COUP-TFII is essential for metanephric mesenchyme formation and kidney precursor cell survival

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SUMMARY
Development of the metanephric kidney in mammals requires complex reciprocal tissue interactions between the ureteric epithelium and the mesenchyme. It is believed that Gdnf, produced in the metanephric mesenchyme, activates Ret signaling in the Wolffian duct to initiate the formation of the metanephros. However, the molecular mechanism for induction of Gdnf in the metanephric mesenchyme is not completely defined. Previous studies demonstrated that during the early stages of kidney development, loss of Osr1, Eya1, Pax2 or Wt1 gene function in the metanephric mesenchyme compromises the formation of the kidney. Moreover, it has been shown that the Hox11-Eya1-Pax2 complex activates the expression of Six2 and Gdnf in the metanephric mesenchyme to drive nephrogenesis. Here, we demonstrate that the orphan nuclear receptor chicken ovalbumin upstream promoter transcription factor II (COUP-TFII, also known as Nr2f2) is required for the specification of the metanephric mesenchyme. Deletion of COUP-TFII at E7.5 results in improper differentiation of the metanephric mesenchyme and absence of essential developmental regulators, such as Eya1, Six2, Pax2 and Gdnf. Importantly, we show that COUP-TFII directly regulates the expression of both Eya1 and Wt1 in the metanephric mesenchyme. Our findings reveal, for the first time, that COUP-TFII plays a central role in the specification of metanephric fate and in the maintenance of metanephric mesenchyme proliferation and survival by acting as a crucial regulator of Eya1 and Wt1 expression.

KEY WORDS: COUP-TFII, Metanephric mesenchyme, Eya1, Wt1, Mouse

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 (mesonephric) 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 (Gfrα1) 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-TFII and COUP-TFI; 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...
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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-TFII<sup>fl/fl</sup>) (Takamoto et al., 2005) with Rosa26-Cre-ERT<sup>2+</sup>, 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<sup>2+;COUP-TFII<sup>fl/fl</sup></sup> males (hereafter referred to as COUP-TFII<sup>fl/fl</sup>) were intercrossed with COUP-TFII<sup>fl/fl</sup> 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-
**TFII<sup>d/d</sup>** mutant embryos, including the nephrogenic 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<sup>d/d</sup>** 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<sup>f/f</sup>** control at E10.5 (Fig. 1F). In COUP-TFII mutants, expression was undetectable (Fig. 1I). These data indicate that the ureteric bud (ub) outgrowth, visualized by E-cadherin staining, was detected from the Wolffian duct of the **COUP-TFII<sup>j/j</sup>** control (K) and **COUP-TFII<sup>d/d</sup>** mutant (L) embryos. As the paired box-containing transcription factor Pax2 is a key regulator of kidney development (Torres et al., 1995), we investigated whether 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 and in the Wolffian duct (wd). **COUP-TFII** is detected in the entire nephrogenic mesenchyme region of **COUP-TFII<sup>d/d</sup>** 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<sup>d/d</sup>** 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 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 induced metanephric mesenchyme and in the Wolffian duct of the metanephros in the controls (Fig. 2E). By contrast, Pax2 expression...
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is totally lost in the nephrogenic mesenchyme area, whereas it is intact in the Wolffian duct of the COUP-TFIId mutants (Fig. 2F). lacZ-positive cells, which denote COUP-TFI ‘expressing cells’ in the COUP-TFIId 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 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 Gdnf, which is essential for promoting the ureteric bud outgrowth (Costantini and Shakya, 2006), we investigated whether Gdnf expression in the nephrogenic mesenchyme cells is also compromised in the mutants. Gdnf is detectable in the cytoplasm of the metanephric mesenchyme in control animals (Fig. 2I), but is not detected in the COUP-TFIId mutants (Fig. 2J). Together, these results strongly implied that COUP-TFIId is likely to regulate the expression of genes essential for metanephric mesenchyme formation and kidney development.

To investigate whether COUP-TFIId mutant embryo at E11, we injected Tam at E7.5 to delete COUP-TFIId and collected the embryos at E10.5. The results obtained from organ cultured of three litters of mice indicate that the ureteric bud was induced to outgrow from the Wolffian duct and started to branch from all ten COUP-TFIId controls (Fig. 2K). By contrast, we found that majority of COUP-TFIId mutants (six out of eight) did not display any ureteric bud outgrowth (Fig. 2L). Therefore, the inability of the mutant mesenchyme to induce ureteric bud outgrowth and branching suggests that the mature kidney will not be able to form in the COUP-TFIId 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-TFIId is also expressed in the mesonephros (also called the mesonephric tubule). COUP-TFIId staining in E10 embryos indicated that COUP-TFIId 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-TFIId 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 mutants in which COUP-TFIId is deleted very early in the germ cells, compared with COUP-TFIId controls (supplementary material Fig. S2C,D). Therefore, even though COUP-TFIId is expressed in the mesonephros, it is not essential for mesonephros formation.

