Pax2/8-regulated Gata3 expression is necessary for morphogenesis and guidance of the nephric duct in the developing kidney

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The mammalian pro- and mesonephros are transient embryonic kidneys essential for urogenital system development. The nephric (Wolffian) duct, which is a central constituent of both structures, elongates caudally along a stereotypical path to reach the hindlimb level where it induces metanephros (adult kidney) formation, while the remaining duct gives rise to the male genital tract (epididymis, vas deferens). The transcription factors Pax2 and Pax8 are essential for the initiation of pro- and mesonephros development. In a cDNA microarray screen for genes specifically expressed in the pro/mesonephros and regulated by Pax proteins, we identified Gata3, a transcription factor gene associated with hypoparathyroidism, deafness and renal anomaly (HDR) syndrome. Gata3 is already expressed in the pronephric anlage, together with Pax2 and Pax8, suggesting that it may be a direct Pax2/8 target gene. Inactivation of Gata3 by insertion of an Ires-GFP reporter gene resulted in a massive increase in nephric duct cellularity, which was accompanied by enhanced cell proliferation and aberrant elongation of the nephric duct. Interestingly, however, the nephrogenic cord extended, with delayed kinetics, along the entire caudal path up to the level of the hindlimb bud, indicating that extension of the nephric duct and cord is controlled by different guidance cues. At the molecular level, the nephric duct of Gata3−/− embryos is characterized by the loss of Ret expression and signaling, which may contribute to the guidance defect of the nephric duct. Together, these results define Gata3 as a key regulator of nephric duct morphogenesis and guidance in the pro/mesonephric kidney.

KEY WORDS: Pax2, Pax8, Gata3, Mesonephros, Nephric (Wolffian) duct, Duct guidance, Kidney development, Mouse

INTRODUCTION

The formation of tubular epithelia from mesenchymal cells or pre-existing ducts is a common feature of organ development. In the mammalian kidney, tubulogenesis first occurs during the formation of the pronephros. This primary embryonic kidney generates a single nephric (Wolffian) duct that elongates caudally towards the cloaca (Saxen, 1987). The nephric duct subsequently forms the second embryonic kidney, the mesonephros, by induction of mesonephric tubules in the adjacent mesenchymal nephric cord. When the nephric duct reaches the metanephric mesenchyme at the level of the hindlimb, interactions between both tissues initiate metanephros development by inducing budding and invasion of the ureter from the duct into the metanephric mesenchyme. The newly formed ureter branches and induces mesenchymal-epithelial transitions in the surrounding mesenchyme, thereby initiating the first of numerous cycles of nephron formation. Later during development, the nephric duct is transformed into the male genital tract (epididymis and vas deferens) or degenerates in female embryos. Hence, unraveling the molecular mechanisms of nephric duct morphogenesis is essential for our understanding of urogenital system development.

Despite the central role of the pro- and mesonephros (referred to as pro/mesonephros) for kidney and genital tract formation, very few genes have so far been found to control the development of these two structures in the mouse. Notably, the Pax2 and Pax8 genes are both necessary and sufficient for the formation of the pronephros and all subsequent kidney structures (Bouchard et al., 2002). Pax2, Pax8 double-mutant embryos fail to specify the nephric lineage, as they neither undergo the initial epithelial-mesenchymal transitions leading to nephric duct formation nor activate early nephric marker genes (Bouchard et al., 2002). Pax2 and Pax8 are closely related members of the Pax family of sequence-specific transcription factors (Chi and Epstein, 2002). Pax8−/− embryos have a severe defect in thyroid development but form normal kidneys (Mansouri et al., 1998). By contrast, Pax2 is necessary for metanephros development (Torres et al., 1995), as it controls the survival of the ureter and late nephric duct (Ostrom et al., 2000; Porteous et al., 2000; Torres et al., 1995; Bouchard, 2004) and induces the mesenchymal-epithelial transitions leading to nephron formation (Rothenpieler and Dressler, 1993). At the molecular level, Pax2 regulates the expression of important nephrogenic molecules such as Wt1 (Debhi et al., 1996) and Gdnf (Brophy et al., 2001). These genes are expressed in the metanephric mesenchyme, and are necessary for metanephros induction and growth (Kuure et al., 2000). These data therefore point to a role of Pax2 in late mesonephros and metanephros development. However, the observation that Pax2−/−Pax8−/− embryos show a more severe phenotype than single-mutant embryos underscores not only the functional redundancy among the two Pax genes (Bouchard et al., 2000; Bouchard et al., 2002), but also suggests an early role for Pax2 and Pax8 in regulating key target genes involved in pro/mesonephros formation (Bouchard et al., 2002; Bouchard, 2004).

