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Original Article

Differentiation of murine embryonic stem cells toward renal lineages by conditioned medium from ureteric bud cells *in vitro*

Xiaohui Ren[1,2], Jingya Zhang[1], Xiaowen Gong[1], Xin Niu[1], Xuejin Zhang[1], Peng Chen[1], and Xuejun Zhang[1*]

[1]Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China
[2]Key Laboratory of Stem Cell Biology, Institute of Health Sciences, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences/ School of Medicine, Shanghai JiaoTong University, Shanghai 200025, China
*Correspondence address. Tel: +86-21-54921403; Fax: +86-21-54921403; E-mail: xjzhang@sibs.ac.cn

The kidney is formed from two tissue populations derived from the intermediate mesoderm, the ureteric bud, and the metanephric mesenchyme. Metanephric mesenchyme is a pluripotent renal stem population, and conversion of renal mesenchyme into epithelia depends on the ureteric bud *in vivo* and *in vitro*. Embryonic stem (ES) cells have been induced to differentiate into a broad spectrum of specialized cell types *in vitro*, including hematopoietic, pancreatic, and neuronal cells. Such ES-derived cells can provide a valuable source of progenitor cells. However, whether ES cells can be stimulated by factors secreted from the fetal renal cells to differentiate into renal precursor cells *in vitro* has not been reported. In this study, we showed that murine ES cells can give rise to embryoid bodies in the absence of leukemia inhibitory factor. Culture conditions were optimized [6 days, 10 ng/ml activin and 10^{-7} M retinoic acid (RA)] to generate maximal mesoderm populations specifically expressing Pax2 and brachyury. Results showed that 72% of the cells were brachyury positive by fluorescent activated cell sorter on Day 6 of EB cell differentiation. Conditioned medium collected from cultures of ureteric bud cells from renal cells of a 13-day-old fetus was added to the culture medium. Mesoderm cells were cultured for up to 10 days before showing expression of renal markers, initiation of nephrogenesis (WT-1 and Pax2), and terminally differentiated renal cell types (POD-1 and E-cadherin). This study suggests that ES cells pre-treated by RA and activin can interact with secreted molecules of the fetal renal cells to specifically differentiate into renal precursor cells. Our results provide an experimental basis for the development of *in vitro* assays to steer differentiation of ES cells toward renal lineages.

Keywords embryonic stem cell; embryoid body; differentiation; ureteric bud; renal lineage

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Introduction

Renal function in mammals is dependent on the organization of diverse epithelial cell types into functional units called uriniferous tubules. Each uriniferous tubule has a nephron and collecting tubule. According to anatomical conventions, the nephron is composed of a filtering glomerulus and a segmented epithelial tubule. Each nephron is contiguous with a collecting tubule that empties into the ureter via the renal calyces and pelvis [1]. Kidney development is mediated by mechanisms of induction, proliferation, and patterning, which provides a comprehensive model system for the study of embryonic organogenesis [2]. Kidney development depends on the interaction of the ureteric bud with the metanephric mesenchyme.

The initial outgrowth of the ureteric bud from the nephric duct is induced by growth factors from the metanephric mesenchyme [3]. The induced metanephric mesenchyme that aggregates at the ureteric bud tips can generate diverse epithelial cell types of the nephron [4]. The majority of ureteric bud cells differentiate into the renal collecting system. Metanephric mesenchymal cells, however, are not restricted to differentiating into nephron epithelia [5–7]. The metanephric blastema may be a pluripotent renal epithelial stem cell population [1].

Embryonic stem (ES) cells have been investigated extensively and were shown to be genuinely pluripotent [8], being able to differentiate into all embryonic cell types [9,10]. When leukemia inhibitory factor (LIF) is withdrawn, ES cells give rise to EBs [11] that can be induced to differentiate into several different cells types of mesodermal, endodermal, and neuroectodermal lineages, including those that specify hematopoietic [12], cardiomyocyte [13], pancreatic [14,15], hepatocyte [16,17], or neuronal fates [18].

Mimicking the differentiation of embryonic tissues within ES cell assay systems will require complex and

sequential combinations of protein signals, particularly when establishing more differentiated organ-specific precursor populations. The signals that specify intermediate mesoderm have received more attention in the past few years [4]. The model proposed by James and Schultheiss suggested that factors within the axial mesoderm suppressed the expression of intermediate mesodermal markers, and this suppression was lost at low bone morphogenetic protein (BMP) concentrations [19]. Activin, retinoic acid (RA), and BMP7, when added in combination to mouse ES (mES) cell culture, were shown to elevate *Pax2* and *WT-1* gene expression and improve embryonic renal tubule cell integration compared with undifferentiated mES cells [20].

