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厚生労働科学研究研究費補助金

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ES細胞からの腎臓細胞誘導法の開発

平成１９年度　総括・分担研究報告書

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総括　研究報告書

ES細胞からの腎臓細胞誘導法の開発

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研究要旨

腎不全は難病指定とされ、社会的負担は大きいにもかかわらず、腎機能を回復させる画期的な治療法はいまだ存在しない。腎臓の再生研究に決定的に欠けているもの、それは腎臓前駆細胞を検定する系である。そこで本計画は、発生期腎臓から前駆細胞を同定する系を確立し、これを基盤として、胚性幹(ES)細胞から腎臓前駆細胞を誘導することを目的とした。我々が単離した核内因子Sall1は、腎臓前駆細胞を含むと考えられる後腎間葉に発現し、そのノックアウトマウスは腎臓を欠損する。この遺伝子座にGFPを導入したマウスの後腎間葉から、GFPが高発現する細胞をFACSで選別し、Wnt4を発現するフィーダー上で培養すると、1個の細胞からコロニーが形成され、このコロニーは糸球体、近位尿細管、遠位尿細管という多系統へ分化することを見いだした。次にこのコロニーアッセイを基盤として、ES細胞から腎臓前駆細胞の誘導を目指したが、野生型ES細胞の単純な分化ではコロニーは形成されなかった。そこで発生期腎臓前駆細胞に発現する転写因子Osr1の遺伝子座にGFPを挿入したESを作成し、さらにアクチビンとレチノイン酸で処理したところGFP陽性細胞が誘導された。今後Osr1-GFPマウスから採取されるin vivoでの腎臓前駆細胞との類似性を確認しつつ、誘導効率の改善を図りたい。

分担研究者氏名・所属機関及び所属機関における職名
小林　千余子　熊本大学発生医学研究センター助手

A. 研究目的

日本で腎不全により人工透析を受ける人は26万人を超え、この10年で2倍となった。現在、慢性腎不全の原因の第1位は糖尿病であり、今後も増える一方である。腎不全は難病指定とされ、その医療費はすべて国庫によって賄われるため、社会的負担は大きい。このような状況にもかかわらず、腎機能を回復させる画期的な治療法はいまだ存在せず、最終的には透析導入となる。腎臓の再生研究に決定的に欠けているもの、それは腎臓前駆細胞を検定する系である。そこで本計画は、発生期腎臓から前駆細胞を同定する系を確立し、これを基盤として、胚性幹(ES)細胞から腎臓前駆細胞を誘導することを目的とした。

B. 研究方法

1) 腎臓前駆細胞アッセイ系の確立

我々が単離した核内因子Sall1は、腎臓前

駆細胞を含むと考えられる後腎間葉に発現し、そのノックアウトマウスは腎臓を欠損する。この遺伝子座にGFPを導入したマウスの後腎間葉から、GFPが高発現する細胞をFACSで選別し、Wnt4を発現するフィーダー上で培養すると、１個の細胞からコロニーが形成され、このコロニーは糸球体、近位尿細管、遠位尿細管という多系統へ分化することを見いだした (図１)。またGFP高発現の細胞群を再凝集させ器官培養すると、５日間で３次元構造を再構築でき、その中には糸球体様構造や尿細管様構造が認められた (図2)。よってSall1を高発現する後腎間葉細胞中に腎臓前駆細胞が存在し、これは３次元立体構造を再構築できることを証明し、平成17年度中に発表した(Osafune et al., Development, 2006)。

2) 遺伝子導入による腎臓前駆細胞誘導の試み

ESの分化系として、培養皿で２次元に展開する方法(RIKENの西川伸一らが開発)を主として使用した。この利点は、分化誘導後再解離が容易でFACSが可能であること、かつ側方中胚葉がflk1陽性画分に、沿軸中胚葉がPDGFR陽性画分に存在することである。腎臓はこの２つに挟まれた中間中胚葉から生じるので、どちらかの画分に重複する形で腎臓前駆細胞を含む集団があると考えた (図 3)。上述のコロニーアッセイを基盤にして、ES細胞からの腎臓前駆細胞誘導を試みたが、どの分画からもコロニーは形成されなかった。

3) 腎臓系譜誘導条件の検討

そこで今度は、野生型ESから胚様体を形成させ、アクチビンとレチノイン酸を添加したところ、非常に効率よく発生期腎臓関連の遺伝子群が発現することを見出した。しかしこれらからコロニーは形成されなかった。この原因としては、誘導される前駆細胞の頻度がまだ少ない、あるいはコロニーを作れる段階まで誘導できていないことが考えられる。そこでまずSall1-GFP ES細胞で前駆細胞の濃縮ができないか検討したが、ES細胞でもSall1が発現するために分離が不可能であった。ちなみにES細胞においては、Sall1ではなくSall4が必須であることを、分担研究者の小林らとともに平成18年度に発表した(Sakaki-Yumoto et al., Development, 2006)。次いで、腎臓発生においてSall1の上流で機能するSix1のGFP ES細胞（Kobayashi et al., Mech Dev, 2007）についても検討したが、発現が筋肉など広汎なため適さないと判断した。

4) Osr1-GFP ES細胞及びマウスの作成

ES細胞のアクチビンとレチノイン酸処理において、転写因子Osr1が極めて再現性よく誘導されることを見出した。この遺伝子は、腎臓が発生してくる中間中胚葉と後腎間葉に強く発現し、その欠失マウスはSall1やSix1/4よりも初期に腎臓発生異常をきたすことが報告されている。そこでこの遺伝子座にGFPを組み込んだESをノックイン法で作成し、さらに得られたマウスにおいて中間中胚葉及び後腎間葉が蛍光発色することを確認した(図4)。GFP陽性の後腎間葉を使ったマイクロアレイからは、Sall1-GFPを凌ぐ興味深い遺伝子プロファイルを得ており、Osr1-GFPが腎臓前駆細胞の極めて有用な指標であ

ることが判明した。

5) Osr1-GFP ESの分化誘導

このES細胞をアクチビンとレチノイン酸で処理するとGFPの強度が増加した。このGFP陽性細胞はPDGFR陽性の沿軸中胚葉画分から派生することも明らかになった（図4）。

（倫理面への配慮）

実験動物は、麻酔下での手術を行い、検体採取時には安楽死させ、無用な苦痛を与えないようガイドラインに従って取り扱う。

C. 研究結果

ES細胞をアクチビンとレチノイン酸で処理すると、腎臓前駆細胞で発現するOsr1陽性の集団が誘導されることが明らかになった。Osr1-GFPマウスから採取されるin vivoでの腎臓前駆細胞との類似性を確認しつつ、誘導効率の改善を図りたい。また最近報告されたヒトiPS細胞を使って同様の試みがヒトでも可能かを検討したい。

D. 考察

腎臓前駆細胞の検定系の確立や、Sall遺伝子のESにおける役割の解明など、科学として相当レベルの貢献はできたと考えている。しかし究極目標であるESからの腎臓細胞誘導は、まだ道半ばである。計画の遅れの原因は、腎臓特異的マーカーが存在しないこと及びコロニーアッセイで検出できるほどESからの分化が進んでいないことが考えられる。ようやくその候補となる細胞集団の単離に成功したので、誘導法の改善を急ぎたい。

E. 結論

ES細胞をアクチビンとレチノイン酸で処理し、腎臓前駆細胞で発現する遺伝子を指標にすることによって、その候補集団の単離が可能になった。

F. 健康危険情報

本研究はヒトを対象とした検討を行っていないので、該当する情報はない。

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○由利俊祐、山口泰華、西中村隆一 Sall4 partially maintains stemness of embryonic stem cells. 第 30 回日本分子生物学会・第 80 回日本生化学会合同大会　2007 年 12 月 12 日、横浜

