA during this time window, they showed a slightly further expanded MCC domain (Figure 3.6B).
These data suggested that RA has dual roles in patterning of the MCC domain and stimulating MCC formation, possibly via the inhibition of *mecom*.

To determine if RA is required for MCC progenitor formation during early nephrogenesis, we exposed embryos to DEAB to inhibit RA synthesis. Wild type embryos treated with DEAB from 75% epiboly to 24 hpf, at the concentration documented to induce proximodistal segmentation changes in the pronephros (Wingert, et al., 2007), displayed a complete loss of MCCs at 24 hpf (Figure 3.6B). Consistent with the notion that RA is required for MCC formation, *lightbulb (lib)* embryos that have a mutation in the RA biosynthesis gene *aldehyde dehydrogenase 1a2* (Wingert and Davidson, 2011) showed a reduction in the MCC domain. Next, we further tested the relationship between *mecom* and RA in MCC formation. Since our findings thus far indicated that *mecom* represses MCCs, and RA represses *mecom*, we hypothesized that DEAB treatment had abrogated MCC formation due to the elevated *mecom* expression (Figure 3.6A). In keeping with this, DEAB-treated *mecom* morphants developed MCCs (Figure 3.6B). These results indicate that alleviating the inhibitory effect of *mecom* on MCC differentiation is sufficient to reverse the MCC deficiency that results from an absence of RA signaling. Taken together, these data suggest that there is an exquisite interplay involving RA and *mecom* that titrates MCC formation: essentially, that RA acts to inhibit *mecom*, while *mecom* inhibits MCC fate choice.
3.3.7 *mecom* and Notch signaling coordinate MCC density in zebrafish pronephros

In the pronephros tubule, MCCs are dispersed among the single-ciliated transporting epithelia in a ‘salt-and-pepper’ pattern (Ma and Jiang, 2007; Liu, et al., 2006). Notch signaling creates this cell distribution by controlling the MCC fate decision through a mechanism of Notch-mediated lateral inhibition (Ma and Jiang, 2007; Liu, et al., 2006). During pronephros differentiation, MCC progenitors expressing the Notch ligand *jag2a* exclusively repress MCC fate in neighboring cells, leading them to acquire a transporting epithelial fate (Ma and Jiang, 2007; Liu, et al., 2006). The γ-secretase inhibitor DAPT has been shown to effectively block Notch signaling and causes increased MCC numbers to form during nephrogenesis (Ma and Jiang, 2007; Liu, et al., 2006). Based on the discovery that RA levels affect MCC formation, we first addressed the relationship between RA and Notch signaling in modulating MCC fate. Changes in RA levels during mid-gastrulation (between 60-70% epiboly) alter proximodistal segmentation (Wingert, et al., 2007; Wingert and Davidson, 2011), while changes in Notch affect MCC differentiation at slightly later times (90% epiboly to bud stages) (Ma and Jiang, 2007; Liu, et al., 2006). Thus, it is very likely that RA acts upstream of Notch in MCC formation. MCC formation was evaluated when embryos were treated with RA or a combination of RA and expression of the Notch1a intracellular domain (NICD) under heat-shock control using a transgenic line Tg(*hsp70:gal4; uas:notch1a-intra*). RA treatment shifted the MCC domain posteriorly in non-transgenic NICD siblings. This was
consistent with the change in proximodistal segment domains after RA treatment in wild types (Figure 3.5), and confirmed our prior observation (Figure 3.6B). Dual RA treatment and NICD heat-shock caused a similar MCC domain posterior shift, but the number of MCCs was reduced. This confirms that Notch acts as a repressor of MCC fate, and is consistent with the notion that RA treatment causes a diminution of Notch signaling that increases MCC formation in the pronephros.

Next, we hypothesized that mecom might interact with Notch to mediate the MCC epithelial fate choices because mecom morphants showed an increase in MCC density similar to the effect of blocking Notch signaling (Ma and Jiang, 2007; Liu, et al., 2006). To compare the effects of mecom and Notch on MCC differentiation, MCC number was evaluated in mecom morphants and DAPT treated wild types using the ciliogenesis marker odf3b. As noted previously, the MCC domain shift in mecom morphants correlated with the alteration in segment sizes, and there was a significant increase of MCC density compared to wild types at 24 hpf (Figure 3.7). To assess MCC quantity, the number of MCCs was quantified per every 10 µm in single nephrons. MCC density in mecom morphants was increased by 50% compared to wild types (Figure 3.7). As reported (Ma and Jiang, 2007; Liu, et al., 2006), wild type embryos treated with DAPT exhibited an increased MCC density (Figure 3.7). In addition, whereas MCCs are regularly dispersed in a salt-and-pepper array within the wild type nephron, large MCC aggregates were observed both in DAPT-treated wild types and mecom morphant nephrons (Figure 3.7). mecom morphants incubated with DAPT did not show any further increase in MCC density compared to mecom
morphants or embryos treated with DAPT treatment alone (Figure 3.7). Further, disrupting Notch signaling in mecom morphants with DAPT did not induce additional condensed MCC numbers (Figure 3.7). Taken together, these results show that there is no additive effect of mecom and Notch signaling in modulating MCC differentiation. As such, we hypothesized that mecom and Notch signaling may collaborate in the same pathway to limit MCC formation, with additional roles of mecom to precisely define MCC territory in the pronephros.

To address whether mecom acts upstream or downstream of Notch signaling in modulating MCC differentiation, we utilized Notch transgenic lines to investigate whether MCC expansion could be ameliorated in mecom morphants with ectopic Notch signaling. We again used the double transgenic line Tg(hsp70:gal4; uas:notch1a-intra) to overexpress NICD with heat-shock induction. NICD activation by heat-shocking transgenic embryos at the bud stage resulted in fewer MCCs compared with control siblings (Figure 3.7), consistent with published results (Liu, et al., 2006). Strikingly, transgenic NICD activation in mecom morphants reduced MCC numbers, thus successfully rescuing the aggregate MCC phenotype (Figure 3.7). These results provide evidence that Notch signaling acts downstream of mecom to restrict MCC formation during the choice between MCC and transporting epithelia fates. Overall, these studies show that mecom is a vital component of the gene regulatory network that controls MCC development.
3.4 Discussion

Herein we uncovered a fundamental role of *mecom* in modulating proximodistal segmentation and MCC fate choice during zebrafish nephrogenesis (Figure 3.8A). Given the presence of *mecom* transcripts in a shifting caudal domain of the renal progenitors during the time period when nephron segmentation is established (through to 24 hpf), we speculate that *mecom* could act in multiple places and times to restrict the PST and promote the DL, respectively. The onset of *mecom* expression was detected at the 3-somite stage in the caudal domain of the renal progenitor field, in a non-overlapping pattern with the *dlc*-expressing rostral domain. This provides molecular evidence for renal progenitor specification several hours earlier in somitogenesis than previously documented (Gerlach and Wingert, 2012). However, this finding is consistent with the observation that modulations in RA levels during mid to late gastrulation have consequences for nephron proximodistal pattern (Gerlach and Wingert, 2012). The *mecom* expression domain later undergoes an incredibly progressive and dynamic shift toward the caudal end of the renal progenitor field before its restriction to the DL-PD at 24 hpf. Based on the presence of *mecom* transcripts in central and caudal renal progenitors, we hypothesize that *mecom* functions in proximodistal segmentation to promote DL induction and restrict the PST field during nephrogenesis (Figure 3.8A).

In addition, we have determined several new roles and relationships between *mecom*, RA and Notch signaling during MCC fate choice along the pronephros (Figure 3.8B). We found that *mecom* morphants exhibit increased
MCC numbers, demonstrating that mecom is needed to repress MCC fate choice. Blocking RA signaling by treating embryos with DEAB completely prevented MCC formation, and this inhibitory effect was alleviated by knocking down mecom. These data suggest that RA signaling promotes/enables MCC formation through mecom inhibition. Further, our studies place mecom upstream of Notch, since MCC expansion in mecom morphants was rescued by inducing Notch signaling. Based on these findings, we conclude that RA is crucial for MCC progenitor formation by limiting mecom activity, and that mecom in turn provides an inhibitory effect on MCC formation through promoting Notch signaling to regulate the balance of MCC-transporting epithelia fate choice (Figure 3.8B). Also, since RA treatment can expand the MCC domain in mecom morphants, this suggests that there could be other factor(s) downstream of RA, possibly in parallel with mecom/Notch, that contribute to the positive role of RA in promoting MCC differentiation (Figure 3.8B). Additional studies are needed to address how mecom coordinates both proximodistal segmentation and MCC fate choice—such as whether these are actually independent or overlapping pathways. Nevertheless, our data suggest that the proper dosage of RA, mecom, and Notch is absolutely essential for normal nephrogenesis.

