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ES細胞からの腎臓細胞誘導法の開発

平成１７年度～１９年度　総合研究報告書

主任研究者　西中村　隆一

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総合研究報告書

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教

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。そこで、腎臓発生カスケードの現時点での最上流Pax2を薬剤制御下に誘導できるESを作成した。Pax2をニワトリ沿軸中胚葉に導入すると異所性の腎管が誘導できることが学会レベルで報告されているためである。しかしこれも陰性の結果であった。

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った。

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。

D. 考察

1) 達成度について

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。

2) 研究成果の学術的・国際的・社会的意義について

今回開発したコロニーアッセイは、腎臓前駆細胞を prospective に同定する初めての系であり、組織や切片のレベルでしか検討されていなかった腎臓形成が、単一細胞レベルで解析できるようになる可能性を秘めている。この研究成果は Development 誌(Impact factor 7.764)の巻頭でも紹介され、2007 年 8 月ハンガリーでの International Developmental Nephrology Workshop において、唯一の日本人演者として招待講演を行った。また ES における Sall4 の機能解明についても、Development 誌の巻頭で紹介され、既に Nature, Nature Cell Biology などを含む 11 個の論文に引用されている。

3) 今後の展望について

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。

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。

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G. 知的所有権の取得状況

1. 特許取得

誘導法について出願を検討している。

2. 実用新案登録

3. その他

図1. 1個の後腎間葉細胞からのコロニー形成

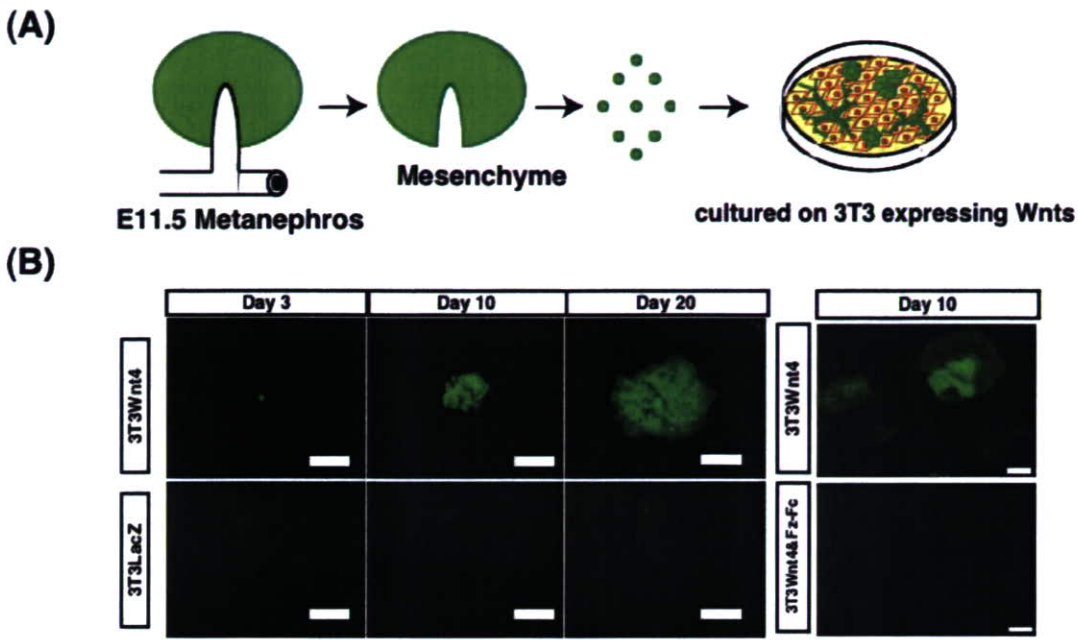


図2. 3次元構造の再構築

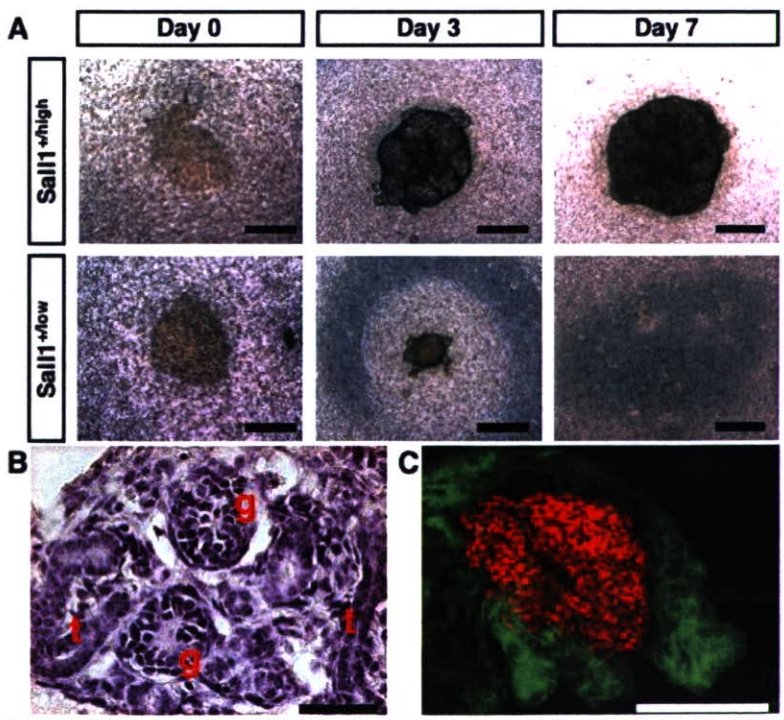


図3. 中間中胚葉及び後腎間葉からの腎臓発生

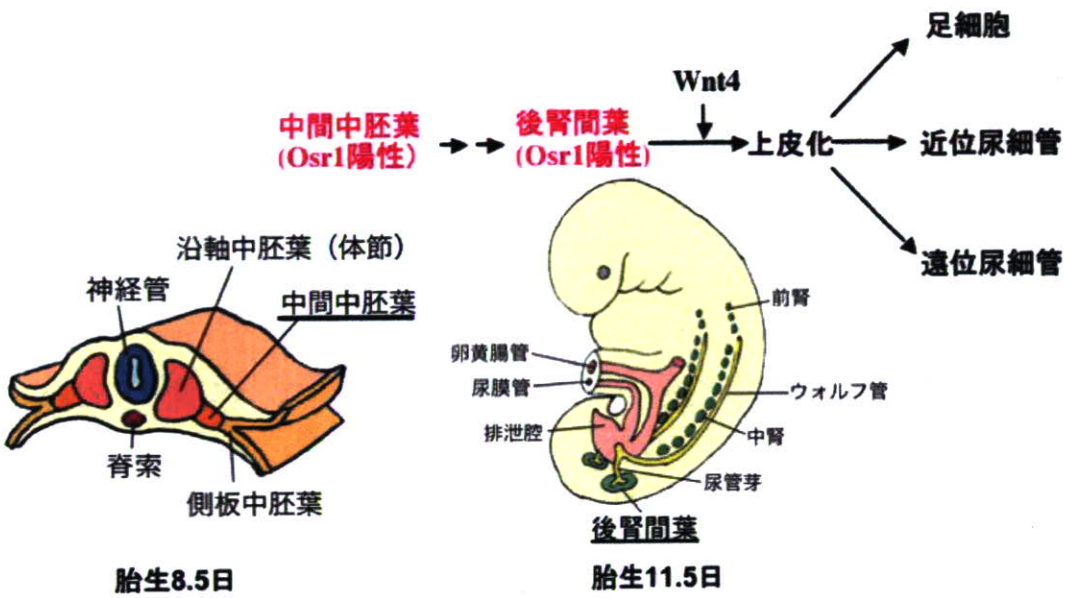
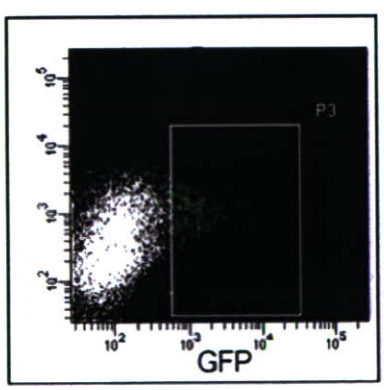
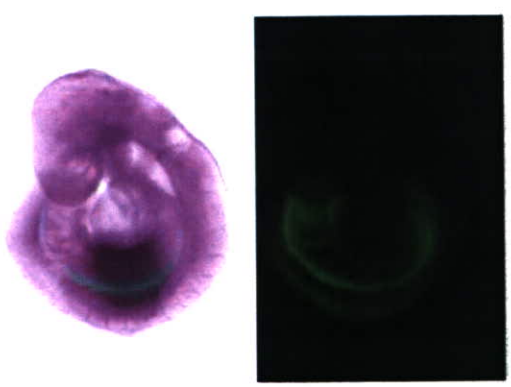


図4. ESからのOsr1-GFP陽性細胞誘導

Osr1-GFP ES

Osr1-GFPマウス





ESからの誘導成功

中間中胚葉の蛍光発色

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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Essential roles of *Sall1* in kidney development

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Essential roles of *Sall1* in kidney development. *SALL1* is a mammalian homologue of the *Drosophila* region-specific homeotic gene *spalt* (*sal*) and heterozygous mutations in *SALL1* in humans lead to Townes-Brocks syndrome. We isolated a mouse homologue of *SALL1* (*Sall1*) and found that mice deficient in *Sall1* die in the perinatal period with kidney agenesis. *Sall1* is expressed in the metanephric mesenchyme surrounding ureteric bud and homozygous deletion of *Sall1* results in an incomplete ureteric bud outgrowth. Therefore, *Sall1* is essential for ureteric bud invasion, the initial key step for metanephros development. We also generated mice in which a green fluorescent protein (*GFP*) gene was inserted into the *Sall1* locus and we isolated the *GFP*-positive population from embryonic kidneys of these mice by fluorescence-activated cell sorting (FACS). We then compared gene expression profiles in the *GFP*-positive and -negative population using microarray analysis, followed by in situ hybridization. We detected many genes known to be important for metanephros development, and genes expressed abundantly in the metanephric mesenchyme. We also found groups of genes which are not known to be expressed in the metanephric mesenchyme. Thus a combination of microarray technology and *Sall1-GFP* mice is useful for systematic identification of genes expressed in the developing kidney.

