INTRODUCTION

The mammalian kidney originates from the intermediate mesoderm and develops through three distinct stages: pronephros, mesonephros and metanephros. Organogenesis of the permanent kidney occurs through reciprocal interactions between the Wolffian duct and the metanephric mesenchyme. At embryonic day (E) 10.5 in mouse, the metanephric mesenchyme secretes glial cell line-derived neurotrophic factor (Gdnf), which induces the Wolffian duct to invade the metanephric mesenchyme and induce adjacent mesenchymal cells to condense. These condensed metanephric mesenchyme cells then induce ureteric bud outgrowth and branching, leading to the formation of nephrons (Dressler, 2006; Saxén, 1987). Specifically, Gdnf promotes ureteric bud outgrowth by binding to its receptor tyrosine kinase (Ret) and co-receptor Gdnf family receptor alpha-1 (Grfα1) on the surface of the ureteric bud (Durbec et al., 1996; Moore et al., 1996; Sainio et al., 1997; Vainio and Lin, 2002).

Although it is well established that the Gdnf-Ret signal transduction pathway initiates metanephric induction, the detailed mechanism of this signal pathway is still not well understood. During the metanephric mesenchyme condensation, many marker genes, including Osr1 (James et al., 2006), Pax2 (Torres et al., 1995), Eya1 (Sajithlal et al., 2005), Wt1 (Kreidberg et al., 1993), Six1 (Xu et al., 2003), Six2 (Self et al., 2006) and the Hox11 paralogous group (Hoxa11, Hoxc11 and Hoxd11) (Wellik et al., 2002), are expressed in the metanephric mesenchyme. Ablation of any of these genes in mice leads to renal agenesis, indicating that these genes are essential for the proper formation of the kidney. Osr1 and Eya1, the two earliest genes expressed in kidney precursor cells, sit atop the signal cascade and activate expression of other marker genes. Osr1 is expressed first. Mice lacking Osr1 do not form metanephric mesenchyme and do not express Eya1, Six2, Pax2, Sall1 and Gdnf. Eya1 is expressed downstream of Osr1 and is required for renal genesis (Xu et al., 1999). Eya proteins have no intrinsic DNA-binding domain but can localize to the nucleus and function as transcriptional co-activators (Ohto et al., 1999). Eya1 not only works with Six1 and Pax2 to regulate Gdnf expression, but also complexes with Hox11 paralogous proteins (including Hoxa11, Hoxc11 and Hoxd11) and Pax2. The Hox11-Eya1-Pax2 complex binds to the Six2 enhancer to induce the expression of Six2, which, in turn, mediates Six2 and Gdnf activation (Gong et al., 2007). Thus, Eya1 acts as a key regulator of Gdnf expression and, hence, determination of metanephric fate within the intermediate mesoderm (Sajithlal et al., 2005).

Chicken ovalbumin upstream promoter-transcription factor I and II (COUP-TFI and COUP-TFII; Nr2f1 and Nr2f2, respectively – Mouse Genome Informatics) are members of the nuclear receptor superfamily (Qu et al., 1994). Although biochemical studies indicate that these two factors have similar DNA-binding and transcriptional activity in vitro, the patterns of COUP-TFI and COUP-TFII expression are distinct from each other. COUP-TFI is expressed in the mesenchyme of developing organs and is shown to play a key role in their organogenesis, cell fate determination, cell differentiation, angiogenesis and metabolic homeostasis (Kim et al., 2009; Kurihara et al., 2007; Li et al., 2009; Lin et al., 2010; Qin et al., 2010; Qin et al., 2008; Tang et al., 2010; Xie et al., 2011; You et al., 2005a; You et al., 2005b). During kidney development, COUP-TFII expression is detectable in the condensed mesenchyme and
COUP-TFII in early nephrogenesis

MATERIALS AND METHODS

Animals

Generation of the floxed COUP-TFII mice and COUP-TFII-lacZ knock-in mice was as described previously (Takamoto et al., 2005). The Rosa26-Cre-ERT2lox/lox knock-in mouse strain was provided by T. Ludwig (Columbia University, New York City, USA) (de Luca et al., 2005). For inducible deletions during embryonic stages, 2 mg tamoxifen (Tam), dissolved in corn oil (Sigma-Aldrich; 10 mg/ml), was injected intraperitoneally into pregnant females at E7.5 or E8.5. Embryos from littersmates were collected at indicated stages. All mouse strains were maintained in a mixed genetic background and received standard rodent chow.

X-gal staining

Whole-mount X-gal staining of embryos (up to E10.5) was performed according to published methods (Takamoto et al., 2005).