Other researchers have reported that ES cells can differentiate into renal lineage via EBs *in vitro* [20,21], and the latest report showed that cells isolated from EBs formed the proximal tubules *in vivo* and without teratoma formation [22]. However, whether ES cells can be stimulated by soluble factors secreted from ureteric bud cells of fetal kidney to differentiate *in vitro* has not yet been reported. Here, we described the induction of mES cells to differentiate toward renal lineages by a two-step procedure, in which activin and RA were added in combination first to ES cell culture to induce mesodermal marker gene expression. Results showed that subsequent treatment with conditioned medium collected from cultures of ureteric bud cells of fetal kidneys could elevate the expression of renal lineage markers in the differentiated ES cells. The induced cells may be very useful for the development of bioartificial organs or cell-based therapies in chronic or acute renal failure.

Materials and Methods

Isolation and characterization of embryonic kidney tissues

Timed-pregnant Imprinting Control Region mice were obtained from SLAC Laboratory Animal Co. of Shanghai Institutes for Biological Sciences (Shanghai, China). The day of appearance of the vaginal plug was designated as gestation Day 0. On gestation Day 13, pregnant mice were sacrificed and metanephric kidney rudiments were dissected from the embryos and incubated with Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, USA), 10% fetal calf serum (Gibco) (DMEM-FCS) containing 0.2% collagenase (200 U/mg; Gibco) for 15 min at 37°C. Rudiments were then washed extensively in ice-cold DMEM-FCS. Ureteric buds were dissected from the collagenase-digested rudiments utilizing fine-tipped minutia pins. Isolated buds and blastemata were kept in DMEM-FCS on ice until use. For quantitative analysis, the isolated ureteric bud and metanephric blastemata fractions were trypsinized for 10 min (0.05% in 0.5 mM EDTA; Gibco). The single-cell suspensions were washed in DMEM-FCS and then phosphate-buffered saline (PBS) by low speed centrifugation and incubated for 30 min at 37°C with fluorescein-conjugated Dolichos Biflorus (FITC-DB, vector; 50 μg/ml; Sigma, St. Louis, USA), a lectin that binds specifically to the ureteric bud [23]. In addition, single-cell suspensions were incubated with FITC-DB in the presence of 1 M *N*-acetyl galactosamine, the sugar moiety to which DB binds. Cell preparations were washed and then fixed in 4% paraformaldehyde for 1 h prior to cell sorting. In addition, whole metanephric kidney rudiments were subjected to collagenase followed by trypsin digestion, and the total cell suspensions were assayed for FITC-DB binding as described above. Data on the cells (a minimum of 25,000/sample) were acquired on a Becton Dickinson fluorescent activated cell sorter (FACS) and analyzed with FlowJo software (version 5.7.2). The ureteric bud cells were grown in DMEM-FCS. At 80% confluence, the cells were rinsed with DMEM and the medium was then replaced with DMEM without FCS. Cells were then incubated for 2 days to generate conditioned media. After the medium was collected, ureteric bud cells were split 1:3, and this cycle was repeated. The number of passages of the ureteric bud cells is within three passages. The conditioned medium was centrifuged to remove cellular debris, filtered through 0.22-μm filters, and stored in −80°C until use for induction of differentiation of mesoderm-derived ES cells.

Cell culture and differentiation of EBs

mES cells (R1) were cultured on a feeder layer of mouse embryonic fibroblasts in DMEM supplemented with 15% FCS, 2 mM L-glutamine (Invitrogen, Carlsbad, USA), 5×10^{-5} M beta-mercaptoethanol (Sigma), and non-essential amino acids (Invitrogen; stock solution diluted 1:100) at 37°C in 5% CO_2 for 2 days. The ES cells were dissociated with 0.05% trypsin plus 0.53 mM EDTA (Gibco) and transferred to a 10-cm bacteriological petri dish coated with 1% agar (BD Falcon, San Jose, USA) to induce formation of EBs. The EBs suspension was cultured in the same medium without LIF for 2 days and then transferred to 6-cm tissue culture plates coated with 0.1% gelatin. The EBs were grown with or without combinations of the following growth factors: 10^{-6}, 10^{-7}, or 10^{-8} RA and 1, 5, or 10 ng/ml activin (Minneapolis, Billerica, USA). The plated EBs were cultured for up to 6 days before mesoderm cells were obtained. To study growth factor-induced modulation of ES cell-derived nephrogenesis, conditioned medium collected from cultures of ureteric bud cells were added to the culture medium and cell differentiation was compared with control cultures without conditioned medium. Mesoderm cells were cultured for up

to 10 days until they differentiated into renal cells (**Fig. 1**). Experiments were done at least in triplicate.