In an attempt to identify these effector molecules, we have searched for mesonephros-specific, Pax2/8-regulated genes by cDNA microarray analysis of FACS-sorted mesonephric cells from wild-type and Pax2−/− embryos. These experiments identified the transcription factor gene Gata3 as an early Pax2/8-regulated gene.
In humans, Gata3 has been associated with the HDR syndrome (Van Esch et al., 2000), a genetic disease characterized by hypoparathyroidism, sensorineural deafness and renal dysplasia (Bilous et al., 1992). In the mouse, homozygous inactivation of Gata3 results in a complex phenotype including the failure to form the metanephros (Lim et al., 2000; Pandolfi et al., 1995). Here, we show that Gata3 is a genetic target of Pax2 and Pax8 in the nephric duct of the pro/mesonephric kidney and that Gata3 is required for the morphogenesis and guidance of the nephric duct along the anteroposterior axis of the embryo.

**MATERIALS AND METHODS**

**Mice**

The Pax2<sup>loxP</sup> and Pax8<sup>neo</sup> alleles and the Pax2<sup>GFP</sup> BAC transgene (#30) were maintained on the C3H/He genetic background and genotyped as described (Bouchard et al., 2000; Bouchard et al., 2002; Pfeffer et al., 2002).

**Generation of Gata3 mutant mice**

The Gata3 targeting vector was assembled in the pSP64 vector with a modified poly linker containing the appropriate restriction sites. Both recombination arms were cloned by PCR from the BAC clone RP23-136M6 with the following primers: long arm (5′ fragment), 5′-GGCTGCTGAAGCTCAACCTAAACAAACACG-3′ and 5′-CTGCTACATGCTCAGCTCCT-3′; short arm, 5′-CAAGGAGCTCATGATGGA-3′. The long arm was assembled using a Drfl site common to both fragments, located 2616 bp from the 5′ end. Exon 4 was cloned by PCR from the same BAC clone with the following primers: 5′-CGAGGAGGCAAGGAACTTCTGTAATTGGTGGTTTATTGTTTTT-3′ (containing a loxP site) and 5′-GCGGTTACATCATGAGCTTCGCAAGA-3′. A 3660 bp KpnI-SalI fragment from the pk11-iresGFP vector was inserted into the modified pSP64 vector. The pk11-ires-GFP vector was generated by modifying the pk11 vector (a gift from Gail Martin), containing a loxP site followed by the pgk neo selection cassette flanked by Frt sites. The modification of pk11 was made by insertion of a Clal-EcoRI fragment containing the splice acceptor of Pax5 exon 2 modified to contain stop codons in all three reading frames, a 1360 bp fragment containing the splice acceptor of Gata3, 5′-ACACGGCCCGCCAAATCTTCTGATATTGATTATTATT-3′ (containing a loxP site) and 5′-GAACTTGTTTATTGCAGCTTAT-3′; 5′-CACGGAGGCTTTTGAAAGCAATCATTACTCAAGA-3′. A 3660 bp KpnI-SalI fragment from the pk11-iresGFP vector was added as a 1940 bp Xhol-SalI fragment from the pRK7 vector, containing a SV40 polyA sequence. These three fragments were added to pk11 using the Clal-HindIII restriction sites located between the first FRT site and the neo gene. The primers used for these modifications are as follows: Pax5 splice acceptor, 5′-GGCTGCTGATGTATGCTATCGGATGGTTA-3′ and 5′-GCGGAATTCATTATTAACACCGAACTTCTGCTACCT-3′; SV40 polyA, 5′-GGCTGCTGGAACCATTTTGTGTGTCAGCTTT-3′ and 5′-GCAGACCGGTATCATGATGAATAAAGATA-3′. The negative selection cassettes were added as a 1940 bp NotI-SalI fragment from the pBS-hsv-tk vector and a 1150 bp HindIII-NotI fragment from the p64-DT vector. HM1 ES cells were electroporated with 15 μg of NotI-linearized plasmid and selected with 250 μg/ml G418 and 2 μM gancyclovir. The selected clones were screened by PCR following by confirmation by Southern blot analysis on the positive clones. Embryos for subsequent microarray analysis. For this purpose, a BAC transgene expressing GFP from the Pax2 locus (Pax2<sup>GFP</sup>) (Pfeffer et al., 2002) was used to label pro/mesonephric cells, which were purified by FACs sorting from the remaining cells of the dissected trunk regions of wild-type or Pax2 mutant embryos at 13, 16- and 20-somite stages. Importantly, these stages correspond to the early period of pro/mesonephros formation, prior to the tissue degeneration observed in Pax2 mutant embryos (Bouchard, 2004; Bouchard et al., 2002; Torres et al., 1995). Sorted GFP<sup>+</sup> cells (2000 to 10,000 cells per embryo) and control GFP<sup>−</sup> cells were subjected to linear RNA amplification and microarray analysis. Pax2-regulated genes were identified as transcripts: (1) that were differentially regulated by Pax2 in the pro/mesonephros at all three stages; (2) that were upregulated between 13 to 16 somites and 13 to 20 somites in the pro/mesonephros; and (3) that were expressed in the pro/mesonephros but not in the surrounding GFP<sup>+</sup> tissue at the 13-.
and 20-somite stages (Table 1). By applying a stringent cut-off of \( \geq 1.7 \)-fold activation in all seven microarray experiments, we identified a single Pax2-regulated transcript out of 26,000 expressed sequence tags (ESTs corresponding to 17,000 UniGene clusters; Table 1). This EST (BG080090) corresponds to a cDNA transcript of the Gata3 gene (UniGene cluster Mm.313866). By contrast, the control house-keeping gene \( \beta2 \)-microglobulin failed to show differential expression in any of the seven microarray experiments (Table 1).