3.4.1 Mechanisms of mecom function during zebrafish nephrogenesis

The molecular mechanism of mecom in regulating pronephron segmentation and epithelial fate decision remains intriguing. In mammals, the Mecom gene is characterized as one of the splice variants of the gene Evi1, a
conserved zinc finger transcription factor crucial for both development and oncogenesis (Wieser, 2007). The Mecom transcript consists of extra sequence from the upstream Mds1 gene, resulting in a 188 amino acid extension encoding a PR domain homologous to the SET protein methyltransferase domain (Wieser, 2007). The SET domain-containing proteins are a family of epigenetic regulators responsible for most histone lysine methylation (Dillon, et al., 2005). A genome-wide survey of zebrafish SET domain-containing gene revealed mecom (referred to as prdm3) as a member of the SET domain-containing proteins (Sun, et al., 2008). It is not known, however, whether the PR domain encoded by the zebrafish mecom sequence possesses histone methyltransferase activity.

The presence of zinc finger domains and their potentials to bind specific DNA sequences suggests mecom may function as a transcription factor to directly modify gene activities. However, target genes of mecom responsible for kidney development have yet to be identified. The presence of several consensus binding sites of co-activators and co-repressors in the Mecom protein argue additional biochemical role of mecom as a transcription cofactor (Wieser, 2007). Indeed, mecom has been shown to participate in chromatin modification by interacting with CtBP corepressor or histone deacetylases (HDACs) (Wieser, 2007). In Drosophila, the mecom homologous gene hamlet controls olfactory receptor neuron diversification via locus-specific histone methylation at the Notch target promoter (Endo, et al., 2012). In vertebrates, the highly related mecom variant evil has been shown to repress BMP/Smad-mediated activation of endogenous genes required for cell fate specification. In this case, Evil and CtBP are
recruited to target gene promoter upon TGFβ stimulation, leading to decreased histone acetylation and transcription (Alliston, et al., 2005). Notably, chromatin modification seems to be crucial for proper nephron development, as inhibition of HDAC in zebrafish embryos results in the expansion of the renal progenitor cell population (de Groh, et al., 2010).

Several studies suggest that mammalian Mecom affects cellular proliferation and differentiation in cell-type specific manners (Wieser, 2007). Therefore, in the developing pronephros, one possibility that we have not yet explored is that mecom fulfills its function by interfering with renal progenitor proliferation and/or differentiation. Further, the persistence of mecom transcripts in the DL and PD at 24 hpf could serve later roles in nephron morphogenesis—which might partly explain renal failure in mecom morphants. Recent studies have reported elevated epithelial proliferation in the distal tubule and duct at 3-4 days post fertilization, which serves as a compensatory mechanism allowing for complex morphological change in the proximal segments driven by collective cell migration toward the glomerulus (Vasilyev, et al., 2009; Vasilyev, et al., 2012). Thus, persistent mecom expression in the DL segment and PD at 24 hpf may account for active cell proliferation in these regions and in turn facilitate nephron morphogenesis post segmentation events. Interestingly, the overexpression of Mecom in Xenopus repressed proximal fates and glomerulus formation, while inactivation of Mecom by fusing the wild type protein with the VP16 activation domain disrupted pronephros duct development (van Campenhout, et al., 2006). In light of our findings, Mecom activity may be a conserved requirement in distal
nephron development, though additional studies are needed to explore this possibility.

3.4.2 The elucidation of new roles that RA plays during zebrafish nephrogenesis

RA is a key modulator of pronephros development in the zebrafish (Gerlach and Wingert, 2012). RA signaling is required between gastrulation and early somitogenesis to induce podocytes and proximal segmental fates and to inhibit expansion of distal segments (Wingert, et al., 2007). RA generated in the anterior paraxial mesoderm (PM) is hypothesized to diffuse to the adjacent renal progenitors that give rise to the pronephros (Wingert, et al., 2007; Wingert and Davidson, 2011). Since the rostral-most PM produces RA first, the current model is that a gradient of RA is generated along the IM, which provides higher levels of RA in the rostral region that induce proximal fates, while low RA levels in the caudal region allow distal segment formation (Wingert, et al., 2007; Wingert and Davidson, 2011). Furthermore, growing evidence suggests transcription factors essential for nephron development act downstream of RA during pronephric segmentation patterning. For example, the homeodomain transcription factor irx3b has been shown to modulate DE formation and PCT/PST boundary establishment downstream of RA (Wingert and Davidson, 2011).

Several studies support the existence of a regulatory relationship between RA and Mecom. In mammalian cell culture studies, all-trans RA was shown to activate the Mecom locus via a consensus RARE located in exon 1a of the human
*Evi1* gene (Bingemann, et al., 2009). Meanwhile, RA also induced *mecom* expression indirectly through unknown mechanisms (Bingemann, et al., 2009). In the *Xenopus* pronephros, exogenous RA is associated with elevated *Mecom* transcript levels (van Campenhout, et al., 2006). In contrast, our analysis reveals that RA signaling inhibits *mecom* expression in the zebrafish pronephros. Since *mecom* is expressed in zebrafish pronephros renal progenitors starting from the onset of nephrogenesis, *mecom* could be a direct or indirect target of RA signaling. A better understanding of the relationship between RA and *mecom* will help to elucidate how these factors and/or their targets interact to precisely define the pronephros segment pattern.

The link between RA signaling and MCC development has not been previously reported. In the zebrafish pronephros, the majority of pronephric epithelial cells bear single apical cilium, while MCCs represent a subset of epithelial cells that display up to 16 apical motile cilia. It has been demonstrated that Notch signaling regulates differentiation of transporting epithelia and MCCs in the zebrafish pronephros via lateral inhibition, where ‘multiciliated progenitor cells’ suppress transporting epithelia to acquire MCC fates possibly by expressing high levels of *jag2*, which interacts locally with Notch3 receptors present on transporting epithelial surface (Ma and Jiang, 2007; Liu, et al., 2006). Our data reveals that MCC fate choice is titrated through a concert of RA, *mecom*, and finally Notch signaling. Further experiments are needed to ascertain how *mecom* promotes Notch activity in renal progenitors. However, our work here adds
several levels of complexity to the knowledge about how the MCC-transporting epithelia balance is regulated.

In conclusion, our studies have revealed new insights into the processes of nephron segmentation and cell fate choice during nephrogenesis. Given the similarities that exist between nephron segmental structure and ontogeny between zebrafish and other vertebrates, future analysis of *Mecom* function during mammalian renal development may provide novel information about nephron patterning in humans that could be relevant to understanding renal stem cell biology, kidney birth defects and other kidney diseases.
Figure 3.1  *mecom* transcripts mark an early caudal domain of the renal progenitors, and the *mecom* expression domain is dynamic during nephrogenesis. (A) Schematic depictions of zebrafish pronephros at 24 hpf, shown in lateral view. Enlargement represents segmental organization of the nephron at 24 hpf. Abbreviations: G (glomerulus), N (neck), PCT (proximal convoluted tubule), PST (proximal straight tubule), DE (distal early), CS (corpuscle of Stannius), DL (distal late), PD (pronephric duct), C (cloaca). (B) At the 2 and 3 somite stages, the renal progenitor field was labeled by *pax2a* (purple) and forming somites were marked by *dlc* (red). Onset of *mecom* expression in renal progenitors could be detected at the 3 somite stage, in a caudal domain exclusive to the *dlc*-expressing rostral domain. Inset shows non-overlapping expression territory of *dlc* (red) and *mecom* (purple) at 3 somites in the renal progenitor field. (C) Expression of *mecom* (purple) and *myod1* (red) at various time points between 6 to 14 somite stages in wild types. At 14 somites, the expression domains of solute transporters *slc4a4a* and *slc12a3* indicate premature patterning of the pronephros proximal versus distal segment regions. (D) Upper panel: genomic structure of zebrafish *mecom202* (dark purple, bottom) and *mecom201* (*evi1*) (light purple, top). The *mecom202*-5’ and *mecom202*-3’ riboprobes (orange filled lines) were designed to distinguish *mecom202* and *mecom201* transcripts by targeting the 5’ and 3’ region exclusively present in *mecom202* transcripts. Lower panels: in 24 hpf wild type embryos, WISH using a full-length *mecom202* probe and *mecom202*-specific probes showed that *mecom* expression was restricted to the DL and PD regions of the pronephros.
Figure 3.2 *mecom* morphants exhibit pericardial edema and symptoms of renal failure. (A) Schematic indicates targeting sites of *mecom* morpholinos (blue lines) blocking splice sites of *mecom* mRNA between exon 3 and 4. Primers (red arrowheads) were designed to amplify the 180 bp linkage region between properly spliced exon 3 and 4. Right panels: cDNA isolated from wild type embryos showed the 180 bp band indicating proper splicing to remove intron 3. In contrast, amplification of this product was abrogated in *mecom* morphants. *eef1a1l1* was used as internal control. (B) *mecom* morphants showed gross developmental defects when compared with mismatch controls. Pericardial edema could be visualized at 30 hpf in *mecom* morphants indicating fluid accumulation (left panels). At 50 hpf, *mecom* morphants displayed severe pericardial edema and body curvature (right panels). (C) Fluorescent 40 kDa dextran was injected into the somite of wild types or *mecom* morphants at 48 hpf. A failure of renal clearance, indicated by dextran accumulation, was observed in the yolk and edema of *mecom* morphants at 72 and 96 hpf.
Figure 3.3 *mecom* knockdown leads to an expanded PST segment. (A) At 24 hpf, WISH indicates proximal segments marked by *slc4a4a* elaborated in *mecom* morphants. Within proximal domains, PCT labeled by *slc20a1a* was not affected comparing with wild type embryos. Knockdown of *mecom* induced a 3-somite expansion in *trpm7*-expressing PST in morphant pronephros at 24 hpf, which could be rescued by co-injecting *mecom* cRNA along with *mecom* morpholinos. (B) Schematic summary of proximal segment organization in wild type, *mecom* morphant, and *mecom* rescued embryos, with PST alterations highlighted in yellow. Abbreviations: P (podocytes), PCT (proximal convoluted tubule), PST (proximal straight tubule), DE (distal early), DL (distal late), PD (pronephric duct).
Figure 3.4  *mecom* knockdown leads to the formation of a reduced DL segment. (A) WISH using distal segment markers showed a 3-somite reduction in DL region by 24 hpf. The distal segments and pronephros ducts were labeled by *clcnk*. Expression of *slc12a1* and *slc12a3* marked DE and DL respectively. Pronephros segment boundaries were evaluated relative to the somites, which were shown by *smyhc1* expression (red). The reduced DL could be ameliorated by co-injection of *mecom* morpholino and *mecom* cRNA. (B) Schematic depiction of distal segment alterations, with DL domains highlighted in orchid. Abbreviations: P (podocytes), PCT (proximal convoluted tubule), PST (proximal straight tubule), DE (distal early), DL (distal late), PD (pronephric duct).
Figure 3.5 *mecom* and RA have opposing roles in PST and DL formation during proximodistal segmentation of the pronephros. (A) Wild type embryos or *mecom* morphants were incubated with 1x10^{-7}M RA, 1.6x10^{-5}M DEAB or DMSO. WISH using the PST marker *trpm7* and DL marker *slc12a3* showed that exogenous RA resulted in a more severe segmental phenotype in the *mecom* morphant pronephros, with further expanded PST and reduced DL. DEAB treatment partially rescued the segmentation phenotype in *mecom* morphants. (B) Schematic summary of segmentation changes in wild type embryos, *mecom* morphants, and wild type embryos or morphants treated with RA or DEAB. Yellow and blue boxes highlight the PST and DL segments, respectively.
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**SUMMARY**

