Wt1a, Foxc1a, and the Notch mediator Rbpj physically interact and regulate the formation of podocytes in zebrafish

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ABSTRACT
Podocytes help form the glomerular blood filtration barrier in the kidney and their injury or loss leads to renal disease. The Wilms’ tumor suppressor-1 (Wt1) and the FoxC1/2 transcription factors, as well as Notch signaling, have been implicated as important regulators of podocyte fate. It is not known whether these factors work in parallel or sequentially on different gene targets, or as higher-order transcriptional complexes on common genes. Here, we use the zebrafish to demonstrate that embryos treated with morpholinos against wt1a, foxc1a, or the Notch transcriptional mediator rbpj develop fewer podocytes, as determined by wt1b, hey1 and nephrin expression, while embryos deficient in any two of these factors completely lack podocytes. From GST-pull-downs and co-immunoprecipitation experiments we show that Wt1a, Foxc1a, and Rbpj can physically interact with each other, whereas only Rbpj binds to the Notch intracellular domain (NICD). In transactivation assays, combinations of Wt1, Foxc1/2, and NICD synergistically induce the Hey1 promoter, and have additive or repressive effects on the Podocalyxin promoter, depending on dosage. Taken together, these data suggest that Wt1, Foxc1/2, and Notch signaling converge on common target genes where they physically interact to regulate a podocyte-specific gene program. These findings further our understanding of the transcriptional circuitry responsible for podocyte formation and differentiation during kidney development.

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Introduction

The vertebrate kidney plays essential roles in osmoregulation, body fluid homeostasis, and waste excretion. The nephron is the functional unit of vertebrate kidneys and is generally comprised of a glomerulus, where the blood filtration occurs, and a tubular epithelium, where the urinary filtrate is modified by absorption and secretion (Hebert et al., 2001). In lower vertebrates, a short ciliated neck segment separates the glomerulus from the proximal segment of the tubule and likely functions in fluid propulsion (Reimschuessel, 2001; Schonheyder and Maunsbach, 1975). Blood enters the glomerulus via a fenestrated capillary bed known as the glomerular tuft. Highly specialized epithelial cells called podocytes envelope the glomerular tuft endothelium and these two cell types, together with an intervening basement membrane, establish the glomerular filtration barrier (Quaggin and Kreidberg, 2008).

Podocytes display an unusual morphology characterized by multiple ‘comb-like’ foot processes that interdigitate with those of neighboring podocytes. Foot processes are linked together by a specialized cell-to-cell junction, called the slit diaphragm, which is made up of transmembrane proteins such as Nephrin and cytosolic proteins such as Podocin (Patrakka and Tryggvason, 2007; Schwarz et al., 2001; Shono et al., 2007). The establishment and/or maintenance of foot processes are dependent upon slit diaphragm components, adhesion complexes such as α3β1 integrin, and the lateral surface expression of Podocalyxin, a highly electronegative sialoglycoprotein (Korhonen et al., 1990; Kreidberg et al., 1996; Pätäri-Sampo et al., 2006; Schnabel et al., 1989). Podocytes are the targets of several pathogenic pathways and damage or congenital defects that disrupt their function are associated with proteinuria and kidney disease (Wiggins, 2007).

The zebrafish embryo, with its simplified two-nephron pronephric kidney, has become a useful genetic and developmental model to study nephron formation and patterning (Wingert and Davidson, 2008). A common, fused glomerulus is connected to each tubule by a short epithelial segment that likely represents the neck segment found in other lower vertebrates. Gene expression analysis has shown that the zebrafish tubule is segmented similarly to that of mammals, being made up of two proximal segments and two distal segments that connect to the cloaca via a short pronephric duct (Wingert et al., 2007). Additional similarities are found in the glomerular podocytes, which show high conservation in gene expression, structure and function compared to mammals (Drummond, 2005; Kramer-Zucker et al., 2005). Progenitors of the zebrafish pronephros arise from bilateral stripes of intermediate mesoderm around the 3-somite stage (11–12 hours post-fertilization; hpf) and epithelialize in situ into podocyte, tubule, and duct epithelial cells (Krauss et al., 1991; Pfeffer...
Podocyte progenitors migrate medially, fuse around 36 hpf, and recruit blood vessels from the overlying dorsal aorta by 48 hpf (Drummond et al., 1998). At present there is some confusion in the literature concerning the precise origin of podocyte progenitors along the anterior–posterior axis. Early studies using laser ablation and fate mapping suggest that podocytes descend from cells adjacent to somites 1–2 (Serluca and Fishman, 2001). However, subsequent molecular marker analyses suggest that podocytes arise from the intermediate mesoderm adjacent to somite three (Bollig et al., 2006).

Aising in close proximity to podocytes are interrenal gland cells, the teleost equivalent of the adrenal gland in mammals (Liu, 2007). The earliest marker of these cells is nr5a1a (also known as fir1b), a member of the Ftz-F1 nuclear receptor family, which initiates at the 22-somite stage (20 hpf) and is critical for interrenal gland specification (Hsu et al., 2003). At present the developmental relationship between podocytes and the interrenal gland is unclear.

One of the earliest markers of podocytes is the Wilms’ tumor suppressor-1 (Wt1) gene, which encodes a zinc finger transcriptional activator/repressor and putative splicing co-factor (Call et al., 1990; Caricase et al., 1996; Drummond et al., 1994). Considerable evidence implicates Wt1 as playing a key role in podocyte differentiation and/or maintenance, although the exact details of these functions have been elusive (Guo et al., 2002; McCaggart et al., 2001; Menke et al., 2003; Moore et al., 1999; Patek et al., 2003; White et al., 2010). In zebrafish, two paralogs of Wt1 exist, wt1a and wt1b. Wt1a is first expressed at the 3-somite stage in a broad domain of the anterior trunk that includes podocyte progenitors (Bollig et al., 2006; Drummond et al., 1998; Serluca and Fishman, 2001; Wingert et al., 2007). Expression of wt1b initiates later at the 10–12 somite stage where it is restricted to a subpopulation of wt1a-positive podocyte progenitors (Bollig et al., 2006; Penner et al., 2007). Morpholino-mediated knockdown of wt1a results in defective glomerular development and a loss of nephrin and podocin expression (Hsu et al., 2003; Penner et al., 2007). In contrast, wt1b-deficient animals show normal podocyte formation suggesting that wt1a is the major regulator of podocyte development.