We next analyzed the expression pattern of Gata3 by in situ hybridization of wild-type embryos to investigate whether this gene is expressed within the known Pax2 and Pax8 expression domains during kidney development. In accordance with our microarray data, we detected strong Gata3 expression in the pronephros anlage at the 10-somite stage (E8.5; Fig. 1A). This gene is expressed within the known hybridization of wild-type embryos to investigate whether this expression is detected in the pronephros anlage (pa; A), in the nephric duct (nd) of the mesonephros (B), and in the collecting duct (cd) and ureter tip (ut) of the metanephros (C,D).

To directly investigate this possibility, we examined the expression of Gata3 in embryos of a Pax2\(^{-/-}\)Pax8\(^{-/-}\) mutant allele series. Whereas Pax2\(^{-/-}\)Pax8\(^{-/-}\) embryos fail to specify the nephric lineage, Pax8 expression is still observed in the developing kidney of Pax2\(^{-/-}\)Pax8\(^{+/+}\) embryos, indicating that one functional Pax8 allele is sufficient to induce pro/mesonephros development (Bouchard et al., 2002) (Fig. 2D). In situ hybridization analysis of tissue sections revealed that Gata3 was similarly expressed in wild-type and Pax2\(^{-/-}\) embryos (Fig. 1A, data not shown). By contrast, Gata3 expression was virtually undetectable in the nephric duct of the mesonephros (Fig. 1B); by E13.5 to E18.5, Gata3 mRNA was present in the ureter tips and collecting duct system of the metanephros, but was absent from the developing nephrons (Fig. 1C,D). This expression pattern is consistent with, and extends, previously published data (George et al., 1994; Manaia et al., 2000). Importantly, the expression domains of Gata3 in the nephric duct and ureter correspond to a subset of the Pax2/Pax8-expressing cells (Bouchard et al., 2002; Dressler et al., 1990) (Fig. 2A,C), suggesting that Gata3 is regulated by Pax2 and Pax8.

**Table 1. Identification of Gata3 as a Pax2-regulated gene by microarray analysis of pro/mesonephric cells**

<table>
<thead>
<tr>
<th>Genotype comparison [wild type versus Pax2(^{-/-}) GFP (+) cells]</th>
<th>Upregulation in time [GFP (+) cells]</th>
<th>Pro/mesonephros expression [GFP (+) versus GFP (–) cells]</th>
</tr>
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<tbody>
<tr>
<td>13 s</td>
<td>16 s</td>
<td>20 s</td>
</tr>
<tr>
<td>Gata3</td>
<td>1.7</td>
<td>2.1</td>
</tr>
<tr>
<td>( \beta2 )-microglobulin</td>
<td>1.1</td>
<td>0.9</td>
</tr>
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Ratios are expressed as control/mutant, late/early or GFP+/GFP–. The embryonic stages are defined by somite number (s).