Histology, immunofluorescence and in situ hybridization

All the histology and immunofluorescence staining of paraffin-embedded slides were performed as described (You et al., 2005a). Antibodies used were: anti-COUP-TFII (R&D, 1:2000), anti-Pax2 (Covance, 1:500), anti-Ki67 (BD Biosciences, 1:400), anti-β-galactosidase (BioGenes, 1:1500), anti-WT1 (Santa Cruz, 1:500), anti-Gdnf (Santa Cruz, 1:200) and anti-Six2 (Proteintech group, 1:400). Non-radioactive in situ hybridization was carried out as described previously (Bramblett et al., 2004). The RNA probe for mouse Osrl was provided by Thomas M. Schultheiss (Harvard University, Boston, USA) and the mouse Eve1 probe was as described (Xu et al., 1999). Cell apoptosis was detected by cleaved Caspase 3 (Cell Signaling, 1:500) staining or by TUNEL assay (Roche, In Situ Cell Death Detection Kit) according to the manufacturer’s instructions.

Kidney organ culture and staining

The metanephric mesenchymes containing the Wolffian duct from 10.5 days post-coitum (dpc) mouse embryos were cultured on transwell filters as described (Shakya et al., 2005). The Wolffian duct and ureteric bud were visualized by staining with anti-E-cadherin (BD Biosciences, 1:200).

Cell lines and transfection

Human embryonic kidney 293 (HEK 293) cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The rat inducible metanephric mesenchyme (RIMM-18) cell line was provided by Alan O. Perantoni (National Institutes of Health, NCI, Bethesda, MD, USA) and the culture conditions were as described (Levashova et al., 2003).

RNA interference and quantitative real-time RT-PCR assay

The siRNA oligonucleotides were purchased from Thermo Fisher Scientific or Applied Biosystems/Ambion. The target sequences for rat siRNA are described in supplementary material Table S1. RNA interference experiments were carried out as described (Lin et al., 2010). The primer sequences are listed in supplementary material Table S2. Means for mRNA levels in control and knockdown cells were compared using Student’s t-test.

Chromatin immunoprecipitation (ChiP) and PCR

ChiP assays were carried out with an EZ ChiP Chromatin Immunoprecipitation Kit (Millipore) by following the manufacturer’s protocol. Primers are shown in supplementary material Table S3.

Luciferase assays

The human EYA1 and WT1 promoter regions, which contained the putative Sp1-binding sites, were amplified from human BAC clone RP11-160C13 (Children’s Hospital Oakland Research Institute, CA, USA) and CTD-2083A15 (Invitrogen) separately. The PCR primers for amplifying the promoter region or for generating the Sp1-binding site mutations are as shown in supplementary material Table S4. The amplified promoter fragments were cloned into the pGL2-basic-luc vector (Promega). HEK 293 cells were transfected with pGL2-Eya1-basic-luc or pGL2-WT1-luc using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

RESULTS

COUP-TFII is expressed specifically in kidney precursor cells

We showed previously that COUP-TFII is expressed in the metanephric blastema at E11.5 and later in the developing nephron, nephrogenic cortex and stromal cells. In the adult kidney, high COUP-TFII expression is also detectable in the distal tubules, podocytes and epithelial cells of Bowman’s capsule (Suh et al., 2006). Nonetheless, the expression pattern of COUP-TFII in kidney precursor cells has not been determined. Here, we employed a COUP-TFII-specific antibody to investigate its expression pattern in the nephrogenic mesoderm from E9.5 to E10 (Fig. 1A-C). At E9.5, COUP-TFII staining was observed in the intermediate mesoderm and other mesenchyme cells surrounding the Wolffian duct (Fig. 1A). Using Pax2 to mark the Wolffian duct, we demonstrated that COUP-TFII is only expressed in the intermediate mesoderm and not in the duct (Fig. 1B). Half a day later, at E10, COUP-TFII expression was observed in the nephrogenic mesenchyme region surrounding the Wolffian duct and in the urogenital ridge (Fig. 1C). These results indicate that COUP-TFII is expressed in kidney precursor cells.

Inducible deletion of COUP-TFII in kidney precursor cells

In order to bypass early embryonic lethality of COUP-TFII homozygous mutant at E10.5, we conditionally inactivated COUP-TFII by crossing the COUP-TFII floxed mouse strain (COUP-TFIIfl/fl) (Takamoto et al., 2005) with Rosa26-Cre-ERT2lox/lox, a strain that harbors a tamoxifen-inducible Cre recombinase under the control of the ubiquitously active ROSA26 promoter (de Luca et al., 2005). Rosa26-Cre-ER(T2)fl/fl males (hereafter referred to as COUP-TFIIfl/fl) were intercrossed with COUP-TFIIfl/fl females, and a single dosage of 2 mg Tam was injected intraperitoneally into pregnant dams to induce COUP-TFII deletion. Specifically, we performed Tam injection at E7.5. In our mouse model, a lacZ gene inserted into the COUP-TFII locus is turned on when the COUP-TFII gene is deleted. One and half a days after Tam treatment, COUP-TFII was deleted throughout the whole body of COUP-
COUP-TFII is essential for metanephric mesenchyme formation but not for the formation of mesonephros