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Figure 3.6 RA negatively regulates *mecom*, which enables MCC formation. (A) Wild type embryos treated with $1 \times 10^{-7}$ M RA, $1.6 \times 10^{-5}$ M DEAB or DMSO. Analysis of *mecom* transcripts using WISH shows that RA treatment diminishes the *mecom* domain while abrogation of RA signaling expands the *mecom* domain. (B) MCCs were labeled by *odf3b* in wild types and *mecom* morphants treated with RA or DEAB. *mecom* knockdown resulted in a caudal shift of MCC domain in the DL and PD regions, and this effect was partially rescued by treating morphant embryos with DEAB. Notably, wild type embryos treated with DEAB had abolished MCC formation, while RA induced ectopic MCC formation in the distal region of the pronephros.
Figure 3.7 *mecom* acts upstream of Notch signaling to modulating MCC differentiation and regulates the MCC domain. (A) Wild type embryos treated with 100 µM DAPT showed a significant increase of MCC number without ectopic MCC formation. *mecom* knockdown led to a caudal expansion of MCC in the DL and PD, and exhibited increased MCC density compared to wild types. A similar condensed MCC arrangement could also be seen in *mecom* morphants treated with DAPT. The overexpression of Notch1a resulted in decreased MCCs in heatshock induced *Tg(hsp70:gal4; uas:notch1a-intra)* embryos. Ectopic MCC formation associated with *mecom* knockdown was abolished by Notch signaling activation, though the domain of MCCs was still expanded. (B) Differentiated MCCs at 24 hpf under 10× magnification in a single nephron from wild types, wild types treated with DAPT, *mecom* morphants and morphants treated with DAPT, and finally wild types and *mecom* morphants with NICD overexpression. Note MCCs displayed a condensed organization/cluster pattern in DAPT-treated wild type and *mecom* morphant pronephros, while DAPT treatment in *mecom* morphant failed to induce further MCC density. Arrows indicate large MCC aggregates observed in DAPT-treated wild types, *mecom* morphants, and DAPT-treated *mecom* morphants, which were absent from the wild type pronephros. For each experiment, at least 20 embryos were examined. (C) Quantification of MCC density in wild types, wild type embryos treated with DAPT, *mecom* morphants, and *mecom* morphants treated with DAPT. Student *t* test revealed significant increase of MCC density in DAPT treated wild types and *mecom* morphants relative to untreated wild types (**p=0.0005). Alterations of MCC density between *mecom* morphants and morphants treated with DAPT did not reach statistical significance. For each experiment, at least 20 embryos were examined.
Figure 3.8 Model of *mecom* function during nephrogenesis. (A) Role(s) of *mecom* during pronephros proximodistal segmentation. At early somitogenesis, forming somites generate a gradient of RA, which diffuses along the IM and modulates proximodistal patterning of the renal progenitors by promoting proximal segmentation and restricting distal fates. Initially expressed in the caudal domain of the renal progenitor field, *mecom* is negatively regulated by RA signaling and executes a contrary role to that of RA by favoring distal tubule formation and/or limiting proximal segmentation. Interplays between RA and *mecom*, as well as other transcription factors and signaling pathways precisely define the patterning of the renal progenitors during nephrogenesis, which develops into a pronephros with at least eight distinct segments by 24 hpf. *mecom* is specifically essential to promote the DL and possibly to restrict the PST. (B) RA, *mecom*, and Notch activities coordinate MCC formation. RA signaling is required for MCCs to develop, and one modality is that RA acts to negatively regulate *mecom* expression. RA likely has other targets that promote MCC formation, which remain unidentified. To further refine the balance between MCC and transporting epithelia fate choice, *mecom* represses ectopic MCC formation by promoting Notch signaling. Notch is the penultimate signal that inhibits MCC induction via lateral inhibition. By 24 hpf, MCCs are dispersed along the pronephric tubules in PST, DE, and DL regions adopting a proper ‘salt-and-pepper’ fashion. Abbreviations: RA (retinoic acid), PCT (proximal convoluted tubule), PST (proximal straight tubule), MCC (multiciliated cell), DE (distal early), DL (distal late), PD (pronephric duct).
A
Proximodistal segmentation:

RA

PCT, PST

PST

mecom

DL

IM

wild type, 24 hpf

B
MCC fate choice:

RA

mecom

Notch

IM

wild type, 24 hpf

PCT

PST

DE

DL

PD

MCC precursor
differentiated MCC
CHAPTER 4:

TBX2A/B TRANSCRIPTION FACTORS DIRECT PRONEPHROS
SEGMENTATION AND CORPUSCLE OF STANNIUS FORMATION IN ZEBRAFISH

4.1 Abstract

The zebrafish embryonic pronephros is an excellent model of kidney development, as it possesses remarkable conservation with other vertebrate nephrons, including functionally distinct regions of the proximal convoluted and straight tubule (PCT, PST), distal tubule segments, and a duct. In addition, endocrine cells called the corpuscle of Stannius (CS) are interpolated between the distal segments. How nephron pattern is established and how the CS forms remains intriguing. Using whole mount in situ hybridization, we found that transcripts encoding the zebrafish orthologues tbx2a and tbx2b were spatially restricted to distal renal progenitors during nephron formation. To elucidate the function of these genes in nephrogenesis, morpholino knockdown studies were performed. tbx2a and tbx2b single and double morphants exhibited a modest expansion in the proximal segments accompanied by a reduction in the distal
domains, indicating that these genes have redundant roles segment patterning. Noticeably, \(tbx2b\) morphants formed significantly larger CS clusters, as shown by the elevated expression of the marker \(stc1\). Further, in preliminary studies we identified expression of Notch pathway components in the developing CS. To study the possible link between \(tbx2b\) and Notch, DAPT treatment was used to block Notch activity in wild type embryos and \(tbx2b\) morphants. DAPT treatment resulted in moderate CS expansion in wild types, while DAPT induced further enlarged CS clusters in \(tbx2b\) morphants. Supporting this result, ectopic activation of Notch signaling in \(Tg(hsp70::Gal4; UAS::NICD)\) led to a reduced CS post heat-shock induction. In addition, ectopic Notch expanded proximal tubule segments at the expense of distal, reducing \(tbx2\) expression domains and therefore suggesting that Notch promotes proximal segments in part by inhibiting \(tbx\) expression. Taken together, these data reveal that interplay between Notch and \(tbx2a/b\) mitigates nephron segmentation, and thus provide novel insights into renal genetic regulatory networks.