More recently, the Notch pathway has been implicated as an important regulator of podocyte cell fate and homeostasis (Cheng and Kopan, 2005; Niranjan et al., 2009). Notch signaling is activated upon cell-to-cell contact, resulting in the interaction between Notch receptors and the Jagged/Delta (Dll) family of ligands. Upon activation, the Notch receptor is proteolytically cleaved, releasing the Notch intracellular domain (NICT) that then translocates to the nucleus. The NICT cannot bind DNA directly but instead complexes with other signaling components (Ninan et al., 2008). The NICD expressed during nephrogenesis and work in subsequent studies (Ninan et al., 2008; Sharma et al., 2010). These results suggest that Notch signaling must be tightly controlled in podocytes and that constitutive Notch activation is pathogenic (Waters et al., 2008). Therefore, understanding how Notch signaling controls podocyte development may have important implications in understanding its role in kidney disease.

In addition to Wt1 and the Notch pathway, the forkhead transcription factor Foxc2 is also involved in podocyte development. Foxc2 is expressed in podocyte progenitors during early stages of mouse nephrogenesis. Podocytes are formed in Foxc2 knockout mice but they remain immature and lack foot processes, slit diaphragms, and expression of certain podocyte markers including Podocin and Mafb (Takimoto et al., 2006). Similar results were found in X. laevis, where knockdown of foxc2 leads to decreased expression of mature podocyte markers and is required together with wt1 for podocyte formation (White et al., 2010). In mice, Foxc1 and Foxc2 interact with the Notch signaling pathway during somitogenesis and cardiovascular development, and recent studies in endothelial cells have shown that Foxc2 can form a transcriptional complex with Rbpj and NIDC (Hayashi and Kume, 2008; Kume et al., 2001). These data raise the possibility that Notch and Foxc factors may interact in a common pathway during podocyte formation as well. Consistent with this, overexpression of NIDC with foxc2 and wt1 induces podocyte markers in Xenopus explants (White et al., 2010).

While Wt1, Foxc, and Notch signaling factors are clearly involved in podocyte formation, the biochemical nature of the interactions between these factors and whether they act directly on common transcriptional targets has been unclear. Here, we use the zebrafish model to examine the requirement of Wt1a, Foxc1a, and Rbpj for podocyte formation and use biochemical methods to analyze the physical interactions and transcriptional complexes formed by these factors. From single and double morpholino knockdowns of wt1a, foxc1a, and rbpj we demonstrate that a deficiency in any two of these factors causes a failure in podocyte specification. Biochemical analyses reveal that each factor can physically interact with the others and influence the transcriptional activity of podocyte gene promoters in vitro. We hypothesize that these factors form higher order transcriptional complexes on common gene targets to induce and maintain podocyte identity.

Materials and methods

Zebrafish care and breeding

Wildtype Tubingen strain zebrafish were bred and maintained using standard zebrafish husbandry (Westerfield, 2000). Developmental staging was done by embryo morphology (Kimmel et al., 1995). The mi6b mutant strain was derived as described in (Itoh et al., 2003) and obtained from Dr. Leonard Zon (Children’s Hospital; Boston, MA).

Whole mount in situ hybridization

Single and two-color whole mount in situ hybridizations were performed as described (Thiss et al., 1993; Topczewska et al., 2001b). Molecular markers were generously provided by members of the zebrafish community or obtained from Open Biosystems or the Zebrafish International Resource Center. Stained embryos were transferred into 90% glycerol, flat-mounted where necessary, and photographed.

Morpholino injections

Morpholinos to wt1a (5′-CACGAAACATCAGAACCATTTTGGAG-3′) (Perner et al., 2007), (rbpja/b) (5′-AAACTCTCCGTGTCACAACAGGCGC-3′) (Sieger et al., 2003), fox1a (5′-GCTAAAGAGACTGAGACCATCAACACCA-3′) (Topczewska et al., 2001b), jag1b (5′-AATTGTGCTACTCACCCACCTTTTG-3′; Transmembrane splice), jag2b (5′-TTGCTACTCACCACCATGGCTA-3′ Transmembrane splice) and the Standard Control were purchased from Gene Tools LLC and resuspended in 1x Danieau solution. Embryos at the 1–2 cell stage were injected with 1 nl of morpholino at 3.5 ng/nl (wt1a, jagged-1b, jagged-2a), 1.75 ng/nl (rbpja/b), and 2.3 ng/nl
(foxc1a). For the double knockdowns, morpholinos were mixed together to preserve the above concentrations and 1 nl was injected. Control embryos were either injected with the Standard Control (5.8 ng/nl) or left uninjected (no differences were found between these two classes of controls). Embryos were incubated at 28.5 °C, fixed in 4% paraformaldehyde, and stored in methanol.

Electron microscopy

Electron microscopy was performed by the Microscopy Core of the Program in Membrane Biology (PMB) at MGH. Briefly, embryos were fixed in 1.5% glutaraldehyde/1% paraformaldehyde/70 mM NaPO4 pH 7.2/3% sucrose, washed in 0.1 M cacodylate buffer pH 7.4 and postfixed in 1% OsO4/1.5% potassium ferrocyanide for 3 h. Embryos were washed, dehydrated, embedded in Epon 812 and then sectioned on a Reichert Ultracut E ultramicrotome and viewed with a JEOL 1011 electron microscope.

Laser ablations

Embryos to be ablated were dechorionated and mounted dorsal side up within an agarose well on a glass depression slide. The embryos were covered by a small volume of E3 embryo buffer and viewed with a Nikon 80i upright compound microscope equipped with a MicroPoint laser system (Photonic Instruments). The microscope was focused on the intermediate mesoderm adjacent to somites 1, 2 or 3 and a 5 × 5 patch of intermediate mesoderm cells (corresponding to the width of the somite) were pulsed under maximum power. Ablated cells either became swollen and burst or contracted abruptly. Ablated embryos were returned to the 28.5 °C incubator and then fixed in 4% paraformaldehyde once the desired developmental stage was obtained.

Glomerular filtration assay

Embryos at 4 dpf were positioned on agarose ramps and injected into circulation with 40 kD fluorescein-labeled dextran (Invitrogen). Injected embryos were returned to the 28.5 °C incubator and fixed the next day in 4% paraformaldehyde. Following dehyration in methanol the embryos were infiltrated in JB-4 resin and 5 μm thick sections were cut through the glomerulus/neck segment. Sections were photographed under DIC optics and epi-fluorescence using a FITC filter set.