Immunofluorescence staining

The following antibodies (Abs) were used (source of supply, catalogue number, (m)onoclonal/(p)olyclonal Ab, and the dilution in PBS): rabbit anti-wt1 (Santa Cruz, Santa Cruz, USA; sc-192, pAb, 1:50); mouse anti-E-cadherin (Santa Cruz; G-10: sc-8426, mAb, 1:50), goat anti-podocin (Santa Cruz; P-16: sc-15007, pAb, 1:50), rabbit anti-Pax2 (Invitrogen; 71−6000, pAb, 1:50). Cells on the glass coverslips were fixed in 4% paraformaldehyde in PBS for 15 min on ice, followed by permeabilization with 0.1% Triton X-100 in PBS for 10 min at room temperature (RT). Cells were blocked for 1 h with 3% BSA in PBS. All primary antibodies were diluted in the same blocking buffer and incubated with samples overnight at 4°C. Cells treated with fluorescently coupled secondary antibody were incubated for 1 h at RT. The nuclei were stained with DAPI (Sigma) for 5 min at RT. All images were captured using conventional fluorescence and/or Laser Confocal Scanning Microscope (Leica TCS SP2 AOBS, Heidelberg, Germany).

DB staining

For DB staining, cells on the glass coverslips or metanephric kidney rudiments sections were incubated with 50 μg/ml of FITC-DB in the presence or absence of 1 M *N*-acetyl-D-galactosamine, the competitive sugar for DB binding. After lectin incubation (37°C for 30 min), cells or sections were washed and fixed. The nuclei were stained with DAPI (Sigma) for 5 min at RT. All images were captured using conventional fluorescence and/or confocal microscopy LSM. Cells were analyzed by FACS.

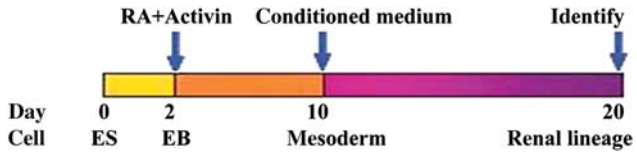

Figure 1 ES cell-derived renal differentiation protocol ES cells were dissociated and transferred to a petri dish coated with 1% agar to induce EB formation. The EB suspension was cultured in the same medium without LIF for 2 days and then transferred to 6-cm tissue culture plates coated with 0.1% gelatin. The EBs were grown without growth factors or in the presence of different combinations of the following growth factors: 10^{-6}, 10^{-7}, or 10^{-8} M of RA and 1, 5 or 10 ng/ml activin. The plated EBs were cultured for up to 7 days before obtaining mesoderm cells. After differentiation, individual properties of ES cell−derived mesoderm cells were assayed by immunofluorescence (anti-brachyury antibody) and sorted by FACS. Conditioned medium collected from cultures of ureteric bud cells of a gestation day 13 fetal kidney was added to the culture medium. Mesoderm cells were cultured for up to 10 days until differentiation into renal cells.

Flow cytometry

Cells were dissociated into single-cell suspension with 0.05% trypsin plus 0.53 mM EDTA, and washed twice with PBS, and resuspended in blocking solution (PBS with 3% BSA) for 1 h. Cells were then incubated with anti-brachyury (H-210) rabbit polyclonal antibody (Santa Cruz; sc-20109, pAb, 1:50) prepared in PBS with 1% BSA at 4 μg/ml for 1 h. Cells were then washed twice with PBS containing 1% BSA, incubated with appropriate secondary antibody conjugated with Alexa 561 for 1 h. Following incubation, cells were washed twice, and resuspended in PBS. The stained cells were analyzed on a Becton Dickinson FACS machine and analyzed with FlowJo software (version 5.7.2).