4.2 Introduction

As described in previous chapters, one major aspect of investigating kidney development is to understand how nephron tubular epithelial cells acquire specific fates during nephrogenesis and form nephrons with a segmented pattern. There is currently a limited understanding of how nephron tubules are patterned into segments in mammalian organisms, due in part to the complexity of mammalian nephrogenesis and kidney anatomy (Costantini and Kopan, 2010).
However, nephron structure is highly conserved among vertebrate species, and there has been an increasing appreciation of using the zebrafish as a significant genetic model for nephrogenesis studies because of the conservation between zebrafish and mammalian nephron cell compositions (Kroeger et al., 2014).

The zebrafish pronephros is segmented into functionally distinct regions including the proximal convoluted and straight tubule (PCT, PST), distal early and late (DE, DL) tubule, and a pronephric duct (PD) (Wingert et al., 2007). Each segment plays discrete and essential roles in renal function, including but not limited to the absorption and secretion of metabolites and electrolytes (Garrett, 1942; Krishnamurthy, 1976; Kaneko et al., 1992). In addition, endocrine cells called the corpuscle of Stannius (CS) are interpolated between the DE and DL segments. As an endocrine organ, the corpuscles of Stannius are responsible for the synthesis and secretion of stanniocalcin 1 (STC1), a glycoprotein hormone that regulates calcium and phosphate homeostasis in fishes through its actions on the gills and kidneys (Elizondo, et al., 2005; Krishnamurthy, 1976; Kaneko et al., 1992). As such, CS is thought to be an important regulator of calcium uptake from the aquatic environment for bony fish (Elizondo et al., 2005). Although the CS and STC are previously considered to be an endocrine system that is unique to fishes, recent evidence has indicated the existence of STC-like proteins in vertebrates other than fishes, in particular, humans (Wagner et al., 1995; Chang et al., 2005). To date, not much is known about how the CS is patterned and what genetic factors regulate CS differentiation. Formation of the CS is reliant on retinoic acid (RA) levels in the zebrafish embryo, where elevated RA leads to the
inhibition of CS formation and the expansion of proximal nephron segment fates (Wingert, et al., 2007). More recently, however, the sim1a transcription factor was discovered to be both necessary and sufficient for CS formation, and shown to act downstream of RA in promoting the CS fate (Cheng and Wingert, 2015).

The zebrafish pronephros model has been proven to be a strong tool to fulfill the gaps with respect to how each nephron segment is precisely established during nephrogenesis – including the identity of the key factors regulating segmentation formation of the nephron. Our previous study using zebrafish embryo as a nephrogenesis model has identified the transcription factor mecom as a key regulator of segmentation formation and epithelial fate choices of the zebrafish pronephros (Li et al., 2014). During zebrafish nephrogenesis, mecom shows dynamic expression in early distal renal progenitors, and is expressed in DL and PD when segmentation is fully established at 24 hpf. Acting downstream of RA, proper function of mecom is crucial for DL differentiation and restricting PST fate. As previous study has indicated that Jagged 2/Notch signaling modulates the number of multiciliated versus transporting epithelial cells in the pronephros via lateral inhibition (Liu et al., 2007; Ma and Jiang, 2007), our result proved further that mecom acted upstream of Notch signaling pathway to balance multiciliated cells (MCC) and transporting epithelia fate decisions. During nephrogenesis in the developing mouse embryo, it has been demonstrated that gamma-secretase activity, which activates Notch signaling is required for maintaining a competent progenitor pool as well as for determining the proximal tubule and podocyte fates (Cheng et al., 2003; Cheng et al., 2007; Georgas et al.,
2009; Kobayashi et al., 2005; Chen et al., 2005). Despite the fact that Notch is involved in MCC differentiation, there has not been evidence to indicate whether the requirement of Notch in regulation of nephron segmentation formation is conserved in the zebrafish pronephros.

In searching for other key nephron segmentation modulators, we noticed expression of the transcription factor \(tbx2b\) in the distal segment of the pronephros during zebrafish nephrogenesis (Slanchev et al., 2011). Interestingly, previous research in \(Xenopus\) showed that \(Tbx2\) was specifically expressed around the forming pronephric nephron in tadpoles (Cho et al., 2011). Ectopic activation of \(Tbx2\) inhibits nephric mesoderm differentiation. Conversely, \(Tbx2\) loss-of-function resulted in an enlarged pronephros. Importantly, \(Tbx2\) repressed expression of the Notch factor \(Hey1\) to control pronephric morphogenesis, suggesting possible link between \(Tbx2\) and Notch in regulation of nephrogenesis in \(Xenopus\) (Cho et al., 2011).

In this study, using whole mount \textit{in situ} hybridization, we found that transcripts encoding the zebrafish \(tbx2\) orthologues \(tbx2a\) and \(tbx2b\) were spatially expressed in the distal territory of the renal progenitor field during nephrogenesis, and are restricted to the DL and PD at 24 hpf. To elucidate the function of these \(tbx2\) genes in regulation of pronephric segmentation formation, morpholino knockdown studies were performed. Knockdown of \(tbx2a/b\) induced a slight expansion in the PCT, while the DL segment was largely reduced. Surprisingly, \(tbx2a/b\) morphants formed significantly larger CS clusters, as shown by the elevated number of \(stc1^+\) cells in the corpuscle. We have also identified
expression of the Notch pathway effector her9 in the developing CS, suggesting possible roles of Notch signaling pathway in regulation of CS formation. To test this hypothesis, the gamma-secretase inhibitor DAPT was used to block Notch activity in wild type embryos, and a double transgenic line Tg(hsp70::Gal4; UAS::NICD) was used to ectopically activate the Notch signaling pathway (Scheer and Campos-Ortega, 1999). Blocking Notch signaling led to an enlarged CS in wild type embryos, while activation of Notch signaling repressed CS differentiation. Of note, NICD transgenic tbx2b morphants exhibited an expanded CS, suggesting that tbx2 transcription factors act downstream of Notch to repress CS formation. Finally, in this study, we have also documented the conserved role of the Notch signaling pathway in promoting proximal fate and restricting distal segmentation, and have identified the time frame during nephrogenesis when this signaling is essential. Taken together, our research has identified tbx2a/b and Notch signaling pathway as key modulators of segmentation formation during zebrafish nephrogenesis, and has shed lights on the role of tbx2b and Notch signaling in regulation of CS differentiation.

4.3 Results

4.3.1 The tbx2 transcripts were expressed in the distal territory of the pronephros during zebrafish nephron development

First, we examined whether tbx2 transcripts were expressed in the renal progenitor field during nephrogenesis of the zebrafish embryo. Zebrafish possesses two tbx2 orthologues, tbx2a and tbx2b, which are 74% identical. To
detect *tbx2* expression in the developing pronephros, whole mount *in situ* hybridization with RNA riboprobes detecting *tbx2a* and *tbx2b* was performed on embryos at various developmental stages. Overall, *tbx2a* and *tbx2b* showed a similar expression pattern in the distal renal progenitor field, though *tbx2b* was expressed at earlier time points and at higher expression levels (Figure 4.1A). As for kidney development, expression of *tbx2b* could be detected in the renal progenitor field starting from 15 somite-stage (ss), with a punctuated pattern in the caudal domain. Expression of *tbx2b* could not be seen prior to this time point. As development progressed, *tbx2b* expression became more robust in the distal region of the forming pronephros, suggesting possible role(s) of *tbx2b* in regulating distal tubule formation. At 24 hpf, when segmentation of the pronephros is fully established, both *tbx2a* and *tbx2b* expression was restricted to the DL and the PD domains within the pronephros (Figure 4.1B). The dynamic expression patterns of *tbx2* transcripts in the renal progenitors during nephrogenesis suggest that these genes could play essential roles for nephron development in zebrafish embryos.

### 4.3.2 Proper function of *tbx2* is crucial for DL segmentation and PCT specification during zebrafish nephrogenesis

To further characterize the role(s) of *tbx2a/b* in nephron patterning formation, we undertook a loss-of-function study utilizing morpholino knockdowns. To specify the function of *tbx2a* and *tbx2b*, morpholinos targeting *tbx2a, tbx2b* and *tbx2a/b* were used. In general, *tbx2* morphant embryos displayed
minor body axis curvature and slight cardiac edema at 24 hpf, while severe edema could be observed at 48 hpf, suggesting fluid imbalance due to tbx2 knockdown (Figure 4.1C). Next, morphant embryos or control embryos were fixed at 24 hpf to examine possible segmentation defects using double whole mount in situ hybridization. To precisely define possible nephron segmentation alterations, the somite marker smyhc1 was used to mark the somites. We found that at 24 hpf, the pan-proximal marker slc4a4a encoding an electrogenic Na-bicarbonate cotransporter, expressed in the PCT and PST segments from somite 4 to 11 in wild-type embryos, was expanded from somite 4 to 12 in tbx2a morphants, tbx2b morphants, and tbx2a/b double morphants (Figure 4.2). Scrutiny of each segment domain revealed that expansion of the proximal tubule was attributed to an elongated PCT. The PCT expresses the sodium-dependent phosphate transporter slc20a1a, a domain that was situated adjacent to somites 4–8 in wild type embryos, while morphant pronephros exhibited PCT expansion from somite 4 to 9. However, the PST labeled by the transient receptor potential cation channel gene trpm7 was unaffected in tbx2 morphants, and only displayed a one-somite shift due to the expanded PCT (Figure 4.2).