○西中村隆一　Sall ファミリーによる腎臓形成機構　第 4 回宮崎サイエンスキャンプ　2008 年 2 月 16 日、宮崎

H. 知的財産権の出願・登録状況

1. 特許取得
   誘導法について出願を検討中
2. 実用新案登録
   なし
3. その他
   なし

図1. 1個の後腎間葉細胞からのコロニー形成

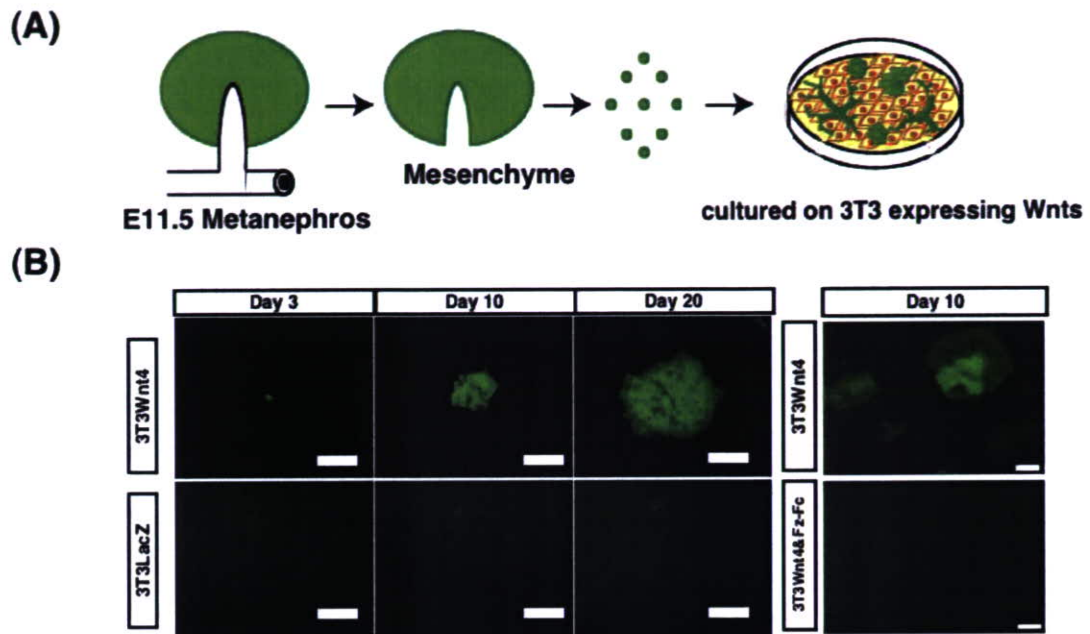


図2. 3次元構造の再構築

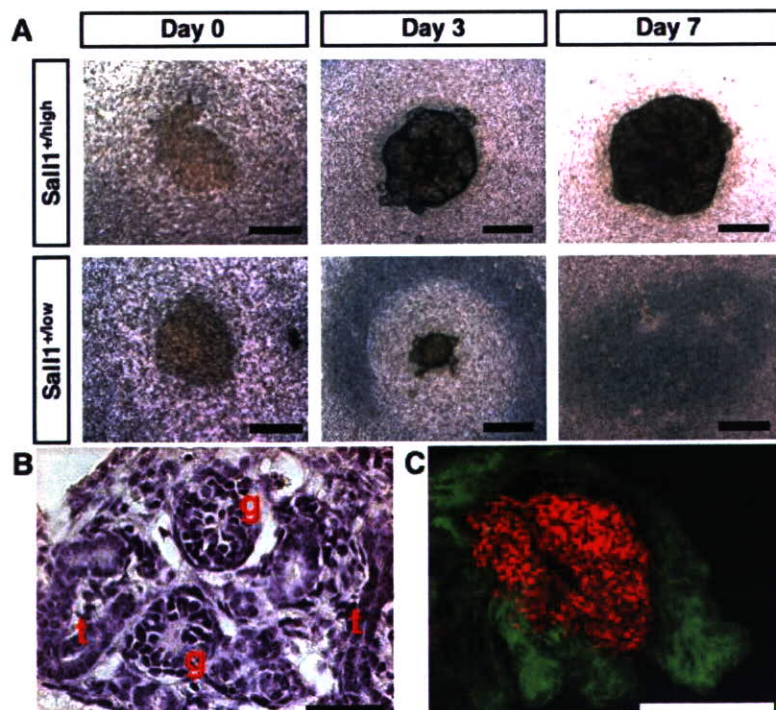


図3. 中間中胚葉及び後腎間葉からの腎臓発生

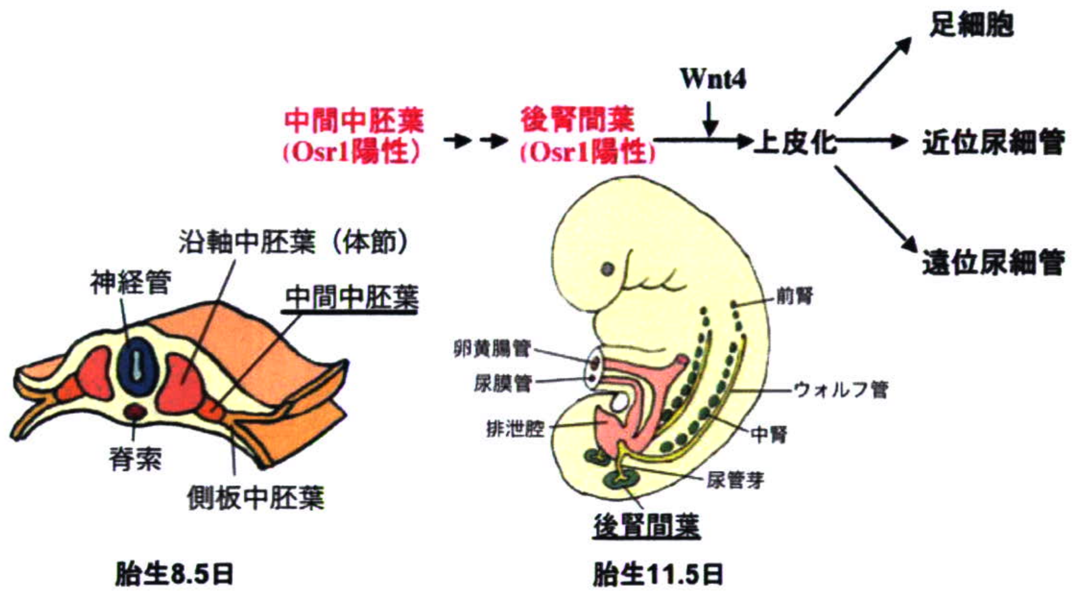
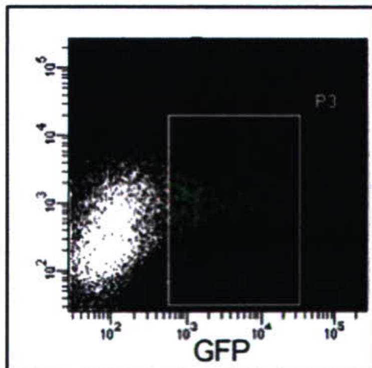


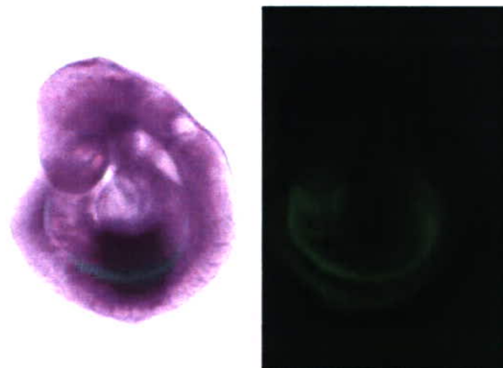
図4. ESからのOsr1-GFP陽性細胞誘導

Osr1-GFP ES



ESからの誘導成功

Osr1-GFPマウス



中間中胚葉の蛍光発色

厚生労働科学研究費補助金（ヒトゲノム・再生医療等研究事業）
分担　研究報告書

ES細胞と腎臓に共通する分子機構の解明

分担研究者　小林　千余子　熊本大学発生医学研究センター　助教

研究要旨

Sall はショウジョウバエからヒトまで保存された zinc フィンガー蛋白で、哺乳類では４種類(Sall1-4)存在する。マウス Sall1 は腎臓発生に必須であり、Sall1 を欠失する後腎間葉の前駆細胞は増殖が低下している。これに対して Sall4 のノックアウトは子宮着床直後に死亡し、胚盤胞の内部細胞塊からの増殖障害が観察された。胚性幹細胞(ES 細胞)は内部細胞塊から由来するため、Sall4 を欠損する ES 細胞を作製したところ、同様に増殖異常が認められた。これらの結果から、腎臓と ES 細胞に Sall ファミリー遺伝子を介する共通の機構が存在することが示唆された。さらに Sall4 欠失 ES 細胞では、胎盤遺伝子の発現上昇が認められ、Sall4 が幹細胞の未分化維持に働いていることが示唆された。