During the formation of the metanephros, the metanephric mesenchyme appears morphologically as an aggregate of the nephrogenic mesenchyme cells at the caudal end of the nephrogenic cord at E10.5 (Fig. 2A,C). This structure is largely missing in COUP-TFII\textsuperscript{id/d} mutant embryos (Fig. 2B,D). In addition, serial sections through the entire metanephric mesenchyme region were examined with littermates of the same somite number. The results clearly show that the metanephric mesenchyme is not formed in the COUP-TFII\textsuperscript{id/d} mutant (supplementary material Fig. S1). As the paired box-containing transcription factor Pax2 is a key regulator of kidney development (Torres et al., 1995), we investigated whether the expression of this gene is compromised. In the mouse kidney, the expression of Pax2 is initiated in the intermediate mesoderm and maintained throughout the development of the pronephros and mesonephros (Bouchard et al., 2000). Pax2 is expressed in the nephrogenic mesenchyme (nm) and urogenital ridge (ur), but not in the Wolffian duct (wd). (A-D) Tamoxifen was administered at E7.5 to induce COUP-TFII deletion and analyzed at E9. The whole-mount X-gal staining indicates that COUP-TFII is deleted throughout the whole mutant embryo, including the nephrogenic mesenchyme (nm) (G). The transverse section also shows deletion of COUP-TFII in the intermediate mesoderm and lateral plate mesoderm of the mutant embryo (E,H). At E10.5, COUP-TFII expression is detected in the urogenital ridge and metanephric mesenchyme (mm) (F). COUP-TFII expression is totally lost in the mutant embryo three days after Tam administration (I). DAPI counterstain for nuclei (blue) was applied for A, C, F and I. Scale bars: 50 \( \mu m \).

COUP-TFII is essential for the formation of the metanephric mesenchyme (Fig. 1D,G). Further examination of X-gal staining of transverse sections at the nephrogenic mesenchyme indicated that deletion of COUP-TFII occurred in the intermediate mesoderm and lateral plate mesoderm of COUP-TFII\textsuperscript{id/d} mutant embryos (Fig. 1E,H). Using the COUP-TFII-specific antibody, we showed that COUP-TFII is highly expressed in the urogenital ridge and the developing condensed nephrogenic mesenchyme, but not in the Wolffian duct of the COUP-TFII\textsuperscript{id/d} control at E10.5 (Fig. 1F). In COUP-TFII mutants, expression was undetectable (Fig. 1I). These data indicate that the tam-inducible deletion system effectively deletes COUP-TFII in kidney progenitor cells. Unfortunately, deletion of COUP-TFII at E7.5 led to embryonic lethality at ~E11, which limits our study to embryos before E11 (data not shown).
is totally lost in the nephrogenic mesenchyme area, whereas it is intact in the Wolffian duct of the COUP-TFIId/d mutants (Fig. 2F). lacZ-positive cells, which denote COUP-TFI ‘expressing’ cells in the COUP-TFIId/d mutants, indicated that some ‘COUP-TFI expressing’ metanephric mesenchyme cells surrounding the Wolffian duct are still present (Fig. 2F).

In addition to Pax2, the homeobox gene Six2 is also specifically expressed in the metanephric mesenchyme before ureteric bud outgrowth (Xu et al., 1999) and is required to activate Gdnf expression by directly binding to its promoter during kidney development (Brodbeck et al., 2004). Therefore, we examined Six2 expression in COUP-TFIId/d mutants. As indicated in Fig. 2G,H, Six2 expression is not detectable in the mutant metanephric mesenchyme. As both Pax2 and Six2 lie upstream of expression in COUP-TFIId/d mutants. As indicated in Fig. 2G,H, Six2 expression is not detectable in the mutant metanephric mesenchyme. As both Pax2 and Six2 lie upstream of expression by directly binding to its promoter during kidney outgrowth (Xu et al., 1999) and is required to activate Gdnf expression (Fig. 2L). Therefore, the inability of the mutant metanephric mesenchyme to induce ureteric bud outgrowth and branching suggests that the mature kidney will not be able to form in the COUP-TFIId/d mutants at later stages.