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

Although the mesonephric mesenchyme markers, such as Pax2 or Six2 expression, are not detected in the COUP-TFIId 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 does indeed regulate metanephric mesenchyme marker gene expression, we deleted COUP-TFIId one day later by injecting Tam at E8.5 and collecting embryos at E10.5. We reasoned that by deleting COUP-TFIId one day later, we would have metanephric mesenchyme formation, but in some cells COUP-TFIId 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{ld} 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{ld} mutant (Fig. 3C,D). Similarly, Wt1 and Six2 expression levels in the metanephric mesenchyme are significantly decreased in the COUP-TFI\textsuperscript{ld} 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 lacZ and found that Six2 colocalizes with lacZ expression in the metanephric mesenchyme of COUP-TFI\textsuperscript{ld} mutant (Fig. 3I). Under high magnification, we examined the Six2-lacZ-positive cells (Fig. 3J) and found that those cells with a higher lacZ signal (i.e. in which COUP-TFI is deleted) have lower Six2 expression (Fig. 3K,L, arrowheads). By contrast, if lacZ expression is low (i.e. COUP-TFI is not deleted), Six2 expression is higher (Fig. 3K,L, arrows).

In order to obtain densitometric levels of Six2 expression, we measured the fluorescence intensity of Six2-positive cells (metanephric mesenchyme cells) in the COUP-TFI\textsuperscript{ld} controls (n=131 cells) and COUP-TFI\textsuperscript{ld} mutants (n=65 cells) shown in Fig. 3G 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{ld} 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-TFI aboliishes 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{ld} 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{ld} mutants (Fig. 4A,B). By contrast, the COUP-TFI\textsuperscript{ld} 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 Wtms tumor suppressor gene (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 Wt1 expression is compromised in COUP-TFI\textsuperscript{ld} mutants. First, we determined whether COUP-TFI and Wt1 colocalize with each other. As shown in supplementary material Fig. S3A-C, we found that COUP-TFI colocalizes with Wt1 in the metanephric mesenchyme and in the urogenital ridge. Next, we investigated whether Wt1 expression level is altered with COUP-TFI deletion. Indeed, we found Wt1 expression to be significantly decreased in the COUP-TFI mutant urogenital ridge and metanephric mesenchyme (deletion cells shown as the lacZ positive cells) (Fig. 4E,F and Fig. 4I). These results indicate that Wt1 lies downstream of COUP-TFI and is regulated by COUP-TFI. Owing to the importance of Wt1 in metanephric mesenchyme cell survival, our findings suggested that loss of Wt1 expression due to COUP-TFI deletion is the likely reason for the increased metanephric mesenchyme apoptosis in COUP-TFI\textsuperscript{ld} 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 Gdnf. We stably expressed the COUP-TFI vectors in this cell line to obtain inducible metanephric mesenchyme cell line (RIMM-18). 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 Gdnf. We stably expressed the COUP-TFI vectors in this cell line to obtain inducible metanephric mesenchyme cell line (RIMM-18).
COUP-TFI mutants by in situ hybridization. TFII is upstream of Osr1 by examining the expression of TFII in the regulatory cascade, we first investigated whether COUP-TFI is located in the stroma mesenchyme. Our laboratory has previously demonstrated that COUP-TFII is expressed in the metanephric mesenchyme, whereas COUP-TFI is located in the stroma mesenchyme. The underlying mechanism of COUP-TF regulation of transcription factors important for kidney development. Knockdown of COUP-TFI and COUP-TFII using specific siRNAs. All mice (supplementary material Fig. S5A). Despite the fact that the expression levels of both COUP-TFI and COUP-TFII are high in RIMM-18 cells. Therefore, during in vitro experiments, we used siRNAs to knock down both COUP-TFI and COUP-TFII to study the underlying mechanism of COUP-TF regulation of transcription factors important for kidney development. Knockdown of COUP-TFI and COUP-TFII significantly decreases Gdnf, Eya1, Six2 and Wt1 (Fig. 5C-F) expression. In order to confirm whether COUP-TFII alone can regulate these transcription factors in RIMM-18 cells, we also used siRNA to knock down COUP-TFII specifically. We found that knockdown of COUP-TFII alone is enough to significantly decrease Eya1 and Wt1 (supplementary material Fig. S4B,C) expression. These results indicate that COUP-TFs can indeed positively regulate genes expressed early in metanephric mesenchyme development.

