D. Malpighian tubules play similar function as tubular parts of the nephron. Main segment of Malpighian tubules is responsible for fluid secretion, lower segment and ureter for reabsorption, and initial segment for storing ions and other metabolic products. Since, Malpighian tubules are enriched with several transporters, fluid transport and ion transport assay offer opportunity to evaluate the function of transporters. Nitrogenous waste products and uric acid gets deposited in lumen of MTs and finally excretes out through alimentary canal. Capability of transporter and functions of MTs can also be judged through uric acid deposition assay. Junctional protein Armadillo (orthologue of vertebrate beta-catenin), tight junctional protein Dlg (orthologue of vertebrate Zonula occludens ZO-1), cell adhesion protein Fas-2 (orthologue of vertebrate neural cell adhesion molecule N-CAM) expresses in MTs and offers opportunity to judge the effects of nephritic syndrome on junctional and adhesion proteins. It will also be helpful to identify new proteins/molecules interacting with these proteins. MTs help in maintaining homeostasis, intestinal pH, regulation and secretion of calcium (Maddrell et al. 1991), immunological defense, and clearance to toxic substances (Beyenbach et al. 2010). Therefore, MTs act as a quality controller for the hemolymph as nephrons control the quality of blood in humans.

E. Diabetic nephropathy

Impairment of kidney function through diabetes is known as diabetic nephropathy. Long-term diabetes leads to chronic kidney diseases and renal failure; 44% cases of renal failures are due to diabetes in the USA (National Diabetes Statistics Report 2014). Since in Drosophila, type 1 (Rulifson et al. 2002) as well as type 2 diabetes (Musselman et al. 2011; Morris et al. 2012, Pendse et al. 2013; Trinh and Boulianne 2013) can be induced, and its renal system similar to nephrons, it offers the best opportunity to study molecular basis of diabetic nephropathy (Na and Cagan 2013; Na et al. 2015; Betz and Conway 2016). Very recently, Delanoue et al. identified that insulin release is triggered by stunted (Sun), a ligand of Methuselah (Mth) receptor, in insulin producing cells of Drosophila brain. Therefore, Mth and Sun delineate a new cross-organ circuitry that modulates physiological insulin level in response to nutrients (Delanoue et al. 2016).

F. Renal Cancer

Fault in regulation of stem cell behavior results in tissue degeneration, premature aging, and cancer formation. Human kidney has a low rate of cellular turnover but has a great ability for tissue regeneration after an ischemic injury. Multipotent stem cells were identified in MTs of adult Drosophila. These stem cells are relatively quiescent and only divide once in one week. However, they can become very active and even develop stem cell tumors upon activating the JAK-STAT signal transduction pathway or expressing the activated form of the Ras oncogene (Singh et al. 2007; Zeng et al. 2010). Therefore, Drosophila MTs provide an excellent in vivo system for understanding the molecular mechanisms of stem cell self-renewal and differentiation which may increase our understanding of the mechanisms underlying cancer formation, aging, and degenerative diseases. It
will be helpful for using stem cells for future regenerative medicine and gene therapy for acute and chronic kidney diseases in humans.

G. Xenobiotic nephrotoxicity

Kidney is the major target for xenobiotics, which includes drugs, industrial chemicals, environmental toxicants, and other compounds. Accurate methods for screening of large number of potential nephrotoxic xenobiotics with diverse chemical structure are currently not available. *Drosophila* can provide in vivo tool for screening of nephrotoxicants affecting glomerulus and tubular part of nephron as nephrocytes play similar function to podocytes (Weavers et al. 2009) and MTs play role similar to tubular part of nephrons. MTs are enriched with several conserved genes that participate in metabolism and detoxification, for example, alcohol dehydrogenase, glutathione transferase, and cytochrome P450. Glutathione transferases play a role in metabolism of xenobiotics, conjugating reduced glutathione to lipophilic substrates, making them more hydrophilic and thus more easily excreted (Dow 2009). Therefore, *Drosophila* renal system offers a good opportunity for dissection of molecular mechanisms of nephrotoxicants.

1.7 Conclusion

As the basic developmental process and genes are conserved in Malpighian tubules and human kidneys, the genetic tools available in *Drosophila* can be utilized to understand the kidney development and associated disorder. Since Malpighian tubules develop from ectodermal and mesodermal cell lineages, it provides opportunity to identify novel genes involved in the integration of cells from different origins that makes mature and functional structure. Different segments of tubules are specialized to perform different functions that provides opportunity to dissect pathways involved in the regulation of absorption, reabsorption and secretion. Additionally Malpighian tubules are immune tissues and express several components of immune pathways hence they can be used to understand innate immune mechanisms pertaining to humoral response. Therefore, *Drosophila* renal system provides an opportunity to decipher molecular basis of many kidney diseases which will be helpful for identifying potential target for the alleviation of these diseases and preventing renal failure.

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Chapter 2
Zebradish Pronephros Development

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Abstract The pronephros is the first kidney type to form in vertebrate embryos. The first step of pronephrogenesis in the zebrafish is the formation of the intermediate mesoderm during gastrulation, which occurs in response to secreted morphogens such as BMPs and Nodals. Patterning of the intermediate mesoderm into proximal and distal cell fates is induced by retinoic acid signaling with downstream transcription factors including wt1a, pax2a, pax8, hnf1b, sim1a, mecom, and irx3b. In the anterior intermediate mesoderm, progenitors of the glomerular blood filter migrate and fuse at the midline and recruit a blood supply. More posteriorly localized tubule progenitors undergo epithelialization and fuse with the cloaca. The Notch signaling pathway regulates the formation of multi-ciliated cells in the tubules and these cells help propel the filtrate to the cloaca. The luminal sheer stress caused by flow down the tubule activates anterior collective migration of the proximal tubules and induces stretching and proliferation of the more distal segments. Ultimately these processes create a simple two-nephron kidney that is capable of reabsorbing and secreting solutes and expelling excess water—processes that are critical to the homeostasis of the body fluids. The zebrafish pronephric kidney provides a simple, yet powerful, model system to better understand the conserved molecular and cellular progresses that drive nephron formation, structure, and function.

2.1 Introduction

The vertebrate kidney functions to maintain the composition of the blood so as to permit healthy bodily function. In order to achieve this, the kidney has evolved one of the most structurally and physiologically complex organ subunits in the body, the
nephron. Each nephron consists of a blood filter (the renal corpuscle containing the glomerular tuft) that selectively transports fluid from the blood vasculature into epithelial tubules where passive and active reabsorption and secretion of solutes such as ions, sugars, amino acids, and water occur. Nitrogenous waste and excess minerals and water are then expelled to the exterior via a duct system.

In amniotic vertebrates, three kidney forms arise during development: the pronephros, the mesonephros, and the metanephros. Each kidney is created sequentially and requires the previous kidney type for its induction. While their topographical complexity increases progressively, each kidney type maintains the nephron as its functional subunit. The pronephros, at least in frogs and fish, is comprised of two nephrons, while the mesonephros contains tens to hundreds of nephrons. The metanephros (found only in mammals, reptiles, and birds) has a much greater nephron number, although this number is highly divergent, even between members of the same species. In humans, healthy metanephric kidneys contain between 200,000 and >2.5 million nephrons, with an average of one million nephrons (Bertram et al. 2011).