A. 研究目的

日本で腎不全により人工透析を受ける人はこの 10 年で２倍となり、そのコストは１兆円と医療費の 3%を占めるに至っている。にもかかわらず、腎機能を回復させる画期的な治療法はいまだ存在せず、最終的には透析導入となる。腎臓の再生は確かに困難な課題であるが、そのヒントはその発生機構にあると考え、我々は腎臓発生の分子機構解明に取り組んでいる。

本計画は、Sall ファミリー遺伝子を中心として、<u>ES 細胞と腎臓に共通の機構を解明することによって、腎臓再生への足がかりを得る</u>ことを目的とする。Sall はショウジョウバエからヒトまで保存された zinc フィンガー蛋白で、哺乳類では４種類(Sall1-4)存在する。我々は以前マウス Sall1 を発生期腎臓から単離し、このノックアウトマウスは腎臓を欠損することを見いだした。腎臓の形成不全を伴うヒト SALL1 の変異も報告されており、種を越えた Sall1 の機能が提唱されている。しかしその分子機構は、発生期腎臓の小ささによる生化学的解析の困難さから解明が進んでいない。一方 Sall4 のノックアウトマウスは胎生 5.5 日という極めて初期に死亡し、胚盤胞の内部細胞塊からの増殖障害が観察された。胚性幹細胞(ES 細胞)は内部細胞塊から由来するため、Sall4 を欠損する ES 細胞を作製することができれば、ES 細胞は試験管内で無限に増殖するため、生化学的にその詳細な機能が解明できると考えた。そしてその知見を腎臓形成にフィードバックすることを目的とした。

B. 研究方法

1) ES 細胞における Sall4 の機能解明

Sall4 のノックアウトは胎生 5.5 日とい

う極めて初期に死亡し、胚盤胞の内部細胞塊からの増殖障害が観察された。内部細胞塊から由来する細胞株が胚性幹細胞(ES 細胞)である。そこで Sall4 を欠損する ES 細胞を作製した。条件付きノックアウト作成の技法で、loxP 配列で Sall4 のエクソンをはさんだ ES 細胞（Flox Sall4）を一旦作成し、そこに Cre を導入することで、Sall4 欠失 ES 細胞を単離した。得られた ES 細胞について、増殖、分化の検討を行った。

2) ES 細胞における Sall1 の機能解析

ES 細胞には Sall1 も発現しており、Sall1 に対しても条件付きノックアウト作成の技法で、ES 細胞及びマウス(Flox Sall1)を作成した。さらに Flox Sall1/Sall4 二重ホモマウスを作成し、その胚盤胞から ES を単離した。そこに Cre を導入して、Sall1/4 二重欠失 ES 細胞を作成した。

（倫理面への配慮）

実験動物は、麻酔下での手術を行い、検体採取時には安楽死させ、無用な苦痛を与えないようガイドラインに従って取り扱った。

C. 研究結果

1) ES細胞におけるSall4の機能解明

条件付きノックアウト作成の技法で、Sall4 欠失 ES 細胞を単離することに成功した。この細胞は非常に増殖が遅く、Sall4 が ES 細胞の増殖に必須であることが明らかになった。これに対して未分化能や形態は保たれており、増殖低下が異常な分化のためではないことが示された (Sakaki-Yumoto et al. 2006)。

これらはフィーダー上で培養した所見であるが、フィーダーを外した場合、Sall4 欠失 ES 細胞では、Oct3/4 の発現を保ったまま長期培養が可能なものの、増殖低下とともに自発的分化傾向がみられ、胎盤方向への分化を促す Cdx2 の発現が上昇した。単一細胞レベルでも、Oct3/4 陽性の幹細胞コロニーの形成率が低下しており、自己複製効率の低下が示唆された。これらの結果は、ES 細胞を未分化な状態で維持することに Sall4 が必須である可能性を示唆する。ES 細胞から Sall4 を含む蛋白複合体を同定したところ、Sall4 は Mi2/NuRD という HDAC を含む複合体に結合しており、Sall4 がヒストンのアセチル化やメチル化を変えて Cdx2 を含む標的遺伝子を制御している機構が考えられた。

2) ES 細胞における Sall1 の機能解析

Sall1/4 二重欠失 ES 細胞においても、増殖低下及び Cdx2 の上昇は認められたものの、Sall4 単独欠失と有意な差はなかった。よって Sall1 は ES 細胞には必須ではないと考えられる。

D. 考察

Sall4 は、ES 細胞において胎盤遺伝子の発現を抑制することによって、未分化状態を安定に維持していると考えられる。ES 細胞は遺伝子導入などが容易でかつ大量に増やせるので、遺伝学的生化学的解析に適している。他グループから Sall4 に Nanog や Oct3/4 といった ES 細胞必須遺伝子が結合するという報告もあり、ES 細胞という未分化な細胞群の維持に Sall4 がど

のような機構で関わっているのかを解析していきたい。それを腎臓に応用することによって、Sall1 の腎臓における機構をも解明したい。

E. 結論

Sall4 は ES 細胞の未分化性を維持していることが示唆された。Sall4 の ES 細胞における分子機構を解明することは、Sall4 に依存する ES 細胞が、Sall1 に制御される腎臓前駆細胞にどのように変化していくかのヒントになると考えられる。

F. 研究発表

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2. 学会発表

なし

G. 知的財産権の出願・登録状況

特になし

研究成果の刊行に関する一覧表

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Six1 and *Six4* are essential for *Gdnf* expression in the metanephric mesenchyme and ureteric bud formation, while *Six1* deficiency alone causes mesonephric-tubule defects

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Abstract

Interaction between the ureteric-bud epithelium and the metanephric mesenchyme is important for kidney development. *Six1* and *Six4* are the mammalian homologs of *Drosophila sine oculis*, and they are coexpressed in the nephrogenic mesenchyme. *Six1*-deficient mice show varying kidney defects, while *Six4*-deficient mice have no apparent abnormalities. Here, we report *Six1/Six4*-deficient mice that we generated in order to elucidate the functions of *Six4* in *Six1*-deficient kidney development. The *Six1/Six4*-deficient mice exhibited more severe kidney phenotypes than the *Six1*-deficient mice; kidney and ureter agenesis was observed in all the neonates examined. The *Six1/Six4*-deficient metanephric mesenchyme cells were directed toward kidney lineage but failed to express *Pax2*, *Pax8*, or *Gdnf*, whereas the expression of these genes was partially reduced or unchanged in the case of *Six1* deficiency. Thus, *Six4* cooperates with *Six1* in the metanephric mesenchyme to regulate the level of *Gdnf* expression; this could explain the absence of the ureteric bud in the *Six1/Six4*-deficient mice. In contrast, *Six1* deficiency alone caused defects in mesonephric-tubule formation, and these defects were not exacerbated in the *Six1/Six4*-deficient mesonephros. These results highlight the fact that *Six1* and *Six4* have collaborative functions in the metanephros but not in the mesonephros.
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Keywords: *Six1*; *Six4*; Kidney development; *Sall1*; *Gdnf*; *Pax2*; *Pax8*

1. Introduction

In mammals, kidney development occurs in three successive steps – initial pronephros formation followed by mesonephros formation and finally, metanephros formation. In mice, development of the first kidney – the pronephros – is initiated between embryonic day 8.0 and 8.5 (E8.0–E8.5) by signals from the paraxial mesoderm and surface ectoderm. These signals induce the intermediate mesoderm to differentiate into the Wolffian duct (nephric duct). Next, the nephrogenic cord – the mesenchymal tissue – is induced at the ventral side of the Wolffian duct (Obara-Ishihara et al., 1999; Mauch et al., 2000; James and Schultheiss, 2003). The Wolffian duct and nephrogenic cord elongate caudally towards the cloaca, and the Wolffian duct converts the adjacent mesenchyme (nephrogenic cord) into mesonephric tubules between E9.0 and E11.0. When the Wolffian duct reaches the level of the developing

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hindlimb at E10.0, the ureteric bud evaginates from the Wolffian duct and invades the surrounding metanephric mesenchyme, which is formed as a bulge at the caudal-most region of the nephrogenic cord. Subsequently, both the metanephric mesenchyme and ureteric bud undergo reciprocal inductive interactions to form the nephrons and collecting ducts of the metanephros – the third and the adult kidney (Grobstein, 1953; Saxen, 1987). Thus, the two mesenchymal tissues – the nephrogenic cord and metanephric mesenchyme – constitute the nephrogenic mesenchyme.