During this stage, the early mesonephros structure also formed. The mesonephros develops when the Wolffian duct reaches the prospective mesonephric mesenchyme and induces adjacent mesenchymal cells to condense and form mesonephric tubules (Saxén, 1987). First, we investigated whether COUP-TFI is also expressed in the mesonephros (also called the mesonephric tubule). COUP-TFI staining in E10 embryos indicated that COUP-TFI is expressed in the mesonephros but not in the Wolffian duct (supplementary material Fig. S2A). Pax2 is expressed in the Wolffian duct and mesonephros. By using Pax2 as a mesonephric tubule marker, we found COUP-TFI colocalizes with Pax2 in the mesonephros but not in the Wolffian duct (supplementary material Fig. S2B). We examined the E9.5 embryos and found that the mesonephros is still intact in the COUP-TFIId/d mutants in which COUP-TFIId/d deletion is very early in the germ cells, compared with COUP-TFIId/d controls (supplementary material Fig. S2C,D). Therefore, even though COUP-TFI is expressed in the mesonephros, it is not essential for mesonephros formation.

**COUP-TFI specifically regulates Eya1, Wt1 and Six2 in the metanephric mesenchyme**

Although the metanephric mesenchyme markers, such as Pax2 or Six2 expression, are not detected in the COUP-TFIId/d mutant embryos (Fig. 3F,H), the presence of lacZ-positive cells indicates that it is still possible that the lack of marker gene expression could be due to the loss of metanephric mesenchyme cells. To demonstrate that COUP-TFIId/d does indeed regulate metanephric mesenchyme marker gene expression, we deleted COUP-TFIId/d one day later by injecting Tam at E8.5 and collecting embryos at E10.5. We reasoned that by deleting COUP-TFIId/d one day later, we would have metanephric mesenchyme formation, but in some cells COUP-TFIId/d would be completely deleted, in some one allele would be deleted,
and in some it would not be deleted at all. In this scenario, a decrease in overall expression of these marker genes per cell would be observed in the formed metanephric mesenchyme. In addition, those cells with COUP-TFI deletion would have lower levels of target gene expression and those cells with no COUP-TFI deletion would have higher levels of target gene expression. As expected, we found that the metanephric mesenchyme was formed in both COUP-TFI-control (Fig. 3A) and COUP-TFI\textsuperscript{dd} mutant (Fig. 3B) embryos, even though the mutant metanephric mesenchyme is smaller and less condensed compared with the control. Using in situ hybridization to examine Eya1 expression, we found that both the number of cells expressing Eya1 and the expression level per cell are decreased in the COUP-TFI\textsuperscript{dd} mutant (Fig. 3C,D). Similarly, Wt1 and Six2 expression levels in the metanephric mesenchyme are significantly decreased in the COUP-TFI\textsuperscript{dd} mutant (Fig. 3F,H) compared with the controls (Fig. 3E,G). To define further whether COUP-TFI specifically regulated Six2 expression in the metanephric mesenchyme, we double labeled Six2 with \textit{lacZ} and found that Six2 colocalizes with \textit{lacZ} expression in the metanephric mesenchyme of COUP-TFI\textsuperscript{dd} mutant (Fig. 3I). Under high magnification, we examined the Six2-\textit{lacZ}-positive cells (Fig. 3J) and found that those cells with a higher \textit{lacZ} signal (i.e. in which COUP-TFI is deleted) have lower Six2 expression (Fig. 3K,L, arrowheads). By contrast, if \textit{lacZ} expression is low (i.e. COUP-TFI is not deleted), Six2 expression is higher (Fig. 3K,L, arrows).

In order to quantify the results, we measured the fluorescence intensity of Six2-positive cells (metanephric mesenchyme cells) in the COUP-TFI\textsuperscript{dd} controls (n=131 cells) and COUP-TFI\textsuperscript{dd} mutants (n=65 cells) shown in Fig. 3G,H and divided by the cell area to obtain densitometric levels of Six2 expression. The result shows that deletion of COUP-TFI significantly decreases Six2 expression in the COUP-TFI\textsuperscript{dd} mutant (Fig. 3M). These data clearly indicate that COUP-TFI does indeed regulate the expression of the metanephric mesenchyme markers Six2, Eya1 and Wt1.

**Deletion of COUP-TFII abolishes Wt1 expression and increases metanephric mesenchyme cell apoptosis**

We observed a reduction in cell number (Fig. 4A,B) in the nephrogenic mesenchyme region of the COUP-TFI\textsuperscript{dd} mutant embryos compared with controls. We investigated whether the loss of mesenchymal cells in this region is due to decreased cell proliferation or increased apoptosis. Embryo-wide comparison of Ki67 staining revealed no obvious differences in overall proliferation in controls versus COUP-TFI\textsuperscript{dd} mutants (Fig. 4A,B). By contrast, the COUP-TFI\textsuperscript{dd} mutant metanephric mesenchyme region showed a significant decrease in Ki67-positive cell numbers (Fig. 4C,D, red circled regions), indicating a specific decrease in proliferation of the metanephric mesenchyme. Furthermore, we observed an increase in the number of apoptotic cells in the metanephric mesenchyme of mutant mice as detected by both cleaved Caspase 3 and tunnel assays (Fig. 4E-H).