Anamniotic vertebrates, such as the teleost fish Danio rerio (zebrafish), do not develop a metanephros. Instead, kidney development ceases upon formation of the mesonephros. Despite this, development of the zebrafish kidney utilizes molecular pathways and cell types that are conserved in the mammalian kidney. The amenability of zebrafish as an experimental model means it has become a powerful tool to unlock the secrets of kidney development and disease. In this chapter, we will describe in detail how the pronephros forms during early development. The studies that have characterized zebrafish pronephrogenesis have been important in aiding our understanding of key processes in mammalian kidney development.

2.2 Pronephric Origins and Anatomical Organization

Early development in the zebrafish embryo involves rapid cleavage of blastomeres before activation of genomic transcription at the mid-blastula transition. Soon after this time point, the single-layered blastula embryo initiates extensive cell movements (epiboly) to form the tri-laminar gastrula embryo. The three germ layers (the ectoderm, the mesoderm, and the endoderm) form between 4 and 10 hours post-fertilization (hpf) (Kimmel et al. 1995). The pronephros is derived from the mesoderm germ layer, and progenitors for the pronephros are first detected at the late gastrula stage (Drummond et al. 1998; Pfeffer et al. 1998). This bilateral stripe of cells is anatomically positioned between paraxial mesoderm and lateral plate mesoderm and thus is termed the intermediate mesoderm. The zebrafish intermediate mesoderm gives rise to erythroid cells, endothelium, and the pronephros (Davidson and Zon 2004). The origin of glomerular precursors is not so easily discernable, but a population of cells expressing Wilms’ tumor suppressor 1a (wt1a), an early marker of glomerular fate, can be detected at the anterior end of the intermediate mesoderm stripe from the 3-somite stage (11 hpf) onwards.
By 48 hpf, the pronephros consists of a fused midline glomerulus linked via a neck segment to paired tubules that run the length of the trunk to the cloaca (Fig. 2.1) (Bollig et al. 2006). Excluding the neck segment, the pronephric tubules are segmented along the anterior–posterior axis into four segments: the proximal convoluted tube (PCT), the proximal straight tube (PST), the distal early tubule (DE), and the distal late segment (DL). The PCT, PST, and DE segments are identifiable by the expression of multiple different solute carriers that perform the transport functions of the nephron (see Fig. 2.1 for examples) (Wingert and Davidson 2008, 2011; Wingert et al. 2007). The DL segment expresses genes encoding solute carriers (such as \( \text{slc12a3} \)) and genes that are also expressed in the mammalian nephric duct (such as \( \text{gata3} \)) suggesting that this segment is a tubule/duct hybrid (Fig. 2.1) (Wingert et al. 2007). At its most posterior end, the DL segment is fused with the cloaca, which acts as an opening to the exterior where waste products are expelled (Pyati et al. 2006).

Fig. 2.1 Early pronephros development. Whole embryo views on the left are lateral views outlining the spatial arrangement of the pronephros at the stages indicated. Schematic images on the right show dorsal views of the complementary stages. Arrows in 6-ss stage embryo highlight caudal migration of the distal tubule to the cloaca. Arrows in 24 hpf embryo are to show the subsequent migration of the P/PEC lineage towards the midline. Arrows in 48 hpf embryo indicate anterior collective cell migration that occurs in the pronephros from 29 hpf. Images are not to scale. Abbreviations: 6-ss, 6-somite stage, P/PEC podocytes and parietal epithelial cells, PCT proximal convoluted tubule, PST proximal straight tubule, DE distal early tubule, DL distal late tubule/duct hybrid segment, MCC multi-ciliated cell.
2.3 Early Embryonic Development Establishes Distinct Mesodermal Territories

The formation of the pronephric glomerulus and tubules is primarily dependent upon early embryonic patterning events that allocate the intermediate mesoderm and its different progenitor subtypes. The establishment of different territories of mesoderm along the rostral-caudal (RC) axis, the left–right axis, and the dorsal-ventral (DV) axis initiates at the blastula stage and continues during gastrulation. In order to comprehend the spatial arrangement of these mesoderm territories in the early embryo, we first need to define an accurate layout of these axes. In this chapter, we assign spatial orientation of embryonic axes based on the fate map of *Xenopus laevis* (Kumano and Smith 2002; Lane and Sheets 2002b). This fate map fits lineage-labeling work in zebrafish (Warga and Nusslein-Volhard 1999; Woo and Fraser 1995) and reconciles previous classical experiments that were misinterpreted. For example, UV treatment of *Xenopus* or zebrafish embryos ablates a cortical rotation in the zygote cytoplasm that is required to establish the RC axis (Elinson and Rowning 1988; Jesuthasan and Stahle 1997; Zust and Dixon 1975). The phenotype caused by UV irradiation was termed “ventralization” even though this treatment favors cranial and anterior trunk fates over posterior trunk and tail fates, thus could be more accurately termed “caudalization.” As such, it can be generally applied that the classic DV axis of the non-axial mesoderm (from which the pronephros descends) should be redefined as the RC axis [sometimes referred to as the anterior-posterior (AP) axis] (Fig. 2.2) (Lane and Sheets 2006). This orientation of the mesoderm is dissimilar to the neurectoderm, whose RC fates (forebrain/midbrain/hindbrain rhombomeres/spinal cord) are generally aligned with the animal vegetal axis (Kozlowski et al. 1997). This highlights the important point that the embryonic axes are initially oriented differently for different germ layers, and it is only at the end of gastrulation, after extensive cell movements, that the axes of all three germ layers become entrained.

2.3.1 Establishing the RC Positioning of the Intermediate Mesoderm

The earliest definitive marker of intermediate mesoderm is *pax2a*, which is expressed from the late gastrula stage in zebrafish (Fig. 2.2) (Thisse and Thisse 2005). Initially, *pax2a* expression is restricted to a caudolateral domain, but eventually *pax2a*+ cells can be observed as a stripe extending around the caudal end of the late gastrula embryo (Fig. 2.2). This expression pattern suggests that the RC axis of the intermediate mesoderm is initially oriented orthogonal to the animal-vegetal axis of the embryo. As such, AP fates in the intermediate mesoderm may be influenced by classic DV patterning signals. Study of patterning across the RC axis (the classic DV axis) was pioneered by Mangold and Spemann who showed
grafts of the blastopore lip of a salamander embryo transplanted to the opposite side of another embryo induced an entire secondary axis (Spemann and Mangold 1923). Importantly, the difference in pigmentation of the donor and engrafted salamanders demonstrated that dorsal structures induced on the ventral side came from the host. This groundbreaking work highlighted the blastopore lip as an “organizer” region (capable of inducing fates in neighboring tissues). Modern molecular analyses have shown that the organizer largely emits antagonizing signals to prevent caudal structures from forming (as reviewed in De Robertis 2009; Langdon and Mullins 2011; Niehrs 2004). Caudally, bone morphogenetic proteins (BMPs) and Wnts are expressed, whereas the more rostrally positioned organizer emits BMP inhibitors (Chordin, Noggin, Follistatin-like 1b) and Wnt inhibitors (Sfrp3, Frzb, Dickkopf1).