During metanephros development, the glial cell line-derived neurotrophic factor (Gdnf) secreted from the mesenchyme acts on the receptor tyrosine kinase Ret and its co-receptor Gfra1, both of which are expressed in the Wolffian duct, and it induces ureteric-bud formation from the Wolffian duct. Thus, mice that are deficient in *Gdnf*, *Ret*, or *Gfra1* show similar phenotypes such as failure of ureteric-bud invasion into the mesenchyme (Durbec et al., 1996; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Sainio et al., 1997b; Cacalano et al., 1998; Enomoto et al., 1998; Sariola and Saarma, 1999). Several mesenchymal transcription factors also play important roles in metanephros formation (reviewed by Vainio and Lin, 2002). *Pax2* is expressed both in the metanephric mesenchyme and ureteric-bud, and *Pax2*-deficient mice show a reduced mesenchyme and failure in ureteric-bud invasion (Torres et al., 1995). *Pax2* has been shown to bind the *Gdnf* promoter, and this may explain the reduced *Gdnf* expression and the ureteric-bud phenotype in the mutant mice (Brophy et al., 2001). *Sall1* is expressed exclusively in the mesenchyme, and mice lacking this gene show phenotypes similar to those of *Pax2*-null mice (Nishinakamura et al., 2001). However, *Gdnf* and *Pax2* expression remains unaffected in *Sall1*-deficient mice, while *Sall1* continues to be expressed in *Pax2*-mutant mice. This suggests that *Sall1* may function independent of the *Pax2–Gdnf* pathway. *Wt1*-deficient mice also exhibit similar phenotypes; however, *Gdnf* expression is not affected in these mice (Kreidberg et al., 1993; Sainio et al., 1997b). Mice lacking *Osr1* do not develop the metanephric mesenchyme, and they do not express *Pax2*, *Gdnf*, or *Sall1*; this implies that *Osr1* is one of the most upstream genes in the metanephric cascade (James and Schultheiss, 2005; Wang et al., 2005; James et al., 2006).

The mesonephros comprises the mesonephric tubules and the Wolffian duct. Two types of mesonephric tubules are formed along the Wolffian duct. The first type comprises a few pairs of cranial mesonephric tubules, which develop as outgrowths from the Wolffian duct and later become epididymal ducts in males. The other type comprises caudal mesonephric tubules, which constitute a majority of the tubules and are formed in the nephrogenic cord – a mesenchymal tissue – upon induction by the Wolffian duct (Sainio et al., 1997a). Further, some of the genes involved in metanephros formation also play important roles in mesonephros development. *Pax2*-deficient mice show defects in mesonephric-tubule formation and Wolffian-duct elongation (Torres et al., 1995). *Wt1*-deficient mice lack the caudal mesonephric tubules but not the cranial tubules (Kreidberg et al., 1993; Sainio et al., 1997a). *Osr1* deficiency leads to asymmetric defects in Wolffian-duct elongation and to failure in mesonephric-tubule formation (James and Schultheiss, 2005; Wang et al., 2005; James et al., 2006). In contrast to these genes that are expressed in the nephrogenic cord (and also in the Wolffian duct in the case of *Pax2*), *Wnt9b* is expressed exclusively in the Wolffian duct and induces the nephrogenic cord to transform into mesonephric-tubule epithelia. Thus, *Wnt9b*-deficient mice lack mesonephric tubules (Carroll et al., 2005).

The initial specification toward the nephrogenic lineage from the intermediate mesoderm is beginning to be elucidated. *Pax2* and *Pax8* are coexpressed in the Wolffian duct and in the pro- and mesonephric tubules, and *Pax2*/*Pax8*-deficient mice exhibit complete absence of the pro-, meso-, and metanephros (Bouchard et al., 2002), suggesting redundant roles of the two transcription factors in kidney-lineage commitment. The homeodomain protein Lim1 is expressed early in the intermediate mesoderm, Wolffian duct, and pro- and mesonephric tubules (Fujii et al., 1994), and is required for kidney development (Kobayashi et al., 2005). In *Lim1*-deficient mice, the intermediate mesoderm is disorganized and fails to express *Pax2* – a prerequisite for kidney development (Tsang et al., 2000).

The *Six* homeobox genes are characterized by conserved six domains and a homeodomain, both of which are required for specific DNA binding. This gene family is essential for compound-eye development, and the prototype of this gene family is *Drosophila sine oculis*. Six members (*Six1–Six6*) of the *Six* gene family have been identified in mice and humans (Kawakami et al., 2000). *Six3* and *Six6* are essential for forebrain formation and eye development (Kobayashi et al., 1998; Loosli et al., 1999; Zuber et al., 1999; Carl et al., 2002; Zhu et al., 2002; Lagutin et al., 2003; Lopez-Rios et al., 2003), whereas *Six5* is involved in cataractogenesis and spermatogenesis (Sarkar et al., 2000, 2004). Several papers, including ours, report that *Six1*-deficient mice show anomalies in the development of various organs such as the inner ear, nose, thymus, kidney, and skeletal muscle (Laclef et al., 2003a,b; Zheng et al., 2003; Ozaki et al., 2004; Zou et al., 2004, 2006). During renal development, *Six1* is required for ureteric-bud invasion into the metanephric mesenchyme although variation among animals exists. In *Six1* deficiency, the metanephric mesenchyme is formed, although it is small. Further, expression of *Sall1* and *Six2* is absent, while that of *Pax2* and *Gdnf* is only partially reduced in this condition (Li et al., 2003; Xu et al., 2003). *Eya1*, a murine homolog of the *Drosophila eyes absent* gene, functions as a coactivator of the *Six* family genes. *Eya1*-deficient mice exhibit more severe kidney phenotypes than *Six1*-null mice – absence of the metanephric mesenchyme and ureteric-bud and complete reduction in the expression levels of *Pax2* and *Gdnf* (Xu et al., 1999; Nica et al., 2006). Thus, molecules other

than *Six1* that possibly cooperate with *Eya1* in kidney development might exist.

Six4, another member of the *Six* family, is separated from the *Six1* gene on the same chromosome by only 100 kb; its expression overlaps with that of *Six1* in many regions such as the neural placodes, Rathke's pouch, dorsal root ganglia, dermomyotome, myotome, limb bud mesenchyme, and myogenic migrating precursors (Grifone et al., 2005). Interestingly, as we previously demonstrated, *Six4*-deficient mice exhibit no major developmental defects and are fertile (Ozaki et al., 2001). Since Six1 and Six4 exhibited a similar binding specificity to the MEF3 site (TCAGGTTC), the roles played by *Six1* and *Six4* in embryogenesis may be redundant (Spitz et al., 1998; Himeda et al., 2004). The first evidence of this assumption was observed during myogenesis; *Six1* and *Six4* contribute to myogenic migration in somites by regulating *Pax3* and *Met* expression (Grifone et al., 2005). By independently generating *Six1*/*Six4*-deficient mice, we here demonstrate that these two transcription factors (*Six1* and *Six4*) have overlapping functions in kidney development. The *Six1*/*Six4*-deficient mice exhibited more severe kidney phenotypes than the *Six1*-deficient mice. The expression of *Pax2*, *Pax8*, and *Gdnf* was completely lost in the *Six1*/*Six4*-deficient metanephric mesenchyme, while it was partially reduced or unchanged in the *Six1*-deficient mice. These results indicate that *Six1* and *Six4* are required for metanephric mesenchyme development.

2. Results

2.1. Six1 and Six4 are coexpressed in the nephrogenic cord and metanephric mesenchyme

To demonstrate the overlapping expression patterns of *Six1* and *Six4* in the mesenchyme of developing renal tissues, we performed in situ hybridization for these two genes by using sections of E9.5 and E10.5 embryos, as described in Fig. 1A and B. In addition to its expression in somites (Oliver et al., 1995), *Six1* was expressed in the nephrogenic cord on E9.5 and in the metanephric mesenchyme on E10.5 but not expressed in the Wolffian duct (Fig. 1C–E). The domains of *Six4* expression were similar to those of *Six1*. *Six4* expression, although weak, was also detected in the nephrogenic cord and metanephric mesenchyme, both of which are mesenchymal components of the developing kidney (Fig. 1F–H).

2.2. Kidney phenotypes are exacerbated in Six1/Six4-deficient mice

Since *Six1* and *Six4* are on the same chromosome and closely linked, we introduced the *Six1*-targeting vector into *Six4*-heterozygous embryonic stem cells, and we generated strains with mutations in both the genes. As recently described in our paper, the resultant *Six1*/*Six4*-heterozygous mice appeared to be normal and fertile. Further, after heterozygous crossing, mice homozygous for *Six1* and *Six4* were born at the expected Mendelian frequency (Konishi et al., 2006). However, the *Six1*/*Six4*-deficient mice died soon after birth and showed developmental defects in various organs; this finding is consistent with a previous report (Grifone et al., 2005).