THREE KIDNEYS DURING DEVELOPMENT

The kidney develops in three stages: pronephros, mesonephros, and metanephros. The nephric duct (Wolffian duct) develops in the craniocaudal direction from the intermediate mesoderm and acts upon the surrounding mesenchyme as an inducer of epithelial transformation to nephric tubules. The pronephric and mesonephric tubules and the anterior portion of the Wolffian duct eventually degenerate, and it is the metanephros that becomes the permanent kidney in mammals.

IDENTIFICATION OF *Sall* GENES USING FROG EMBRYOS

The animal cap is a tiny portion of the presumptive ectoderm of *Xenopus* embryos in the blastula stage. In the presence of activin, animal caps differentiate into a variety of tissues. A combination of activin plus retinoic acid induces pronephric tubules efficiently and selectively [1]. We used this animal cap system to identify molecules expressed in pronephros and potentially in mesonephros and metanephros. Thousands of animal caps treated with activin plus retinoic acid were collected at various time points and subjected for a varitety of subtraction procedures. One of the obtained molecules was *Xsal-3*, which is homologous to *Drosophila* region-specific homeotic gene *spalt* (*sal*) and has multiple double-zinc finger motifs characteristic of the *sal* gene family [2]. We also isolated a mouse homologue (*Sall1*) and found it to be expressed in otic vesicles, limb buds, anus, hearts, and kidneys (metanephric mesenchyme) [3].

Sall1 IS ESSENTIAL FOR KIDNEY DEVELOPMENT

When we generated *Sall1* knockout mice, all of homozygous mice died within 24 hours after birth, and kidney agenesis or severe dysgenesis were present (Fig. 1) [3]. About one third had no kidneys or ureters, bilaterally (Fig. 1B). The remaining mice had either unilateral kidney agenesis, or bilateral hypolasia (Fig. 1C). At day 11.5 of gestation, the ureteric bud invades the metanephric mesenchyme and subsequent reciprocal interaction between these two tissues leads to development of a metanephric kidney (Fig. 1D). In *Sall1*-null mice, morphologically distinct metanephric mesenchyme was formed, albeit the size being reduced (Fig. 1E). In contrast, the ureteric bud formed but failed to invade the metanephric mesenchyme. Thus, loss of *Sall1* leads to a failure of ureteric bud invasion into the mesenchyme, the initial key step for metanephros development.