Fig. 2.2 Axis orientation in the zebrafish gastrula stage embryo. Top left panel is a schematic representation of the mesendoderm layer (hypoblast) of a zebrafish embryo with the positions of proximal and distal pronephric progenitors shown. The same positions are shown on a late gastrula embryo labeled with zulu, a pan mesodermal marker that is highly expressed in the posterior lateral mesoderm from which the pronephros descends. Bottom panels are a stage series of zebrafish embryos stained for pax2a, the earliest marker of pronephric fate. The arrows indicate regions of pronephric pax2a expression at the stages indicated. All images are lateral views apart from the tailbud stage pax2a stained embryo, which is a sagittal view from the posterior.
In zebrafish, when rostral, lateral or caudal regions of the embryonic margin (the equatorial ring of tissue where gastrulation movements initiate) are transplanted to the animal pole of blastula stage embryos, tissue induction occurs in, and around, the explant (Fauny et al. 2009). The rostral margin was found to induce axial mesendoderm, the lateral margin induces anterior somites and proximal pronephros, and the caudal margin induces posterior somites, distal pronephros, and tail. This finding showed that the entire embryonic margin in zebrafish has organizer activity. Startlingly, these transplants are recapitulated by injection of mRNAs encoding BMP and Nodal, and the ratio of BMP to Nodal determines the RC identity of the tissue. When 25 times more \( \text{bmp2b} \) mRNA was injected relative to \( \text{nodal-related} \) 2 mRNA, caudal fates (mostly tail) were preferred. In 1:1 mRNA injections, posterior head and anterior trunk fates were preferred (Fauny et al. 2009). In an elegant follow-up experiment, BMP and Nodal mRNAs were separately injected into two opposing animal pole blastomeres at the 128-cell stage, creating distinct clones of cells that secreted these factors (Xu et al. 2014). Unlike when injecting both BMP and Nodal mRNAs into the same blastomere, injection into separate blastomeres permitted a variation of concentrations to form. This led to the creation of an entire secondary axis, confirming that BMP and Nodal are sufficient to form the entire zebrafish embryonic axis. Thus, formation of an intermediate mesoderm of appropriate size and position along the RC axis is likely dependent upon the ratio of BMP to Nodal at the blastula and early gastrula stages of development.

### 2.3.2 Establishing the DV Positioning of the Intermediate Mesoderm

While BMP and Nodal are required for determining the size of rostral versus caudal domains in the early embryo, a similar understanding of pathways regulating dorsal versus ventral fates is lacking. The DV axis specifies the separation of paraxial mesoderm, intermediate mesoderm, and lateral plate mesoderm. In zebrafish and Xenopus studies, misexpression of early kidney markers, such as \( \text{lhx1} \) or \( \text{pax2} \), promotes ectopic kidney formation but only in paraxial mesoderm and not in lateral plate mesoderm (Bedell et al. 2012; Carroll and Vize 1999). Additionally, perturbation of anterior somite formation in Xenopus embryos precludes pronephrogenesis (Seufert et al. 1999). These findings suggest that the paraxial mesoderm contains an inductive signal for intermediate mesoderm formation. In chick embryonic development, a gradient of BMP4 across the DV axis has been suggested to separate the different mesodermal subdomains across this axis (James and Schulteiss 2003, 2005; Obara-Ishihara et al. 1999). However, the signal from the paraxial mesoderm to induce intermediate mesoderm in zebrafish has not been discovered. Instead, other mechanisms for DV patterning of the mesoderm have been proposed. The size of the DV extent of mesendoderm versus ectoderm in the
blastula embryo is dependent upon the AV gradient of Nodal, which has been directly visualized and quantitated at blastula stages (Harvey and Smith 2009). This study found that nodal signaling is highest at the margin and lowest at the animal pole. In *squint* (*sqt*) and *cyclops* (*cyc*) mutants that are deficient in *nodal-related 1* (*ndr1*) and *nodal-related 2* (*ndr2*), respectively, no mesendoderm forms (Dougan et al. 2003). This fits with the known function of nodals in mesendoderm induction (Rodaway et al. 1999). Recently, a novel LIM-domain binding protein, Ldb2, was shown to negatively modulate ndr1 activity (Gu et al. 2015). In *ldb2* morphants, the size of the mesendoderm increased at the expense of ectoderm. Nodal is therefore required for determining the relative amounts of ectoderm versus mesendoderm that form across the AV axis. One potential model for DV patterning of the mesoderm that arises from these studies is that the concentration of Nodal could also directly induce different DV fates across this axis. This model is challenging to test, as it will be difficult to separate the role of Nodal in mesendoderm induction from a parallel role in DV patterning. The *Progressive Critical Intervals* model proposes that DV and RC fates are assigned simultaneously but progressively along the embryo during gastrula stages (Tucker et al. 2008). This model fits well for the neurectoderm, where the forebrain is specified first, then the midbrain, then finally the hindbrain and spinal cord, but further work is needed to understand if this model applies to the mesoderm.

### 2.3.3 Specification of Blood Versus Kidney Fates in the Intermediate Mesoderm

In addition to its contribution to the kidney anlage, the intermediate mesoderm gives rise to erythroid and endothelium precursors. The splitting of the intermediate mesoderm into kidney versus blood appears dissimilar in *Xenopus* and zebrafish. For *Xenopus*, kidney and blood fates are assigned along the RC axis, creating a caudolateral *pax8*+ pronephric anlage and caudoventral blood anlage that becomes the ventral blood island (Kyuno et al. 2008; Lane and Sheets 2002a). In zebrafish, the intermediate mesoderm is split along the DV axis (Davidson and Zon 2004; de Jong et al. 2010). Early zebrafish red blood progenitors express *pax2a*, but later in development a medial domain of the intermediate mesoderm stripe is specified as blood and migrates towards the midline to form the inner cell mass. In *spadetail* (*spt*) mutants, which lack the T-box transcription factor *tbx16*, erythroid precursors are lost and the pronephros is expanded (Warga et al. 2013). This phenotype is suggested to be a result of elevated *fgf8a* expression in the posterior trunk of *spt* mutants, which Warga et al. (2013) propose may favor kidney fate over blood fate. Zebrafish studies into the *odd-skipped related* (*osr*) class of zinc finger transcription factors showed *osr1* expression in the endoderm during gastrulation is also important in establishing the ratio of kidney versus blood/vascular lineages (Mudumana et al. 2008). Intriguingly, the amount of endoderm in the embryo is negatively
regulated by osr1, and osr1-depleted embryos have ectopic angioblast formation at the expense of anterior kidney fates. This suggests that the endoderm contains an inductive signal that promotes blood/vascular fates over kidney fates in zebrafish.

2.4 Development of the Renal Corpuscle

The next stage in pronephros development involves the expression of genes within the intermediate mesoderm that specify distinct nephron cell types. These genes are largely conserved between zebrafish and mammals. In this section, we will investigate the developmental pathways involved in the formation of the renal corpuscle (the blood filter).

2.4.1 Formation of the Renal Corpuscle Ultrastructure

The rostral-most cells of the intermediate mesoderm contribute two epithelial cell populations that will establish the renal corpuscle: podocytes and parietal epithelial cells (aka Bowman’s capsule) that we herein refer to collectively as P/PECs. The wt1a gene is the earliest marker of the P/PEC lineage. Transcripts for wt1a are initially detected in a broad domain before restricting to being highly expressed in P/PEC progenitors lateral to somite 3 between the 18-somite to 24 hpf stages (Bollig et al. 2006; Drummond et al. 1998; O’Brien et al. 2011; Serluca and Fishman 2001). Other early markers of the P/PEC lineage include the transcription factors wt1b, mafb, hey1, and lhx1a (O’Brien et al. 2011) (see Sect. 2.4.2 for a detailed discussion of the roles these factors play in glomerulogenesis). At the 48 hpf stage, wt1a, wt1b, and mafb expression persist in the P/PEC lineage, but between 24 and 48 hpf, hey1 and lhx1a levels reduce and markers of podocyte differentiation begin to be expressed (such as podocalyxin, nephrin, podocin, and integrina3).