**Gata3 is necessary for mesonephros formation**

The results presented above suggest that Gata3 may be an important effector of Pax gene function during pro/mesonephros development. To investigate this possibility, we inactivated the Gata3 locus in embryonic stem (ES) cells by deletion of exon 4, coding for the first DNA-binding zinc finger, and by simultaneous insertion of an Ires-GFP minigene (Fig. 3A). Heterozygous Gata3\(^{GFP^+}\) mice express...
The Gata3 gene was targeted by insertion of loxP sites (grey triangles) flanking exon 4 (encoding the first zinc finger), followed by a cassette, flanked by fl sites (grey rectangles), containing a translation stop codon, an internal ribosome entry site (ires) driving the GFP gene, and the neomycin selection gene (neo). The GFP and neo genes were followed by SV40 polyadenylation signals. The targeting construct additionally contained the thymidine kinase (tk) and diphtheria toxin A (DT-A) genes at the 5’ and 3’ ends, respectively, for selection against random genomic integration. Mice with the original targeted allele (Gata3ex4Gfp) were mated with the More-cre germline deleter strain (Tallquist and Soriano, 2000) to excise exon 4 and generate the Gata3Gfp allele (referred to as Gata3 allele). The different alleles were detected by Southern blot analysis of BamHI-digested genomic DNA with the indicated probe. The exons are numbered according to Pandolfi et al. (Pandolfi et al., 1995). (B) Southern blot analysis of wild-type (+/+), Gata3ex4Gfp (ex4Gfp+) and Gata3Gfp (Gfp+) tail DNA digested with BamHII. (C) GFP expression of the targeted Gata3 gene in Gata3Gfp+ embryo at E9.5. Broken line indicates the contour of the embryo. ba, branchial arches; op, otic placode; nd, nephric duct; va, vitelline artery.

Fig. 3. Inactivation of the Gata3 gene. (A) The Gata3 gene was targeted by insertion of loxP sites (grey triangles) flanking exon 4 (encoding the first zinc finger), followed by a cassette, flanked by fl sites (grey rectangles), containing a translation stop codon, an internal ribosome entry site (ires) driving the GFP gene, and the neomycin selection gene (neo). The GFP and neo genes were followed by SV40 polyadenylation signals. The targeting construct additionally contained the thymidine kinase (tk) and diphtheria toxin A (DT-A) genes at the 5’ and 3’ ends, respectively, for selection against random genomic integration. Mice with the original targeted allele (Gata3ex4Gfp) were mated with the More-cre germline deleter strain (Tallquist and Soriano, 2000) to excise exon 4 and generate the Gata3Gfp allele (referred to as Gata3 allele). The different alleles were detected by Southern blot analysis of BamHI-digested genomic DNA with the indicated probe. The exons are numbered according to Pandolfi et al. (Pandolfi et al., 1995). (B) Southern blot analysis of wild-type (+/+), Gata3ex4Gfp (ex4Gfp+) and Gata3Gfp (Gfp+) tail DNA digested with BamHII. (C) GFP expression of the targeted Gata3 gene in Gata3Gfp+ embryo at E9.5. Broken line indicates the contour of the embryo. ba, branchial arches; op, otic placode; nd, nephric duct; va, vitelline artery.

GFP in all endogenous Gata3 expression domains, including the nephric duct of the pro/mesonephros (Fig. 3C). Homozygous Gata3Gfp/Gfp embryos died around E11.0 (data not shown), consistent with previously published Gata3 mutant mice (Lim et al., 2000; Pandolfi et al., 1995). Hence, the Gata3Gfp allele corresponds to a null allele, which we subsequently refer to as Gata3.

To study the role of Gata3 in the kidney, we initially looked at the progression of mesonephros development in Gata3 mutant embryos at E9.0–E9.5 by whole-mount in situ hybridization with a Pax2 probe. At the 17-somite stage, the mesonephros of wild-type embryos extends over 12 somites (Fig. 4A). At the same stage, the mesonephros of Gata3–/– embryos showed, however, a marked delay in caudal extension (Fig. 4A,B), which became even more prominent at the 21-somite stage (Fig. 4C,D). By 27 somites, the wild-type nephric duct has reached the cloaca, while a second row of Pax2-positive cells was apparent in the adjacent nephric cord (Fig. 4E, insert). In Gata3–/– embryos, a single and discontinuous row of Pax2-positive cells extended caudally, which eventually reached the tip of the tail (Fig. 4F, insert). Hence, the absence of Gata3 causes a severe growth defect of the mesonephros.