KIDNEY ABNORMALITIES CAUSED BY HUMAN *SALL1* MUTATIONS

Humans and mice have four known *sal*-related genes, respectively (*SALL1-4* for humans and *Sall1-4* for mice). Mutations in *SALL1* have been associated with Townes-Brocks syndrome, an autosomal-dominant disease with features of dysplastic ears, preaxial polydactyly, imperforate anus, and, less commonly, kidney and heart anomalies [4]. Mice deficient in *Sall1* show kidney agenesis or

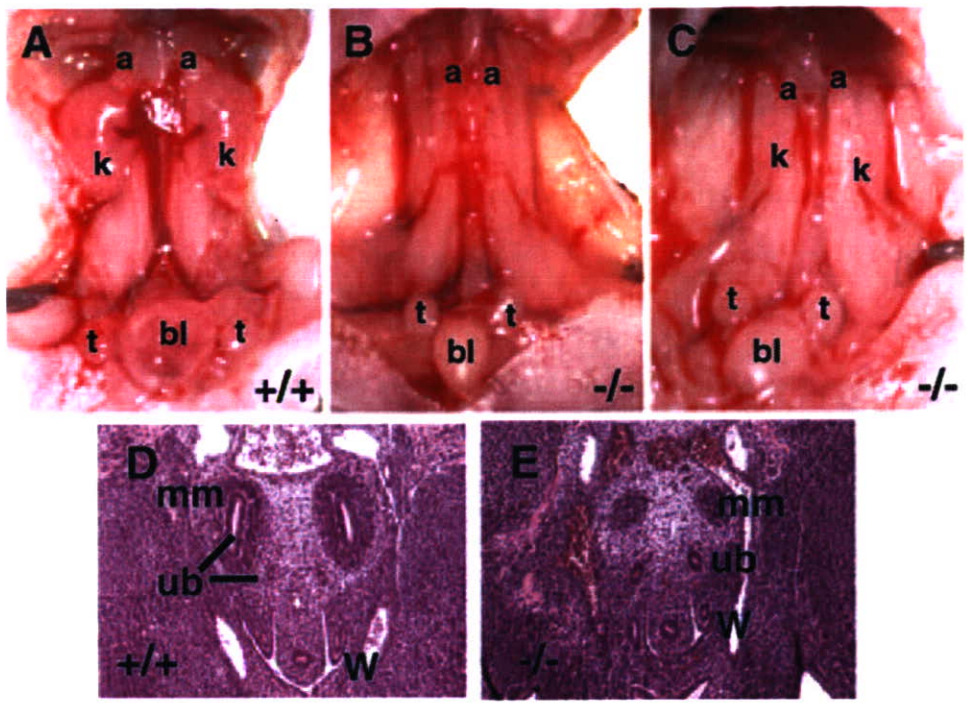

Fig. 1. Kidney phenotypes in *Sall1*-deficient mice. (*A*) Kidneys (k) of wild-type newborn. Urinary bladder (bl) is filled with urine. (*B*) Kidneys of *Sall1*-deficient newborn. Note kidneys are absent and the urinary bladder is not inflated with urine. Other organs, such as adrenal glands (a) and testis (t), are normal. (*C*) Kidneys of another *Sall1*-deficient newborn with severe bilateral kidney hypoplasia. Urine is absent in the bladder. (*D*) Metanephros in wild-type mice at 11.5 days past coitus (dpc). Ureteric bud (ub) branches from Wolffian duct (W) and metanephric mesenchyme (mm) are condensed around the bulging ureteric bud. (*E*) Metanephros in *Sall1*-deficient mice at 11.5 dpc. Metanephric mesenchyme is formed but reduced in size and is not invaded by the ureteric bud.

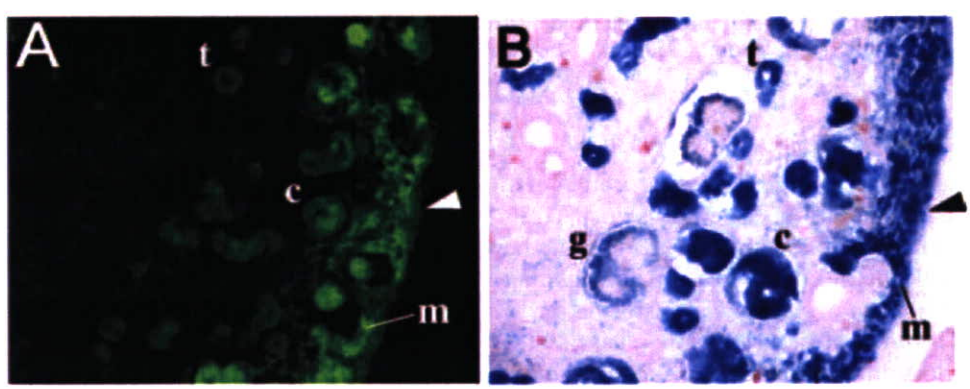

Fig. 2. Generation of *Sall1*-green fluorescence protein *(GFP)* knockin mice. (*A*) *GFP* expression in embryonic kidney of heterozygous *Sall1-GFP* knockin mice. (*B*) 5-bomo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining of embryonic kidney in heterozygous *Sall1-LacZ* knockin mice. Arrowhead is the stroma; m is condensed mesenchyme; c is comma-shaped bodies; t is tubles; and g is glomerulus.

severe dysgenesis, but other phenotypes observed in human disease are not apparent, as described above [3]. This discrepancy could be explained by truncated SALL1 proteins by human mutations, possibly functioning in a dominant-negative manner, as mutant mice that produce a truncated Sall1 protein exhibit more severe defects than *Sall1-null* mice, including renal agenesis, exencephaly, limb, and anal deformities [5]. *Sall2*-deficient mice show no apparent phenotypes, and mice lacking both *Sall1* and *Sall2* show kidney phenotypes comparable to those of *Sall1* knockout [6]. *Sall3*-null mice die on the first postnatal day and deficiencies in cranial nerves and abnormalities in the oral structures are present [7]. Mutations of *SALL4* cause an autosomal-dominant disorder Okihiro syndrome, characterized by limb deformity and eye movement deficits, and, less commonly, anorectal and kidney anomalies [8, 9], and we are currently generating *Sall4*-deficient mice. Generation of mice lacking all of the *Sall* genes would be necessary to address developmental roles of *Sall* family.