This commencement of molecular differentiation can be correlated to the physical formation of the renal corpuscle. Between the 24 and 40 hpf stages, the bilateral P/PEC populations migrate to, and fuse at, the midline beneath the dorsal aorta and notochord (Drummond et al. 1998; Majumdar and Drummond 2000). Between 40 and 48 hpf, capillaries extending out from the dorsal aorta enter the mass of P/PECs and form the glomerular tuft. Small molecular weight fluorescent dextrans (10 kDa) are able to filter through the glomerulus by the 48 hpf stage, indicating the onset of blood filtration function (Drummond et al. 1998). However, the renal corpuscle is leaky at this early stage and does not fully mature until 4 dpf when it has an upper size limit of 70 kDa (Ichimura et al. 2012; Kramer-Zucker et al. 2005b).

The glomerular filtration barrier is established by the cumulative functionality of three components: the fenestrated endothelium, the glomerular basement
membrane (GBM), and the podocyte slit diaphragm. The fenestrations in the afferent capillaries of the glomerulus are trans-cellular passages that permit contents of the blood to pass through the surrounding GBM, but are too small to allow large proteins and blood cells to egress from the blood. The GBM consists of a feltwork of proteins such as laminin and collagen and is dually created by the podocytes and the endothelial cells of the capillaries. While the GBM filters only molecules of a certain size and charge, it is thought that the slit diaphragm created by podocytes also determines which components of the blood can pass into the tubules. The podocytes envelope the endothelium with a complex cytoarchitecture of interdigitating foot processes. The slit diaphragm forms as a protein “zipper” between the foot processes. The glomerulus is surrounded by a Bowman’s capsule, which is created by PECs. The common ontogeny of the P/PEC lineage in zebrafish indicates these cells must be separated later in development by an as yet unknown mechanism. The final cellular component of the renal corpuscle is the mesangial cell, which is a pericyte-like cell type whose function is to provide contractile support to the glomerulus (Ichimura et al. 2012). When pericytes enter the glomerular tuft in zebrafish is not known, but potential candidate markers to examine in the future include acta2, timp3, and adamtsl (Schrimpf et al. 2012; Whitesell et al. 2014).

2.4.2 Molecular Regulation of Renal Corpuscle Formation

2.4.2.1 The Role of the WT1 Transcription Factors in Determining Podocyte Fate and Function

Knockdown of wt1b has no effect on P/PEC specification and development, whereas knockdown of wt1a reduces nephrin and podocin expression (Bollig et al. 2006; O’Brien et al. 2011; Perner et al. 2007; Schnerwitzki et al. 2014). wt1a has been shown to interact synergistically with forkhead box c1a (foxc1a) and notch mediator recombination signal binding protein for immunoglobulin kappa J (rbpj) as double knockdowns of wt1a/foxc1a or wt1a/rbpj completely prevents expression of early podocyte markers such as wt1b, whereas singly depleted wt1a, foxc1a or rbpj embryos only reduce wt1b expression (O’Brien et al. 2011). Glutathione S-transferase pull-down assays indicated that wt1a, foxc1a, and NotchICD3 can physically interact (O’Brien et al. 2011). While further analysis is required to decipher the importance of these interactions and to determine if wt1a is able to interact with other transcription factors and Notch pathway molecules, it appears that cross talk between wt1a and the Notch signaling pathway is important for the formation of the P/PEC lineage, most likely through fate determination and control of proliferation.
2.4.2.2 Retinoic Acid Patterns the Intermediate Mesoderm to Promote P/PEC Fates

A conserved 299 bp element of the wt1a promoter (approximately 4.2 kb upstream from the start codon) was found to be necessary and sufficient to drive transgenic eGFP expression in a spatial and temporal manner that recapitulated endogenous wt1a expression (Bollig et al. 2009). Within this region of the wt1a promoter is a Retinoic Acid Response Element. The Retinoic Acid (RA) signaling pathway is dependent on the concentration of free RA in the cell, which will bind to RA receptors (RAR/RXR). When bound to RA, RARs/RXRs translocate to the nucleus and interact with DNA binding sites in the genome to modulate gene expression. Embryos treated with an inhibitor of RA synthesis (diethylaminobenzaldehyde) fail to express wt1a, wt1b, and mafba in the intermediate mesoderm (Wingert et al. 2007). Conversely, application of RA to wt1a::eGFP transgenic embryos greatly increased the expression of eGFP (Bollig et al. 2009). These results support a model whereby RA induces wt1a expression and, together with notch, wt1a specifies the P/PEC lineage from the intermediate mesoderm.

2.4.2.3 pax2a Restricts the Size of the Glomerulus

In the pax2a mutant no isthmus (noi), the wt1b+ P/PEC lineage appears to expand into the neck segment, which suggests a requirement for pax2a in appropriate formation of the renal corpuscle and neck lineages (Majumdar et al. 2000). In mice, Wt1 is a negative regulator of Pax2 (Ryan et al. 1995), and a similar epistatic relationship between wt1a and pax2a may be present in zebrafish as the anterior pax2a expression domain initially overlaps with wt1a in P/PECs, but then restricts to the neck segment at 24 hpf (O’Brien et al. 2011). In addition, pax2a overexpression reduces wt1a expression and promotes formation of an aglomerular pronephros (Bedell et al. 2012). These data suggest that pax2a and wt1a are negative regulators of each other. Such genetic interactions may be important for the appropriate formation of the glomerulus/neck/proximal tubule boundaries.

2.4.2.4 Odd-Skipped Related 1 and Lhx1a as Downstream Regulators of Podocyte Fate

Podocyte progenitors co-express wt1a and osr1 (Tomar et al. 2014). In embryos depleted of osr1, wt1a expression is normal, but nephrin and podocin are not expressed. However, in wt1a-depleted embryos, osr1 expression is reduced (Tomar et al. 2014). In summary, these results suggest osr1 acts downstream of wt1a to regulate nephrin and podocin expression. Interestingly, osr1 morphant embryos fail to express lhx1a in P/PECs, and the effects of osr1 depletion on nephrin and podocin expression can be rescued by forced expression of an activated
form of \textit{lhx1a} (ldb1-lhx1a) (Tomar et al. 2014). Taken together, it appears that the following hierarchical transcriptional pathway operates during P/PEC formation: \textit{RA} > \textit{wt1a} > \textit{osr1} > \textit{lhx1a} > \textit{podocin/nephrin}.