Further, by examining the kidney abnormalities in the *Six1*/*Six4*-deficient mice, we noted that they exhibited more severe phenotypes than the *Six1*/*Six4*-heterozygous or *Six1*-deficient mice. None of the *Six1*/*Six4*-heterozygous neonate mice showed developmental defects in the kidneys and ureters (Fig. 2A, Table 1). Of the *Six1*-deficient mice, 30% exhibited uni- or bilateral renal hypoplasia (Fig. 2B), and the remaining 70% exhibited kidney agenesis and had short ureters (Fig. 2C). In contrast, ureters and bilateral kidneys failed to develop in all the *Six1*/*Six4*-deficient mice, whereas the adrenal glands, urinary bladder, and genital tracts were formed without any apparent defects (Fig. 2D, Table 1).

Next, in the E11.5 embryos, we examined ureteric-bud invasion into the metanephric mesenchyme and the subsequent metanephric-mesenchyme condensation, an essential step for kidney and ureter formation. We stained the kidneys with an antibody against E-cadherin, which is expressed in the Wolffian duct and ureteric bud. A whole-mount view of the E-cadherin staining revealed that the ureteric bud originated from the Wolffian duct and branched in the *Six1*/*Six4*-heterozygous embryos (Fig. 2E). In the *Six1*-deficient embryos, the ureteric bud was formed but showed defects in elongation and branching (Fig. 2F and G). In contrast, ureteric-bud formation was never detected in the *Six1*/*Six4*-deficient embryos (Fig. 2H). Hematoxylin-eosin (HE)-stained sections of the E11.5 *Six1*/*Six4*-heterozygous embryos revealed no developmental defects in the invasion of the ureteric bud and condensation of the metanephric mesenchyme adjacent to the ureteric bud (Fig. 2I). In the *Six1*-deficient embryos, ureteric-bud invasion partially occurred, but variations were observed with regard to ureteric bud development and metanephric-mesenchyme condensation (Fig. 2J). In contrast, none of the *Six1*/*Six4*-deficient embryos showed ureteric-bud invasion and subsequent metanephric-mesenchyme condensation (Fig. 2K).

Further, prior to ureteric-bud invasion into the metanephric mesenchyme, we examined the metanephric mesenchyme at E10.5 by performing HE staining of the sections. The metanephric mesenchyme appeared as a distinct cell cluster adjacent to the Wolffian duct in the *Six1*/*Six4*-heterozygous (Fig. 2L and L′) and *Six1*-deficient embryos (Fig. 2M and M′), whereas this cell cluster was not observed in the *Six1*/*Six4*-deficient embryos (Fig. 2N and N′; arrowhead). Thus, the absence of *Six4* exacerbated *Six1*-deficient kidney phenotypes, suggesting that the transcription factors Six1 and Six4 cooperatively regulate the early stages of metanephros development and ureteric bud formation.

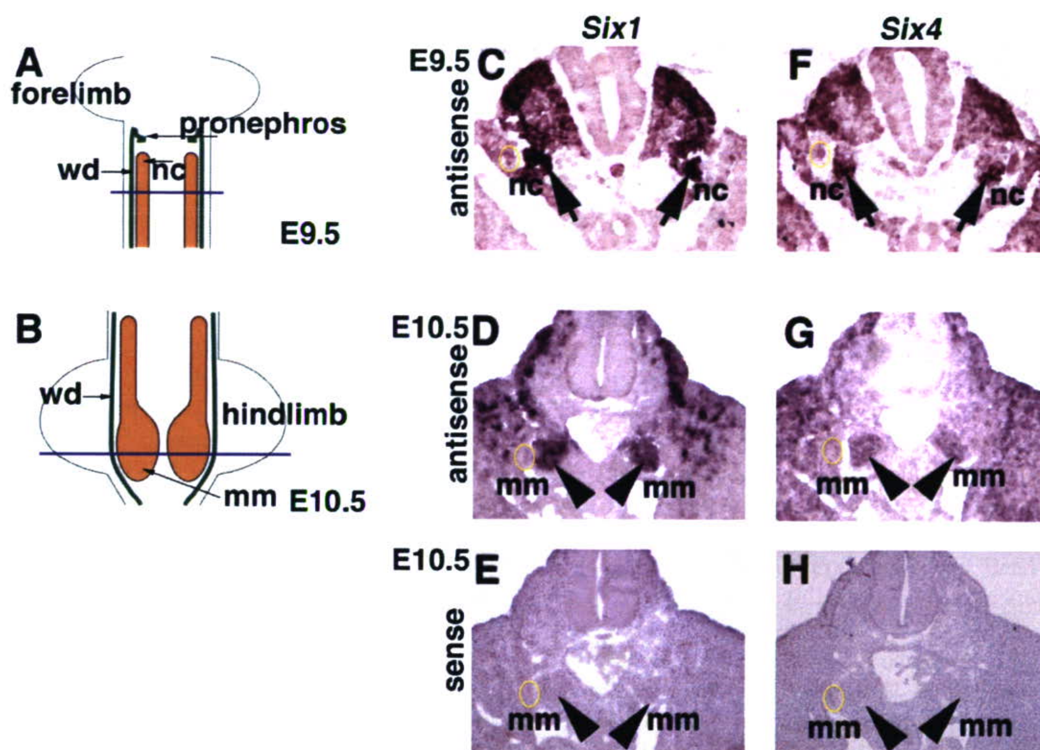

Fig. 1. Overlapping expression patterns of *Six1* and *Six4* in the nephrogenic mesenchyme. (A and B) Nephrogenic cord at E9.5 (A) and metanephric mesenchyme at E10.5 (B). Blue lines in A and B represent the planes along which the sections in C–H were sliced. (C–E) *Six1* expression in the developing kidneys in the E9.5 (C) and E10.5 (D) embryos. The sense probe produced no signals (E). (F–H) *Six4* expression in the developing kidneys in the E9.5 (F) and E10.5 (G) embryos. The sense probe produced no signals (H). *Six1* and *Six4* are coexpressed in the nephrogenic cord (arrow) in E9.5 embryos as well as in the metanephric mesenchyme (arrowhead) in the E10.5 embryos. The yellow circle indicates the Wolffian duct (nc, nephrogenic cord; mm, metanephric mesenchyme).

2.3. Pax2, Sall1, and Gdnf were absent in the Six1/Six4-deficient metanephric mesenchyme

To examine apoptosis, we carried out a TdT-mediated dUTP-digoxigenin nick end labeling (TUNEL) assay; however, apoptosis-positive cells were not detected in the metanephric mesenchyme of the *Six1/Six4*-heterozygous, *Six1*-deficient, or *Six1/Six4*-deficient embryos (Fig. 3A–C), suggesting that the absence of the mesenchymal cluster in *Six1/Six4* deficiency was not due to increased apoptosis in this region. Furthermore,expression of *EGFP* driven by the *Six1* locus (refer to Section 4), *Osr1*, and *Wt1*, which are known to be expressed at an early stage in kidney precursors (Kreidberg et al., 1993; James et al., 2006), was detected in the metanephric mesenchyme region of the *Six1/Six4*-deficient embryos as well as in the *Six1/Six4*-heterozygous and *Six1*-deficient embryos (Fig. 3D–L). This implied that the *Six1/Six4*-deficient metanephric mesenchymal cells were directed toward kidney lineage. In contrast, *Gdnf*, *Sall1*, and *Pax2* expression was severely affected in the *Six1/Six4*-deficient embryos. *Gdnf* was expressed in the metanephric mesenchyme in the *Six1/Six4*-heterozygous embryos (Fig. 3M), and it was weakly expressed in the *Six1*-deficient embryos (Fig. 3N). However, *Gdnf* expression was absent in the *Six1/Six4*-deficient embryos (Fig. 3O). These results are consistent with the complete absence of the ureteric bud in the *Six1/Six4*-deficient embryos and with its partial impairment in the *Six1*-deficient embryos because *Gdnf* is a critical regulator of ureteric budding. *Sall1* was expressed in the metanephric mesenchyme in the *Six1/Six4*-heterozygous embryos (Fig. 3P), but its expression was markedly lower in the *Six1*-deficient embryos and absent in the *Six1/Six4*-deficient embryos (Fig. 3Q and R). *Pax2* was expressed both in the metanephric mesenchyme and Wolffian duct in the *Six1/Six4*-heterozygous embryos (Fig. 3S). *Pax2* expression was lowered in the metanephric mesenchyme but not in the Wolffian duct in the *Six1*-deficient embryos, whereas it was completely absent in the *Six1/Six4*-deficient embryos (Fig. 3T and U). These results genetically place *Six1* and *Six4* downstream of *Osr1* and *Wt1* and upstream of *Gdnf*, *Sall1*, and *Pax2*. These results also imply that *Six1* and *Six4* redundantly regulate *Pax2* and *Gdnf* expression in the metanephric mesenchyme.