Preparation of proteins for GST-pulldowns

Zebrafish cDNA from 72 hpf embryos was made using the SuperScript II First-Strand Synthesis System (Invitrogen). Rbpj, wt1α and NICD3 were each amplified from 2 μl of 72 hpf cDNA using gene specific primers with BamHI (5′) and EcoRI (3′) restriction sites (NICD3 and rbpj), BamHI (5′) and Xhol (3′) (wt1α), or EcoRI (5′) and Xhol (3′) (foxc1a) and subsequently cloned into the pcPS2 vector (NICD3 5′: TTATAAGGATC-CACCATGTTGATTGGCCGGCGAAGCG; NICD3 3′: CGACGGGAGATTT-CAGAAAAACACCTGATCT; rbpj 5′: ATGGGTTGATCCAGGGCTGCC; TGGTGACA; rbpj 3′: GGTACCAATTCGGAAGACCAATCCGG; wt1α 5′: ATCCAAGAGATCATGTCCTGATGTTCTTGAG; wt1α 3′: ATGACCCGAGGGGCAATGTTGCTGGCTGG; FoxC2 5′: ATCTGGGATC-GAGGTGATCCTTTGGTTTTCCTCAG; FoxC2 3′: GCACTGAATTCTTAGGACAC-ATGAAGTCCGGGGCTG; Rbpj 5′: ATCTGGGATC-GAGGTGATCCTTTGGTTTTCCTCAG; Rbpj 3′: GCACTGAATTCTTAGGACAC-ATGAAGTCCGGGGCTG). Proteins were produced according to manufacturer’s instructions with the following modification: 1.5 μl of Transcend tRNA (Biotinylated lysine-tRNA complex, Promega) was added to each reaction and the other components adjusted as necessary.

GST-pulldowns

For each pulldown condition, 25 μl of the appropriate in vitro translated protein (from a 50 μl total reaction volume) was added to each tube containing 30 μl of the GST-tagged protein or GST control protein bound to the glutathione-agarose. Total reaction volumes were brought to 125 μl with wash buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.25% TX–100) and incubated for 1 h at 4 °C with rotation. Supernatant was removed and beads were washed 4× with wash buffer. Beads were resuspended in 30 μl of 1X SDS-PAGE sample buffer. 20 μl of each supernatant was mixed with the appropriate volume of 4X sample buffer. Samples were heated at 95 °C for 5 minutes and run on a 10% SDS-PAGE gel.

Cell culture and transfections

HEK293T or NIH3T3 cells were cultured at 37 °C, 5% CO2 in DMEM containing 10% FBS, 100 units/ml penicillin and 100 mg/ml streptomycin. For co-IPs, two 10 cm2 plates were transfected for each condition. For luciferase reporter assays, one 2.0 cm2 well of a 24-well plate was transfected for each condition. Cells were transfected using TransIT Transfection Reagent (Mirus) according to the manufacturer’s recommendations and subsequently incubated for 48 h.

Co-immunoprecipitations

HEK293T cells were transfected as indicated above with the appropriate combination of the following constructs: pCMX-N/RBPJ (Riken BRC DNA Bank, No. 3021), pEF-BOS neo-mNotch1 R AMIC (Riken No. 6771), pCMV-Tag2B-Rbpj, pCMV-Tag2B-FoxC2, pCS2-Wt1 or pCS2-FoxC2. pCMV-Tag2B-Rbpj was constructed by amplifying Rbpj from the pCMX-N/RBPJ plasmid using gene specific primers with BamHI (5′) and EcoRI (3′) restriction sites (Rbpj 5′: ATCTGGGATC-GAGGTGATCCTTTGGTTTTCCTCAG; Rbpj 3′: GCACTGAATTCTTAGGACAC-ATGAAGTCCGGGGCTG). The resulting product was cloned into the pCMV-Tag2B vector (Strategene). pCMV-Tag2B-FoxC2, pCS2-FoxC2 and pCS2-Wt1 were constructed by amplifying FoxC2 or Wt1 from mouse kidney cDNA. cDNA was made from adult male kidney total RNA (Agilent) using the methods listed above. Gene specific primers with BamHI (5′) and EcoRI (3′) were used to clone FoxC2 or Wt1 into
each vector (Foxc2 5′: ATTAGGATCCGCCACCATGCAAGCGCTG- TACTGGTA; Foxc2 3′: CGCAGATATCTGATTATTTGTCGACTTCTA; Wt1 5′: TATTGGATCCGCCACCATGCAAGCTTCTGTCGAC GWT1 3′: GACGAATTCCTAAGCGCCGCTTGAATTC)

Cells were harvested 48 hours post-transfection. Nuclear extracts were made using the Nuclear Extract Kit (Active Motif) and following the manufacturer’s instructions. The resulting extract from each transfection combination was split in half and incubated with either normal rabbit antibody, anti-FLAG antibody (Sigma; F7425) or anti-c-Myc antibody (Sigma; M4439) crosslinked to Protein G Mag Sepharose (GE Healthcare). Antibodies were crosslinked using buffers supplied in the Protein A/G SpinTrap Buffer Kit (GE Healthcare) in combination with the crosslink protocol supplied with the magnetic sepharose. Cross-linked beads were preincubated with 1 mg/ml BSA to reduce nonspecific binding. Extracts were incubated with the antibody-crosslinked beads 4–16 h at 4°C with rotation. The supernatant was removed and the beads washed 4X with the IP low buffer (supplied with Nuclear Extract Kit and prepared as indicated; 2nd wash contained 1 mg/ml BSA). The proteins were eluted from the antibodies with 2 × 50 μl of 0.1 M glycine pH 2.0, 5 min each. Elutions were pooled and mixed with 4X SDS sample buffer. 15 μl of supernatant was mixed with 4X SDS sample buffer. Samples were heated at 95°C for 5 min and run on a 10% SDS-PAGE gel.

Western blotting

Proteins separated on SDS-PAGE gels were transferred to nitrocellulose and Western blotted according to standard protocols. For GST pull-downs, the amount of GST or GST-tagged protein bound to beads was visualized by Ponceau S stain. Non-tagged proteins in the pulldown assays were detected by Streptavidin Alkaline Phosphatase (Promega) in combination with the AP Conjugate Substrate Kit (BioRad). For co-IPs, the following antibodies were used to detect proteins: anti-c-Myc (NICD), anti-FLAG (FLAG-Rbpj or FLAG-FoxC2), anti-Rbpj (H-50; Santa Cruz Biotech), anti-FoxC2-ChIP Grade (Abcam), anti-Wt1 (Abcam), anti-c-Myc (NICD), anti-FLAG (FLAG-Rbpj or FLAG-FoxC2), anti-Rbpj (H-50; Santa Cruz Biotech). The appropriate AP-linked secondary was used in combination with the AP Conjugate Substrate Kit to detect immunoprecipitated proteins.