The Wilms tumor suppressor gene (\textit{Wt1}) is expressed in the metanephric mesenchyme and has been shown to be essential for the survival of metanephric mesenchymal cells (Kreidberg et al., 1993; Kuure et al., 2000; Moore et al., 1999). Therefore, we investigated whether \textit{Wt1} expression is compromised in COUP-TFI\textsuperscript{dd} mutants. First, we determined whether COUP-TFI and \textit{Wt1} colocalize with each other. As shown in supplementary material Fig. S3A-C, we found that COUP-TFI colocalizes with \textit{Wt1} in the metanephric mesenchyme and in the urogenital ridge. Next, we investigated whether \textit{Wt1} expression level is altered with COUP-TFI deletion. Indeed, we found \textit{Wt1} expression to be significantly decreased in the COUP-TFII mutant urogenital ridge and metanephric mesenchyme (deletion cells shown as the \textit{lacZ}-positive cells) (Fig. 4E,F and 4I,J). These results indicate \textit{Wt1} lies downstream of COUP-TFI and is regulated by COUP-TFI. Owing to the importance of \textit{Wt1} in metanephric mesenchyme cell survival, our findings suggested that loss of \textit{Wt1} expression due to COUP-TFI deletion is the likely reason for the increased metanephric mesenchyme apoptosis in COUP-TFI\textsuperscript{dd} mutants.

**COUP-TFs regulate the expression of Eya1, Six2, Wt1 and Gdnf in metanephric mesenchyme cells**

In order to study the detailed molecular mechanism by which COUP-TFI controls regulatory genes in the metanephric mesenchyme, we employed the conditionally immortalized rat inducible metanephric mesenchyme cell line (RIMM-18). RIMM-18 cells were generated from rat mesenchymal cells by transfection with a vector encoding an estradiol-dependent E1A-ER fusion protein and
COUP-TFII mutants by in situ hybridization. TFII is upstream of Osr1 by examining the expression of Odd-skipped related 1 (Osr1) is also required for nephrogenic mesenchyme development. Similar to COUP-TFII, knockdown of both COUP-TFI and COUP-TFII using specific siRNAs. All Wt1 mRNA measurements were normalized to 18S rRNA. Error bars indicate s.d. **P<0.005.

To test further whether COUP-TFII binding to the Eya1 promoter is regulated by COUP-TFII expression in RIMM-18 cell culture experiments and found that simultaneous knockdown of COUP-TFI and COUP-TFII by siRNA does not affect COUP-TFII expression (supplementary material Fig. S5G-I). These results indicate that Osr1 and COUP-TFII act in parallel with one another and are both required for Eya1 activation to control nephrogenic mesenchyme differentiation and nephrogenesis. To support this conclusion further, we employed RIMM-18 cell culture experiments and found that simultaneous knockdown of COUP-TFI and COUP-TFII by siRNA does not affect COUP-TFII expression (supplementary material Fig. S5G-I). Similarly, knockdown of Osr1 had no significant effect on COUP-TFI or COUP-TFII expression (supplementary material Fig. S5J-L). These results support the conclusion that COUP-TFII and Osr1 act in parallel to regulate Eya1 expression.

**COUP-TFII directly regulates Eya1 gene expression by interacting with Sp1**

Eya1 has been shown to specify the nephrogenic mesenchyme fate of Eya1 and is indispensable for nephrogenesis of the metanephric mesenchyme. In order to determine whether Eya1 expression is regulated by COUP-TFII at the transcriptional level, we carried out in situ hybridization to see whether Eya1 is regulated by COUP-TFII at the mRNA level. Indeed, Eya1 mRNA is detected in the metanephric mesenchyme of COUP-TFII mutants but is absent in COUP-TFII mutants (Fig. 6A,B). This indicates that Eya1 is a COUP-TFII target and is likely to be regulated by COUP-TFII at the transcriptional level.

To support this conclusion further, we carried out chromatin immunoprecipitation (ChIP) assays to determine whether COUP-TFII is recruited to the promoter/enhancer region of the Eya1 gene. Our laboratories have previously demonstrated that COUP-TFII acts as a positive regulator, enhancing target gene expression by interacting with Sp1 at Sp1-binding sites (Kim et al., 2009; Lin et al., 2010; Pipaon et al., 1999; Qin et al., 2010). To assess whether COUP-TFII regulates Eya1 expression through its interaction with Sp1, we searched for the evolutionarily conserved Sp1-binding sites in human, mouse and rat sequences upstream of the transcription start site (Fig. 6C, black boxes). We then performed ChIP assays to evaluate whether endogenous COUP-TFII is recruited to the Sp1 sites of the Eya1 promoter. Indeed, we found COUP-TFII is preferentially recruited to the region lacking Sp1-binding sites (Fig. 6C, Sp1). In parallel, Sp1 was also specifically recruited to the same region as COUP-TFII, but not to the region without Sp1-binding sites (Fig. 6C, Sp1). To substantiate that COUP-TFII was recruited by Sp1 to the Eya1 promoter, we knocked down endogenous Sp1 expression with Sp1-specific siRNA (si-Sp1). Recruitment of COUP-TFII and Sp1 to the Eya1 promoter was, in fact, significantly reduced in Sp1-knockdown cells compared with controls (Fig. 6D,E).