2.4. Six1 and Six4 regulate Pax8 in the metanephric mesenchyme

Together with *Pax2*, *Pax8* is also known to play a role in pro- and mesonephros development from the intermediate

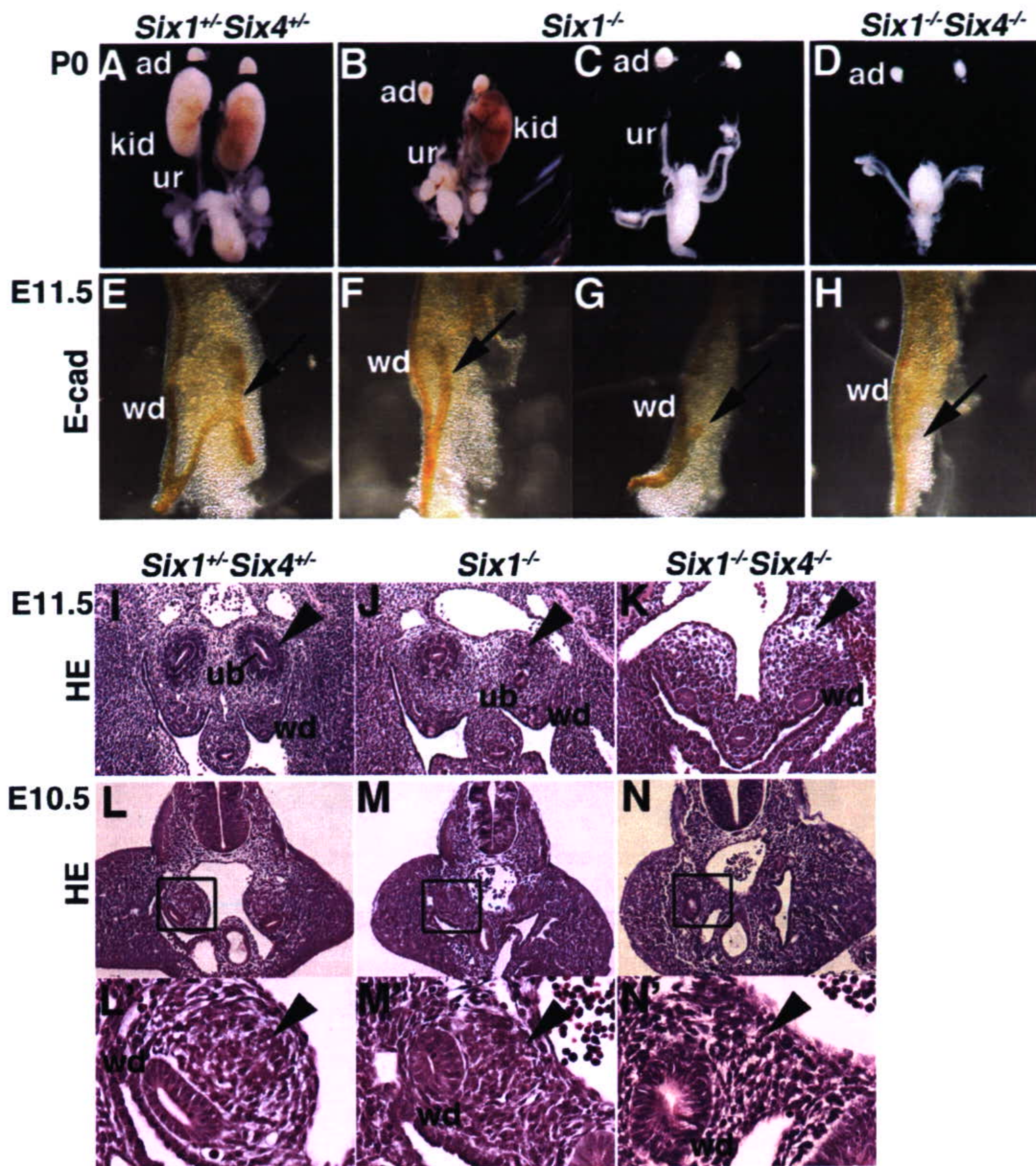

Fig. 2. *Six1/Six4*-deficient mice exhibit more severe renal defects than *Six1*-deficient mice. (A–D) Whole-mount views of urogenital tissues of the *Six1/Six4*-heterozygous (A), *Six1*-deficient (B and C), and *Six1/Six4*-deficient (D) newborn mice. Of the *Six1*-deficient mice, 30% exhibited uni- or bilateral renal hypoplasia (B) and the rest exhibited kidney agenesis (C). In contrast, all the *Six1/Six4*-deficient mice completely lacked kidney and ureter formation (D). (E–H) Whole-mount views of E-cadherin staining in the metanephric region of the E11.5 *Six1/Six4*- heterozygous (E), *Six1*-deficient (F and G), and *Six1/Six4*-deficient (H) embryos. The ureteric buds (arrow) were short and unbranched in the *Six1*-deficient embryos, while they were absent in the *Six1/Six4*-deficient embryos. (I–K) HE staining of the metanephric region of the E11.5 *Six1/Six4*-heterozygous (I), *Six1*-deficient (J), and *Six1/Six4*-deficient (K) embryos. The condensed mesenchyme (black arrowhead) was reduced, and the invasion of the ureteric bud into the mesenchyme was partially impaired in the *Six1*-deficient embryos (J). The mesenchymal cell cluster and the ureteric bud were not detected in the *Six1/Six4*-deficient embryos (K). (L–N) HE staining of the metanephric region of the E10.5 *Six1/Six4*-heterozygous (L), *Six1*-deficient (M), and *Six1/Six4*-deficient (N) embryos. (L′–N′) The lower panels are at a higher magnification than the upper panels (L–N). The mesenchymal cell cluster (arrowhead) was not detected in the *Six1/Six4*-deficient embryos (kid, kidney; ur, ureter; ad, adrenal gland; wd, Wolffian duct; ub, ureteric bud).

Table 1
Kidney abnormalities in newborn mutants

Genotype	Normal kidney	Hypoplastic kidney[a]	Kidney agenesis[b]	Kidney & ureter agenesis
$Six1^{+/-}Six4^{+/-}$	8/8	0/8	0/8	0/8
$Six1^{-/-}$	0/20	6/20	14/20	0/20
$Six1^{-/-}Six4^{-/-}$	0/15	0/15	0/15	15/15

[a] Bilateral hypoplastic kidneys or unilateral agenesis.
[b] A lack of bilateral kidneys but not ureters.