To determine how the abnormal PCT in tbx2 morphants affected distal segment patterning, we used pronephric distal markers and smyhc1. Indeed, the increased PCT in the morphant pronephros was accompanied by a robust reduction in the distal pronephros domain (Figure 4.3). In wild type embryos at 24 hpf, the expression pattern of the chloride conductance channel gene clcnk could be seen in the DE, DL, and PD regions from somites 12 to 18. In contrast, the
clcnk expression domain was reduced in the tbx2 morphants, being located from somites 13 to 18 starting adjacent to somite 13. Interestingly, a proximally located one-somite gap between the DE and DL region could be seen in more than 90% of morphant embryos (Figure 4.3, insert), and this phenotype will be discussed later. The DE domain marked by slc12a1, which encodes a Na/K/Cl cotransporter, had a similar length in wild types and tbx2 morphants. Surprisingly, the distal pronephros region was reduced specifically at the cost of the DL segment. The DL domain indicated by slc12a3, a Na/Cl transporter gene, showed a significant reduction in tbx2 morphants and was restricted to somites 16–17, whereas wild type embryos showed slc12a3 expression from somites 14 to 17. We conclude from these findings that tbx2 is essential for normal nephron segmentation, specifically by promoting DL differentiation and restricting PCT development.

4.3.3 Notch signaling and tbx2 genes cooperate to repress CS development of the zebrafish pronephros

The corpuscles of Stannius, to date found only in bony fishes, are sac-like bodies located adjacent to the kidney. The gene stc1, encoding a glycoprotein, is a marker that can be used to specifically detect the CS (Elizondo, et al., 2005; Krishnamurthy, 1976; Kaneko et al., 1992; Elizondo, et al, 2010; Camp et al., 2003). In wild type embryos, the corpuscles are comprised of a small group of cells that emerge from the renal progenitor field, approximately located next to the 14\textsuperscript{th} somite between the DE and the DL segments. By examination of the CS
marker stc1, we found that in wild type embryos, the corpuscles possessed about 4 cells per nephron on average (Figure 4.4). However, morpholino knockdown of tbx2 transcripts induced a significant expansion in the size of the corpuscles in morphant animals (Figure 4.4). To determine if the increased size of the corpuscles was due to enlarged cell sizes or elevated cell number in the corpuscles, we quantified the number of corpuscle cells in both wild type and morphant embryos (Figure 4.4). Notably, whereas tbx2a knockdown only induced a slight up-regulation of the corpuscle number in the morphant pronephros, tbx2b morphants and tbx2a/b morphant exhibited dramatic increase in the number of stc1+ cells per corpuscle. Thorough quantification indicated that the number of stc1+ cells in the corpuscles increased by ~50% in tbx2b morphants and tbx2a/b double morphants. This data suggest while tbx2a may have minor roles mediating corpuscle development, tbx2b acts as a dominant inhibitor of corpuscle formation, most likely by limiting the number of stc1+ cells in the CS.

In searching for cofactors of tbx2a/b in regulation of the CS formation, we noticed that the Notch signaling pathway effector her9 was expressed in the corpuscles at 24 hpf (Figure 4.5A), suggesting that Notch could also be contributing to CS development during zebrafish nephrogenesis. Several studies have demonstrated essential roles of the Notch signaling pathway in mediation of the renal progenitor fate decisions during nephron patterning formation (Cheng et al., 2003; Cheng et al., 2007; Georgas et al., 2009; Kobayashi et al., 2005; Chen et al., 2005). Therefore, we tested if Notch was also responsible for CS differentiation in nephrogenesis. To do this, we utilized the double transgenic line
Tg(hsp70::GAL4, UAS::NICD) to ectopically activate Notch signaling through heat-shock induction. Conversely, to block the Notch signaling pathway, wild type embryos were treated with the gamma-secretase inhibitor DAPT. We found that heat-shock treatment of the transgenic embryos performed at 15 ss induced the most significant phenotype in the CS, based on examination of the marker stc1, while the overall appearance of the embryo was normal (Figure 4.5A). DAPT treatment was conducted by incubating wild type embryos in 100µM DAPT starting from 15 ss through 24 hpf. Surprisingly, ectopic activation of Notch resulted in a striking reduction in the CS formation in double transgenic embryos, while their siblings showed normal CS development as wild type embryos (Figure 4.5A). In contrast, blocking Notch by DAPT induced a largely expanded CS in wild type embryos. Again, alterations in the size of the CS were due to changes in the number of stc1+ cells, as shown by cell number quantification (see chart in Figure 4.5A). This data suggest that Notch inhibits CS formation by limiting the number of corpuscle cells during zebrafish nephrogenesis.

To investigate whether tbx2 cooperates with Notch in regulation of the CS development, we applied morpholino knockdown of tbx2b in Tg(hsp70::GAL4, UAS::NICD) embryos and performed heat-shock induction at 15 ss. Interestingly, whereas tbx2b morphant siblings showed up-regulation in the corpuscle similar to tbx2b morphants, heat-shock induction of Notch signaling in Tg(hsp70::GAL4, UAS::NICD) tbx2b morphants fail to show reduction in CS formation (Figure
suggesting that \textit{tbx2b} functions downstream of Notch in inhibition of CS development.

\textbf{4.3.4 Notch signaling also involves in segmental patterning by promoting proximal fates of the pronephros}

It has been unknown whether the Notch signaling pathway was also involved in proximal tubule segment patterning during zebrafish nephrogenesis, even though its function in nephron segmentation has been demonstrated in other mammals (Cheng et al., 2003; Cheng et al., 2007; Georgas et al., 2009; Kobayashi et al., 2005; Chen et al., 2005). In previous studies, disrupting Notch activity during early gastrulation fail to induce significant segmentation defects in zebrafish pronephros (unpublished data), raising a fundamental concern as to whether zebrafish nephrogenesis shared conserved genetic networks as other species. However, as the function of Notch in regulation of CS formation is highly temporal-sensitive, we next hypothesized that the role of Notch in proximal tubule induction requires precise control of timing.

Indeed, when Notch signaling was ectopically activated at 15 ss, a time point when advanced patterning of the pronephros started to form (Gerlach and Wingert, 2013), nephron segmentation was disrupted with a preferred proximal fate and repressed distal fate (Figure 4.6). Compared to sibling controls, \textit{Tg(hsp70::GAL4, UAS::NICD)} embryos heat-shocked at 15 ss displayed a 1-somite expansion in the PCT segment, shown by the PCT marker \textit{slc20a1a}. The proximal \textit{trpm7} domain also expanded by 1-somite, accompanied with a slight
caudal shift (Figure 4.6, upper panels). In contrast, the distal DE and DL segment domains, labeled by slc12a1 and slc12a3 respectively, both exhibited a caudal shift and reduction in domain length (Figure 4.6, lower panels). These results indicate that Notch signaling is capable of promoting proximal segmentation and repressing distal domains, suggesting that Notch is a conserved pathway in modulating nephron patterning among zebrafish and other organisms.

4.4 Discussion

Using whole mount \textit{in situ} hybridization, we found that transcripts encoding the zebrafish orthologous \textit{tbx2a} and \textit{tbx2b} were spatially restricted to distal renal progenitors during nephron formation. Morpholino knockdown studies revealed that \textit{tbx2a} and \textit{tbx2b} single and double morphants exhibited a modest expansion in the proximal segments accompanied by a reduction in the distal domains, indicating that these genes have redundant roles in segment patterning. Noticeably, \textit{tbx2b} and double \textit{tbx2a/b} morphants formed significantly larger CS clusters, as shown by the elevated expression of the marker \textit{stc1}. Further, we identified expression of Notch pathway components \textit{her9} in the developing CS, suggesting a possible role of Notch in regulation of CS formation. Indeed, blocking Notch by DAPT resulted in CS expansion in wild-types. Ectopic activation of Notch signaling in \textit{Tg(hsp70::Gal4; UAS::NICD)} led to a dramatically reduced CS post heat-shock induction. However, knocking down \textit{tbx2b} in transgenic embryos failed to repress CS formation post heat-shock induction, suggesting that \textit{tbx2b} acts downstream of Notch to inhibit CS
development. Additionally, we have also characterized a new role of Notch in regulating segmentation patterning of the zebrafish pronephros, which was previously unknown in the field. Ectopic Notch activation at the 15 ss expanded proximal tubule segments at the expense of distal domains, and therefore suggests that Notch signaling favors proximal segments and represses distal fate. This data indicates that Notch is a conserved pathway in regulating renal progenitor fate decisions among species.