We next determined the cellular identity of the Pax2-positive cells observed in the caudal Gata3–/– mesonephros. In wild-type E9.5 embryos, Pax2 is expressed in cells of both the nephric duct and cord. E-cadherin expression is, however, restricted to the nephric duct, whereas Wt1 expression is excluded from the duct, but present in the intermediate mesoderm (including cord cells; Fig. 5A,C). Strikingly, E-cadherin+ nephric duct cells were absent in Gata3–/– embryos, while the caudal mesonephric cells were located exclusively in the Pax2+ Wt1+ nephric cord (Fig. 5B,D). The loss of the nephric duct was further confirmed by the absence of laminin staining in this region of E9.5 Gata3–/– embryos (data not shown). The absence of the nephric duct led to increased apoptosis in the nephric cord (Fig. 5E,F). This cell death may be responsible for the discontinuity in Pax2 expression, which is observed in Gata3–/– embryos at 27 somites (Fig. 4E,F). By E10.5, the nephric duct and ureteric bud were missing at the level of the metanephric mesenchyme in Gata3–/– embryos, as revealed by the absence of expression of Pax2 and the epithelial marker genes Enx2, Brn1 (Pou3f3 – Mouse Genome Informatics) and Sim1 (Fig. 5G,H,I,J, data not shown). Owing to the absence of a caudal nephric duct,
metanephros induction could never be detected in these embryos (Fig. 5G-L, data not shown). Surprisingly, however, cells expressing Wt1, Eya1 and Pax2 were still detected in the region of the metanephric mesenchyme, indicating that the expression of these genes does not depend on inductive signals from the nephric duct (Fig. 5G,H,K,L, data not shown). We therefore conclude that Gata3 is critically important for the caudal extension of the nephric duct but not of nephric cord cells.

**Ectopic formation and aberrant guidance of the nephric duct in Gata3−/− embryos**

We next followed nephric duct formation in Gata3 mutant embryos at E9.0-E9.5 by Pax2 whole-mount in situ hybridization and confocal imaging. In wild-type embryos, the nephric duct was visible as a single tube extending towards the tail region, with the confocal imaging. In wild-type embryos, the nephric duct was stained the duct but not the mesonephric tubules at this stage. In contrast to the single epithelial duct observed in wild-type embryos (Fig. 6A), Gata3−/− embryos harbored a large cluster of E-cadherin+ ductal structures (Fig. 6F, data not shown). These E-cadherin+ cells also expressed the GFP protein from the targeted Gata3 locus, further confirming their nephric duct origin (Fig. 6G,H). Interestingly, in about 65% of Gata3−/− mesonephros (n=52), the nephric duct initiated caudal extension but moved away from the nephric cord into an aberrant elongation path (Fig. 6K-N). As for the multiple disorganized ducts (Fig. 6C-J), all misguided ducts in these embryos extended to and fused with the surface ectoderm, as revealed by the expression of the epithelial marker gene Brn1 (Fig. 6O,P). Importantly, despite the observed variability in mesonephros dysgenesis, Gata3−/− nephric ducts invariably failed to reach the cloaca region. Together, these results unequivocally demonstrate an important role for Gata3 in controlling the morphogenesis and guidance of the nephric duct.

**Altered proliferation and signaling in the nephric duct of Gata3 mutant embryos**

The massive increase in nephric duct tissue suggested a possible role for Gata3 in cell proliferation control. To test this hypothesis, we determined the cellularity and mitotic index of the E-cadherin+ nephric duct cells in wild-type and Gata3−/− embryos. The mitotic index was determined as the ratio of phospho-histone H3 signals per 50 nephric duct cells (E-cadherin+). These analyses revealed a 2.6-fold increase in the number of nephric duct cells and a 4.6-fold increase in cell proliferation in the mesonephros of Gata3−/− embryos compared with wild-type controls (Table 2, Fig. 7A,B).