IDENTIFICATION OF KIDNEY MESENCHYMAL GENES BY A COMBINATION OF MICROARRAY ANALYSIS AND *Sall1-GFP* KNOCKIN MICE

In the embryonic kidney, *Sall1* is expressed abundantly in mesenchyme-derived structures from condensed mesenchyme, S-shaped, comma-shaped bodies, to renal tubules and podocytes (Fig. 2). We generated mice in which *GFP* gene was inserted into the *Sall1* locus and we isolated the *GFP*-positive population from embryonic kidneys of these mice by FACS [10]. The *GFP*-positive population indeed expressed mesenchymal genes, while the negative population expressed genes in the ureteric bud. To systematically search for genes expressed in the mesenchyme-derived cells, we compared gene expression profiles in the *GFP*-positive and *GFP*-negative populations using microarray analysis, followed by in situ hybridization. We detected many genes known to be important for metanephros development, including *Sall1*, *GDNF*, *Raldh2*, *Pax8*, and *FoxD1*, and genes expressed abundantly in the metanephric mesenchyme such as *Unc4.1*, *Six2*, *Osr-2*, and *PDGFc*. We also found groups of genes, including *SSB-4*, *Smarcd3*, *μ-Crystallin*, and *TRB-2*, which are not known to be expressed in the metanephric mesenchyme. Therefore, a combination of microarray technology and *Sall1-GFP* mice is useful for systematic identification of genes expressed in the developing kidney. To find essential genes from this large list, efficient and rapid screening is needed. Recently emerging siRNA technology is one potent method, but generating knockout mice of each candidate gene is necessary for proof.

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Identification of multipotent progenitors in the embryonic mouse kidney by a novel colony-forming assay

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Renal stem or progenitor cells with a multilineage differentiation potential remain to be isolated, and the differentiation mechanism of these cell types in kidney development or regeneration processes is unknown. In an attempt to resolve this issue, we set up an in vitro culture system using NIH3T3 cells stably expressing *Wnt4* (3T3Wnt4) as a feeder layer, in which a single renal progenitor in the metanephric mesenchyme forms colonies consisting of several types of epithelial cells that exist in glomeruli and renal tubules. We found that only cells strongly expressing *Sall1* (Sall1-GFP^{high} cells), a zinc-finger nuclear factor essential for kidney development, form colonies, and that they reconstitute a three-dimensional kidney structure in an organ culture setting. We also found that Rac- and JNK-dependent planar cell polarity (PCP) pathways downstream of Wnt4 positively regulate the colony size, and that the JNK pathway is also involved in mesenchymal-to-epithelial transformation of colony-forming progenitors. Thus our colony-forming assay, which identifies multipotent progenitors in the embryonic mouse kidney, can be used for examining mechanisms of renal progenitor differentiation.

KEY WORDS: Progenitor, Kidney, Colony-forming assay, *Sall1*, Wnt, PCP, JNK, Rho, Mouse

INTRODUCTION

Mammalian adult kidney, metanephros, is formed by reciprocally inductive interaction between two precursor tissues derived from the intermediate mesoderm, the metanephric mesenchyme and the ureteric bud. The ureteric bud induces the metanephric mesenchyme to differentiate into the epithelia of glomeruli and renal tubules, endothelial and stromal cells (Saxen, 1987). Inductive signals have been vigorously investigated, and several factors have been elucidated that trigger epithelialization of metanephric mesenchyme in explant culture system; the members of Wnt family (Herzlinger et al., 1994; Kispert et al., 1998), leukemia inhibitory factor (LIF) (Barasch et al., 1999; Plisov et al., 2001), and transforming growth factor β2 (TGFβ2) (Plisov et al., 2001). These studies have also suggested the presence of clonal cells in mesenchymal rudiments, which sequentially form renal condensation, comma (C)- and S-shaped bodies, and terminally epithelia of glomeruli and renal tubules, and the existence of single epithelial precursors responding to LIF was demonstrated in mesenchyme (Barasch et al., 1999). One previous report suggested retrospectively the presence of multipotent cells in embryonic kidneys, demonstrating that cells in several portions of nephron were derived from a single stem cell using *lacZ* gene transduction with retrovirus into a single cell of mesenchyme (Herzlinger et al., 1992). However, none has isolated prospectively the renal progenitor cells with a multilineage differentiation potential from the embryonic kidney, and none has examined their differentiation mechanisms in a single cell culture. There has been a lack of assay systems that specifically identify renal progenitors, as in cases of the neurosphere method for neural stem cells (Reynolds et al., 1992) and the colony assay for hematopoietic progenitors (Pluznik and Sachs, 1965; Bradley and Metcalf, 1966).

We previously generated mice in which the green fluorescence protein gene (*GFP*) was knocked into the locus of *Sall1* (*Sall1-GFP* mice), a zinc finger nuclear factor that is expressed in the metanephric mesenchyme and that is essential for kidney development (Nishinakamura et al., 2001; Takasato et al., 2004). *Sall1* is also expressed in the subventricular zone of the central nervous system and progress zones of limb buds, where neural and mesenchymal stem cells reside, respectively, leading to speculation that *Sall1* might have some association with stem cells in several organs, including the kidney.

Targeted disruption of *Wnt4* results in kidney agenesis and impairs mesenchymal-to-epithelial transformation (Stark et al., 1994), and co-culture with 3T3Wnt4 induces tubulogenesis in the mesenchyme rudiment in organ culture (Kispert et al., 1998), suggesting both essential and sufficient roles of Wnt4 for epithelial differentiation of metanephric mesenchyme. Recently, Wnt9b expressed in the ureteric bud was shown to function upstream of Wnt4 (Carroll et al., 2005). Thus, we attempted to set up assay systems that can identify and characterize the progenitor cells with multipotent differentiation potential from uninduced metanephric mesenchyme using Wnt4 signal. Wnt genes are known to regulate multiple cellular functions using at least three intracellular signaling branches: the β-catenin pathway (canonical pathway), in which stabilized β-catenin interacts with members of the lymphoid enhancer factor/T cell factor (LEF/TCF) family of transcription factors and activates gene expression in the nucleus (Wodarz and Nusse, 1998; Miller et al., 1999); the planar cell polarity (PCP) pathway, which involves Jun N-terminal kinase (JNK) and the Rho family of small guanosine triphosphatases (GTPases) and which directs cytoskeletal rearrangements, coordinated polarization within the plane of epithelial sheets, and morphogenetic movements during development (Veeman et al., 2003; Wallingford et al., 2002); and the Wnt/Ca^{2+} pathway, which leads to release of intracellular calcium and is implicated in *Xenopus* ventralization and in the

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regulation of embryonic cell movements (Miller et al., 1999; Veeman et al., 2003; Wallingford et al., 2002). Mechanisms by which Wnt pathways mediate cellular effects in kidney development are poorly understood.