Results

Isolation and characterization of embryonic kidney tissues

Isolated ureteric buds were dissociated into single-cell suspensions, incubated with FITC-DB and analyzed as described in Materials and Methods. Isolated ureteric buds presented as a single homogeneous population of DB-positive cells and exhibited fluorescent staining intensities approximately 1 log unit greater than negative controls. Data from 25,000 cells were acquired and analyzed, showing that 69% of this cell population stained positive [**Fig. 2(A−C)**]. On gestation Day 13, the mouse metanephric kidney rudiment is composed of the two primordia that form the kidney, the ureteric bud and the surrounding metanephric mesenchyme. By light microscopy, ureteric bud cells were observed to be organized into a single tubule with a dilated ampulla. All ureteric bud cells bound the lectin DB, but cells of the metanephric blastema exhibited a dispersed, spindle-shaped morphology and were not labeled by DB [**Fig. 2(D−F)**].

Cells differentiated from ES cells expressed mesoderm marker molecules after RA and activin treatment

RA and activin in combination are able to expand the pronephric field in the *Xenopus* embryo [20,24]. Experiments with the RA and activin combination generated high levels of Pax2 protein [20]. *Brachyury*, or *T*, is one of the few genes in mouse known to play a role in the specification and differentiation of the early mesoderm at gastrulation [25]. Pax2 is one of the earliest markers in the intermediate mesoderm [20]. To examine the ability of ES cells to differentiate into intermediate mesoderm under controlled conditions, we first formed EBs in culture over a 2-day period. Subsequently, the EBs were cultured with increasing concentrations of RA and activin and grown for an additional 5 days before being analyzed for brachyury and Pax2 protein expression (**Fig. 1**). The concentrations of RA