**Table 2. Cellularity and mitotic index of control and Gata3−/− nephric ducts**

<table>
<thead>
<tr>
<th>Control</th>
<th>Average nd cells per section (s.d.)</th>
<th>Average mitotic cells per 50 duct cells (s.d.)</th>
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<tbody>
<tr>
<td>Gata3−/−</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=4)</td>
<td>11* (3.5)</td>
<td>0.7** (0.7)</td>
</tr>
<tr>
<td>(n=6)</td>
<td>29* (7.0)</td>
<td>3.1** (1.3)</td>
</tr>
</tbody>
</table>

*P<0.002, **P<0.01 (Student's t-test). Nephric duct cells are defined by E-cadherin staining. s.d., standard deviation.
hybridization with a Pax2 probe reveals the presence of Gata3–/– proteins showed any significant difference in expression between the nephric duct marker E-cadherin. (G, H) Same section as in E, F, showing the colocalization of E-cadherin with GFP expressed from the targeted Gata3 locus. (I, J) Whole-mount in situ hybridization with a Pax2 cRNA probe reveals the presence of multiple nephric ducts (nd) growing towards the ectoderm at the level of the mesonephric tubules (mst) in Gata3–/– embryos. (K, L) Gata3–/– embryos in which a nephric duct grew caudally showed a guidance defect, as revealed by whole-mount in situ hybridization with a Pax2 cRNA probe. (M, N) Immunohistochemistry with an anti-Pax2 antibody showed an abnormally large distance between the nephric duct and nephric cord in Gata3–/– embryos. (O, P) Brn1 cRNA in situ staining of a representative E10.5 embryo in which the duct turned abruptly in the direction of the surface ectoderm (broken line) and fused with it. da, dorsal aorta; ec, ectoderm; ms, mesonephros; mst, mesonephric tubules; nc, nephric cord; nd, nephric duct.

These results clearly implicate Gata3 in the regulation of mesonephric cell proliferation. The ectopic nephric structure also showed a mild increase in apoptosis, possibly reflecting the abnormal environment of these cells (data not shown).

We next investigated the molecular basis for the nephric duct elongation defect of Gata3–/– embryos. Several mechanisms have been implicated in duct guidance during embryogenesis. These include extracellular matrix attachment, axon-like guidance and morphogen signaling (Drawbridge et al., 2003; Eichmann et al., 2005; Myat, 2005). We thus studied the composition of the extracellular matrix and expression of integrin receptors during mesonephros development by immunostaining for pan-laminin, laminin B2 (γ1), β1 integrin, α3 integrin, α6 integrin and α5β1 integrin (fibronectin receptor) expression. Surprisingly, none of the proteins showed any significant difference in expression between Gata3–/– and wild-type embryos (data not shown). We then investigated the expression of axon guidance molecules known to be present in the kidney. These included Gdnf, Ret, Slit2, neuropilin, ephrins and Eph proteins. Although most of these molecules were normally expressed in the Gata3–/– mesonephros (data not shown), transcripts of the Gdnf receptor gene Ret were undetectable in the nephric duct of Gata3–/– embryos in contrast to control embryos (Fig. 7C,D). Gdnf itself remained, however, expressed in the mesenchyme adjacent to the Gata3–/– duct (data not shown). Importantly, Wnt11 expression, which is regulated by Ret signaling (Majumdar et al., 2003; Grieshammer et al., 2004), was also lost in the Gata3–/– nephric duct (Fig. 7E,F). Hence, these data indicate that Gata3 controls Ret expression and signaling in the nephric duct.