In this study, we established a novel colony-forming assay system using 3T3-expressing Wnt4 to identify renal progenitors in the metanephric mesenchyme. Combining our colony-forming assay with flow cytometry, we found that these progenitors could be enriched by using *Sall1* as a marker. We also examined the effects of Wnt downstream branches on the renal progenitors.

MATERIALS AND METHODS

In vitro colony-forming assay

Metanephric mesenchyme of embryonic day (E) 11.5 mice was isolated surgically from embryonic kidney rudiment. The mesenchyme was incubated in 0.05% trypsin-EDTA at 37°C for 10 minutes and then transferred to DMEM with 10% fetal calf serum. Mesenchymal cells were then mechanically dissociated by gentle aspiration through repeated pipetting. Metanephros of E14.5 and 17.5 embryos was incubated in 1 mg/ml Dispase (Invitrogen) at 37°C for 30 minutes then mechanically dissociated by repeated pipetting. NIH3T3 cells stably expressing Wnt3a, Wnt4 and *lacZ* (Kispert et al., 1998) were mitotically inactivated with mitomycin C before use. Single mesenchymal cells were sorted by FACS Vantage (Becton Dickinson) and plated onto these feeder cells at a low density (5×10^3 cells/well of 6-well plates), then cultured in DMEM/F12 with 5% knockout serum replacement (Invitrogen), 10 μg/ml insulin, 6.7 μg/l sodium selenite, 5.5 μg/ml transferrin, 1×10^{-7} mol/l dexamethasone, 10 mmol/l nicotinamide, 2 mmol/l L-glutamine, 50 μmol/l β-mercaptoethanol, 5 mmol/l HEPES and penicillin/streptomycin.

RT-PCR

Primers used for PCR were as follows:

Pax2, 5′-AGGGCATCTGCGATAATGAC-3′ and 5′-CTCGCGTTTC-CTCTTCTCAC-3′;

Lim1 (*Lhx1* – Mouse Genome Informatics), 5′-TGGACC-GTTTCCTCTTGAAC-3′ and 5′-TGTTCTCTTTGGCGACACTG-3′;

Eya1, 5′-CGGTCGACCTCTATGGAAATGCAGGATCTAAC-3′ and 5′-AACTTCGGTGCCATTGGGAGTC-3′;

Sall1, 5′-TCTCCAGTGTGAGTTCTCTCG-3′ and 5′-GTACACG-TTTCTCCTCAGGAC-3′;

Wt1, 5′-ACCCAGGCTGCAATAAGAGA-3′ and 5′-GCTGAAGG-GCTTTTCACTTG-3′;

Hoxa11, 5′-GGATTTTGATGAGCGTGGTC-3′ and 5′-GAGTAG-CAGTGGGCCAGATT-3′;

glial cell line derived neurotrophic factor (*Gdnf*): 5′-CCCGA-AGATTATCCTGACCA-3′ and 5′-TAGCCCAAACCCAAGTCAGT-3′;

integrin α8, 5′-GGCGAAAGTGCAGTCCTAAA-3′ and 5′-GAAG-GAGACATTCGGAGTGG-3′;

integrin α3, 5′-CGGCCTGTCATCAATATCCT-3′ and 5′-CGAA-CATTGTCCATCAGCAG-3′;

neural cell adhesion molecule (*Ncam*), 5′-ACGTCCGGTTCA-TAGTCCTG-3′ and 5′-CTATGGGTTCCCCATCCTTT-3′;

E-cadherin (cadherin 1 – Mouse Genome Informatics), 5′-GCA-CTCTTCTCCTGGTCCTG-3′ and 5′-GTTGACCGTCCCTTCACAGT-3′;

K-cadherin (cadherin 6 – Mouse Genome Informatics), 5′-CTAGT-GGCTTCCCAGCAAAG-3′ and 5′-CGTGACTTGGACCACAAATG-3′;

Ret, 5′-GCGTCAGGGAGATGGTAAAG-3′ and 5′-CATCAGGG-AAACAGTTGCAG-3′;

Hoxb7, 5′-TTCCCCGAACAAACTTCTTG-3′ and 5′-CGGAGAGG-TTCTGCTCAAAG-3′;

α-actinin-4, 5′-TGGTGCAACTCTCATCTTCG-3′ and 5′-CCGCA-GCTTGTCATACTCAA-3′;

CD2-associating protein (CD2-AP), 5′-AGGAATTCAGCCACAT-CCAC-3′ and 5′-CCTGAGCGTTGTGAGTTTCA-3′;

P-cadherin (cadherin 3 – Mouse Genome Informatics), 5′-CAC-ACGACCTCATGTTCACC-3′ and 5′-GAAATGGTCCCCATCATCAC-3′;

podoplanin, 5′-TCTACTGGCAAGGCACCTCT-3′ and 5′-GCTCTT-TAGGGCGAGACCTT-3′;

podocalyxin-like, 5′-ACTACATTGCCCGTCTCCAC-3′ and 5′-AAA-TCCTCAGCTGGCTTGAA-3′;

aquaporin 1 (*Aqp1*), 5′-CCTCCAGGCACAGTCTTCTC-3′ and 5′-CAGTGGCCTCCTGACTCTTC-3′;

chloride channel 5 (*Clcn5*), 5′-TGGGCTCTTCTGTTTGCTTT-3′ and 5′-GCGAAGAAAGAACGCCATAG-3′;

cubilin (intrinsic factor cobalamin receptor), 5′-CAACCTT-GCCCGTGTTCTAT-3′ and 5′-GTCTGAGTCATCGCTGTGGA-3′;

megalin (low density lipoprotein receptor-related protein 2 – Mouse Genome Informatics), 5′-CAGGGACTCCTCTGACGAAG-3′ and 5′-CCTCTCCTTCTGGACAGTCG-3′;

sodium glucose transporter 1 (*Sglt1*; *Slc5a1* – Mouse Genome Informatics), 5′-GCCATCATCCTCTTCGTCAT-3′ and 5′-ACCACTG-TCCTCCACAAAGG-3′;

Brn1 (*Pou3f3* – Mouse Genome Informatics), 5′-TCTATGGCA-ACGTGTTCTCG-3′ and 5′-CGTCATGCGTTTTTCCTTTT-3′;

Na-K-2Cl co-transporter 2 (*Nkcc2*; *Slc12a1* – Mouse Genome Informatics), 5′-CATGGCATTCATTCTCATCG-3′ and 5′-GCAGAGG-CCACTATTCTTCG-3′;