Luciferase reporter assays

NIH3T3 cells were transfected as indicated above with the appropriate combination of the following constructs: pCMX-N/RBPj, pEF-BOSneo-mNotch1 RAMIC, pcS2-Wt1, pcS2-FoxC2, pcS2-FoxC1, pcS2-Rbpj, pcS2-Wt1a, pcS2-NICD3 or pcS2-focx1a. Transfections were carried out using 0.5 μg or 2.5 μg of each construct. The synthetic Notch luciferase reporter (TP1-luc; 50 μg/p-transfection), Riken No. 6776, 0.1 μg), Hey1 luciferase reporter (0.1 μg; Maier and Gessler, 2000) or Podocalyxin luciferase reporter (0.1 μg; Palmer et al., 2001) was cotransfected with the pRL-TK Renilla internal control vector (Promega, 10 ng) for each condition. The amount of total DNA was adjusted as necessary with empty pcS2 vector. After 48 h, the cells were lysed and processed using the Dual Luciferase Reporter Assay Kit (Promega). Each extract sample was diluted 1:100 in the supplied Passive Lysis Buffer and the luciferase assays carried out using a Berthold Centro XS II luminometer in conjunction with the MikroWin2000 program. Triplicates of each condition were analyzed for luciferase activity. Each experiment was repeated a minimum of three times.

Results

Podocyte development and relationship to neck, proximal tubule, and interrenal gland cells

To better characterize and clarify podocyte development in the zebrafish embryo, we began by analyzing podocyte gene expression from progenitor to differentiated cell (Fig. 1A). The expression patterns of renal transcription factors expressed during early stages of podocyte formation (wt1a, wt1b, mafba, hey1, lhx1a and pax2a) and markers of mature podocytes (nephrin, podocin, podocalyxin, and integrinα3) were assessed (Fig. 1B). These genes were examined from the 15-somite stage, around the time that podocyte progenitors first arise from the intermediate mesoderm, through to 48 hpf, when glomerular filtration has initiated (Drummond et al., 1998; Serluca and Fishman, 2001). As previously described, wt1a is expressed broadly at early stages and becomes highly expressed in podocyte progenitors between the 18 somite to 24 hpf stages (Fig. 1B; Bollig et al., 2006; Drummond et al., 1998; Serluca and Fishman, 2001). By contrast, the other early markers (wt1b, mafba, hey1 and lhx1a) show more restricted expression domains in the intermediate mesoderm, specifically marking presumptive podocyte progenitors. Expression of wt1a, wt1b, and mafba persists in podocytes as they merge at the midline and form the pronephric glomerulus (36 hpf and 48 hpf, Fig. 1B). In contrast, lhx1a and hey1 transcripts are downregulated in podocytes starting around 24 hpf and are significantly reduced by 36 hpf. Similarly, expression of pax2a, which is initially expressed throughout the intermediate mesoderm, is lost in podocytes around the 24 hpf stage but maintained in the presumptive neck region. Weak expression of podocalyxin and nephrin initiates at 24 hpf followed by podocin and integrinα3 by 36 hpf (Fig. 1B). We conclude from this analysis that podocyte progenitors arise around the 15-somite stage, initiate terminal differentiation at 24 hpf, and that their maturation is associated with an upregulation of wt1a and a corresponding downregulation of lhx1a and pax2a.

To analyze the spatial arrangement of podocytes, neck, proximal tubule cells and the interrenal gland in more detail, we examined the expression domains of wt1b, pax2a, cadherin-17 (cdh17; a marker of non-podocyte renal epithelia), slc20a1a (a marker of the first proximal tubule segment) and the interrenal gland gene nr5a1a at 24 and 36 hpf (Horsfield et al., 2002; Hsu et al., 2003; Wingert et al., 2007). At 24 hpf, wt1b+ podocytes were found closely associated with cdih17+ epithelial cells posteriorly and nr5a1a+ interrenal gland progenitors medially with little to no overlap in these expression domains (Fig. 1C). Pax2a expression was observed in podocytes as well as the anteriormost region of cdih17+ cells, but showed little overlap with slc20a1a+ tubule cells. By 36 hpf, the number of nr5a1a+ cells was markedly expanded and formed a distinct mass near the midline just posterior to the podocyte clusters. Expression of pax2a in the presumptive neck segment was juxtaposed between the podocytes and the slc20a1a+ proximal tubule segment (Fig. 1C). To confirm the identity of the pax2+ neck cells, we analyzed cell morphology and the expression of genes required for cilia motility (Essner et al., 2005; Thissie et al., 2001; Yu et al., 2008). FoxJ1a, dnah9, eefc1 and capsl were expressed in a ‘salt and pepper’ pattern in the neck region at 48 hpf, as well as a few scattered cells in the rostral portion of the proximal tubule (Fig. S1A). Transmission electron micrographs showed that this region lacks a brush border (which characterizes the proximal tubule) and contains multiciliated cells similar to those observed in the neck segments of other fish species (Fig. S1B,C; Elger et al., 2000; Hentschel and Elger, 1989; Reimischwessel, 2001). Additionally, these cells took up minimal amounts of fluorescent 40 kDa dextran from circulation compared to the proximal tubule cells, confirming a functional distinction between the neck region and proximal tubule (Fig. S1D).

Determination of podocyte progenitor origin and candidate factors responsible for their specification

The finding that podocyte and neck progenitors initially express pax2a and are closely associated with each other during pronephric development led us to investigate their origin from the intermediate mesoderm prior to podocyte specification. At the 8-somite stage, pax2a transcripts are found from the level of somite 3 to the tailbud and mark the cells in the intermediate mesoderm that will give rise to...
the pronephros (Fig. 2A, B). Based on comparing the expression domains of pax2a and wtf1a relative to the somite marker myod, as well as double in situ hybridization for wtf1a and pax2a transcripts, we determined that the expression domain of wtf1a overlaps with the anterior-most pax2a+ cells adjacent to somite 3 with weaker co-expression at the level of somite 4 (Fig. 2B, C; see also Serluca and Fishman, 2001). We noted that by the 15-somite stage, expression of pax2a becomes prominent in the anterior-most intermediate mesoderm (herein referred to as pax2a+[High] cells, Fig. 1B). Double in situ hybridization for pax2a and wtf1b transcripts revealed that within this pax2a+[High] population, the anterior cells express wtf1b and become displaced medially, consistent with their identity as podocyte progenitors (arrow, Fig. 2C). The posterior pax2a+[High] population, which remains lateral, presumably comprises neck progenitors. Taken together, these observations suggest that the intermediate mesoderm adjacent to somite 3, which co-expresses pax2a and wtf1a, gives rise to discrete populations of podocyte and neck progenitors by the 15-somite stage.