To test further whether COUP-TFII binding to the Eya1 promoter leads to activation of transcription, we performed luciferase reporter assays in HEK293 cells using a 1.8-kb (–731 bp to 1079 bp) human EYA1 promoter fragment that included the three conserved Sp1-binding sites. Luciferase reporter activity was significantly increased...
when COUP-TFII was expressed compared with control cells (Fig. 5F). Next, we mutated these three conserved Sp1-binding sites as depicted in supplementary material Fig. S4A. We generated reporters with three single Sp1-binding sites or with all three sites mutated (supplementary material Fig. S6A; pGL2-Eya1-M1, pGL2-Eya1-M2, pGL2-M3 and the triple-mutation pGL2-Eya1-M123). In the presence of COUP-TFII expression plasmid, activation of all four mutant luciferase reporters was significantly diminished versus the intact reporter (Fig. 6F). This result indicates that COUP-TFII works through Sp1 on the Sp1-binding sites to activate Eya1 expression. Collectively, these results substantiate a model in which COUP-TFII is recruited to the Eya1 promoter in an Sp1-dependent manner to directly activate Eya1 transcription.

COUP-TFII interacts synergistically with Sp1 to regulate Wt1 expression directly

Next, we investigated whether COUP-TFII also directly regulates the transcription of Wt1 through its interactions with Sp1. We identified two evolutionarily conserved Sp1-binding sites surrounding the Wt1 promoter (Fig. 7A, black boxes). ChIP analysis showed that COUP-TFII is indeed preferentially recruited to Sp1-binding sites at the Wt1 promoter but is not recruited to the region lacking Sp1-binding sites (Fig. 7A). Sp1 was recruited to the same regions as COUP-TFII. To determine whether recruitment of COUP-TFII to the Wt1 promoter is Sp1-dependent, we knocked down endogenous Sp1 by siRNA. With knockdown, COUP-TFII recruitment to the Wt1 promoter was significantly reduced (Fig. 7B,C).

To confirm whether COUP-TFII binding to the Wt1 promoter leads to transcription activation, we performed luciferase reporter assays using a 1.1-kb (–736 bp to 377 bp) human WT1 promoter fragment that contains the two conserved Sp1-binding sites. As shown in Fig. 7D, Wt1-luciferase reporter (pGL2-Wt1) activity was significantly increased by COUP-TFII. In addition, when Sp1-binding sites were mutated in the Wt1-luciferase reporter (supplementary material Fig. S6B; pGL2-Wt1-M1, pGL2-Wt1-M2, and the double-mutation pGL2-Wt1-M12) COUP-TFII-dependent luciferase activity decreased dramatically (Fig. 7D). Taken together, these results strengthen our proposed model that recruitment of COUP-TFII to the Wt1 promoter is Sp1-dependent, and that COUP-TFII directly activates Wt1 transcription.

DISCUSSION

COUP-TFII directly regulates transcription of Eya1 to modulate Gdnf expression during mesenchepelic induction

COUP-TFII is expressed in the mesenchyme of developing organs and has been shown to play a key role in their organogenesis. In this study, we found that COUP-TFII is expressed in the kidney
suggesting that COUP-TFII directly regulates expression in Eya1 expression. Furthermore, expression of COUP-TFII can enhance Eya1 expression, by direct regulation of Eya1 expression. These results place COUP-TFII as an upstream regulator of these important regulatory factors.

Our in situ hybridization data showed that Eya1 expression is significantly decreased in the COUP-TFII mutant (Fig. 3C,D), suggesting that COUP-TFII directly regulates Eya1 expression in the metanephric mesenchyme. Indeed, ChIP assays showed that COUP-TFII is recruited to the conserved Sp1-binding site of the Eya1 promoter by tethering to Sp1 for direct regulation of Eya1 expression. Furthermore, expression of COUP-TFII can enhance Eya1 promoter-driven reporter activity. Collectively, these results clearly indicate that COUP-TFII promotes Gdnf signaling cascade by direct regulation of Eya1 expression and, thus, induces metanephric mesenchyme fate in kidney progenitor cells. It should be noted that in Eya1 knockout mice, ureteric buds fail to grow out into the metanephric mesenchyme (Sajithlal et al., 2005). Similar to Eya1, we observed no ureteric bud outgrowth in six out of eight COUP-TFII mutants using the kidney organ cultures (Fig. 2L). It is not clear, however, whether the remaining two COUP-TFII mutants have ureteric bud outgrowth (supplementary material S7A,B, arrowheads). In any event, if they indeed have ureteric bud outgrowth, it is possible that COUP-TFII was not deleted early enough in these two particular mutants, resulting in partially committed metanephric mesenchyme and subsequent induction of ureteric bud outgrowth. As the majority of mutants do not have ureteric buds outgrowth, it indicates that COUP-TFII is required for the proper formation of metanephric mesenchyme and subsequent induction of ureteric bud outgrowth.

