

Integration of Embryonic Stem Cells in Metanephric Kidney Organ Culture

Brooke M. Steenhard,* Kathryn S. Isom,* Patricia Cazcarro,* Judy H. Dunmore,[†]
Alan R. Godwin,[†] Patricia L. St. John,* Dale R. Abrahamson*

Departments of Anatomy and Cell Biology*, and [†]Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, Kansas

Many stages of nephrogenesis can be studied using cultured embryonic kidneys, but there is no efficient technique available to readily knockdown or overexpress transgenes for rapid evaluation of resulting phenotypes. Embryonic stem (ES) cells have unlimited developmental potential and can be manipulated at the molecular genetic level by a variety of methods. The aim of this study was to determine if ES cells could respond to developmental signals within the mouse embryonic day 12 to embryonic day 13 (E12 to E13) kidney microenvironment and incorporate into kidney structures. ROSA26 ES cells were shown to express β -galactosidase ubiquitously when cultured in the presence of leukemia inhibitory factor to suppress differentiation. When these cells were microinjected into E12 to E13 metanephroi and then placed in transwell organ culture, ES cell-derived, β -galactosidase-positive cells were identified in epithelial structures resembling tubules. On rare occasions, individual ES cells were observed in structures resembling glomerular tufts. Electron microscopy showed that the ES cell-derived tubules were surrounded by basement membrane and had apical microvilli and junctional complexes. Marker analysis revealed that a subset of these epithelial tubules bound *Lotus tetragonolobus* and expressed α_1 Na⁺/K⁺ ATPase. ES cells were infected before injection with a cytomegalovirus promoter-green fluorescence protein (GFP) adenovirus and GFP expression was found as early as 18 h, persisting for up to 48 h in cultured kidneys. This ES cell technology may achieve the objective of obtaining a versatile cell culture system in which molecular interventions can be used *in vitro* and consequences of these perturbations on the normal kidney development program *in vivo* can be studied.

J Am Soc Nephrol 16: 1623–1631, 2005. doi: 10.1681/ASN.2004070584

The development of the metanephric kidney begins when inductive signals are shared between the metanephric mesenchyme and the ureteric bud (1). The mesenchyme responds by aggregating at the tips of the ureteric bud, forming a vesicle that sequentially transforms into a comma shape, followed by an S-shaped figure. Endothelial precursors migrate into the vascular cleft of comma- and S-shaped figures and, together with developing podocytes, begin to synthesize the glomerular basement membrane (2). Subsequent cellular differentiation occurs, which ultimately produces the distinct segments of the mature nephron.

Many aspects of kidney development occur when isolated embryonic kidneys are placed into organ culture, or when isolated tissues are cocultured using transwell filters to separate metanephric mesenchyme from the ureteric bud (3). Namely, aggregation, formation of comma-shaped and S-shaped figures, development of avascular glomerular tufts, and segmentation of tubules all occur. In addition, transplantation of embryonic kidneys into newborn mouse kidney cortex or anterior eye

chambers of adult hosts allows the study of vascularization of the nephron (4–6). Consequently, these organ culture and transplantation experiments have been valuable for understanding aspects of renal induction, branching morphogenesis, tubulogenesis, and vascularization.

We sought to exploit the organ culture model system further by introducing cells into embryonic mouse kidneys and establish if they would respond to metanephric inductive signals by developing into kidney structures. Specifically, we desired to inject a developmentally uncommitted cell, and one that was amenable to genetic modification. Embryonic stem (ES) cells are multipotent and differentiate into a variety of cell types in response to growth factors in culture. However, the culture conditions necessary to drive differentiation toward kidney are not well-established (7,8). Human ES cells do have the capacity to differentiate into kidney structures when injected into immunosuppressed mice to form teratomas (9,10). In regeneration models of kidney repair, only adult stem cells have been used as a source of cells to repopulate the injured kidney (11,12). Treatment of renal diseases has primarily focused on either organ transplantations or gene therapy (13,14), given the existing hurdles of ES cell therapy (15). Application of ES cell therapy has been used successfully in many other disease models such as Parkinson disease, diabetes, and hepatectomy (16–18). In those experiments, ES cells not only expressed markers of differentiation but also restored physiologic functions lack-

Received July 22, 2004. Accepted March 14, 2005.

Published online ahead of print. Publication date available at www.jasn.org.

Address correspondence to: Dr. Dale R. Abrahamson, Department of Anatomy and Cell Biology, University of Kansas Medical Center, MS #3038, 3901 Rainbow Boulevard, Kansas City, KS 66160. Phone: 913-588-7000; Fax: 913-588-2710; E-mail: dabrahamson@kumc.edu

ing in the host animal, offering great promise for the therapeutic use of ES cells (19). ES cells are also capable of undergoing genetic modifications by a variety of means such as homologous recombination (20), viral infection (21), or RNA interference (22). We therefore elected to microinject ES cells into embryonic mouse kidney. We then assessed whether these exogenous cells integrated into structures that formed in metanephric organ culture.