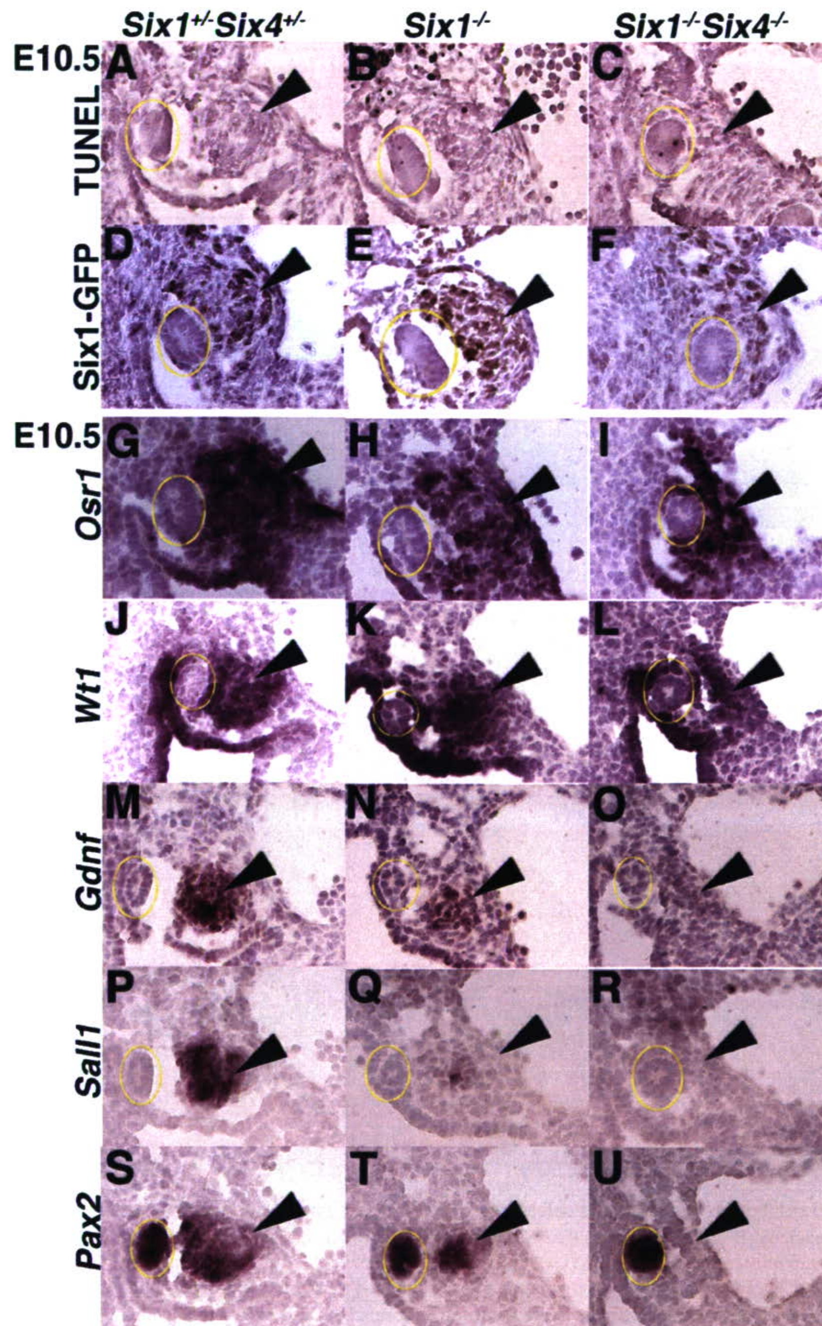

Fig. 3. *Pax2*, *Sall1*, and *Gdnf* expression is absent in the *Six1*/*Six4*-deficient metanephric mesenchyme. (A–C) TUNEL analysis of the metanephric region in the E10.5 *Six1*/*Six4*-heterozygous (A), *Six1*-deficient (B), and *Six1*/*Six4*-deficient (C) embryos. TUNEL-positive cells were not detected in the *Six1*/*Six4*-deficient metanephric mesenchyme (arrowhead). (D–F) EGFP staining in the metanephric region in the E10.5 *Six1*/*Six4*-heterozygous (D), *Six1*-deficient (E), and *Six1*/*Six4*-deficient (F) embryos. *EGFP* was knocked in the *Six1* locus. EGFP-positive cells were detected in the *Six1*/*Six4*-deficient metanephric mesenchyme. (G–I) In situ hybridization of *Osr1* in the metanephric region of the E10.5 *Six1*/*Six4*-heterozygous (G), *Six1*-deficient (H), and *Six1*/*Six4*-deficient (I) embryos. *Osr1* expression in the metanephric mesenchyme was unaffected in the *Six1*/*Six4*-deficient embryos. (J–L) *Wt1* expression in the metanephric region of the E10.5 *Six1*/*Six4*-heterozygous (J), *Six1*-deficient (K), and *Six1*/*Six4*-deficient (L) embryos. *Wt1* expression was intact in the mutant mice. (M–O) *Gdnf* expression in the metanephric region of the E10.5 *Six1*/*Six4*-heterozygous (M), *Six1*-deficient (N), and *Six1*/*Six4*-deficient (O) embryos. *Gdnf* expression was reduced in the *Six1*-deficient embryos and absent in the *Six1*/*Six4*-deficient mesenchyme. (P–R) *Sall1* expression in the metanephric region of the E10.5 *Six1*/*Six4*-heterozygous (P), *Six1*-deficient (Q), and *Six1*/*Six4*-deficient (R) embryos. *Sall1* expression was reduced in the *Six1*-deficient embryos and absent in the *Six1*/*Six4*-deficient mesenchyme. (S–U) *Pax2* expression in the metanephric region of the E10.5 *Six1*/*Six4*-heterozygous (S), *Six1*-deficient (T), and *Six1*/*Six4*-deficient (U) embryos. *Pax2* expression was reduced in the *Six1*-deficient mesenchyme and absent in the *Six1*/*Six4*-deficient mesenchyme. *Pax2* expression in the Wolffian duct remained intact in the mutant mice. The yellow circle indicates the Wolffian duct.

mesoderm (Bouchard et al., 2002). *Pax8* expression was not detected in the metanephric region at E9.5 or E10.5, while it was detected in the mesonephric region (Fig. 4A–F). However, at E10.0, *Pax8* was expressed in the metanephric mesenchyme and Wolffian duct (Fig. 4E, arrowhead) as well as in the nephrogenic cord and Wolffian duct in the mesonephric region (Fig. 4B, arrow). These results show that *Pax8* was transiently expressed in the metanephric region at E10.0.