### 4.4.1 tbx2a/b play critical roles in renal progenitor patterning

The *tbx2* transcription factor subfamily belongs to the T-box transcription factor gene family, which is known by the highly conserved T-box DNA binding motif common to all members of the T-box gene family (Douglas et al., 2013). *tbx2* genes play critical roles during embryonic organogenesis by mediating progenitor fate decisions and regulating cellular proliferation (Douglas et al., 2013; Sylva et al., 2013; Abrahams et al., 2010). With respect to kidney development, a previous study in *Xenopus* has identified expression of *Tbx2* in a region surrounding the pronephric territory (Cho et al., 2011). Our study identified expression of *tbx2a* and *tbx2b* in the distal region of the renal progenitor field during zebrafish nephrogenesis, suggesting the possibility of conserved roles of *tbx2* in vertebrate kidney organogenesis. Of note, previous analysis has identified TBX2 expression in human fetal kidney and adult kidney (Law et al., 1995). Studies in mice embryos also described expression of *Tbx2* in the Wolffian duct, which is part of the mesonephric system (Douglas et al., 2013).
Despite these reports of tbx2 expression in the urinary tract system among various organisms, the molecular mechanisms underlying how tbx2 impacts kidney development remain intriguing. In tadpoles, overexpressing Tbx2 inhibited nephric mesoderm formation. In contrast, Tbx2 inactivation expanded the boundary of the pronephric nephron, resulting in an enlarged pronephros. These results suggested a role of Tbx2 in determining the size of the nephron (Cho et al., 2011). In contrast, our research has suggested distinctive roles of tbx2a/b in regulating nephron segmentation patterning and CS differentiation during zebrafish nephrogenesis. Here, we demonstrated that disrupting tbx2 function induced expansion in proximal tubule and reduction in distal segments, suggesting a role of tbx2 in promoting distal renal fates. As such, our research identified tbx2 as a new regulator of nephron segmentation formation in the whole genetic network of nephron patterning. It remains unclear if these are conserved roles in nephron patterning in mammals and other species.

4.4.2 tbx2b and induction of the CS in teleosts

The CS are endocrine glands unique to bony fish. Firstly discovered in 1839, the CS are are morphologically distinguishable as cream-colored bodies situated midway along the ventral surface of the kidneys in adult salmonids (Garrett, 1942; Krishnamurthy, 1976). The CS has been well studied for its secretion of Stanniocalcin (STC), a homodimeric glycoprotein hormone with an integral role in calcium and phosphate homeostasis in fishes (McCudden et al., 2011). STC in fish acts to prevent hyperkalemia and is released into the
bloodstream in response to serum Ca\(^{2+}\) elevations, whereas in kidney it stimulates phosphate reabsorption in order to balance excess Ca\(^{2+}\) in the serum (Lu et al., 1994). Despite relatively intensive studies on the CS gland and STC, not much is known about how the CS is formed and what molecular mechanisms regulate its differentiation.

Recent findings by our lab have identified the transcription factor \textit{sim1a} as a critical modulator of CS differentiation, as disrupting \textit{sim1a} function totally abrogated CS formation in zebrafish pronephros (Cheng and Wingert, 2015). In the present study, evidence on the roles of \textit{tbx2} and Notch signaling added further knowledge on the regulation of CS development during zebrafish nephrogenesis. Interestingly, as a patterning factor, \textit{sim1a} is also involved in negotiating the PCT/PST boundary. While a major function of \textit{tbx2} is to promote DL formation and restricting PCT fate, it is reasonable to believe that nephron patterning requires precise execution, and possibly collaboration between, these developmental factors.

4.4.3 Notch signaling pathway is involved in nephron segmentation formation during zebrafish nephrogenesis

Previous studies in mammalian models have suggested a crucial role of the Notch signaling pathway in proximal-distal axis establishment and development of the mammalian nephron. Malfunction of Notch signaling components caused severe deficiency in the formation of proximal segments and the glomerulus (McCright, 2003). Functional studies performed in mouse
metanephros organ culture showed that blocking Notch signaling pathway during early kidney development abrogated glomeruli formation and proximal tubule differentiation (Cheng et al., 2003). Supporting this result, mice with a hypomorphic allele for the Notch receptor Notch2 also exhibited podocyte and proximal segmentation defects (McCright, 2003). These results lead to a model where Notch signaling pathway is necessary for promoting the proximal fates including podocyte and proximal segments during mammalian nephrogenesis (Sirin and Susztak, 2012). O'Brien et al. showed that depletion of a Notch transcriptional mediator in zebrafish embryos induced fewer podocyte formation, suggesting involvement of Notch in regulating podocyte specification (O'Brien et al., 2011). However, until the present work there was no direct evidence that Notch signaling pathway is also involved in zebrafish nephron proximo-distal segmentation patterning.

Interestingly, previous research has demonstrated a role of Notch in zebrafish nephron epithelial fate decisions. For example, Notch has been linked to multiciliated cell (MCC) differentiation during zebrafish pronephric development (Liu et al., 2007; Ma and Jiang, 2007) by repressing ciliogenesis gene expression in transporting epithelia. In this study, we prove further that Notch signaling is also crucial for proximal-distal renal progenitor patterning during zebrafish nephrogenesis. Ectopic activation of Notch signaling at 15 ss induced expansion in proximal segments and the distal domains of the pronephros is reduced due to activated Notch. This data suggested a conserved role of Notch across species by promoting proximal segmentation and repressing distal fates, which adds a new
and important aspect to our understanding of zebrafish nephrogenesis. Supporting this idea, previous research has identified expression of the Notch signaling components \textit{dlc} and \textit{jagged2} in the proximal region of the renal progenitor field during early somitogenesis (Wingert and Davidson, 2008). However, disrupting Notch signaling during early gastrulation failed to induce segmentation defects in zebrafish embryos, proposing that proper function of Notch requires strict control of timing.

As such, it remains intriguing what are the upstream signaling factors mediating Notch function. Previous research has characterized the RA signaling as a crucial upstream factor regulating nephron segmentation (Wingert et al., 2007; Wingert and Davidson, 2008). Exogenous RA induced the formation of a proximalized renal progenitor field at late gastrulation. Downstream of RA, key transcription factors including \textit{irx3b}, \textit{mecom} and \textit{sim1a} further control nephron tubule patterning, and all show temporal and spatial regulation (Wingert and Davidson, 2008; Li et al., 2014; Cheng and Wingert, 2015). Hence, it would be interesting to examine the epistatic relationship between RA and Notch signaling in modulating renal progenitor patterning.