Materials and Methods

ES Cell Culture

ROSA26 embryonic stem cells, a kind gift from Dr. Philippe Soriano (Fred Hutchinson Cancer Research Center, Seattle, WA) (23), were maintained in the presence of mouse embryonic fibroblasts according to standard methods in KO+KOSR media (Knockout DMEM containing 15% Knockout Serum Replacement [Invitrogen, Carlsbad, CA] and supplemented with 1000 U/ml leukemia inhibitory factor (LIF) [Esgro; Chemicon, Temecula, CA] 2 mM L-glutamine, nonessential amino acids, 0.1 mM β -mercaptoethanol, 50 U/ml penicillin, and 50 μ g/ml streptomycin). Mouse embryonic fibroblasts were depleted from ES cell cultures by three rounds of plating on gelatin in the presence of 15% FBS. Frozen stocks of the fibroblast-depleted ES cells were plated in KO+KOSR media for 2 d before injection. ES cells were positive for Oct 4 (data not shown), a stem cell marker, as determined by anti-Oct 4 antibody labeling (Santa Cruz Biotechnology, Santa Cruz, CA).

Animals

Outbred CD-1 mice were obtained from our colony founded with stocks originating from Charles River (Wilmington, MA). All animal husbandry and procedures were in adherence to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Microinjections

Embryonic day 12 to 13 (E12 to E13) mouse kidneys were dissected from timed pregnant CD-1 females and placed in DMEM/F12-defined media (supplemented with 2 mM L-glutamine, 10 mM HEPES, 5 μ g/ml insulin, 5 μ g/ml transferrin, 2.8 nM selenium, 25 ng/ml prostaglandin E, 32 pg/ml T3, 250 U/ml penicillin, and 250 μ g/ml streptomycin) on ice until the time of injection (24). ROSA26 ES cells were trypsinized, counted, and loaded into a beveled glass capillary injection pipette (World Precision Instruments, Sarasota, FL) at a concentration of 100 cells/nl. Immediately before injection, kidneys were placed on small pieces of polyethylene terephthalate track-etched membranes (Falcon cell culture inserts, 0.4 μ m pore size). Each kidney received three microinjections (5 nl each) using a Pico-injector (Harvard Apparatus, Holliston, MA). Membranes with adherent kidneys were floated on a drop of media for 1 h at 37°C before transferring the kidneys to a new cell culture insert or adding additional culture media. Kidneys were grown in organ culture on membranes (as either inserts or free floating membranes) for 3 to 5 d at 37°C and 95% air, 5% CO₂.

β -Galactosidase Development

After organ culture, kidneys were fixed in freshly prepared 0.2% paraformaldehyde (in PBS containing 0.1 M PIPES, pH 6.9, 2 mM MgCl₂), cryoprotected in 30% sucrose, and frozen in Tissue-Tek OCT embedding compound. Frozen serial sections, 8 μ m thick, were postfixed in 0.2% paraformaldehyde, rinsed twice in PBS containing 2 mM MgCl₂, and incubated in detergent rinse (0.1 M phosphate buffer, pH 7.3, 2 mM MgCl₂, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40) for 10 min on ice (25). β -galactosidase histochemistry was performed

overnight at 37°C in color development solution (detergent rinse with 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 20 mM Tris, pH 7.3, and 1 mg/ml X-gal). Slides were postfixed in 4% paraformaldehyde, dehydrated through graded ethanol, cleared in xylene, and coverslipped. In some cases, kidneys underwent color development as whole mounts before OCT embedding and serial sectioning.

Morphometric Analysis

Three kidneys from six daily time points (day 0 to day 5) were serially sectioned and developed for β -galactosidase activity. Every third section through the entire kidney was imaged and imported into Photoshop CS. Total kidney section area and areas occupied by blue, β -galactosidase-positive ES cells were manually selected and measured. Data are presented as a percentage of blue per total kidney area. During the same analysis, each blue structure was classified as single cells, dispersed cells, dead/dying cells, or tubules. These four phenotypes were expressed as a percentage of total blue structures.

Antibody and Lectin Labeling

Serial frozen sections of injected and cultured embryonic kidney were used for antibody or lectin labeling. Slides were fixed for 10 min in cold 100% methanol and rinsed twice with PBS. For antibody labeling, a 1:100 dilution of either rabbit anti-WT1 (Santa Cruz Biotechnology), sheep anti-laminin (26), or 10 μ g/ml of goat anti-type IV collagen (SouthernBiotech, Birmingham, AL) IgGs were incubated on frozen sections at room temperature for 1 h. In other cases, sections were fixed in 0.2% paraformaldehyde, permeabilized with 0.5% TritonX-100/PBS, and double-labeled with a 1:50 dilution of rabbit anti-WT1 and 10 μ g/ml mouse anti- β -galactosidase (Promega, Madison, WI), or a 1:3000 dilution of mouse anti-Na⁺/K⁺ ATPase α_1 (Sigma, St. Louis, MO) and 10 μ g/ml mouse anti- β -galactosidase, followed with isotype-specific secondary antibodies. After three washes with PBS, slides were treated with the appropriate secondary antibody for 1 h (anti-rabbit IgG-Alexa 488, anti-rabbit IgG-Alexa 594, anti-mouse IgG2a-Alexa 488, anti-mouse IgG1-Alexa 594, anti-goat IgG-Alexa 488 [Molecular Probes, Eugene, OR] or anti-sheep IgG rhodamine [MP Biomedicals, Irvine, CA]). Other slides were also treated for 1 h with *Lotus tetragonolobus* (LTA) lectin-FITC (1:100 dilution) (Vector Laboratories, Burlingame, CA). Lectin staining was blocked by preincubation of slides with 0.5 M L-fucose. All slides were washed three times with PBS before mounting in Prolong antifade mounting media (Molecular Probes).