To clarify the origin of podocyte and neck progenitors, we performed laser ablation experiments on 8-somite stage embryos. Cells targeted by the laser either lysed immediately or, after repeated exposure to laser pulses, became swollen and necrotic (Fig. 2D). Ablation of the intermediate mesoderm adjacent to the 1st and 2nd somites embryos failed to significantly affect podocyte cell number based on pax2a expression at 10-somites and nephrin expression at 48 hpf (n = 3/3 for both; Fig. 2E). This result is in agreement with the ablation studies by Serluca and Fishman (2001). By contrast, ablation of the intermediate mesoderm adjacent to the 3rd somite resulted in a significant shortening in the pax2a expression domain on the ablated side of the embryo (n = 3/3; Fig. 2E). A small number of pax2a+ cells were found on the anterior side of the ablated area (arrow, top panel, Fig. 2E). These cells likely represent intermediate mesoderm that lies at, or just anterior to, the boundary between somites 2 and 3. We next examined ablated embryos at the 36 hpf stage for wtf1b and cdh17 transcripts, which distinguish podocytes, neck, and proximal tubule populations. On the ablated side of the embryo we observed a near loss of podocytes (arrow, bottom panel, Fig. 2E) and an absence of neck cells (n = 3/3; Fig. 2E). Interestingly, based on nephrin expression at 48 hpf, a loss of podocytes on one side of the embryo did not appear to perturb the development of the podocytes on the unablated side (n = 3/3; Fig. 2E). We conclude that the majority of podocytes and all of the neck cells are derived from intermediate mesoderm adjacent to the 3rd somite at the 8-somite stage.

To determine the pathways responsible for podocyte specification we examined the expression of components of the Notch signaling pathway and the foxc transcription factors, two strong candidates based on prior analyses in other species (Cheng et al., 2003, 2007; Takemoto et al., 2006; White et al., 2010). Consistent with other reports, we found that the Notch ligands jagged-1b (jag1b) and jagged-2a (jag2a) are strongly expressed in the anterior intermediate mesoderm at the 8-somite stage, extending from the level of somite 3 to 7 (Fig. 2B; Ma and Jiang, 2007; Wingert et al., 2007; Zecchin et al., 2005). Transcripts for notch1a, notch3 and rhp/ja/b are more broadly expressed and also include the intermediate mesoderm (Echeverri and Oates, 2007; Ma and Jiang, 2007; Sieger et al., 2003). A Foxc2 homologue does not exist in zebrafish, instead two foxc1 paralogs are found (foxc1a and foxc1b) with only foxc1a showing expression in the intermediate mesoderm (Topczewska et al., 2007).
one of these three factors developed a reduced number of podocytes, as assessed at 15 somites, compared to control conditions do so independently of podocytes that form under wt1a-deficient conditions do so independently of wt1b function. In rbpj morphants at 36 hpf, the podocytes expressed nephrin and podocalyxin indicating some maturation occurred, although the clusters appeared malformed and often failed to fuse at the midline (Fig. 3; nephrin: n = 33/33; podocalyxin: n = 27/28). Expression of podocin was more variable, being absent in approximately half of the rbpj morphants (data not shown). In correlation with rbpj morphants, podocytes were also reduced in mind bomb (mib) mutants (defective in Notch signaling) and jagged-1b or jagged-2a morphants, indicating that the rbpj-deficient phenotype likely results from a loss of Notch target gene activation rather than de-repression caused by rbpj loss (Fig. S2E, F). Similar to wt1a morphants, wt1b- deficient embryos at 3 dpf, suggesting that podocyte survival is also compromised in these animals (data not shown). Knockdown of foxc1a resulted in relatively normal nephrin and podocalyxin expression in podocytes at 36 hpf, indicating that podocyte differentiation was not significantly perturbed by foxc1a deficiency (Fig. 3; nephrin: n = 16/16; podocalyxin: n = 21/21). In summary, these results indicate that wt1a, foxc1a and Notch signaling are required for early podocyte development,
while wt1a and Notch may have additional roles during later stages of podocyte maturation/survival.

Because no single knockdown caused a complete failure of podocyte specification, we tested whether combinatorial knockdowns between wt1a, rbpj and foxc1a would lead to a more severe effect on podocyte development, as recently shown in Xenopus (White et al., 2010). Embryos double-deficient in wt1a and rbpj showed an absence of early podocyte marker gene expression (wt1b, lhx1a), as well as more mature markers at 36 hpf (nephrin and podocalyxin) consistent with a failure to specify podocytes (Fig. 3 and Fig. S2A; wt1b: n = 30/30 at 15 somites; nephrin: n = 26/26; podocalyxin: n = 27/28). Embryos double-deficient in foxc1a and wt1a also showed a complete failure of podocyte specification (Fig. 3 and Fig. S2A; wt1b: n = 32/32; nephrin: n = 35/36; podocalyxin: n = 32/32). Consistent with these data, embryos double-deficient in foxc1a and rbpj lacked podocytes (Fig. 3 and Fig. S2A; wt1b: n = 19/20; nephrin: n = 17/17; podocalyxin: n = 34/34). The loss of podocytes in wt1a/rbpj and the wt1a/foxc1a double knockdowns could be caused by a general loss of the anteriormost region of the intermediate mesoderm. To investigate this, we examined the effect of each morpholino combination on the pax2a +High population, comprising presumptive podocyte and neck progenitors, at the 15-somite stage. Although expression of pax2a was reduced in these cells in the morphants (Fig. 3), double staining for pax2a and myod showed that the intermediate mesoderm was not truncated, indicating that that the podocyte and neck defects were not simply due to a loss of cells adjacent to somite three (data not shown). In further support of this, we failed to observe elevated apoptosis in the anterior intermediate mesoderm in the morphants and co-injection of anti-apoptotic factors (p53 morpholinos or bcl2 mRNA) did not rescue podocyte cell number (data not shown). Therefore the loss of podocytes in the double morphants was most likely caused by a failure in cell fate specification rather than by cell death. Taken together, these data suggest that all three factors contribute to podocyte specification.

Differential regulation of neck and interrenal fate by wt1a, foxc1a and rbpj

Due to the development of podocytes and neck cells from a similar region of the intermediate mesoderm that expresses wt1a, foxc1a and Notch signaling factors, we assessed the effect of double knockdowns on neck development. Embryos deficient in both rbpj and foxc1a develop a relatively normal pax2a + neck segment (n = 19/19). However, double knockdown of wt1a and rbpj or wt1a and foxc1a caused a complete or near loss of pax2a expression in the neck region.
at 48 hpf (Fig. 3; wt1a/rbpj morphants: n = 17/18; wt1a/foxc1a morphants n = 20/23). Transcripts for fox1a and dnah9 were also significantly reduced at 48 hpf, suggesting that the neck fails to differentiate in wt1a/rbpj and wt1a/foxc1a double-deficient animals (Fig. S3A and data not shown). An analysis of wt1b, cdh17, and slc20a1a transcripts at the 24 hpf stage revealed that despite the loss of podocyte and neck markers, the first proximal tubule segment marked by cdh17 and slc20a1a transcripts is unaffected in single and double-deficient embryos (Fig. 3). From these data, we conclude that while all factors contribute to podocyte specification, their combinatorial knockdown leads to differing effects on neck development and no effect on proximal tubule formation.