Clck2 (*Clcnkb* – Mouse Genome Informatics), 5′-CCTCTCA-CTTCTCCGTCTGG-3′ and 5′-AAGAAAGTCCGCTGGCTGTA-3′;

polycystin 2, 5′-GGTGGTGGCAAACTGAACTT-3′ and 5′-TCTC-CAGCTTGACAATCACG-3′;

renal outer medulla K channel 2 (*Romk2*), 5′-TGGTCTCCAAA-GATGGAAGG-3′ and 5′-ATGGCACCACACATGAAAGA3′;

epithelial Na channel (*ENaC*; *Scnn1g* – Mouse Genome Informatics), 5′-GCCTCACTGCTTTCAAGGAC-3′ and 5′-CCAAGTGGGATACT-GGGCTA-3′;

Na/Ca exchanger, 5′-TGTGTTTACGTGGTCCCTGA-3′ and 5′-TGG-AAGCTGGTCTGTCTCCT-3′;

polycystin 1, 5′-CTCTGTGCCCTTCTGAGTCC-3′ and 5′-TGGATCC-ATTCCTTCAAAGC-3′;

Foxd1, 5′-CTGGTGAAGCCTCCCTACTC-3′ and 5′-GCCGTTGTC-GAACATGTCTG-3′; *Flk1* (*Kdr* – Mouse Genome Informatics), 5′-GCATGGAAGAGGATTCTGGA-3′ and 5′-CAAGGACCATCCCAC-TGTCT-3′;

VE-cadherin (cadherin 5 – Mouse Genome Informatics), 5′-ACCGGATGACCAAGTACAGC-3′ and 5′-TTCTGGTTTTCTGGC-AGCTT-3′;

Cd45 (*Ptprc* – Mouse Genome Informatics), 5′-CCACCAGGGACT-GACAAGTT-3′ and 5′-TAGGCTTAGGCGTTTCTGGA-3′; and

glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), 5′-TGATGA-CATCAAGAAGGTGGTGAAG-3′ and 5′-TCCTTGGAGGCCATGTA-GGCCAT-3′.

PCR cycles were as follows: *Gapdh*, initial denaturation at 94°C for 2.5 minutes, followed by 22 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and final extension at 72°C for 10 minutes; other genes, initial denaturation at 94°C for 2.5 minutes, followed by 28-33 cycles of 94°C for 30 seconds, 58°C for 1 minute, 72°C for 30 seconds, and final extension at 72°C for 10 minutes.

Organ culture

In order to examine the in vitro differentiating potential of cell populations included in the metanephric mesenchyme, each cell population was separated by flow cytometry and was pelleted down by low-speed centrifugation (380 ***g***). The resultant cell pellet (1×10^4 cells per pellet) was cultured on 3T3Wnt4 cells at air-fluid interface on a polycarbonate filter (0.4 μm, Nucleopore) supplied with DMEM plus 10% fetal calf serum at 37°C, 5% carbon dioxide. 3T3Wnt4 cells (50,000 cells in 50 μl medium) were seeded on the filter 24 hours before the experiments, as described (Kispert et al., 1998). To examine the influence of reagents on tubulogenesis, two metanephroi or mesenchyme rudiments from E11.5 embryos were cultured on a polycarbonate filter. For the culture of mesenchyme rudiments, 3T3Wnt4 cells were used as described above.

Retroviral infection

The cDNA clones of the active mutant form of β-catenin (*pUC-EF-1α-β-catenin^{SA}-3HA*) (Miyagishi et al., 2000), the full length of rat axin (*pBSKS-rAxin*) (Ikeda et al., 1998), and both constitutively-active and dominant-negative mutant forms of human *Rac1* and *RhoA* with N-terminus flag tag [*pCAGIP-flag-Rac1 (Val)*, *pCAGIP-flag-Rac1 (Asn)*, *pCAGIP-flag-RhoA (Val)*, *pCAGIP-flag-RhoA (Asn)*] were subcloned into retroviral vector *pMY-IRES-EGFP* (Kitamura et al., 2003). To produce recombinant retrovirus, these plasmid vectors were transfected into the virus packaging cell line PLAT-E (Morita et al., 2000) using FuGENE (Roche), and supernatant from the transfected cells was collected to infect cells of the metanephric mesenchyme. The viral supernatant was centrifuged at 20,000 ***g*** overnight at 4°C to concentrate the virus. To infect mesenchymal cells with the retrovirus, dissociated mesenchymal cells were resuspended into the concentrated virus supernatant with adding polybrene. The suspension was centrifuged 1400 ***g*** for 4 hours at room temperature. After washing with PBS, mesenchymal cells were plated onto 3T3 feeder cells.

Immunocytochemistry and lectin staining

The colonies formed on 3T3Wnt4 feeder were fixed with 4% paraformaldehyde in PBS for 20 minutes at 4°C. After washing with PBS, PBS containing 2% skimmed milk and 0.1% Triton-X was incubated as a blocking solution for 1 hour at room temperature. The fixed dishes were incubated with primary antibodies overnight at 4°C followed by incubating with secondary antibodies for 1 hour at room temperature. The following antibodies were used: rabbit anti-Pax2 (Babco), rabbit anti-WT1 (Santa Cruz), mouse anti-E-cadherin (Becton Dickinson), rabbit anti-AQP1 (Chemicon), and rabbit anti-phosphorylated JNK1 and 2 (Biosource). Rhodamine-conjugated anti-rabbit IgG (H+L) and anti-mouse IgG (Chemicon) were used as secondary antibodies. To examine the expression of a proximal renal tubule-specific marker, fluorescein isothiocyanate (FITC)-conjugated *Lotus Tetragonobulus* lectin (LTL; Vector Labs) was used. After each step, the cultured cells were washed three times with PBS containing 0.1% Triton-X. For detection of Sall1, mesenchymal cells derived from *Sall1-GFP* heterozygote embryos were cultured on 3T3 feeder and subjected to GFP immunostaining procedure using rabbit anti-GFP (Molecular Probes). Rhodamine-conjugated peanut agglutinin (PNA; Vector Labs) staining was done as described (Gilbert et al., 1994). Organ culture tissues were fixed with 4% paraformaldehyde in PBS for 1 hour at 4°C and incubated in PBS including 0.1% saponin (Sigma) for 1 hour at 37°C, then the same staining procedure was carried out. Staining with rabbit anti-secreted frizzled-related protein 2 (sFRP2; Santa Cruz) and FITC-conjugated *Dolichos biflorus* agglutinin (DBA; Vector Labs) were also used on sections of paraffin-embedded explants to examine the effect of reagents on tubule formation and branching, respectively.