In conclusion, in this study we identified the T-box transcription factors \textit{tbx2a} and \textit{tbx2b} as crucial regulators of nephron patterning and renal progenitor differentiation during zebrafish nephrogenesis. The specification and patterning of the nephron progenitor is a complex process that requires integrative regionalization of each progenitor population, while highly specialized nephron epithelial cells in each domain carry out distinct physiological functions. Whereas
applications of zebrafish and other amphibian models continue to advance the knowledge of nephrogenesis, a large gap remains on the broad picture of the molecular control behind renal progenitor specification. Discoveries of the key modulators during nephron patterning and the molecular mechanisms underlying renal progenitor commitment are needed to better understand the processes of nephrogenesis and kidney ontogeny.
Figure 4.1 *tbx2* transcripts are specifically expressed in a caudal domain of the renal progenitor field during nephrogenesis, and knockdown of *tbx2a* and/or *tbx2b* led to disrupted function of the pronephros. (A) Schematic depiction of zebrafish pronephric segmentation at 24 hours post fertilization (hpf). Segment abbreviations: P (podocytes), N (neck), PCT (proximal convoluted tubule), PST (proximal straight tubule), DE (distal early tubule), CS (corpuscle of Stannius), and DL (distal late tubule). (B) Using whole mount *in situ* hybridization, expression of the *tbx2b* transcript could be detected in the distal region of the pronephros starting from the 15 somite stage (ss) (arrow head). At 24 hpf, *tbx2b* expression is restricted to the DL and the PD domains of the pronephros. At 24 hpf, expression of *tbx2a* in the pronephros were detected in the distal and duct region, though with lower expression level compared to *tbx2b*. (B, bottom panel) (C) Morpholino knockdown of *tbx2a*, *tbx2b* or *tbx2a/b* induced cardiac edema at 48 hpf (arrow head). Morphant embryos also displayed malformation in the brain and body curvature.
Figure 4.2 *tbx2* knockdown leads to slight expansion in the PCT segment in *morphant* pronephros. Upper panel: At 24 hours post fertilization (hpf), whole mount *in situ* hybridization was used to label segmental domains in wild types and *tbx* morphants. Proximal segments marked by *slc4a4a* elaborated in *tbx2* morphants. Within proximal domains, morpholino knockdown of *tbx2a*, *tbx2b*, or *tbx2a/b* induced a consistent 1-somite expansion in the *slc20a1a*-expressing PCT segment in morphant pronephros at 24 hpf. The PST segment, labeled by transcripts encoding *trpm7*, was not affected compared with wild type embryos. Representative results are based on examination of at least 20 embryos per marker. Morphant pronephros structures showed consistent segmentation disruption based on somite counting of at least 5 embryos per marker. Lower panel: Schematic summary of proximal segment organization in wild type and *tbx2* morphants, with PCT alterations highlighted in orange and PST in yellow. Abbreviations: PCT (proximal convoluted tubule), and PST (proximal straight tubule).
Figure 4.3 *tbx2* knockdown leads to the formation of a significantly reduced size of the DL tubule segment. Upper panel: Whole mount *in situ* hybridization using distal segment markers showed a 2-somite reduction in the DL region by 24 hpf. The distal segments and pronephros ducts were labeled by *clcnk*. Expression of *slc12a1* and *slc12a3* marked the DE and DL, respectively. Pronephros segment boundaries were evaluated relative to the somites, which were shown by *smyhc1* expression (red). Of note, an approximately one-somite gap region could be detected between the DE and DL in the morphant pronephros, which was not present in the pronephros of wild type embryos. Lower panel: Schematic depiction of distal segment alterations, with DL domains highlighted in orchid. Abbreviations: DE (distal early tubule), DL (distal late tubule), and PD (pronephric duct).
Figure 4.4 *tbx2* genes regulate CS formation by inhibiting the number of *stc1*+ cells during zebrafish nephrogenesis. The CS was labeled by whole mount *in situ* hybridization to detect transcripts encoding *stc1* at the 24 hours post fertilization (hpf) stage. In wild type embryos, each corpuscle is comprised of about 4-6 *stc1*+ cells per nephron (upper panels), while knocking down *tbx2* transcripts induced a significant up-regulation of the *stc1*+ cell number in the corpuscles (lower 3 panels). Notably, knock down of *tbx2b* and double knockdown of *tbx2a/b* led to most severe phenotype while disruption of *tbx2a* only showed minor increase of the CS, suggesting prevailing role of *tbx2b* in regulation of the CS development by repressing *stc1*+ cell differentiation. Quantification of the corpuscle cell number is shown in the chart on bottom. For each experiment, at least 15 embryos were examined. (*P = 0.072, **P = 0.000*)
Figure 4.5 *tbx2b* acts downstream of Notch to inhibit CS differentiation. (A) At 24 hpf, whole mount *in situ* hybridization identified expression of *her9*, a Notch signaling pathway factor, in the CS of zebrafish pronephros, suggesting a possible role for Notch signaling in CS development (upper left panel). *Tg(hsp70::Gal4; UAS::NICD)* embryos and siblings were heat-shocked at the 15 somite stage (ss) and then raised to 24 hours post fertilization (hpf). Post heat-shock induction, *Tg(hsp70::Gal4; UAS::NICD)* embryos exhibited a dramatic reduction of the CS, while siblings showed normal CS clusters. To block Notch signaling, wild type embryos were incubated in 100 µM DAPT/E3 solution from the 15 ss to the 24 hpf stage. DAPT treatment resulted in significant elevation in the corpuscle (right panels). (B) Heat-shocked *Tg(hsp70::Gal4; UAS::NICD)* *tbx2b* morphant exhibited significant expansion in the CS sized shown by *stc1* expression. To quantify changes in the CS, *stc1* cells were counted for each experiment (left chart, *P*=0.000). Representative images were based on thorough examination of at least 20 embryos per treatment.
Figure 4.6 Notch promotes proximal tubule differentiation and represses distal fates prior to segmentation establishment. Tg(hsp70::Gal4; UAS::NICD) embryos and siblings were heat-shocked at the 15 somite stage (ss) by incubating embryos at 37°C for an hour. Embryos were then transferred to normal temperature and raised to 24 hours post fertilization (hpf) to examine segmentation formation by whole mount in situ hybridization. Notch activation in transgenic embryos induced expansion in both PCT and PST segments, while distal segments, especially the DL, were reduced. Pronephric segmentation in siblings appeared normal as wild type embryos. Heat-shock prior to 15 ss fail to affect segmentation patterning in Tg(hsp70::Gal4; UAS::NICD) embryos, suggesting that the effect of Notch signaling to nephron patterning is temporally sensitive. For each segmentation marker, at least 5 embryos were genotyped.
**Figure 4.7 Working model.** (A) During zebrafish nephrogenesis, our data proposes a model where the *tbx2* transcription factors regulate patterning of the pronephros by restricting the PCT domain and promoting the DL segment. Meanwhile, a new role of Notch signaling pathway is revealed in segmentation establishment of the zebrafish pronephros, where it promotes proximal fates and represses distal segmentation. (B) *tbx2* transcription factors balance the CS size by limiting the number of *stc1*+ cells downstream of Notch signaling pathway. However, it remains unclear if there was direct epistatic relationship between these two factors. Notch signaling pathway also acts as a key modulator repressing CS differentiation upstream of *tbx2b* shown by abolished CS formation in heat-shocked *Tg(hsp70::Gal4; UAS::NICD)* embryos. However, it remains unclear if other signaling factors were also involved downstream of Notch in regulating CS development.
CHAPTER 5:

CONCLUSIONS AND FUTURE PERSPECTIVES

Using both mammalian and amphibian models, continuous progress has been made in the identification of genetic factors involved in the establishment of nephron segmentation. While the broader context of proximal and distal specification has been well studied, questions remain on how terminal determination of the various nephron segments is accomplished. Emerging evidence within the field indicates that this complex progress requires elegant communication between individual cell types during pattern formation of the nephron, highlighting regional responses of renal progenitors to stimuli including morphogens and various signaling pathways. Understanding terminally differentiated states of nephron segmentation will help researchers to pinpoint the molecular mechanisms underlying the developmental programs of nephrogenesis, which will provide valuable insights on the origin of congenital renal system anomalies.
5.1 From development to regeneration - understanding the cellular basis of kidney regeneration

While congenital and acquired birth defects of the kidney organ consist of a broad spectrum of kidney disease, other acquired renal diseases ranging from acute kidney injury (AKI) to chronic kidney disease (CKD) could also lead to severe organ failure known as end-stage kidney disease (ESRD) (Schedl, 2007; Bonventre and Yang, 2011; Murugan and Kellum, 2011; Venkatachalam et al., 2010; El Nahas and Bello, 2005). Currently, there is no effective treatment for ESRD other than the kidney replacement therapies of dialysis or transplantation. Both of these interventions are intensive, and lead to a considerable burden not only on existing healthcare systems, but also on patients and their families (Weiner, 2009). Hence, great effort has been made in searching for alternative treatments to reduce the cost of kidney diseases and improve life of patients suffering from renal conditions.

In the past decade, cutting-edge findings from the field of stem cell biology have harnessed new technology for regenerative medicine, which has been broadly applied as an emerging technique to cure diseases in various organs (Stocum, 2011). Stem cells represent a unique group of cells that are able to replenish through self-renewal, while producing offspring that differentiate into specialized cell types via asymmetric cell division (Stocum, 2001). Stem cells are vital to normal development as well as tissue regeneration in adult organs (Weissman, 2000). It is currently well accepted that a small amount of stem cells, maintained from the stem cell pool during embryogenesis, give rise to tissue-
specific adult stem cells that are active throughout life. Adult stem cells exhibit limited potency compared to embryonic stem cells (ES), while their multipotency could generate organ-specific cell types during the process of tissue regeneration (Hsu and Fuchs, 2012). Adult stem cells replace tissue to regulate normal homeostasis, while they also response to damage to sustain organ integrity. As such, adult stem cells play crucial roles in organ turnover and disease/injury conditions (Burness and Sipkins, 2010; Sharpless and DePinho, 2007).

Increasing evidence argues that human kidneys are capable to regenerate intrinsically after damage (Fioretto and Steffes, 1998; Fioretto et al., 2006). To date, it remains unclear how nephrons response to renal injury under AKI or CKD conditions. In particular, a riveting and unresolved controversy centers on whether kidneys retain any stem cell population. If the kidney does not, the central question then remains as to how differentiated renal cells are involved in renal regeneration phenomena. Interestingly, fate-tracing experiments in murine AKI models suggested that differentiated nephron tubular cells serve as a major source of nephron turnover post renal injury (Humphreys et al., 2011). In contrast, a recent study suggested that human CD133+ CD24+ CD106− tubular progenitors located in both proximal and distal segments contributed to the regenerating population in both AKI and chronic tubular damaged kidneys (Angelotti et al., 2012). As such, it remains controversial whether the origin of renal regeneration came from differentiated tubular cells or renal stem cells (Li and Wingert, 2013). Clearly, better understanding of this controversy requires close examination of the
cellular components of the kidney, together with advanced knowledge of the developmental programs underlying various nephrogenesis processes.