**COUP-TFII acts in parallel with Osr1 to regulate Eya1 expression during metanephric induction**

Osr1 is known to be the earliest marker for kidney development in the intermediate mesoderm. Mice lacking Osr1 do not form metanephric mesenchyme and do not express many factors essential for metanephric mesenchyme formation (James et al., 2006; Wang et al., 2005). These phenotypes bear strong resemblance to that of the COUP-TFII knockout mice. To address whether these two genes work in the same pathway, we examined whether Osr1 expression is affected in the COUP-TFII knockout. Our results clearly show that Osr1 expression in the nephrogenic mesenchyme region is not altered in the COUP-TFII mutant. Similarly, COUP-TFII expression in the metanephric mesenchyme region remains the same in the Osr1 knockout embryo. Together, these results indicate that COUP-TFII and Osr1 act independently to regulate kidney morphogenesis. This notion is further supported by in vitro cell culture experiments, in which we showed that COUP-TFII and Osr1 do not regulate each other’s expression. Interestingly, whereas Wt1 is expressed in the metanephric mesenchyme of Osr1 null mice (James et al., 2006), Wt1 expression in the COUP-TFII knockout mutant is totally lost. This result indicates that although both COUP-TFII and Osr1 are expressed in the intermediate mesoderm and both regulate Eya1, Pax2 and Six2 expression, they do not regulate each other to control the expression of those key factors important for metanephric mesenchyme formation. Based on all these results, our working model is that COUP-TFII and Osr1 act in parallel to regulate Eya1 and its downstream target Gdnf to specify metanephric mesenchyme (Fig. 8).

**COUP-TFII is essential for survival of the metanephric mesenchyme and for nephron differentiation**

Wt1 mutant mesenchyme cannot be induced to form nephric tubules (Kreidberg et al., 1993), suggesting that Wt1 is cell-autonomously required for nephron differentiation. Subsequently,
it was shown that kidney precursor cells undergo apoptosis in Wt1-deficient mutant mice, consistent with the notion that Wt1 is essential for the early stage of kidney development (Davies et al., 2004; Kreidberg et al., 1993). The colocalization of COUP-TFII and Wt1 in the metanephric mesenchyme and urogenital ridge and the similar phenotypes exhibited by COUP-TFII and Wt1 mutants in terms of decreased cell numbers in the metanephric mesenchyme strongly implicate that these two factors function in the same pathway. As the expression of Wt1 is drastically decreased in strongly implicate that these two factors function in the same

Fig. 8. COUP-TFII directly regulates Eya1 and Wt1 expression to specify metanephric mesenchyme cell fate and maintain metanephric mesenchyme precursor cell survival. COUP-TFII acts in parallel with Osr1 to regulate Eya1 transcription. COUP-TFII in the intermediate mesoderm kidney precursor cells specifies metanephric mesenchyme fate by directly regulating Eya1 expression. Eya1 regulates Pdx2 expression, forms a complex with Pdx2 and Hox11 paralogous proteins (including Hoxa11, Hoxc11 and Hoxd11) and binds directly to the Six2 enhancer to regulate the expression of Six2 and its downstream target Gdnf. Gdnf, a crucial factor for the specification of the metanephric mesenchyme, will then induce ureteric bud outgrowth from the Wolffian duct and initiate kidney organogenesis. In addition, COUP-TFII is essential for the survival of the metanephric mesenchyme precursor cells through its direct regulation of Wt1 gene expression. Wt1 plays an anti-apoptotic role to maintain metanephric mesenchyme cell survival. MM, metanephric mesenchyme; UB, ureteric bud.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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References
Li, L., Xie, X., Qin, J., Jeha, G. S., Saha, P. K., Yan, J., Haueter, C. M., Chan, L., Tsai, S. Y. and Tsai, M. J. (2009). The nuclear orphan receptor COUP-TFII plays an essential role in adipo genesis, glucose homeostasis, and energy metabolism. Cell Metab. 9, 77-87.
COUP-TFII in early nephrogenesis