RESULTS

In vitro colony formation from E11.5 metanephric mesenchyme

We cultured single cells from the metanephric mesenchyme of E11.5 embryos, using 3T3Wnt4 as a feeder layer in a serum-free condition. The metanephric mesenchyme of transgenic mice ubiquitously expressing enhanced green fluorescence protein (EGFP; Okabe et al., 1997) was used to distinguish mesenchyme-derived cells from feeder cells, and single cells sorted by flow cytometry were cultured at a low cell density on 3T3Wnt4. This culture condition resulted in the formation of sheet-like colonies not formed on 3T3*lacZ* (Fig. 1A, upper panels), while scattered fibroblast-like cells were observed in both conditions (Fig. 1A, lower panels, arrows). Colonies were not formed in the presence of frizzled (Fz)-Fc chimeric protein, a Wnt inhibitor, thus confirming an essential role of Wnt4 for colony formation (Fig. 1B). Colonies were not formed by culturing in the conditioned medium from 3T3Wnt4 without feeder cells (data not shown). Colonies were also formed on 3T3Wnt3a, but not in feeder-free conditions using a purified recombinant Wnt3a protein (data not shown). These data suggested the requirement of other signals from 3T3 cells, in addition to the Wnt signals for the colony formation. In the presence of serum, colonies were not formed even on 3T3Wnt4, and some factors in the serum might prevent colony formation (data not shown). When colonies on 3T3Wnt4 were dissociated and plated onto fresh feeder cells at day 10 of culture, few colonies were obtained, and maintenance of these colonies could not be achieved (data not shown). When we tried colony-formation by using polycarbonate filters, which separate mesenchymal cells from the feeder layer, colonies were formed but the number of colonies formed was much smaller than that formed by directly culturing on feeder cells (data not shown).

To characterize the molecular profiles of the colonies, genes expressed in the metanephric mesenchyme were examined by RT-PCR using RNA from the colonies together with 3T3Wnt4 (Fig. 1C). All the mesenchymal genes examined (*Pax2*, *Lim1*, *Eya1*, *Sall1*, *WT1*, *Hoxa11*, *Gdnf*, integrin α8, integrin α3, *Ncam*, E-cadherin and K-cadherin) were expressed, and the expression continued to day 20 (Fig. 1C, lanes 3-5). By contrast, when cultured on 3T3*lacZ*, the expression of these genes was below the detection level (lanes 7-9). The expression of ureteric bud markers (*Ret* and *Hoxb7*) were not detected in mesenchyme separated from ureteric bud, suggesting that the separation was successful (lane 1). To determine the potential for differentiation within the colonies, markers for terminally differentiated epithelia in glomeruli (podocyte), proximal or distal tubules, and the loop of Henle were also examined (glomeruli: α-actinin-4, *CD2-AP*, P-cadherin, podoplanin and podocalyxin; proximal tubule: *Aqp1*, *Clc5*, cubilin, megalin and *Sglt1*; Henle's loop: *Brn1* and *Nkcc2*; Henle's loop or distal tubule: *Clck2*, polycystin 2, and *Romk2*; distal tubule: *ENaC*, Na/Ca exchanger and polycystin 1. These markers encode: (1) cytoskeletal or structural proteins: α-actinin-4, *CD2-AP*, P-cadherin, podoplanin and podocalyxin; (2) transcription factor: *Brn1*; (3) water or ion channels: *Aqp1*, *Clc5*, *Clck2*, *Romk2*, *ENaC*, and polycystin1 and 2; and (4) transporters: cubilin, megalin, *Sglt1*, *Nkcc2* and Na/Ca exchanger. As shown in Fig. 1C, almost all the genes examined were expressed at day 20 on 3T3Wnt4 (lane 5), while these markers were not expressed on 3T3*lacZ* (lanes 7-9). To ascertain that these genes were expressed by the colony-forming cells, colonies were formed from GFP transgenic mesenchyme, and cells expressing GFP were separated from feeder layers by using flow cytometry sorting. RT-PCR using RNA from these cells suggested that the marker genes examined were indeed expressed by colony-forming cells (lane 10). Furthermore, we made use of immunocytochemistry and found that Pax2 (Fig. 1D-F), E-cadherin (Fig. 1G-I), Sall1 (Fig. 1J,K), and Aqp1 (Fig. 1L,M) were expressed on colonies. The expression of Pax2 and E-cadherin was not detected on immunocytochemistry at day 3, and was subsequently upregulated by day 10, which was consistent with the result of RT-PCR (Fig. 1D,E,G,H). These data suggest that dissociated cells from the metanephric mesenchyme form colonies on 3T3Wnt4 feeder cells in serum-free conditions, and that these colonies contain differentiated epithelia expressing marker genes for epithelia in glomeruli (podocyte), proximal or distal tubules, and the loop of Henle.

Colonies are derived from a single multipotent renal progenitor

To confirm that these colonies were derived from a single cell, each single cell sorted from the EGFP transgenic mesenchyme was cultured in an individual well of 96-well plates coated with 3T3Wnt4. The sheet-like colony was found in 166 wells out of a total of 1632 (10.2%) from three independent experiments (Fig. 2A).