2.4.2.5 Podocalyxin Is Necessary for Proper Formation of the Slit Diaphragm

Podocalyxin is a highly sialylated glycoprotein that localizes specifically to the apical pole of podocytes by 34 hpf (Ichimura et al. 2013). The exact mechanism for how podocalyxin regulates foot process formation is not fully understood. In \textit{Podocalyxin}-deficient mice, regular foot processes do not form and the cell body attaches directly to the GBM (Doyonnas et al. 2001). A similar phenotype is observed in zebrafish injected with a splicing morpholino targeting the \textit{podocalyxin} mucin-domain encoding exon 2 (Ichimura et al. 2013). This mucin domain is extensively glycosylated and sialylated. The presence of sialic acid in the mucin domain creates a negative charge on the apical surface of the podocyte that is hypothesized to generate and maintain spacing between foot processes by charge repulsion and by acting as an anti-adhesive (Schnabel et al. 1989; Takeda et al. 2000).

2.4.2.6 A Role for von Hippel–Lindau in Glomerular vegfa Signaling

The \textit{von Hippel–Lindau} (\textit{vhl}) tumor suppressor gene encodes an E3-ubiquitin ligase that under normal oxygen levels targets the \textit{hypoxia-inducing factor} (\textit{hif}) for degradation (Haase 2006). The \textit{vhl}/\textit{hif} oxygen-sensing pathway enables organisms to sense and adapt to a low-oxygen environment. Depletion of zebrafish genes that regulate the \textit{vhl}/\textit{hif} pathway, such as \textit{proly 4-hyroxylation} (Hyyräinen et al. 2010) or \textit{vhl} (Chen et al. 2015), results in disrupted glomerular development. Knockdown of \textit{vhl} creates an embryo that is unable to recognize oxygen levels in the body. Consequently, \textit{hif} protein is not degraded and pathological hypoxia-driven angiogenesis results. Vascular endothelial growth factor-a (\textit{Vegf-a}) is required for endothelial cell differentiation and angiogenesis during vertebrate development and in \textit{vhl} morphants, \textit{vegfa} expression increases (Chen et al. 2015). It is hypothesized that this increased \textit{vegfa} expression causes overgrowth of the vasculature, which is likely to disrupt vascularization of the glomerulus of \textit{vhl} morphants.

2.5 Genes Involved in Pronephros Tubule Formation

The tubules are essential for reabsorption of solutes, amino acids, glucose, and water. Their formation involves a number of key developmental processes, including molecular patterning, mesenchymal-to-epithelial transitions, tubulogenesis, cell
migration, and tissue morphogenesis. In this section, we will concentrate on the genes expressed in the intermediate mesoderm that are involved in the earliest stages of pronephric tubule formation.

2.5.1 Pax2a and Pax8: Critical Regulators of Nephric Specification

In mice and zebrafish, the paired-box transcription factors Pax2 (pax2a in zebrafish) and Pax8 are the earliest expressed genes that label the intermediate mesoderm (Bouchard et al. 2000; Thisse and Thisse 2004). In mice lacking Pax2 and Pax8, neither the pronephros nor later nephric structures form (Bouchard et al. 2002). This same phenotype is observed in zebrafish depleted of pax2a and pax8 (Naylor et al. 2013). Pax2 or Pax8 singly deficient mice and zebrafish embryos do not develop such a dramatic early kidney agenesis phenotype, consistent with these related factors having redundant functions in the specification of the intermediate mesoderm (Ikenaga et al. 2011; Majumdar et al. 2000; Torres et al. 1995). pax2a and pax8 are expressed throughout the intermediate mesoderm up to the ~15-somite stage (16 hpf). Subsequently, levels of pax8 expression reduce, but pax2a remains strongly expressed in the neck region, multi-ciliated cells in the PST and DE tubule segments and throughout the DL segment. Singly deficient pax2a zebrafish develop edema and die at around 5 dpf, most likely as a consequence of perturbed glomerulus, neck and tubule differentiation, and variable failure in the fusion of the DL segment to the cloaca (Majumdar et al. 2000). Thus, while pax2a and pax8 share functionality, differing expression patterns result in pax2a playing a more dominant role during later events in nephrogenesis.

The biochemical activities of Pax2 in the cell and how these actions affect nephrogenesis remain an ongoing area of investigation. In zebrafish, the hepatocyte nuclear factor 1b (hnf1b) transcription factor has been reported to suppress pax2a expression in the proximal tubule (Naylor et al. 2013). Zebrafish embryos deficient in hnf1ba and hnf1bb paralogs maintain pax2a expression and ectopically express glomerular markers such as nephrin and podocin in the proximal tubule. A similar phenotype is observed in embryos depleted of atypical protein kinase C iota and zeta (prkcι, prkcζ) where pax2a expression increases and wt1a and wt1b are ectopically expressed in the tubule (Gerlach and Wingert 2014). Interestingly, this phenotype can be rescued by pax2a depletion, suggesting that prkcι and prkcζ support epithelial identity by inhibiting pax2a expression. Together, it could be deduced from these studies that hnf1ba/b and prkcι/ζ regulate pax2a levels in the tubule, and this activity may be important for the suppression of glomerular fate in the proximal tubule.

pax2a has also been shown to participate in a negative feedback loop by inducing the expression of plac8 onzin related protein 1 (ponzr1), a poorly studied gene that acts to antagonize pax2a expression. Morpholino-mediated knockdown of
*ponzr1* causes persistent expression of *pax2a* in P/PECs, resulting in the downregulation of *wt1a* and the formation of an agglomerular pronephros (Bedell et al. 2012).

### 2.5.2 The Role of Odd-Skipped Genes in Tubule Formation

The Odd-skipped-related class of transcription factors are zinc finger proteins that have been shown to be important in embryonic patterning and tissue morphogenesis. In *Odd1°/°* mutant mice, heart development is disrupted and nephrogenesis is perturbed as the metanephric mesenchyme fails to form (Wang et al. 2005). In chick, *Odd1* has been shown to be important in tubule differentiation (James et al. 2006). As described in Sect. 2.3.3, knockdown of *osr1* in zebrafish inhibits the formation of the proximal tubule and concomitantly promotes angioblast fate (Mudumana et al. 2008).

While *osr1* is not thought to be expressed in the pronephric tubules, the closely related *osr2* gene is found in the anteriormost portion of the pronephros (Neto et al. 2012; Tena et al. 2007). Double knockdown of *osr1* and *osr2* inhibits formation of the proximal tubule in zebrafish and causes severe edema (Tena et al. 2007). Interestingly, RA signaling activates *osr2* expression in the intermediate mesoderm and *osr1/2* are required for inducing *wnt2ba* expression in this region (Neto et al. 2012). While *wnt2ba* knockdown has no effect on kidney development, it does inhibit pectoral fin formation, suggesting a relay mechanism is in place between the anterior paraxial/intermediate mesoderm and lateral plate mesoderm to regulate fin development. These findings nicely highlight how cross talk between different tissues, in this case kidney and fin precursors, is fundamentally important for organogenesis.

### 2.5.3 *hnf1b* as a Critical Regulator of Pronephric Tubule Differentiation

The *hnf1ba* and *hnf1bb* transcription factors are expressed in the zebrafish intermediate mesoderm that will form the pronephric tubules from the 5-somite stage of development (12 hpf) onwards (Naylor et al. 2013; Sun and Hopkins 2001). In embryos depleted of both *hnf1ba* and *hnf1bb*, nearly all markers of tubule differentiation fail to be expressed (Naylor et al. 2013). These include the many segment-specific solute carriers (such as *slc4a4*, *slc12a1*, and *slc12a3*), later expressed transcription factors (such as *irx3b*) and cell adhesion molecules (such as *cdh17*). In addition, *hnf1ba/b*-deficient embryos maintain expression of early acting transcription factors such as *pax2a*, *pax8*, *lhx1a*, and *jag1b*. Despite these early markers not being restricted, the pronephros still undergoes a degree of epithelialization and
tubulogenesis, though the lumen does not inflate. As such, this study highlights the importance of Hnf1b factors for tubule differentiation, but not tubulogenesis. This integral role for Hnf1b in zebrafish pronephric tubule differentiation is conserved in mice as conditional deletion of Hnf1b in metanephric nephrons results in a similar phenotype (Heliot et al. 2013; Massa et al. 2013).