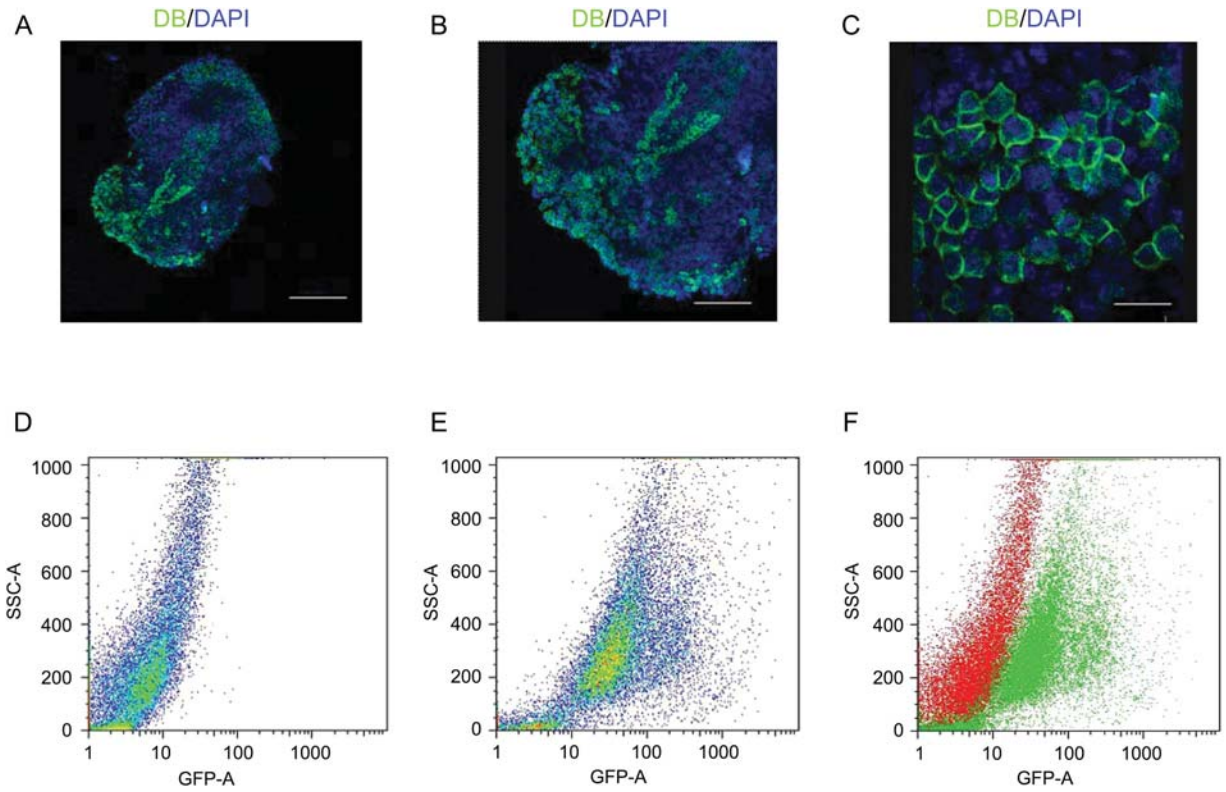

Figure 2 Morphology of the gestation Day 13 mouse kidney rudiment and isolation of ureteric bud by FACS (A–C) Morphology of the gestation Day 13 mouse kidney rudiment. Mouse kidney rudiment isolated from a gestation Day 13 embryo was processed for FITC-DB and Hoechst 33258 staining. The ureteric bud cells were labeled with FITC-DB (green), while metanephric mesenchymal cells did not bind this lectin (scale bar = 150 μm, 100 μm, 30 μm in A, B, C, respectively). Positive cells were not observed when sections were incubated in an excess of the competing sugar for DB, *N*-acetyl-D-galactosamine. (D–F) Kidney rudiments isolated from gestation Day 13 mice embryos were dissociated into single-cell suspensions and incubated with 50 μg/ml FITC-DB, in the presence (D) or in the absence (E) of 1 M N-acetyl-D-galactosamine. Cells were analyzed by FACS. The result showed that 69% of the isolated ureteric bud cells were DB positive. (F) Merged images from (D) and (E) using Flowjo software.

and activin ranged from 10^{-6} to 10^{-8} M and 1–10 ng/ml, respectively. Experiments with RA (10^{-7} M) + activin (10 ng/ml) in combination generated the highest levels of Pax2 and brachyury protein (data not shown). Brachyury [**Fig. 3(A)**] and Pax2 [**Fig. 3(C)**] staining was evident in EBs that were cultured in a cocktail of RA and activin but not in control EBs **[Fig. 3(B,D)]**. FACS analysis of the differentiated cells induced with RA (10^{-7}M) + activin (10 ng/ml) showed that, compared with the negative control [**Fig. 3(E)**], 72% of the cells were brachyury positive by Day 6 of EB differentiation [**Fig. 3(F)**].

Cells differentiated from mesoderm cells express renal lineage marker molecules after conditioned medium treatment

Conditioned medium collected from cultures of ureteric bud cells of a gestation Day 13 fetal kidney was added to the culture medium of the ES cells pre-treated with RA (10^{-7} M) and activin (10 ng/ml). The mesoderm cells were cultured for up to 10 days before expressing renal markers. Results showed that the differentiated cells expressed marker genes characteristic for initiation of nephrogenesis (WT-1 and Pax2) and terminally differentiated renal cell types (POD-1 and E-cadherin) (**Fig. 4**). The studies suggested that the specificity of DB binding to the ureteric bud/forming collecting system is maintained throughout development and in the mature rodent kidney [23,26]. The cells differentiated from ES cells cultured with conditioned medium were FITC-DB positive, suggesting that they possess characteristics of the kidney collecting duct system (**Fig. 5**).

Discussion

In this study, we examined the ability of ES cells to differentiate into the renal progenitor cells of the intermediate mesoderm. RA and activin were used to stimulate the expression of early intermediate mesodermal markers [20]. Brachyury is one of the few genes in the mouse known to play a role in the specification and differentiation of early mesoderm at gastrulation [25]. Kubo *et al*. [27] confirmed that activin-induced brachyury-positive cells provide a unique population of enriched endoderm progenitors. Furthermore, Morizane *et al*. [28] indicated that activin can