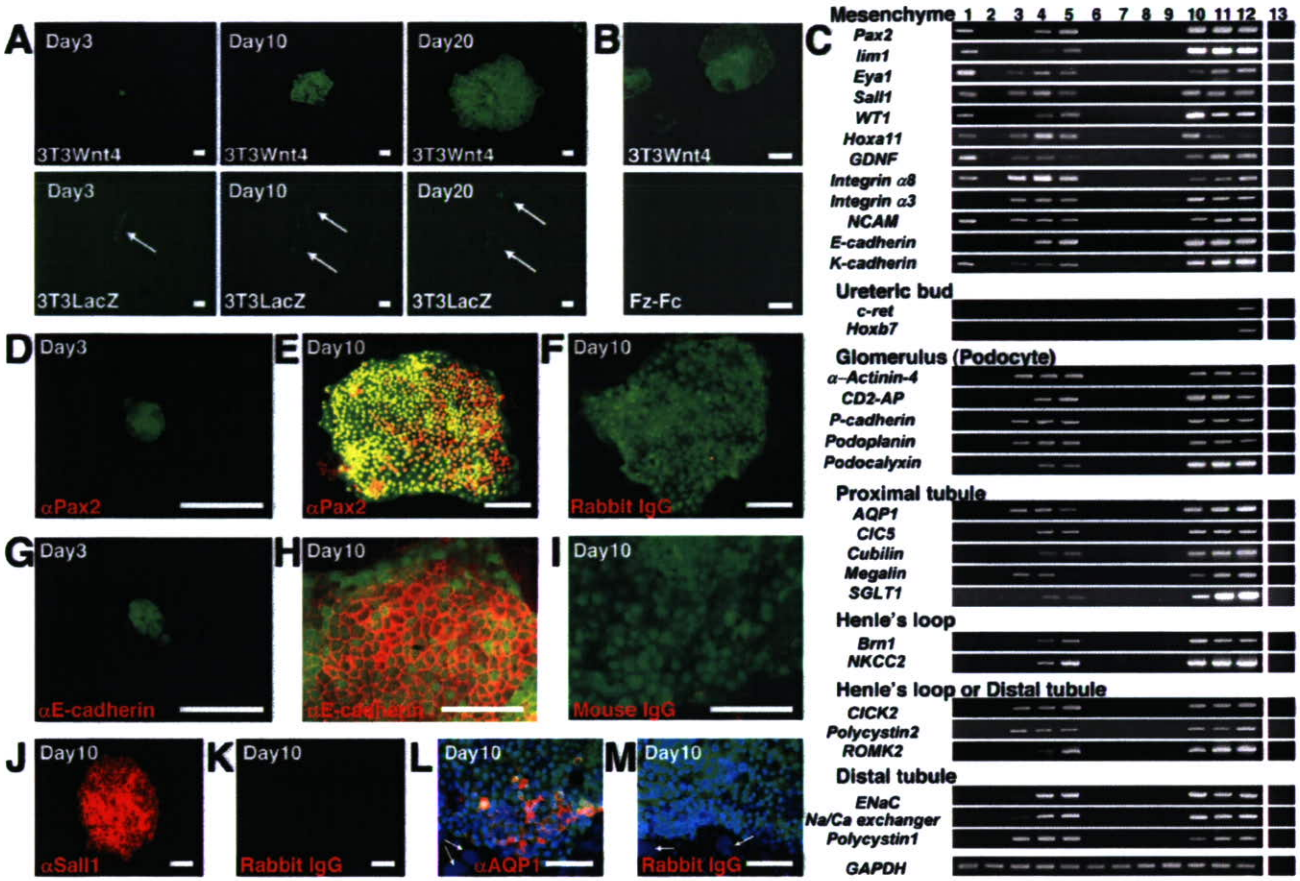

Fig. 1. In vitro colony formation from E11.5 metanephric mesenchyme. (**A**) Sheet-like colonies were formed on 3T3Wnt4, but not on 3T3*lacZ*. Arrows: fibroblast-like cells. (**B**) Colonies were not formed on 3T3Wnt4 with the addition of Fz-Fc chimeric protein. (**C**) RT-PCR analyses of genes expressed in metanephros and fully differentiated epithelia in glomeruli (podocyte), proximal and distal tubules, and the loop of Henle. Lane 1: E11.5 metanephric mesenchyme; 2: 3T3Wnt4 alone; 3: mesenchyme-derived cells cultured on 3T3Wnt4 at day 3; 4: at day 10; 5: at day 20; 6: 3T3*lacZ* alone; 7: mesenchyme-derived cells cultured on 3T3*lacZ* at day 3, 8: at day 10, 9: at day 20, 10: mesenchyme-derived cells at day 10 separated from 3T3Wnt4 feeder cells; 11: organ culture of E11.5 mesenchyme rudiments at day 10; 12: embryonic kidney (E17.5); 13: no RT reaction on mesenchyme-derived cells cultured on 3T3Wnt4 at day 10. (**D-M**) Immunocytochemistry of colonies for Pax2 (D-F), E-cadherin (G-I), Sall1 (J,K) and Aqp1 (L,M). (D-I) The expression of Pax2 and E-cadherin (red) was not detected at day 3 (D,G, respectively) but was observed at day 10 (E,H). (J,K) Sall1 expression (red) at day 10. (L,M) Aqp1 (red, proximal tubule marker) was expressed in some cells of the colony. Feeder cells have larger nuclei (DAPI, blue; arrows) than cells consisting of colonies. Control staining with rabbit (F,K,M) and mouse (I) IgGs. Mesenchyme of *Sall1-GFP* knock-in mice was used for J and K to visualize Sall1 expression using anti-GFP immunostaining, while EGFP transgenic mesenchyme was used for D-I,L,M. Scale bars: 50 μm.

To examine the multilineage differentiation of single cell-derived colonies, RT-PCR was done for 22 independent wells containing a colony at day 20. The representative data from three colonies are shown in Fig. 2B (lanes 1-3). Although variation existed between colonies, all the colonies expressed markers for each of the three segments: glomerular podocytes, proximal tubules and Henle's loop or distal tubules. Double staining using PNA and LTL, specific to glomerular podocytes and the proximal renal tubule, respectively, showed that adult kidney (8 weeks old) contained three kinds of cells; single-positive for PNA (those in the glomerulus); single-positive for LTL (those in the proximal renal tubule); and double-negative for LTL or PNA (Fig. 2C, left panel). Similarly, a single cell-derived colony at day 20 contained these three kinds of cells (Fig. 2C, right panel). With a combination of LTL and E-cadherin, at least three cell types were observed in adult kidney (Fig. 2D, left panel) and in a single cell-derived colony (right panel): cells strongly expressing only E-cadherin characteristic of distal renal tubules (Fig. 2D, arrows), and LTL-positive or -negative cells, with a faint expression of E-cadherin in the cell boundary. These results suggest that a colony was derived from a single progenitor, with multipotent differentiating capacity into epithelial cells in glomeruli, proximal and distal tubule, and the loop of Henle.

Colony-forming progenitors exist in the Sall1-GFP^{high} subpopulation of the metanephros

We next attempted to identify prospectively the renal progenitor cells using *Sall1-GFP* knock-in mice (Takasato et al., 2004). As *Sall1* is expressed in mesenchyme-derived tissues, GFP was detected in the mesenchyme around the ureteric bud at E11.5 in the *Sall1-GFP* heterozygous mouse (Fig. 3A, arrows). At E17.5, GFP-expressing cells were observed in the mesenchyme near the surface, as well as in C- or S-shaped bodies, and parts of renal tubules (Fig. 3B). By flow-cytometrical analysis, three subpopulations were fractionated based on the expression of Sall1-GFP: Sall1-GFP^{high},