2.5.4 Roles for Notch/Jagged in Pronephric Tubule Differentiation

The Notch signaling pathway is a paracrine-signaling network that is activated by binding of the trans-membrane Notch receptor to its Jagged/Delta ligands (Guruharsha et al. 2012). This binding permits γ-secretase to cleave the Notch receptor, releasing the Notch intra-cellular domain (NICD). NICD then translocates to the nucleus where it forms a core transcriptional complex with Suppressor of Hairless (a DNA-binding protein) and Mastermind (a nuclear effector protein required to stabilize the transcription complex). This complex then modulates the expression of downstream effector genes. Commonly, Notch signaling is utilized in a “lateral inhibition” pathway where a group of cells signal locally to each other in order to control which cells will adopt one of two different fates. In the zebrafish pronephros, multi-ciliated cells (MCCs) are important for promoting fluid flow through the lumen of the tubule and are present in a “salt and pepper” pattern in the intermediate tubule region (comprising the PST and DE tubule segments) (Fig. 2.1).

This dispersed positioning of MCCs between solute transporter cells is mediated by a Notch lateral inhibition mechanism. From the 5-somite stage (12 hpf), jag2a is expressed throughout the anterior portion of the pronephric tubules (which will give rise to the PCT, PST, and DE segments) (Thisse and Thisse 2005). Similarly, notch1a and notch3 are expressed in the pronephros from as early as the 10-somite stage (14 hpf) (Ma and Jiang 2007). From the 20-somite stage (19 hpf), jag2a expression restricts to a “salt and pepper” pattern in the intermediate region of the pronephros (within the PST and DE segments). In mindbomb$^{-/-}$ mutants (mib$^{-/-}$), jag2a expression does not restrict and is uniformly expressed in the intermediate region of the tubule. These embryos do not express markers associated with solute transport function, such as trpm7, Na$^+$/K$^+$/ATPase, or slc13a1, and instead have ectopic expression of MCC markers, such as odf3b and rfx2 (Liu et al. 2007; Ma and Jiang 2007). Injection of NICD mRNA induces the opposite phenotype to mib$^{-/-}$ mutants, inhibiting odf3b expression and promoting transporter fates over MCCs. Thus, a Notch signaling lateral inhibition pathway determines MCC or solute transporter cell fate in the pronephric tubule. Knockdown of ETS transcription factors etv4 or etv5a also reduces the number of MCCs, but not when embryos are treated with the γ-secretase inhibitor DAPT (Marra and Wingert 2016). This result suggests a novel role for etv4 and etv5a downstream of Notch signaling to promote MCC fate in the pronephros.
2.5.5 Roles for Cilia in Pronephrogenesis

Cilia are membrane-bounded, centriole-derived, microtubule-containing organelles that project out from the cell surface. The ciliary cytoskeleton (axoneme) is arranged into two major patterns: 9+2, in which microtubule doublets surround a central pair of singlet microtubules or 9+0, where the central microtubules are absent (Satir and Christensen 2007). The two microtubule patterns are indicative of the functional role of a cilium. 9+2 cilia are motile and present in bundles on the apical surface of epithelial cells, whereas 9+0 cilia are nonmotile, sometimes referred to as primary or sensory cilia, which are able to alter intracellular biochemistry based on extracellular cues. Over 600 cilia proteins have been identified (Pazour 2004), and dysfunction of a number of these proteins can cause an array of diseases collectively termed ciliopathies. These broad set of developmental and adult diseases include polycystic kidney disease, nephronophthisis (NPHP), Bardet–Biedl Syndrome, Joubert syndrome, and Meckel Gruber syndrome (Hildebrandt et al. 2011). The zebrafish kidney is particularly vulnerable to aberrant ciliogenesis as both multi-ciliated cells (MCCs) and nonmotile mono-ciliated cells are required for pronephros development.

2.5.5.1 MCCs Drive Fluid Flow in the Zebrafish Pronephros

9+2 cilia are found in MCCs across the animal kingdom, where they act to promote and direct fluid flow (Brooks and Wallingford 2014). For example, in humans, MCCs are important for cerebrospinal fluid flow in the spinal cord and ventricles of the brain (Sawamoto et al. 2006), for ovum transport in the fallopian tubes (Lyons et al. 2006), and for clearance of mucus in the airways (Wanner et al. 1996). In zebrafish, mutants with defective ciliogenesis develop pronephric cysts during early development (Drummond 2005; Obara et al. 2006; Sullivan-Brown et al. 2008). Examples of such mutants include oval (containing a single point mutation in ift88 (Tsujikawa and Malicki 2004)), double bubble [unknown mutation (Drummond et al. 1998)], fleer [which has a nonsense point mutation that truncates the ift70 protein (Pathak et al. 2007)], and a number of cilia genes [including pkd2, ruvbl1, lrec6l, and arl13b] found through the Hopkins retroviral insertion mutagenesis screen (Sun et al. 2004). Similarly, morpholino-mediated knockdown of intraflagellar transport proteins ift88, ift57 (Kramer-Zucker et al. 2005a), and ttc26 (Zhang et al. 2012) causes pronephric cysts. The deglutamylase ccp5, a critical regulator of microtubule glutamylation (which is essential for ciliogenesis), also causes pronephric cysts when it is depleted (Pathak et al. 2014). Ciliogenesis and cilia function requires the highly conserved family of GTP-binding proteins called Septins (Kim et al. 2010). The pronephric tubules have enriched expression of sept7b, and morpholino knockdown results in fewer and shorter cilia as well as cyst formation (Dash et al. 2014). Such “cystic” phenotypes are believed to be due to fluid accumulation and distension of the pronephric tubules/neck region,
consistent with the notion that MCCs drive fluid flow in the lumen of the pronephric tubule (Kramer-Zucker et al. 2005a). MCCs are not normally found in adult mammalian nephrons, perhaps because normal blood pressure is sufficient to propel fluid through the glomerulus and tubules of the nephron [normal systolic blood pressure in humans is ~110 mmHg, whereas in zebrafish it is ~0.68 mmHg (Hu et al. 2001)]. As such, mammalian renal ciliopathies are associated with nonmotile cilia dysfunction.