We also investigated whether knockdown of wt1a, rbpj and foxc1a influenced the development of interrenal gland progenitors. In wt1a and rbpj single morphants, the number of nr5a1a+ interrenal progenitors was increased (Fig. 3; wt1a morphants: n = 25/30; rbpj morphants: n = 23/27, respectively). An even greater expansion was observed in double-deficient embryos with ectopic nr5a1a+ cells extending posteriorly (Fig. 3; n = 33/36), indicating that wt1a and rbpj play a role in suppressing interrenal gland formation. Precocious expression of nr5a1a was not observed in the morphants prior to the normal onset of expression, demonstrating that the timing of interrenal gland formation was not affected by wt1a and rbpj deficiency (data not shown). In contrast to the expansion seen in the wt1a and rbpj single- or double-knockdowns, foxc1a morphants did not show an increase in interrenal gland cells (Fig. 3; n = 18/18). In embryos double-deficient in foxc1a and rbpj, the interrenal gland was enlarged to a similar extent as that observed in rbpj morphants (Fig. 3; n = 17/20). However surprisingly, nr5a1a+ interrenal gland cells were absent in embryos double deficient for foxc1a and rbpj (Fig. 3; n = 14/16). This phenotype was also found at later stages (48 hpf) suggesting that it is not simply caused by delayed interrenal gland development (data not shown). The loss of the interrenal lineage in wt1a/foxc1a morphants suggests these factors may act redundantly in the specification of the interrenal lineage. Together, these results demonstrate that complex interactions exist between foxc1a, wt1a, and rbpj in the intermediate mesoderm tissue that gives rise to podocytes, neck cells, and the interrenal gland.

Wt1, FoxC1/2 and Notch signaling factors physically interact and regulate common transcriptional targets

The complete loss of podocytes in our combinatorial knockdowns suggests that wt1a, foxc1a and Notch signaling cooperatively regulate podocyte development. We therefore assessed whether expression of hey1, a direct downstream target of the Notch pathway, is affected by the knockdown of rbpj, wt1a or foxc1a, or any combination of these factors. As expected, rbpj morphants show reduced, although not completely lost, expression of hey1 at 15 somites (Fig. 3 and Fig. S3B; n = 16/16). In wt1a and foxc1a single morphants, a similar level of hey1 downregulation was observed (wt1a morphants: n = 15/15; foxc1a morphants n = 17/17). When combinatorial knockdowns were performed, all double morpholino treated embryos completely lost hey1 expression consistent with the notion that wt1a, foxc1a and Notch signaling act together on common targets such as hey1 during podocyte development (Fig. 3 and Fig. S3B).

We next tested whether wt1a, foxc1a, rbpj, and the intracellular domain of Notch3 (NICD3) could physically interact to form protein-protein complexes. To do this, we performed glutathione S-transferase (GST) tagged in vitro pull-down assays. GST-rbpj bound to NICD3 as expected, however it was also able to complex with foxc1a and wt1a (both KTS + and KTS- isoforms; Fig. 4A and data not shown). GST-foxc1a was unable to interact with NICD3 but could bind to rbpj and wt1a. Similarly, GST-wt1a failed to bind to NICD3 but could pull-down rbpj and foxc1a. Previous studies in mouse have shown that GST-Rbpg can interact with Foxc2 and NICD1 concomitantly, suggestive of a multimeric complex (Hayashi and Kume, 2008). We confirmed a similar result with the zebrafish proteins, with GST-Rbpg able to pull-down both foxc1a and NICD3, although we cannot rule-out that two mutually distinct complexes (rbpj-NICD3 and rbpj-foxc1a) are forming in these experiments (Fig. 4A). Taken together, these data reveal that wt1a, foxc1a, and rbpj can physically interact with one another and suggest that multiple combinations of these transcription factors may play a role in the regulation of podocyte development.

To confirm the GST pull-down results we conducted co-immuno-precipitation (co-IP) experiments in cultured cells. Because antibodies to the zebrafish proteins are not available, and to confirm that similar complexes can form with mammalian proteins, we overexpressed various combinations of murine Rbpj, NICD1, FoxC2 and Wt1 (KTS-) in HEK293T cells. Consistent with our pull-down data, we found that FoxC2 and NICD1 could be individually and simultaneously co-IP’d from nuclear extracts with Flag-Rbpj (Fig. 4B, top and bottom panels, respectively). Additionally, interactions between Wt1 and Rbpj or Wt1 and Foxc2 were observed using Flagg-Rbpj, Flagg-Wt1 or Flagg-FoxC2 to reciprocally co-IP these complexes (Fig. 4B, top panel). In correlation with our pull-down data, NICD1 could only directly complex with Rbpj (Fig. 4B, top panel). The co-IP data therefore supports our pull-down results and suggests that multiple combinations of these transcription factors can complex in cells, and that the physical interactions between Wt1, Rbpj, and FoxC1/2 are conserved in mammals.

Transcription factors that form complexes can have both positive and negative effects on promoter activation. To determine how Wt1, FoxC1/2 and Notch signaling interact, we examined the effect of combinations of these factors on the mouse Hey1 promoter (Maier and Gessler, 2000). Co-transfection of NIH3T3 cells with a Hey1 promoter luciferase reporter together with NICD1, foxc1a, FoxC2, or Wt1 showed that each factor alone was capable of inducing the reporter 1.5–4-fold above the vector only control (Fig. 4C). Over-expressing NICD1 with equal amounts of foxc1a or FoxC2 or Wt1 failed to enhance promoter activation above the level seen with NICD1 alone (data not shown). However, when a 5-fold excess of foxc1a or FoxC2 or Wt1 was combined with NICD1, the Hey1 promoter was activated between 7 and 11 fold above the control. This result suggests that when FoxC1/2 and Wt1 are present in excess over NICD1 they can cooperate to activate the Hey1 promoter (Fig. 4C). In the case of the NICD1 + foxc1a and NICD1 + Wt1 combinations, the effect on the reporter was synergistic. A similar synergy was also seen when foxc1a or FoxC2 was co-expressed with Wt1 in the absence of exogenous NICD1 (Fig. 4C). Triple transfections (NICD1 + foxc1a/FoxC2+Wt1) further induced the Hey1 promoter 13–15-fold above the control, demonstrating that the strongest synergistic induction occurred when all three factors were present together. Taken together, these data are consistent with our in vivo data and support a model whereby NICD, Rbpj, FoxC1/2, and Wt1 interact to form one or more transcriptional complexes that synergize to activate common podocyte gene targets.