**COUP-TFII signaling is independent of Osr1 in the nephrogenic mesenchyme**

Similar to COUP-TFI, Odd-skipped related 1 (Osr1) is also required for the formation of the metanephric mesenchyme and is upstream of Eya1. In order to define the relationship between Osr1 and COUP-TFII in the regulatory cascade, we first investigated whether COUP-TFII is upstream of Osr1 by examining the expression of Osr1 in COUP-TFII mutants by in situ hybridization. Osr1 is broadly expressed in the nephrogenic mesenchyme regions in the control mice (supplementary material Fig. S5A). Despite the fact that the Coup-TFD/d mutant has fewer mesenchyme cells, the Osr1 expression level in the mesenchyme is equivalent to that of the control (supplementary material Fig. S5B). Next, we examined COUP-TFII expression in Osr1 knockout mice to determine whether COUP-TFII is downstream of Osr1. The COUP-TFII expression level is similar in the urogenital ridge of Osr1 knockout and wild-type mice (supplementary material Fig. S5C-F). Although Osr1 knockouts lack the metanephric mesenchyme, COUP-TFII expression in the nephrogenic mesenchyme surrounding the Wolfian duct is similar compared with wild-type controls (supplementary material Fig. S5C-F). These results indicate that Osr1 and COUP-TFII act in parallel with one another and are both required for Eya1 activation to control metanephric 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 expression of Osr1 (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 metanephric mesenchyme fate and is indispensable for metanephric mesenchyme formation. In order to determine whether Eya1 expression is regulated by COUP-TFI 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/d/d mutants but is absent in Coup-TFI/d/d mutants (Fig. 6A,B). This indicates that Eya1 is a COUP-TFII target and is likely to be regulated by COUP-TFI at the transcriptional level.

To support this conclusion further, we carried out chromatin immunoprecipitation (ChIP) assays to determine whether COUP-TFI is recruited to the promoter/enhancer region of the Eya1 gene locus. 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-TFI regulates Eya1 expression through its interaction with Sp1, we searched for the evolutionarily conserved Sp1-binding sites in human, mouse and rat sequences on and surrounding the Eya1 gene and found three conserved sites upstream of the transcription start site (Fig. 6C, black boxes). We then performed ChIP assays to evaluate whether endogenous COUP-TFI is recruited to the Sp1 sites of the Eya1 promoter. Indeed, we found COUP-TFI is preferentially recruited to the promoter regions containing Sp1-binding sites but not recruited to the region lacking Sp1-binding sites (Fig. 6C, Sp1). In parallel, Sp1 was also specifically recruited to the same region as COUP-TFI, but not to the region without Sp1-binding sites (Fig. 6C, Sp1). To substantiate that COUP-TFI was recruited by Sp1 to the Eya1 promoter, we knocked down endogenous Sp1 expression with Sp1-specific siRNA (si-Sp1). Recruitment of COUP-TFI 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-TFI 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-TFI expression plasmid, activation of all four mutant luciferase reporters was significantly diminished versus the intact reporter (Fig. 6F). This result indicates that COUP-TFI works through Sp1 on the Sp1-binding sites to activate Eya1 expression.

Collectively, these results substantiate a model in which COUP-TFI is recruited to the Eya1 promoter in an Sp1-dependent manner to directly activate Eya1 transcription.

**COUP-TFI interacts synergistically with Sp1 to regulate Wt1 expression directly**

Next, we investigated whether COUP-TFI 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-TFI 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-TFI. To determine whether recruitment of COUP-TFI to the Wt1 promoter is Sp1-dependent, we knocked down endogenous Sp1 by siRNA. With knockdown, COUP-TFI recruitment to the Wt1 promoter was significantly reduced (Fig. 7B,C).

To confirm whether COUP-TFI 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-TFI. In addition, when Sp1-binding sites were mutated in the Wt1-luciferase reporter (supplementary material Fig. S6B; pGL2-Wt1-M1, pGL2-Wt1-M2, pGL2-Eya1-M3 and the double-mutation pGL2-Wt1-M123 (250 ng each) were measured after co-transfection with or without COUP-TFI expression plasmid (50 ng) into HEK 293 cells.

**DISCUSSION**

COUP-TFI directly regulates transcription of Eya1 to modulate Gdnf expression during metanephric induction

COUP-TFI 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-TFI is expressed in the kidney
suggesting that COUP-TFII directly regulates expression in Eya1 expression. Furthermore, expression of COUP-TFII can enhance Eya1 expression and, thus, induces the metanephric mesenchyme. Indeed, ChIP assays showed that COUP-TFII is recruited to the conserved Sp1-binding site of the metanephric mesenchyme. Together, these results indicate that COUP-TFII promotes Gdnf signaling cascade by direct regulation of Eya1 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 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 these factors important for metanephric mesenchyme formation. Based on 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).
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 COUP-TFII mutant mice in the metanephric mesenchyme and urogenital ridge (Fig. 3E,F and Fig. 4LJ), it suggests that COUP-TFII is upstream of Wt1 in the signaling cascade. This notion is supported by ChIP analysis, which showed that COUP-TFII is recruited by Sp1 to the conserved Sp1-binding site in the Wt1 promoter to regulate Wt1 transcription directly. Therefore, Wt1 mediates COUP-TFII function to maintain metanephric mesenchyme cell differentiation and survival (Fig. 8).

In summary, we have shown that COUP-TFII has two major roles during metanephric mesenchyme formation. First, COUP-TFII directly regulates Eya1 transcription to specify the kidney precursor cell differentiation into the mature metanephric mesenchyme. Second, COUP-TFII directly regulates Wt1 expression to maintain metanephric mesenchyme survival and differentiation into the mature nephron.

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COUP-TFII in early nephrogenesis