2.5.5.2 Nonmotile Cilia Are Important for Distal Tubule Morphogenesis

Apart from MCCs, all other cells in the pronephric tubules contain a solitary nonmotile cilium that can sense both physical and biochemical extracellular signals. Discerning between the pronephric phenotypes associated with aberrant motile versus nonmotile cilia function remains an ongoing challenge given the shared assembly mechanisms for both cilia types (Ishikawa and Marshall 2011). As such, factors that impede cilia assembly will prevent function of both cilia forms. Despite this, numerous signaling pathways connect nonmotile cilia to proper pronephros morphogenesis (Hossain et al. 2007; Makita et al. 2008; Tian et al. 2007). Downstream co-activators of the Hippo pathway, yap and taz, are expressed in the distal tubules of the zebrafish pronephros, and their depletion causes pronephric cyst phenotypes (He et al. 2015; Skouloudaki et al. 2009; Zhang et al. 2015). These morphants have reduced cilia number and length in the PST and DE tubule segments where MCCs reside, but also in the DL segment that contains mono-ciliated cells. Such ciliary defects are correlated with aberrant cell migration and apical-basal polarity in the distal tubule. Similarly, morpholino knockdown of nphp4 reduces cilia number and length in the DL segment and perturbs cell migration and DL fusion with the cloaca (Burckle et al. 2011; Slanchev et al. 2011). These studies found that the perturbed cloacal rearrangements in nphp4 morphants, which are attributed to the nonmotile cilia defect, result in a failure in non-canonical Wnt signaling, a pathway implicated in the orientation of cells within a single plane (Gao 2012). Taken together, these results favor a model whereby mono-ciliated sensory cilia in the zebrafish distal nephron are required to mediate the cellular rearrangements needed to fuse the pronephric tubules to the cloaca.

2.6 Pronephric Tubule Segmentation

In all vertebrates, the nephron is segmented along its axis in order to enable efficient reabsorption/secretion of solutes. The proximal tubule is considered the “workhorse” of the nephron as it performs the bulk reabsorption of these solutes. More distal segments are involved in fine-tuning the filtrate, in particular by reabsorption
of sodium, chloride, and bicarbonate ions. The mammalian nephron contains additional nephron segments that are not found in zebrafish nephrons, such as the loop of Henle and principal and intercalated cells of the distal nephron/collecting duct. Nevertheless, zebrafish tubule segments are well conserved functionally in comparison to mammals, and many transporter genes show similar expression patterns in both phyla (Wingert et al. 2007). In this section, we will provide an overview of our current understanding of how the zebrafish pronephric tubule is patterned in response to RA signaling.

### 2.6.1 Retinoic Acid and Establishment of the Tubule Segmentation Pattern

In Sect. 2.3.1, we showed that opposing gradients of BMP and Nodal establish the RC axis of the gastrulating zebrafish embryo. Factors acting downstream of BMP/Nodal are likely involved in committing cells to particular fates along this axis. One candidate is RA, which has been shown to be important for the relative sizes of the PCT/PST/DE/DL tubule segments (Wingert et al. 2007). In embryos where RA synthesis is inhibited by treatment with diethylaminobenzaldehyde (DEAB), pronephroi form with larger distal segments at the expense of proximal segments (Wingert et al. 2007). Inhibiting RA synthesis can completely preclude formation of all proximal segments (PCT, PST, and DE) if DEAB treatment is commenced from early gastrula stages. Progressively later treatments have gradually reduced effects and treatment from the 8-somite stage has no effect on pronephric fate. Thus, RA is important in regulating pronephric tubule fate during gastrulation and early somitogenesis stages of development.

RA is synthesized from retinol by retinaldehyde dehydrogenase (also called aldehyde dehydrogenase). At gastrula stages of development, a major retinaldehyde dehydrogenase expressed in the zebrafish embryo is \textit{aldh1a2}. \textit{aldh1a2} is initially expressed in all cells of the embryonic margin but restricts to the rostral side of the embryo by the ~60% epiboly stage (6 hpf) (Grandel et al. 2002). From mid-to-late gastrulation, the RA catabolic enzyme \textit{cyp26a1} (a member of the cytochrome p450 family of enzymes) begins to be expressed on the caudal side of the embryo (Kudoh et al. 2001; Thisse et al. 2001). These expression patterns support the interpretation that there is an RA “source” on the rostral side of the embryo and an RA “sink” on the caudal side of the embryo. This suggestion is aided by analyses that directly observed RA abundance in the zebrafish embryo using a fluorescent resonance energy transfer-based system called GEPRAN (Shimozono et al. 2013). GEPRAN zebrafish embryos show a clear accumulation of RA on the rostral side of the late gastrula embryo, which appears to be maintained in the anterior trunk region at early somitogenesis stages of development. Taken together, the timing of RA proximalizing actions (during gastrulation), the high expression of RA synthesis genes rostrally and RA catabolic genes
caudally, as well as the accumulation of RA rostrally when observed by GEPRAX analysis suggest RA acts to pattern kidney fates across the RC axis of the embryo during gastrula stages. As RA can act as a morphogen, it can further be proposed that PCT cells form in response to high levels of RA, PST cells in response to medium levels of RA, DE cells in response to low RA levels, and the DL segment in the absence of RA (Wingert et al. 2007).

### 2.6.2 A Role for sim1a in the Formation of the PST Segment

RA is the major determinant of pronephric segment identity; however, the downstream effectors of RA are a focus of ongoing research. One possible mechanism by which RA affects gene expression is via the modulation of transcription factor activities. The single-minded family bHLH transcription factor 1a (sim1a) gene is dynamically expressed from early stages of zebrafish pronephros development. At the 2-somite stage (11 hpf), it is expressed in the caudal region of the intermediate mesoderm, but later in development [at the 22-somite stage (20 hpf)] it is expressed in a more proximal subdomain before restricting to a distal population of cells that contribute to a kidney-derived endocrine gland called the Corpuscles of Stannius (Cheng and Wingert 2015). Intriguingly, sim1a knockdown prevents formation of the PST segment and concomitantly expands the PCT segment. When overexpressed, the opposite phenotype is observed, and PST segment size increases at the expense of PCT fates. The expression pattern of sim1a is influenced by RA levels, suggesting RA induces sim1a expression, and sim1a acts downstream of RA to mediate the boundary between the PCT and PST segments.

### 2.6.3 mecom as an RA Inhibitor that Regulates Formation of the DL Segment

Another transcription factor whose expression is regulated by RA is mecom (mds1/evi1 complex). This gene is expressed in the caudal non-RA responsive region of the intermediate mesoderm that gives rise to the DL segment (Li et al. 2014). In mecom morphants, the size of the DL segment reduces and the PCT and PST segments expand caudally (Li et al. 2014). Surprisingly, Mecom morphants have increased numbers of MCCs. As discussed in Sect. 2.5.4, Notch signaling via lateral inhibition is required to induce MCC fate in the intermediate region of the pronephros tubule. Li et al. suggest that Mecom interacts with the Notch signaling although it is also possible that the increased numbers of MCCs in mecom-deficient embryos is caused indirectly, such as, by increased proliferation of the MCC-bearing PST and DE segments in response to a shortened DL segment.
2.6.4 *irx3b* as a Determinant of DE Segment Formation

The *Iroquois* (*Irx*) gene family encode homeodomain transcription factors that are regulators of tissue patterning and cell fate specification. In zebrafish, *irx3b* is expressed in the PST and DE segments from the 15-somite stage (16 hpf). In *irx3b* zebrafish morphants, *slc12a1* expression is lost in the DE segment and markers of the proximal tubule are expanded distally (Wingert and Davidson 2011). In *Xenopus*, *irx3* knockdown also disrupts the formation of the segment equivalent to the DE (Alarcon et al. 2008; Reggiani et al. 2007). In mice, *Irxl* and *Irx2* are downregulated in *Hnf1b*-deficient nephrons, and this is associated with the proximal tubule and loop of Henle segments failing to form, consistent with *Irxl/2* being potential regulators of segmentation.