DISCUSSION

The pro/mesonephros and its major constituent, the nephric duct, are central components of both kidney and genital system development (Capel, 2000; Saxen, 1987). The molecular mechanisms regulating nephric duct formation are, however, largely unknown. We have previously shown that the transcription factors Pax2 and Pax8 are both necessary and sufficient for the initiation of nephric duct formation by facilitating epithelial-mesenchymal transitions in the intermediate mesoderm (Bouchard et al., 2002). To date, no other mouse mutant was shown to regulate the early onset of pro/mesonephros morphogenesis, while most gene mutations with a strong kidney phenotype affect the later process of ureteric bud formation and subsequent metanephros development (Kuure et al., 2000; Bouchard, 2004). Here, we have used FACS sorting and cDNA microarray analysis of mesonephric cells combined with expression validation in wild-type and Pax2, Pax8 mutant embryos for the identification of genes that are regulated by the two Pax proteins during pro/mesonephros formation. These experiments identified the transcription factor gene Gata3 as the earliest known genetic target of Pax proteins in kidney development. Gene inactivation in the mouse revealed a crucial role for Gata3 in controlling nephric duct morphogenesis and guidance. The nephric ducts of Gata3–/– embryos are hypercellular and fail to elongate along the normal rostrocaudal path, thereby preventing metanephros induction. These defects may partly reflect the role of Gata3 in nephric duct cell proliferation and activation of the receptor gene Ret, which is an essential component of the GDNF signaling pathway involved in ureteric bud formation and nephric duct...
Fig. 7. Regulation of cell proliferation and gene expression by Gata3. Control wild-type or Gata3+/– embryos (A,C,E) and Gata3–/– embryos (B,D,F) were analyzed by immunohistochemistry with anti-phosphorylated-histone H3 and anti-E-cadherin antibodies (A,B) and by in situ hybridization with Ret (C,D) and Wnt11 (E,F) cRNA probes. (A,B) The number of mitotic cells (white arrows) and the cellularity (broken lines) of the nephric duct were significantly higher in Gata3–/– compared with control embryos. Sections A,B were counterstained with DAPI (blue). (C,D) Ret expression was absent from Gata3–/– hypercellular nephric ducts. Strong Ret expression is still present in the Gata3–/– spinal cord (sc). (E,F) Expression of the Ret-regulated Wnt11 gene was completely lost in Gata3–/– nephric ducts in contrast to control embryos. Broken lines in A,B,D,F demarcate the epithelial nephric duct, as defined by E-cadherin staining. da, dorsal aorta; ec, ectoderm; nd, nephric duct; sc, spinal cord.

The Gata3 mutation causes two major defects in pro/mesonephros development. First, Gata3–/– embryos fail to form as a single nephric duct, but instead generate multiple disorganized ducts that accumulate in the intermediate mesoderm and frequently extend to the surface ectoderm. The molecular explanation for this phenotype is still unclear. One possibility is that the number of progenitors committed to the nephric lineage is increased in Gata3–/– embryos, resulting in the simultaneous formation of multiple pronephric ducts. Alternatively, the number of pronephric progenitors remains normal in the mutant, but the subsequent differentiation of these cells is defective. Finaly, the initial specification and differentiation events are unaffected but the steps of tubulogenesis and cell proliferation are disturbed. We favor the last hypothesis based on the following evidence: (1) Pax2 and Pax8 regulate Gata3 in a cell-autonomous manner and yet their mutation leads to a loss rather than an increase in nephric progenitors (Bouchard et al., 2002); (2) we could not detect any obvious increase in the cellularity or ectopic locations of the pronephros in the early Gata3–/– embryo; (3) Gata3–/– nephric duct cells showed normal expression of a number of cell fate and differentiation markers such as Pax2, Pax8, Brn1, Wnt9b, Emc2, Sim1, E-cadherin, laminin and various integrins; (4) the increased mitotic index observed in Gata3–/– mesonephros points to a defect in cell cycle control. In support of this notion, Gata3 was recently shown to control the proliferation of epithelial cells and Gata3 gene mutations were identified in breast tumors (Usary et al., 2004).

The second major aspect of the Gata3–/– phenotype is a defect in rostrocaudal guidance of the nephric duct. Most experiments addressing nephric duct guidance were performed in the amphibian Axolotl (Ambystoma mexicanum), which develops a functional pronephros and is amenable to classical tissue recombination experiments. Axolotl is an interesting model system as its pronephros shares a number of features with the mouse pro/mesonephros (Drawbridge et al., 2003). One of the guidance mechanisms identified in Axolotl depends on the deposition of an ectoderm-derived extracellular matrix containing laminin 1, which is recognized by the α6β1 integrin receptor of nephric duct cells (Drawbridge et al., 1995; Morris et al., 2003). However, we did not observe any significant difference in the expression of laminins and integrin receptors (α6β1, α3, α5β1) between wild-type and Gata3–/– embryos (data not shown). Hence, Gata3 is unlikely to control nephric duct guidance by regulating the expression of extracellular matrix components and integrins.