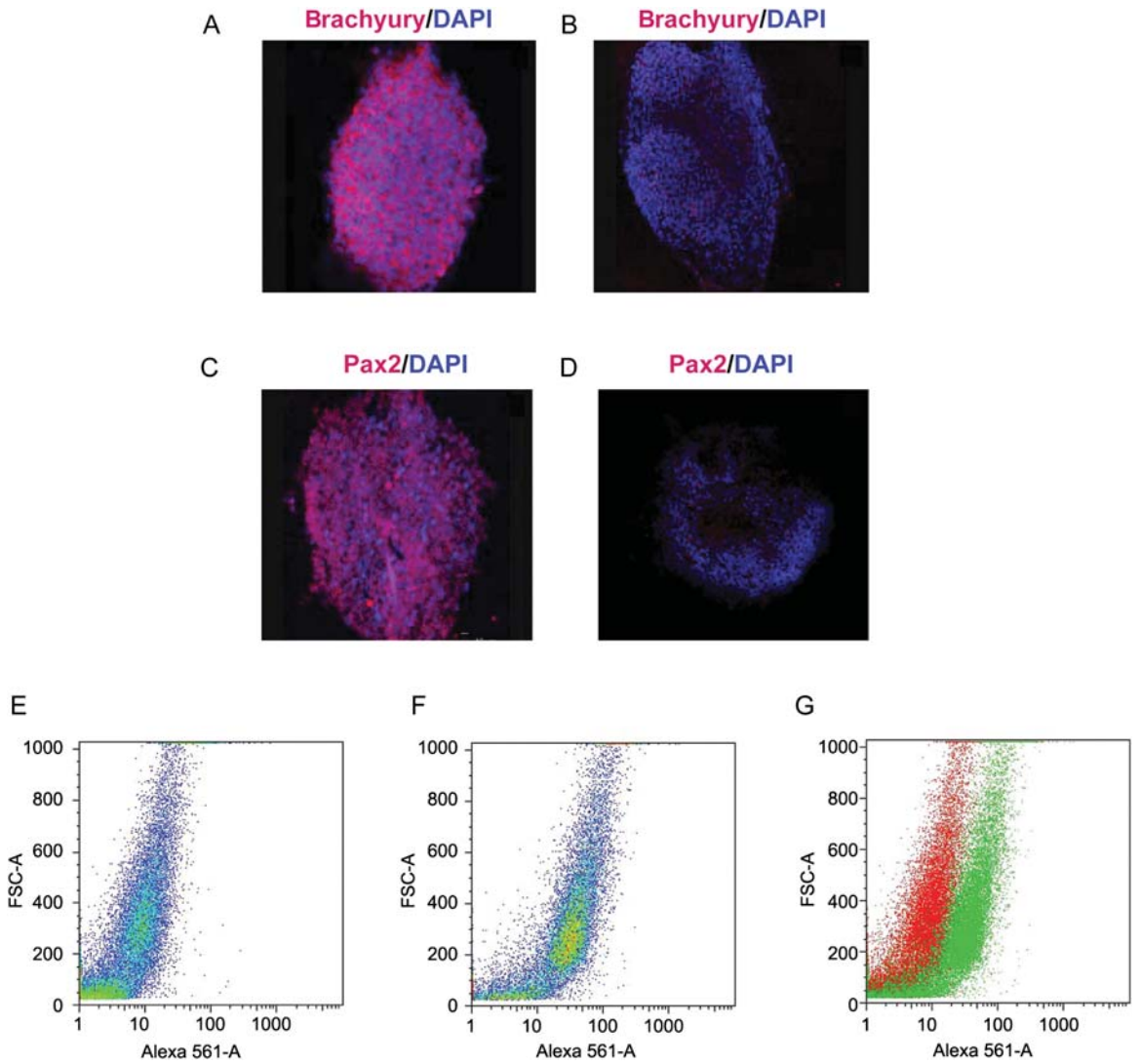

Figure 3 The cells differentiated from ES cells cultured with a combination of RA and activin express markers of intermediate mesoderm The cells differentiated from ES cells cultured with (A, C) or without (B, D) a combination of RA (10^{-7} M) and activin (10 ng/ml) were stained for brachyury (A, B) and Pax2 (C, D), markers characteristic for initiation of differentiation of intermediate mesoderm. The cells were positive for both brachyury (in A, red) and Pax2 (in C, red) with the combination treatment, while the cells without the combination treatment were negative for both brachyury and Pax2 in (B) and (D), respectively. The nuclei were stained with DAPI. These data correlated with those obtained by cell sorting after the differentiated cells were induced with a combination of RA (10^{-7} M) and activin (10 ng/ml). (E) Negative control. (F) On Day 6 of EBs cells differentiation, 72% of the cells were brachyury positive. (G) Merged images from E and F with software Flowjo.

enhance the differentiation of ES cells not only to mesoderm but also to tubular cells. Therefore, Pax2 is essential for multiple steps during intermediate mesoderm development, including the differentiation of both ductal and mesenchymal components of mesonephros and metanephros and their derivatives. It is a primary regulator of urogenital development and it may control a regulatory hierarchy of genes involved in the differentiation of intermediate mesoderm [29]. Our results showed that a combination of RA and activin effectively induced the expression of the intermediate mesodermal markers, brachyury and Pax2, in EBs. These results are consistent with the observation that both RA and activin can induce pronephric markers [20,21,27,28,30]. This represents a first important step in generating endoderm renal populations from brachyury- and Pax2-positive cells.