The transcriptional synergy between Wt1, FoxC1/2, and Notch signaling may arise from a single factor, such as Rbpj, acting as a core DNA binding factor to which the others are tethered. To explore this, we investigated the effects of NICD1, foxc1a, and Wt1 on an established synthetic Notch responsive reporter promoter (pGa981-6), which comprises 12 tandem Rbpj sites upstream of a minimal promoter (Kurooka et al., 1998). While NICD1 induced the Notch reporter ~7-fold over the vector only control, co-transfection with foxc1a or Wt1 significantly inhibited transactivation with foxc1a being more potent than Wt1 (Fig. 4C). These results suggest that Wt1 and FoxC1/2 do not cooperate with Notch signaling by interacting with a DNA-bound Rbpj/NICD1 activation complex. Furthermore, these findings raise the possibility that in vivo, Wt1 and FoxC1/2 and may have antagonistic effects on Notch signaling, depending on the context of the promoter. We next examined the transcriptional effects of Wt1, FoxC1a and Notch signaling on the promoter from the Podocalyxin gene, a Wt1 target that represents a more mature marker of podocytes (Fig. 1B; Palmer et al., 2001). NICD1 had little effect on the Podocalyxin
promoter, consistent with the notion that Notch signaling may only be active on genes expressed in podocyte progenitors. By contrast, both Wt1 and foxc1a were capable of inducing the Podocalyxin promoter between 2 and 3-fold over the vector-only control (Fig. 4C). Co-transfection of foxc1a and Wt1 at a 2:1 ratio showed additive effects, with the promoter being induced 5–6-fold over the control. Surprisingly, we found that foxc1a suppressed activation of the Podocalyxin promoter when co-transfected in 5-fold excess over Wt1 (Fig. 4C). This suggests that the dosage of FoxC1/2 and Wt1 may be important for determining whether target genes are activated or repressed in vivo. Our observation that wt1a expression is upregulated in podocyte progenitors between 18 and 24 hpf, just prior to the onset of podocalyxin and nephrin, is consistent with this hypothesis (Fig. 1B).

Discussion

In this report we have identified novel interactions among transcriptional pathways that are important for kidney development. Using morpholinos to knockdown wt1a, foxc1a, and rbpj in isolation, we revealed that each factor contributes to podocyte specification but deficiency in any one factor alone is not sufficient to completely block podocyte formation. However, when any two of these genes are targeted, we observed a complete failure in podocyte development, as assessed by the lack of expression of the earliest molecular markers of podocyte cell fate. Additionally, we found that wt1a, foxc1a, and rbpj are involved in the development of two cell lineages that arise next to podocytes: ciliated neck cells, which link the glomerulus to the proximal tubule, and interrenal gland cells. We demonstrate that physical interactions occur between Wt1, FoxC1/2 and Rbpj, suggestive of the formation of multimeric transcriptional complexes. Consistent with this, Wt1 and Foxc1a synergize with Notch signaling to induce expression of the Hey1 promoter. Together, these observations suggest that Wt1, FoxC1/2, and Rbpj form a protein interaction network that controls podocyte formation and maintenance.

Our work is consistent with results from mouse and Xenopus that have shown that podocyte differentiation depends on multiple transcriptional inputs from factors that include Wt1, FoxC1/2, Mafb, Lmx1, Hey1, and Notch and retinoic acid signaling (Bollig et al., 2009; Cheng et al., 2003, 2007; Haldin et al., 2008; Sadl et al., 2002; Taelman et al., 2006; Takemoto et al., 2006; White et al., 2010; Wingert et al., 2007). A framework of the molecular circuitry governing podocyte identity is beginning to be constructed (White et al., 2010), although there is still much to be done to confirm the direct targets, feed-back/forward interactions, and the linear or parallel action of these factors. Our data suggests that Wt1, FoxC1/2, and Notch signaling converge on common gene targets such as Hey1, to synergistically activate gene expression. The finding that wt1a, foxc1a, and rbpj can physically interact supports a model whereby these factors assemble into one or
more multimeric transcriptional complexes on DNA. The tethering of the transcription factors to a single DNA binding site appears unlikely, as podocyte specification does not rely on a single factor. Instead, we favor a model in which wt1a, foxc1a, and an rbpj/NICD activation complex interact to form an ‘enhanceosome’ that recruits the transcriptional machinery to the target promoter. (Fig. 5). In this model, a deficiency of one factor compromises promoter activation, whereas a loss of any two factors is sufficient to abrogate gene activation.

Our data, together with recent observations by others, suggest that the regulation of HESR family genes by FoxC1/2, Wt1, and Notch may be a general phenomenon. For example, FoxC2 can bind and synergistically induce the Hey2 promoter in endothelial cells (Hayashi and Kume, 2008) whereas Wt1 binds to the mouse Hey-like (HeyL) promoter in the kidney. Knocking down Wt1 in kidney explants reduces HeyL expression, consistent with a dual input from Wt1 and Notch signaling during metanephros development (Hartwig et al., 2010). Until now, cooperativity and complex formation between FoxC1/2 factors and Wt1 has been unappreciated. It will therefore be interesting to compare ChIP-chip/seq datasets for FoxC2 and Wt1 to identify candidate promoters co-regulated by these factors (Hartwig et al., 2010; Norrmén et al., 2009).

The observation that high levels of foxc1a relative to wt1 inhibit transactivation of the Podocalyxin promoter, whereas lower levels of foxc1a have a positive, additive effect with wt1 raise the possibility that transcription factor dosage is an important contributor to the podocyte regulatory program. We noted that wt1a expression increases just prior to the onset of nephrin and podocalyxin, two genes that are direct targets of Wt1 (Guo et al., 2004; Palmer et al., 2001; Wagner et al., 2004). We speculate that while low levels of wt1a are sufficient to synergize with foxc1a and Notch signaling during the early stages of podocyte development, higher levels of wt1a may be necessary to activate the expression of mature podocyte genes. Thus, tight control over wt1a expression may be critical to ensure the proper temporal execution of the podocyte program. Two candidate regulators of wt1a are pax2a, which has previously been shown to inhibit wt1a expression in the neck region, and hey1, which represses Wt1 in frogs (Majumdar et al., 2000; White et al., 2010). Consistent

![Fig. 5. Model of transcriptional regulation by Wt1, FoxC1/2 and Notch signaling during podocyte development. In podocyte progenitors, where high levels of Notch signaling are proposed to occur, Wt1 and FoxC1/2 complex with Rbpj and other members of the Notch transcriptional complex such as NICD and Mastermind-like (Maml). This multi-factor complex enhances the transcription of Notch targets such as Hey1. As podocytes mature, Notch signaling decreases and Wt1 levels increase. This increase in Wt1 levels activates transcription of mature targets such as Podocalyxin, which is enhanced by FoxC1/2 complexing with Wt1 on the promoter. Wt1 levels may antagonize Notch signaling by sequestering Rbpj, preventing transcription of Notch targets concomitantly with the decrease in Notch signaling.](image-url)
with this role, both genes are downregulated just prior to the upregulation of wtla. Transcripts for pax2a and hey1 are decreased in wtla, foxc1a, and rbpj single and double morphants, highlighting a potential negative feedback loop involving wtla. This loop may be important in the pathogenesis of glomerular diseases as a reactivation of Notch signaling in podocytes is associated with an induction of Hey1, Pax2 and a concomitant downregulation of Wt1 (Murea et al., 2010; Niranjan et al., 2008; Sharma et al., 2010; Waters et al., 2008). Thus, one of the key pathogenic effects of Notch in podocytes may be the re-establishment of an embryonic pathway that acts to lower Wt1 levels below a threshold needed to maintain the expression of slit-diaphragm genes such as Nephrin.