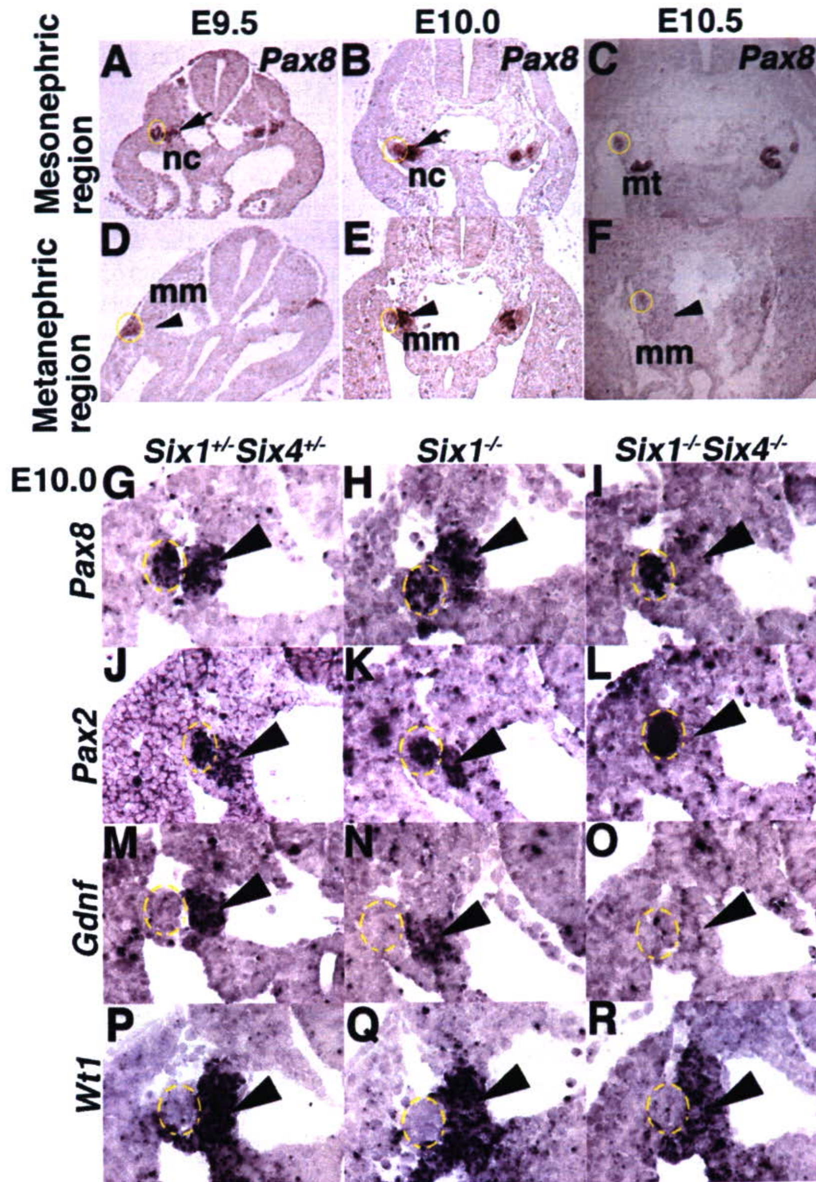

Fig. 4. *Pax8* expression is absent in the metanephric mesenchyme in *Six1*/*Six4*-deficient mice. (A–F) Transient expression of *Pax8* in the metanephric mesenchyme. *Pax8* expression in either the mesonephric (A–C) or the metanephric region (D–F) of the wild-type embryos at E9.5 (A and D), E10.0 (B and E), and E10.5 (C and F). *Pax8* expression was detected in the nephrogenic cord (arrow) and Wolffian duct (yellow circle) in the E9.5 (A) and E10.0 (B) embryos, and it was also detected in the mesonephric tubules and Wolffian duct in the E10.5 embryos (C). *Pax8* was not expressed in the metanephric mesenchyme (arrowhead) at E9.5 (D) and E10.5 (F), while it was expressed in the metanephric mesenchyme at E10.0 (E). (G–I) *Pax8* expression in the E10.0 *Six1*/*Six4*-heterozygous (G), *Six1*-deficient (H), and *Six1*/*Six4*-deficient (I) embryos. *Pax8* was expressed in the metanephric mesenchyme (arrowhead) and Wolffian duct (yellow circle) in the *Six1*/*Six4*-heterozygous (G) and *Six1*-deficient (H) embryos but not in the mesenchyme in the *Six1*/*Six4*-deficient (H) embryos. (J–L) *Pax2* expression in the E10.0 *Six1*/*Six4*-heterozygous (J), *Six1*-deficient (K), and *Six1*/*Six4*-deficient (L) embryos. *Pax2* was expressed in the metanephric mesenchyme and Wolffian duct in the *Six1*/*Six4*-heterozygous (J) and *Six1*-deficient (K) embryos but not in the mesenchyme in the *Six1*/*Six4*-deficient embryos (L). (M–O) *Gdnf* expression in the E10.0 *Six1*/*Six4*-heterozygous (M), *Six1*-deficient (N), and *Six1*/*Six4*-deficient (O) embryos. *Gdnf* expression was detected in the metanephric region in the *Six1*/*Six4*-heterozygous (M) and *Six1*-deficient (N) embryos but not in the *Six1*/*Six4*-deficient (O) embryos. (P–R) *Wt1* expression in the metanephric region of the E10.0 *Six1*/*Six4*-heterozygous (P), *Six1*-deficient (Q), and *Six1*/*Six4*-deficient (R) embryos. *Wt1* expression was maintained in the mutant mice (nc, nephrogenic cord; mt, mesonephric tubules; mm, metanephric mesenchyme).

Next, we examined *Pax8* expression in the mutant embryos at E10.0. Similar to *Pax8* expression in the wild type, it was expressed in the Wolffian duct and metanephric mesenchyme in the *Six1*/*Six4*-heterozygous and *Six1*-deficient embryos (Fig. 4G and H). In contrast, *Pax8* expression was absent in the mesenchyme but not in the Wolffian duct in the *Six1*/*Six4*-deficient embryos (Fig. 4I). Similar to the *Pax2* and *Gdnf* expression detected in the embryos examined at E10.0, the expression of these genes, although low, was also detected in the *Six1*-deficient embryos (Fig. 4K and N). However, it was completely absent in the *Six1*/*Six4*-deficient embryos (Fig. 4L and O). In contrast, *Wt1* expression was unaffected in both the *Six*-deficient and *Six1*/*Six4*-deficient embryos (Fig. 4P–R). These results suggest that *Six1* and *Six4* regulate *Pax8* as well as *Pax2*, which may possibly explain the exacerbated metanephric phenotypes of the *Six1*/*Six4*-deficient embryos.

2.5. *Six1 deficiency alone causes defects in the caudal mesonephric tubules*

Pax2 and *Pax8* are known to be expressed in the mesonephric tubules of mouse embryos (Bouchard et al., 2002; Carroll et al., 2005). Therefore, we examined mesonephric-tubule formation by performing HE staining of sagittal sections of the E10.5 embryos. Mesonephric tubules were histologically detected in the *Six1*/*Six4*-heterozygous embryos (Fig. 5A). In contrast, they were not detected in the *Six1*- and *Six1*/*Six4*-deficient embryos (Fig. 5B and C). To confirm these findings, we performed whole-mount in situ hybridization to detect *Lim1*, which is expressed in the mesonephric tubules and Wolffian duct (Fujii et al., 1994). Mesonephric tubules and the Wolffian duct were detected in the *Six1*/*Six4*-heterozygous embryos (Fig. 5D). In contrast, caudal mesonephric tubules (black arrow), which are formed in the nephrogenic cord on induction by the Wolffian duct, could not be detected in the *Six1*- and *Six1*/*Six4*-deficient embryos (Fig. 5E and F); however, cranial tubules (white arrowhead), which develop as an outgrowth from the Wolffian duct, were detected in these embryos. To eliminate the possibility that mesonephric-tubule formation is merely delayed, we also examined the embryos at E11.5. Although mesonephric tubules were detected in the *Six1*/*Six4*-heterozygous embryos (Fig. 5G), only few tubules were detected in the *Six1*- and *Six1*/*Six4*-deficient embryos (Fig. 5H and I). To confirm these results, we performed staining for E-cadherin, which is expressed in the mesonephric tubules and Wolffian duct. Mesonephric tubules were detected along the Wolffian duct in the *Six1*/*Six4*-heterozygous embryos (Fig. 5J). In contrast, caudal mesonephric tubules (black arrow) were not formed in the *Six1*- and *Six1*/*Six4*-deficient embryos, while cranial mesonephric tubules (white arrowhead), which are derived from the Wolffian duct, were present (Fig. 5K and L). These results indicate that *Six1* deficiency alone causes mesonephric-tubule defects and that the *Six1*/*Six4*-deficient embryos exhibit a phenotype comparable to that of the *Six1*-deficient embryos.

2.6. *Pax2 expression is absent in the nephrogenic cord in Six1-deficient mice*

Next, we investigated the expression of the genes essential for mesonephric-tubule formation by performing in situ hybridization in the nephrogenic cord at E9.5. *Osr1* expression was detected in the *Six1*/*Six4*-heterozygous embryos and was maintained in the *Six1*- and *Six1*/*Six4*-deficient embryos (Fig. 6A–C). *Wt1* was also expressed in both the mutants and *Six1*/*Six4*-heterozygous embryos (Fig. 6D–F). In contrast, *Pax2*, which was expressed in the Wolffian duct and nephrogenic cord in the *Six1*/*Six4*-heterozygous embryos, could not be detected in the nephrogenic cord of the *Six1*- and *Six1*/*Six4*-deficient embryos. However *Pax2* expression was maintained in the Wolffian duct (Fig. 6G–I). *Pax8* was also detected in the Wolffian duct and nephrogenic cord in the *Six1*/*Six4*-heterozygous embryos and the *Six1*-deficient embryos retained *Pax8* expression in the nephrogenic cord, while the *Six1*/*Six4*-deficient cords lacked the expression (Fig. 6J–L). These results suggest that *Pax2* and *Pax8* are regulated by *Six1* and *Six4* in the nephrogenic cord, similar to the metanephric mesenchyme. However, *Six1*-mediated *Pax2* activation but not *Six1*/*Six4*-dependent *Pax8* upregulation may play a central role in mesonephric tubule formation, unlike the situation in the metanephros. This is because *Six1* deficiency alone is sufficient to affect *Pax2* expression and cause mesonephric-tubule defects. *Wnt9b*, which was expressed in the Wolffian duct, was unaffected in both mutant embryos (Fig. 6M–O); this indicates that the kidney phenotypes could not be accounted for by *Wnt9b* deficiency in the Wolffian duct.

3. Discussion

In this paper, we demonstrate that *Six1* and *Six4* have redundant roles in kidney development, and that inactivation of both genes leads to more severe metanephric phenotypes than those of *Six1*-deficient mice. In the *Six1*/*Six4*-deficient mice, a distinct cell cluster is absent in the metanephric mesenchyme, and the ureteric bud is not formed. However, both these components are present in the *Six1*-deficient mice, although they are impaired. In contrast, mesonephric tubules are absent in both the *Six1*- and *Six1*/*Six4*-deficient mice, suggesting that *Six4* plays a minor role in mesonephros formation.

The expression of *Pax2* and *Gdnf* is partially lowered in the *Six1*-deficient mice and absent in the *Six1*/*Six4*-deficient mice. In contrast, *Pax8* expression is absent only in the double mutant mice. These results indicate that *Pax2* and *Gdnf* are regulated mainly by *Six1* and partially by *Six4*, while *Pax8* expression is maintained when either *Six1* or *Six4* is present. *Sall1* is almost absent in the