In searching for the source of kidney regeneration, lineage tracing has become a powerful tool to identify the cellular basis of nephron progeny (Li and Wingert, 2013), which requires cell-type specific promoter to label possible progenitors or differentiated epithelial cells. However, a significant current challenge has to do with the limited number of specific promoters that can be used to generate transgenic models to track regenerative resources (Li and Wingert, 2013). Therefore, it is imperative to identify key developmental modulators of nephrogenesis whose promoter could be tailored for making transgenic animals, which could be of great application for better understanding kidney regeneration.

Another implication for identifying nephron developmental regulators is that these nephrogenesis factors could be used directly as markers to isolate potential kidney stem cells or regenerative tubular cells. For example, the renal developmental gene factor *Paired box 2* (*Pax2*), normally expressed in renal progenitors during nephrogenesis, was adopted as a marker labeling renal tubule progenitors in mature nephrons (Bussolati et al, 2005). The reported renal tubule progenitors were isolated based on tubule cells expressing the antigen *CD133* (a hematopoietic stem cell marker) and *Pax2* from human kidney cortex samples (Bussolati et al, 2005). These cells exhibited self-renewal and could differentiate into tubule-like structures *in vitro*, and incorporated into damaged nephron tubules post tubulonecrosis (Bussolati et al, 2005).
Tissue repair in adult organs has been known to involve re-activation of gene programming previously essential to the process of organogenesis (Hopkins et al., 2009). For instance, re-expression of genes responding to kidney injury has been reported in both animal models and human renal diseases, such as ischaemia–reperfusion injury and diabetes (Devarajan et al., 2003; Walsh et al., 2008). However, regeneration requires accurate control of developmental pathways, while inappropriate gene activity could lead to renal malfunctions rather than overcoming kidney damages. For example, excessive activation of the Notch signaling pathway in podocytes could result in apoptosis and proteinuria, while blocking Notch signaling prevented the onset of glomerulosclerosis and proteinuria (Niranjan et al., 2008). As such, achieving an in-depth understanding of developmental factors and the molecular mechanisms underlying kidney ontogeny could benefit our insight of renal regeneration, which could have great implications for applying regenerative medicine approaches to a whole host of kidney diseases.

5.2 Using zebrafish as a model to study nephrogenesis

Studies using zebrafish pronephros as a developmental model have unveiled a number of exciting aspects concerning nephron patterning and renal progenitor specification. The conserved kidney anatomy of zebrafish pronephros serves as an excellent model to identify developmental pathways, while comprehensive understanding of gene programming during nephrogenesis may shed lights on the pathology of congenital kidney diseases and uncover new
treatment approaches. Especially, the identification of pathways or genetic factors involved in nephron development may facilitate the field of stem cell research to harness regenerative pathways to trigger in vivo tissue repair.

5.2.1 New genome-editing technology in zebrafish

One of the major loss-of-function approaches applied in the zebrafish model is morpholino knockdown. Morpholino oligomers have been widely used for many years in the zebrafish community to knock down the function of target genes transiently. Antisense morpholino molecules are designed based on nucleic acid bases that are linked to morpholine rings and a non-charged phosphorodiamidate backbone, such that morpholinos would not interact electrostatically to protein, hence causing less toxicity, meanwhile being resistant to nucleases (Summerton, 2007). However, morpholino induced phenotypes have been debatable since there is a lack of generally accepted guidelines to control morpholino side effects (Schulte-Merker and Stainier, 2014). Therefore, it has been difficult to determine between specific and non-specific knockdowns caused by the non-specific binding to unintended targets (Eisen and Smith, 2008). To circumvent this dilemma, recently, the surprisingly efficient alternative for reverse genetics has been introduced in zebrafish to generate target-specific mutants (Hwang et al., 2013).

In the past decades, the CRISPR/Cas9 genome-editing platform has been adapted in vivo in zebrafish by its powerful ability to introduce site-specific insertion or deletion mutations. In zebrafish, CRISPR-associated (Cas) genetic
elements (Cas9) can be directed to user-defined genomic target sites via synthetic guide RNAs (sgRNAs), enabling random or homology-directed sequence alterations, long-range chromosomal deletions, simultaneous disruption of multiple genes, and targeted integration of several kilobases of DNA (Gonzales and Yeh, 2014). This genome-editing system has been simplified in zebrafish models and is comprised of only two components including Cas9 endonuclease and a customized synthetic single guide RNA (sgRNA) designed based on target genes. As such, the sgRNA guides the Cas9 endonuclease to bind to and unwind a specific 20-nt genomic target site to induce error-prone nonhomologous end joining–mediated repair (Hwang et al., 2013; Gonzales and Yeh, 2014).

Over the past years, the CRISPR/Cas9 system has proven to be a compelling and efficient genome-editing technique applied in zebrafish by its broad adaptability and ability to generate site-specific mutations. However, to date, the CRISPR/Cas9 system has not yet been used to generate kidney-specific mutations in zebrafish. In light of the growing debates over the reliability of morpholino induced knockdown, there will be enormous potential to employ the CRISPR/Cas technique to create mutations of key kidney developmental factors.

5.2.2 Zebrafish – a conserved model of nephrogenesis

The zebrafish is a powerful vertebrate model for functional genomic analysis, the study of human disease pathogenesis, and for the discovery of new drugs (Campbell et al., 2013; Helenius and Yeh, 2012; Lieschke and Currie, 2007). The key advantage of the zebrafish model lies in its intermediate
evolutionary relationship to humans, between mammalian model systems and invertebrate model systems. The common vertebrate ancestry gives the zebrafish greater genetic and anatomical similarity to humans – not only that orthologous genes carry similar functions, most of the organ systems and structures between zebrafish and humans are homologous as well (Kettleborough et al., 2013; Lieschke and Currie, 2007; Santoriello and Zon, 2012).

The genetic and anatomical similarity between mammalian and zebrafish model also applies to the kidney organ. Indeed, recent research has demonstrated that the segmental nature of the nephrons is fundamentally conserved across pro-, meso- and metanephric nephrons in mammals and popular amphibian animal models such as *Danio rerio* and *Xenopus* (Wingert and Davidson, 2008; Wessely and Tran, 2011; Kroeger and Wingert, 2014). Recent work from our group emphasizing the role of *sim1a* in zebrafish nephrogenesis further proved the conserved requirement of genetic modulators during kidney development across species. The *sim1/2* transcription factors are found across vertebrates, including fish, frog, chick, mouse, and human (Ema, et al., 1996; Fan, et al., 1996; Chrast, et al., 1997; Coumailleau, et al., 2000; Wen, et al., 2002; Coumailleau and Duprez, 2009). Notably, *Sim1* expression has been previously reported in the mice embryonic kidney, with spatial expression in the mesonephric ducts (Fan, et al., 1996; Ema, et al., 1996). Our functional analysis revealed that, during zebrafish pronephric patterning formation, *sim1a* activity is required and partially sufficient to induce PST segmentation, while it may inhibit PCT fate and/or negotiate the PCT/PST specification (Cheng and Wingert, 2014). Supporting the idea that key
nephron patterning factors are conserved across species, more recent finding form the genitourinary developmental molecular anatomy project (GUDMAP) showed Sim1 expression in the comma- and S-shaped body during mouse metanephric nephrogenesis (McMahon, et al., 2008; Harding, et al., 2011). At the S-shaped body stage, Sim1 transcripts could be detected within the medial region, which is developing into the future loop of Henle located between the early proximal and early distal tubules. Whereas functional study of how Sim1 may be involved in mammalian nephron patterning has not been conducted, the conserved expression profile of Sim1 in both zebrafish and mice model is intriguing, and argues that Sim1 is required for nephron segmentation establishment across species. Interestingly, a genome-scale in situ analysis of mammalian transcriptional regulatory factors reported expression of murine Mecom in nascent nephron S-shaped bodies in the developing metanephric kidney (Yu, et al., 2012), thus suggesting mecom could be involved in nephron patterning in mammalian kidney development as well.

In conclusion, in spite of current understanding of nephron development, numerous questions remain in the field of nephrology. During vertebrate nephrogenesis, growth, morphogenesis and patterning transform renal progenitors into a highly specialized tubular structure with distinct cell types positioned along a proximal to distal axis of functional arrangements. Appropriate balancing of the expansion and differentiation of nephron progenitors is crucial for segmentation commitment, which in turn is needed to fulfill proper nephron function. Using zebrafish embryonic pronephros as a model, this thesis focused on a small aspect
of the regulatory processes that assign the renal mesenchymal progenitors into a
segmented nephron. However, future work is needed to identify other key
regulators of nephron patterning and renal progenitor commitment that serve
crucial roles across vertebrate species. Knowledge of nephron development may
shed new light on the mechanisms of kidney regeneration in humans, as such
providing a way to open novel avenues for the treatments of kidney diseases.
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