These data suggested that the RA and activin-treated EBs were primed to respond to inductive signals, similar to the metanephric mesenchyme. The metanephric mesenchyme by itself does not form tubules unless induced by the ureteric bud or a heterologous inducer, such as the spinal cord or exogenous Wnt proteins [31]. Therefore, the ureteric bud 'induces' mesenchymal cells to proliferate and convert to epithelia [32]. Although it has long been known that rescue from apoptosis [33,34], and epithelialization [32] of the metanephrogenic mesenchyme requires signaling by the ureteric bud, the signaling molecules remain to be identified.

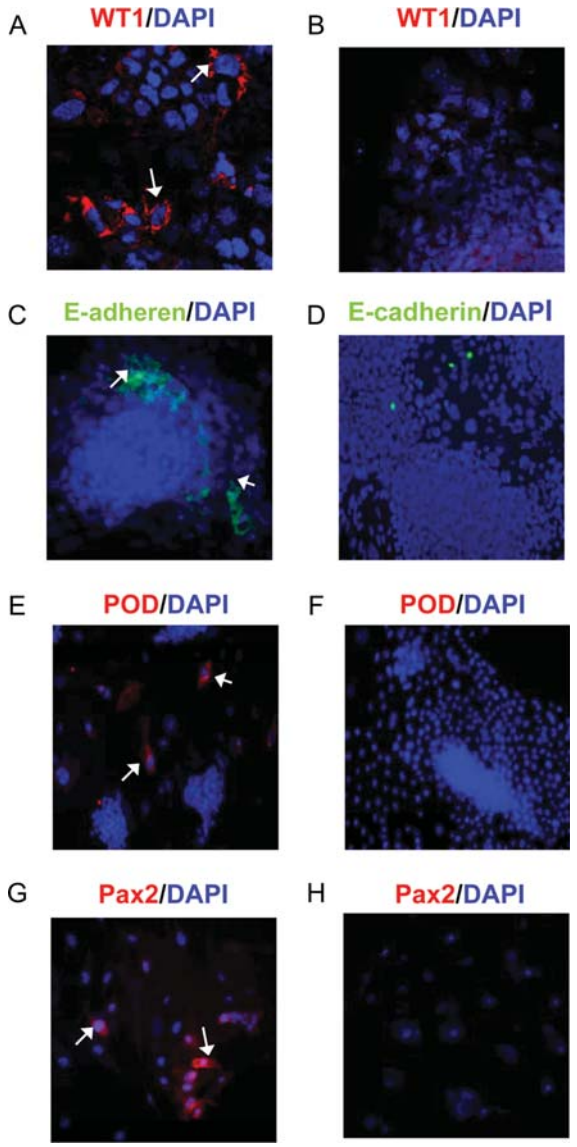

Figure 4 Immunostaining showing the expression of renal lineage genes in differentiated cells The cells differentiated from ES cells cultured by conditioned medium from fetal renal cells expressed marker genes characteristic for initiation of nephrogenesis (WT-1 and Pax2) and terminally differentiated renal cell types (POD-1 and E-cadherin). (A, C, E, G) Cells that were cultured in conditioned medium immunostained for WT-1, E-cadherin, POD-1 and Pax2, respectively. The arrows indicate positive cells. (B, D, F, H) Control cells cultured without conditioned medium were immunostained for WT-1, E-cadherin, POD-1 and Pax2, respectively. The results showed that cells were all negative. The nuclei were stained with DAPI.

Carroll *et al*. [35] suggested that ureteric buds secretes several initial signals that lead to the survival, proliferation, and aggregation of the metanephric mesenchyme. Meanwhile Barasch *et al*. [36] believed that nephrogenesis is stimulated by two distinct ureteric signals, secreted molecules that rescue the mesenchyme from apoptosis, whereas diffusion-limited basolateral molecules trigger mesenchymal/epithelial conversion. Karavanova *et al*. [2] showed that metanephric mesenchyme can be induced to

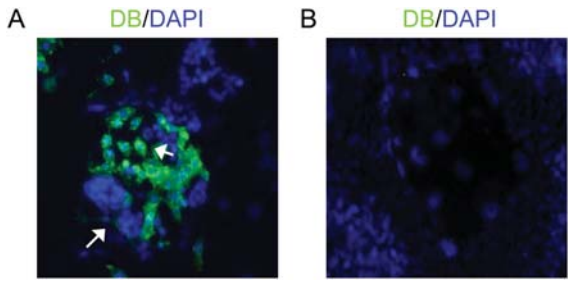

Figure 5 FITC-DB staining showing the expression of renal lineage genes in differentiated cells (A) The cells differentiated from ES cells cultured by conditioned medium collected from cultures of fetal renal cells. (B) Control cells cultured without conditioned medium. The nuclei were stained with DAPI.

differentiate without contact with an inductive tissue, and that these processes can apparently be triggered by soluble factors in the filtered medium. However, whether brachyury-positive cells from the stimulated ES cells could interact with secreted molecules from the fetal renal cells and proceed onto a specific differentiation pathway towards a renal cell line has not yet been reported.