### Table S1. The target sequences for rat siRNA

<table>
<thead>
<tr>
<th></th>
<th>Sequence</th>
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<tbody>
<tr>
<td>rCOUP-TFI single</td>
<td>GUUUCUUCAAGAGGAGCGUtt</td>
</tr>
<tr>
<td>rCOUP-TFII-1</td>
<td>GGAGGAACCUGAGCUACAC</td>
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<tr>
<td>rCOUP-TFII-2</td>
<td>CCUCAGUCAUAGAGCAAUU</td>
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| rCOUP-TFII pooled| GUACCUGUCCGGAUAUUU  
                      | GGAGGAACCUGAGCUACAC  
                      | CCUCAGUCAUAGAGCAAUU  
                      | CAUACAUUGGCAAUUCAAUU |
| rSp1 single      | GGAUGGUUCUGGUCAAAUA                                                      |
| rOsrl sense      | GCAGCUUACCAUUACUCUTT  
<pre><code>                  | AGAGUAUUGGUAAAGCUGCAG                                                     |
</code></pre>
<p>|      antisense   |                                                                           |</p>
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<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Gene</th>
<th>Primer sequences</th>
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<tr>
<td>rCOUP-TFI</td>
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<td>rSix2</td>
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<td>R: ATGGTAAACCAGGCTGTCGT</td>
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<tr>
<td>rCOUP-TFII</td>
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<td>R: CAGGTACGAGTGCCAGTTGA</td>
<td></td>
<td>R: GGTCTCTGTGGTTGAAAGGAA</td>
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<td>rGdnf</td>
<td>F: CGAAAACAGGAGGAACCTGA</td>
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<td>F: CTGCCCAACCTGTATGGTTT</td>
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<td>R: TAGGTGAATGACGTGTTGGA</td>
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<td>rEya1</td>
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<td>18s RNA</td>
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<td></td>
<td>R: ATGGTAAACCAGGCTGTCGT</td>
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<td>R: CAGGACTTAATCAACGCAA</td>
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F, forward; R, reverse
**Table S3.** The primer sequences for ChIP

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<td></td>
<td>R: GAGATTGCTTTGCGTGTGTTGA</td>
</tr>
<tr>
<td>rEya1-neg</td>
<td>F: TGCCAAAACAAAAACACAACAA</td>
</tr>
<tr>
<td></td>
<td>R: CTGGGCTAACGTCAGGATTC</td>
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<tr>
<td>rWt1</td>
<td>F: CAGCTTCCAAAGCTCAAAAT</td>
</tr>
<tr>
<td></td>
<td>R: GAGGGTGCTCCGAGAAGAAA</td>
</tr>
<tr>
<td>rWt1-neg</td>
<td>F: TTCCAGGTCATGCACCTCAAG</td>
</tr>
<tr>
<td></td>
<td>R: ATTGAAGAAGGCATGGGCTA</td>
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F, forward; R, reverse
**Table S4.** The primer sequences for promoter constructs

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>Eya1-pro-Kpn-5′</td>
<td>AAAGGTACCTTCTGGAGAAGGGCAGTG</td>
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<tr>
<td>Eya1-pro-Xho-3′</td>
<td>AAACTCGAGTGCCAGTTGAAAAA</td>
</tr>
<tr>
<td>Eya1-XhoI-5′-M1</td>
<td>TTTTTTTATCTATATTTCAGCTGGTGATAAC</td>
</tr>
<tr>
<td>Eya1-KpnI-3′-M1</td>
<td>GATAAAAGAAAAAAACTTTGACTCTGACAGTTTC</td>
</tr>
<tr>
<td>Eya1-XhoI-5′-M2</td>
<td>TTTTAAAAAAAAATCCATTCCAGCTGTCAT</td>
</tr>
<tr>
<td>Eya1-KpnI-3′-M2</td>
<td>TTTTTTTTTTTAATCAACATGCAAAGCAGCCT</td>
</tr>
<tr>
<td>Eya1-XhoI-5′-M3</td>
<td>TTTTAAAAAAAAATACCTGCAGGAAACACAGT</td>
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<tr>
<td>Eya1-KpnI-3′-M3</td>
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</tr>
<tr>
<td>WT1-pro-KpnI-5′</td>
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</tr>
<tr>
<td>WT1-pro-BglII-3′</td>
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</tr>
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<tr>
<td>WT1-KpnI'-3′-M1</td>
<td>TTATTTTTGAGTCTCTGCTCAATTTTTTTTTCAGAAAGGAGCAGAA</td>
</tr>
<tr>
<td>WT1-BglII-5′-M2</td>
<td>GGAAAAAAATACCCAGCTGCAGGGGCGCC</td>
</tr>
<tr>
<td>WT1-KpnI-3′-M2</td>
<td>TTATTTTTTTCAGGGAGCCCTCTTCG</td>
</tr>
</tbody>
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