Although Wt1, foxc1a and Notch signaling synergized in the context of the Hey1 promoter, we found that Wt1 and foxc1a inhibited the ability of NICD1 to activate a synthetic Notch reporter driven by Rbpj sites. This result suggests that promoter context influences whether these factors interact in a positive or inhibitory fashion. An inhibitory effect of Wt1 and foxc1a on the activation of Notch genes driven by Rbpj sites may provide a novel mechanism to prevent activation of these targets in mature podocytes. This may be a general function of Fox family transcription factors as we found that foxj1, a master regulator of motile cilia genes that has an antagonistic relationship with the Notch pathway, can also complex with Rbpj (unpublished observation; Morimoto et al., 2010; Murphy et al., 1997; Yu et al., 2008). Given that no Wt1 or FoxC sites exist in the Notch reporter, the mechanism of inhibition may be via sequestration of Rbpj. Sequestration may be occurring in solution or on DNA and further study is needed to resolve this issue as well as determine whether Wt1 and FoxC1/2 compete with NICD for binding to Rbpj. These results provide further support for the notion that the levels of Wt1 and FoxC1/2 are critical for establishing/maintaining podocyte identity via the modulation of Notch and podocyte target genes.

In addition to controlling podocyte development, we also found that combinations of wtla, foxc1a, and rbpj are required for the differentiation of the neck segment, a stretch of tubular epithelium that contains multiciliated cells. Neck segments are found in a range of fish and reptilian species where they are postulated to help propel fluid down the nephron. We found that neck cells arise immediately adjacent to podocytes and initially co-express wtla, foxc1a, pax2a, as well as components of the Notch pathway. The equivalent cells in the frog pronephros are the nephrostomes, the ciliated openings to the cloacal duct, which link the nephron to the coeleom (Vize et al., 2003). Recently it has been shown that overexpression of NICD in Xenopus embryos induces ectopic podocyte and nephrostome development at the expense of the tubules (Naylor and Jones, 2009). Our results support this observation but further demonstrate a requirement of wtla and foxc1a for neck formation, possibly via the formation of an rbpj/NICD/wtla/foxc1a enhancer. Some of the Notch target genes in fish are the wt4d gene, which is also capable of inducing ectopic podocyte and neck fates (Naylor and Jones, 2009). Zebrafish have two wt4d paralogs, however neither is expressed in a spatial or temporal fashion consistent with a role in podocyte or neck development (Liu et al., 2000; Ungar et al., 1995). The cause for this discrepancy is unclear at present and possibly another Wnt target exists in zebrafish. Together, these results suggest that podocytes and neck/nephrostome cells are closely related lineages that are regulated by similar developmental pathways. Understanding how a common Notch/Wt1a/Foxc1a pathway is able to direct the fate of these two distinct cell types will require further study.

Our ablation experiments demonstrated that almost all podocyte progenitors arise from the intermediate mesoderm adjacent to somite 3 with little contribution from the intermediate mesoderm adjacent to somites 1 and 2. This result is consistent with our expression analysis, which showed overlapping expression domains of wtla, foxc1a, pax2a, and notch components at the level of somite 3. Similar conclusions can be drawn from the study of Tg(wt1b:gfp) embryos in vivo, where GFP + podocyte progenitors migrate medially under somite 3 (Perner et al., 2007 and our unpublished observations). Cell lineage tracing experiments have shown that the intermediate mesoderm adjacent to somites 1 and 2 can give rise to labeled cells in the glomerulus, whereas cells adjacent to somite 3 contribute mostly to the neck region (Serluca and Fishman, 2001). It is difficult to reconcile these data with our ablation analysis but may be related to technical differences between laser-induced activation of a caged lineage tracer and laser-induced cell ablation. This is supported by the fact that ablation of the intermediate mesoderm adjacent to somites 1 and 2, where the caged lineage tracer was activated, does not lead to any loss of podocytes (noted in both the study by Serluca and Fishman, 2001, and here). Alternatively, because only histology was used to assess cell identity in the fate mapping study, there is the possibility that some of the labeled cells may correspond to extra-renal lineages derived from the intermediate mesoderm, such as endothelial cells.

Interrenal gland cells represent a third cell lineage arising from the podocyte/neck region of the kidney. The teleost interrenal gland, like its mammalian counterpart the adrenal gland, produces steroid hormones important for the homeostasis of glucose and electrolytes. In zebrafish, mrsat1a + interrenal cells arise from wtla-expressing cells adjacent to podocyte progenitors at the 22-somite stage but do not co-express early podocyte markers such as wt1b or pax2a (this study and unpublished observations; Hsu et al., 2003). Our finding that the number of interrenal cells is expanded in wtla- and rbpj-single and double deficient embryos indicates that these transcription factors are required to suppress interrenal fate. The origin of the ectopic mrsat1a-expressing cells in these animals is not known. It is possible that the cells that fail to adopt a podocyte fate in wtla/rbpj-deficient embryos acquire an interrenal identity instead. An inhibitory effect of wtla on interrenal development is contrary to previously reported results, where a reduction in interrenal cell number was seen following wtla knockdown (Hsu et al., 2003). These differing results may be related to morpholino dosage and/or efficacy. Surprisingly, we found that embryos double-deficient in both wtla and foxc1a displayed a complete lack of mrsat1a expression, revealing an additional layer of complexity and suggesting that these factors may be redundantly required for the specification of the interrenal lineage. The role of wtla as a repressor and inducer of interrenal cell fate is challenging to resolve but could reflect opposing activities in two different cell types (interrenal progenitors versus podocyte progenitors).

In summary, we have taken advantage of the zebrafish model where multiple factors can be knocked down at once to demonstrate that wtla, foxc1a, and rbpj interact in surprisingly complex ways at both the physical and molecular level to regulate the generation of pronephric and interrenal fates. This work furthers our understanding of the complicated transcriptional circuitry that governs podocyte identity and has important implications for the study of glomerular diseases where altered Notch signaling and Wt1 levels induce podocyte dysfunction. Whether developmental pathways are being recapitulated in these kidney diseases will be an important point of future study, as new treatments may arise based on our knowledge of normal podocyte development during kidney organogenesis.

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References