When we examined the isolated ureteric buds by FACS after incubation with FITC-DB, we observed that 69% of isolated ureteric buds bound the ureteric bud-specific marker, DB. This percentage is compatible with the initial purity of isolated ureteric bud preparations. In this study, conditioned medium from ureteric bud cells of embryonic kidneys, when added to pre-treated ES cell cultures, were shown to induce renal lineages markers Pax2, WT-1, Pod-1, E-cadherin gene expressions, and FITC-DB binding.

Pax2 is expressed in both ductal and mesenchymal components derived from the intermediate mesoderm during the development of pronephros, mesonephros, and metanephros [37]. Previous *in vitro* experiments have suggested a role for Pax2 in the conversion of mesenchyme to epithelium during metanephros development [38]. Our results showed that Pax2 is expressed in both steps of the differentiation process: induction by RA + activin, and treatment with conditioned medium. The *WT-1* gene is expressed in condensing metanephric mesenchyme and localizes in the distal regions of comma- and S-shaped bodies that differentiate to form podocytes of the glomerulus [39]. The co-localization of WT-1 and podocalyxin during kidney development, and the reported transcriptional activation of podocalyxin by WT-1 *in vitro* [40], suggest possible *in vivo* regulation. Pod-1, also known as epicardin and capsulin, is expressed in mesenchymal cells at the site of epithelial–mesenchymal interaction and later within podocytes as they differentiate. Inhibition of Pod-1 expression resulted in decreased mesenchymal cell condensation around the ureteric bud and a 40% decrease in ureteric branching [41].

E-cadherin is a cell adhesion protein localized at the adherens junction that mediates cell–cell interactions [42],

and its expression serves as a marker for epithelial differentiation [43,44]. The expression of E-cadherin also plays a key role in normal development during the mesenchymal–epithelial transition [45,46]. E-cadherin appears in the induced metanephric mesenchyme once condensation into epithelia begins [47,48], at the same time when WT-1 expression rises in the developing kidney [49]. Findings by Hosono *et al*. [42] suggested that *E-cadherin* is a gene coordinately regulated with *WT-1* during kidney development, whose expression can be activated in response to stable or transient expression of *WT-1*.

Our results showed that cells differentiated from EBs expressed renal lineage markers, Pax2, WT-1, Pod-1, and E-cadherin. The results also indicated that mesoderm cells derived from EBs were induced to differentiate into renal lineage cells when cultured in conditioned medium from ureteric bud cells. Additionally, the specificity of DB binding to the ureteric bud/forming collecting system is maintained throughout development and in the mature rodent kidney [23,26]. The ES cells cultured with conditioned medium were FITC-DB positive, suggesting that we were able to obtain differentiated ES cells, which possessed the characteristics of the kidney duct collecting system in our study. Although the cells were still a heterogeneous mixture and had a low efficiency of induction, many of the cells expressed more differentiated markers found in the derivatives of EBs when treated with the conditioned medium from fetal renal cells. Thus, we believe that pretreatment of EBs with the RA/activin nephrogenic cocktail and conditioned medium predisposes EBs to differentiate along the renal epithelial lineage similar to the potential of the intermediate mesoderm, although they do not generate true epithelial cells *in vitro*. This induction assay will be useful for the generation of mesoderm-derived cell populations with implications for future renal cell therapeutic/integration assays.

To date, only a handful of reports have suggested renal progenitor cell differentiation from ES cells, highlighting the future need for improvements in this field [21]. The high efficiency of ES/EB cells integration into embryonic renal tubule epithelia has been reported [20,21,50,51]. However, it has also been reported that teratomas can form when these cells are administered to adult animals, suggesting that one of the potential dangers of ES cell-based therapy [21]. Thus, it is critically important to find additional specific surface markers of kidney progenitor populations to enhance cell isolation.

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