Another duct guidance cue identified in Axolotl is GDNF, which activates signaling through the dimeric GFRα1-Ret receptor (Drawbridge et al., 2000). GDNF-soaked beads were sufficient to attract the elongating duct in 81% of experimental animals, while subependymal injection of GDNF inhibited duct extension, indicating that the duct cells move along a gradient of GDNF expression (Drawbridge et al., 2000). A similar GDNF gradient was recently identified in the mouse mesonephros, suggesting that GDNF is also a guidance cue for nephric duct extension in mammals (Grieshammer et al., 2004; Kume et al., 2000). Interestingly, GDNF signaling was also shown to act as a chemotactant for kidney epithelial cells (Tang et al., 1998) and to have axon guidance properties in the CNS (Ledda et al., 2002). Consistent with these data, the loss of Ret expression and signaling may contribute to the misguidance of the nephric duct in Gata3–/– embryos. It is, however, important to note that the inactivation of components of the GDNF signaling pathway did not result in duct guidance defects in the mouse (Pichler et al., 1996; Schuchardt et al., 1994). Some redundancy in the GDNF signaling pathway may, however, exist as the loss of metanephros development was not fully penetrant in Gdnf, Gfra or Ret mutant mice (Cacalano et al., 1998; Enomoto et al., 1998; Moore et al., 1996; Sanchez et al., 1996; Schuchardt et al., 1994). In addition to positive guidance cues, our observation that Gata3–/– nephric ducts invariably grew in the direction of the surface ectoderm reveals the presence of an attraction signal emanating from this tissue. In wild-type embryos, Gata3 would counteract this unknown signal, thereby allowing the nephric duct to follow its normal path. Taken together, the combination of positive and negative cues directing nephric duct elongation is highly reminiscent of axon guidance mechanisms and suggests that both processes may share similar molecular mechanisms.

Surprisingly, the Pax2+ E-cadherin–nephric cord cells of Gata3–/– embryos still extended through the intermediate mesoderm along the entire rostrocaudal axis even in the absence of an elongating duct. The nephric cord cells do not express Gata3 (Manaia et al., 2000) (this study) and were thought to follow the nephric duct by receiving guidance (Schuchardt et al., 1994; Drawbridge et al., 2000). These findings establish a transcriptional cascade whereby Pax proteins activate Gata3 to regulate specific aspects of the pro/mesonephros developmental program.
guidance cues and differentiation signals from the duct (Saxen, 1987). Our results unequivocally demonstrate that the two major cell populations of the mesonephros use independent guidance systems for caudal extension, although the mechanism underlying nephric cord formation is still elusive.

Expression of the Pax2 gene was previously observed in the unduced metanephric mesenchyme of Ret mutant embryos, which develop a nephric duct in the absence of ureteric bud formation (Brophy et al., 2001). However, it was still conceivable that the nephric duct could induce Pax2 expression in the adjacent metanephric mesenchyme. The presence of Pax22 mesenchymal cells in Gata3−/− embryos demonstrates that metanephric mesenchyme cells can express Pax2 in a ureteric bud- and nephric duct-independent manner. Hence, these Pax22 cells are likely to originate from the early pro/mesonephros by caudal migration.

**Transcriptional control of pro/mesonephros development**

By identifying Gata3 as a Pax2/8-regulated gene, we provide the first insight into the transcriptional cascade controlling early pro/mesonephros development. Our data do not allow a discrimination between direct or indirect regulation of Gata3 by the two Pax proteins. Direct regulation would, however, be consistent with the fact that Gata3 expression is activated in the pronephros anlage soon after the initiation of Pax2 and Pax8 transcription. A urogenital-specific regulatory element has been mapped to a region located between −35 and −150 kb upstream of the start codon of Gata3 (Lakshmanan et al., 1999). Interestingly, a bioinformatic analysis of 185 kb Gata3 genomic region revealed two elements containing putative Pax2/8-binding sites, which are conserved in mouse, human and chick. Functional analysis will be required to demonstrate that these conserved elements are involved in the Pax2/8-dependent regulation of Gata3.

In contrast to Pax22Pax82 embryos in which the whole nephric lineage fails to be specified, Gata3−/− embryos are competent to undergo mesenchymal-epithelial transitions to form the nephric duct, which in turn is competent to induce mesonephric tubule formation in the surrounding mesenchyme. On the one hand, these phenotypic differences indicate that Pax genes regulate other aspects of pro/mesonephros development, independently of Gata3 regulation. Indeed, additional Pax2-regulated genes were identified by less stringent analysis of our microarray data (M.B., unpublished). On the other hand, a search for Gata3 target genes may lead to the identification of new molecules involved in the morphogenesis and guidance of the nephric duct.

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