In zebrafish, the related *Iroquois* family member, *irx1*, is expressed in a similar domain to *irx3b*. However, knockdown of *irx1* does not cause an overt tubule segmentation phenotype. Expression of *irx1* is negatively regulated by the homeobox transcription factors *mnx1* and *mnx2b* that are expressed in mutually exclusive regions of the intermediate mesoderm to *irx1* (Ott et al. 2015). When *mnx1* and *mnx2b* are knocked down, *irx1* becomes ectopically expressed in the DL segment and these embryos exhibit defects in cilia arrangement and apical microvilli morphology (Ott et al. 2015). Taken together, these observations indicate that the *Iroquois* genes are conserved regulators of tubule segmentation identity and morphogenesis in zebrafish, frogs, and mammals.

2.7 Mechanobiological Regulation of Pronephrogenesis

It is increasingly being recognized that mechanical forces are required for the development of nephric structures. The process of mechanotransduction involves the interpretation by a cell of mechanical inputs that lead to changes in its biochemistry. During early development, the intermediate mesoderm undergoes extensive remodeling, during which cells experience a number of mechanical stresses. Between the 12-somite and 20-somite stage (15–19 hpf), the intermediate mesoderm undergoes mesenchymal-to-epithelial transitions (MET) and forms a tubule (Gerlach and Wingert 2014). In addition, there is a degree of caudal migration of the DL segment towards the cloaca in an analogous manner to nephric duct migration observed in *Xenopus*, chick, and mouse (Slanchev et al. 2011). Such caudal migration means cells will be stretched, a mechanical process that can alter rates of proliferation in the pronephros (Vasilyev et al. 2012). Also, at the onset of luminal flow, the apical surfaces of the pronephric tubules experience sheer stress. How cells in the pronephros respond to these multiple mechanical inputs has become a new area of interest in the field.
2.7.1 Shear Stress Induction of Collective Cell Migration in the Pronephric Tubule

Collective cell migration is the movement of multiple cells in a specific direction with the major feature being that if these cells were individually isolated, they would not migrate as efficiently (Mayor and Etienne-Manneville 2016). In the zebrafish pronephros, collective cell migration plays a key role in tissue morphogenesis. From the 29 hpf stage of development, the cells of the pronephros collectively move rostrally (Vasilyev et al. 2009). This migration compacts and convolutes the proximal tubule. The initiation of collective cell migration in the zebrafish pronephros correlates with the onset of fluid flow in the lumen of the tubule. When fluid flow is halted by simple obstruction, collective migration halts and the proximal tubule fails to convolute (Vasilyev et al. 2009). In follow-up experiments, it was shown that the capacity of the pronephros to accommodate such a dramatic compaction in the proximal segments is afforded by the corresponding proliferation of the DE and DL segments. This can be clearly seen when older embryos are compared to young larvae: at 24 hpf the size of the DL segment is roughly three somite widths (Wingert et al. 2007) but by the 8 mm stage (~21 days post fertilization), it is much longer, spanning between the posterior end of the swim bladder to the region just proximal to the cloaca (Diep et al. 2015). Cell division is observed in the DE and DL tubule segments between the 48 and 72 hpf stages of development, and this can be inhibited by anterior obstruction of the tubule (to halt collective cell migration) or by treating embryos with the Phosphoinositide-3 Kinase (Pi3K) inhibitor LY294002. Treatment with LY294002 produced pronephric tubules that still underwent collective cell migration towards the glomerulus but distally the cells became severely stretched (Vasilyev et al. 2012). These data have led to a model in which shear stress induced by fluid flow promotes rostral cell migration, and this compaction is accommodated by mechanical stretch-induced cell proliferation in the more distal segments.

2.7.2 Vascular Shear Stress Is Required for Capillary Formation in the Glomerulus

As discussed in Sect. 2.4.1, the process of glomerular capillary tuft formation involves the invasion of dorsal aorta endothelial cells into the fused mass of P/PEC progenitors at the midline (Carmeliet et al. 1996; Pham et al. 2001). This process requires vascular sheer stress as zebrafish mutants that have reduced or no blood flow fail to form a glomerular capillary tuft (Bedell et al. 2012; Drummond and Davidson 2010; Majumdar and Drummond 1999; Rottbauer et al. 2001; Sehnert et al. 2002). Studies have implicated matrix-metalloproteinase 2 (MMP2) in this process. Zebrafish embryos treated with an MMP2 inhibitor do not form a glomerulus but have otherwise normal blood circulation (Serluca et al. 2002). As
mmp-2 is expressed by smooth muscle cells and endothelium in response to stretching it suggests that blood flow-induced sheer stress activates mmp-2, which then regulates vascularization of the renal corpuscle (Bassiouny et al. 1998; Singhal et al. 1996; Yasuda et al. 1996).

2.8 Summary

The pronephros descends from the intermediate mesoderm, and its formation involves the concerted actions of secreted morphogens, transcription factors, and cellular rearrangements including migration, stretch, and epithelialization. Pattern- ing of the intermediate mesoderm into different proximal and distal cell fates occurs in response to RA signaling and downstream acting transcription factors such as wt1a, pax2a, pax8, hnf1b, sim1a, mecom, and irx3b. Glomerular progenitors migrate and fuse at the midline and recruit a blood supply while tubule progenitors undergo epithelialization and fuse with the cloaca. The Notch signaling pathway regulates the formation of multi-ciliated cells in the tubules that help propel the urine down the tubule. The luminal sheer stress caused by this flow activates anterior collective migration of the proximal tubules and induces stretching and proliferation of the more distal segments. Ultimately these processes create a simple two-nephron kidney capable of reclaiming and balancing vital metabolites and expelling excess water and waste products to the exterior. This simple, yet dynamic, kidney provides a powerful model system to better understand the conserved molecular and cellular progresses that drive nephron formation, structure, and function.

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Chapter 3
Zebras as a Model of Kidney Disease

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Abstract Animal models have been an invaluable means to advance biomedical research as they provide experimental avenues for cellular and molecular investigations of disease pathology. The zebrafish (Danio rerio) is a good alternative to mammalian models that can be used to apply powerful genetic experimental methods normally used in invertebrates to answer questions about vertebrate development and disease. In the case of the kidney, the zebrafish has proven itself to be an applicable and versatile experimental system, mainly due to the simplicity of its pronephros, which contains two nephrons that possess conserved structural and physiological aspects with mammalian nephrons. Numerous genes that were not previously related to kidney conditions have now been linked to renal diseases by applying genetic screening with the zebrafish. In fact, a large collection of mutations that affect nephron formation and function were generated through phenotype-based forward screens. Complementary reverse genetic approaches have also been insightful, with methods spanning the use of antisense morpholino oligonucleotides to genome editing approaches such as the CRISPR/Cas9 system, to selectively knock down or knock out genes of interest to see if they produce kidney phenotypes. Acute kidney injury (AKI) has also been easily modeled in the zebrafish by injecting nephrotoxins, directly inducing damage through surgical intervention, or by generating transgenic lines that express compounds in a tissue-specific manner that when exposed to certain drugs promote an apoptotic response within cells. In this chapter, we provide an overview of these various approaches as well as discuss many of the contributions that have been achieved through the use of zebrafish to model kidney disease.
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