Electron Microscopy

ROSA26 ES cell-injected organ cultured mouse kidneys were washed three times with PBS containing 2 mM MgCl₂, dislodged from the transwell with gentle pipetting of fluid, and then transferred to a glass sample vial. Tissue was fixed for 2 h on ice with 2% paraformaldehyde/0.4% glutaraldehyde in PBS containing 2 mM MgCl₂, followed by three washes in buffer. Samples were developed overnight with Bluo-gal staining solution (20 mM potassium ferricyanide/ferrocyanide, 2 mM MgCl₂, 2 mM Bluo-gal [Invitrogen], pH 7.3 at 37°C). After washing three times with buffer, samples were refixed with Karnovsky's fixative for 30 min, washed three times with 0.1 M sodium-cacodylate in 3.5% sucrose, pH 7.3, and then postfixed for 1.5 h with Palade's OsO₄. Tissues were dehydrated through graded ethanol and propylene oxide and embedded in Polybed 812 with overnight polymerization at 60°C. Ultrathin sections were stained with 4% uranyl acetate and Reynold's lead citrate.

Adenovirus Infection

ROSA26 ES cells were grown in 10-cm tissue culture plates for 1 d before adenovirus infection. Media was replaced with 5 ml of

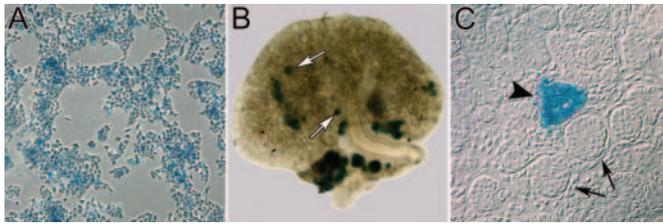


Figure 1. ROSA26 embryonic stem (ES) cells displayed β -galactosidase activity in both undifferentiated cultures (A) and after injection into E13 mouse kidneys (B, C). After injection of approximately 1500 ROSA26 ES cells and after 5 d of organ culture, numerous β -galactosidase–positive clusters were seen within the kidney (white arrows, B). A plastic section of an injected kidney (C) showed that normal organ culture development occurred after ES cell injection. Tubules and glomerular epithelial tufts (black arrows) were apparent, as were areas of blue cells (arrowheads). Often, the structures containing blue cells resembled epithelial tubules. Such tubules were usually uniformly blue and apparently did not contain nontransgenic (unstained) cells.

KO+KOSR containing 200 active virus particles per cell of Ad5-CMV5–green fluorescence protein (GFP) adenovirus (Invivogen, San Diego, CA). The plates were manually rotated every 20 min for 2 h at 37°C, after which an additional 5 ml of KO+KOSR (without virus) was added. The next day, virus-infected ES cells were trypsinized, counted, and microinjected into E12 mouse kidneys as described. GFP fluorescence was viewed daily during the length of organ culture on a Nikon

inverted microscope with a FITC filter (B-2E/C). Control kidneys (cells injected without virus infection) were imaged with the same exposure times used for the virus-infected ES cell-injected kidneys. At various time points during culture, kidneys were fixed for β -galactosidase color development as described. Serial sections were either color developed or reacted with an anti-GFP Alexa-488–conjugated antibody (Molecular Probes; 1:200 dilution).

Results

ES Cells Develop into Tubules after Metanephric Culture

We sought to investigate the capacity of ES cells to adopt a renal differentiation program when placed in the developing kidney. Mouse ROSA26 ES cells contain a knock-in of the *lacZ* gene, which encodes β -galactosidase and is ubiquitously expressed (23,27). These cells were grown for 2 d in media containing LIF to suppress differentiation. When undifferentiated ROSA26 ES cells were developed for β -galactosidase activity, they turned intensely blue, as expected, which reflected transgene expression (Figure 1A). Undifferentiated ES cells were trypsinized, counted, and microinjected into E13 normal mouse kidneys. When microinjected kidneys were processed for β -galactosidase after 5 d of organ culture, blue structures were apparent in both whole-mount and sectioned tissue (Figure 1, B and C). Typical tubulogenesis and “glomerular” epithelial tuft formation occurred in organ culture in close proximity to ES cell–derived structures (Figure 1C, arrows). The most common ES cell–derived structures in 5-d cultures were aggregates of blue cells resembling tubules (Figure 1C).

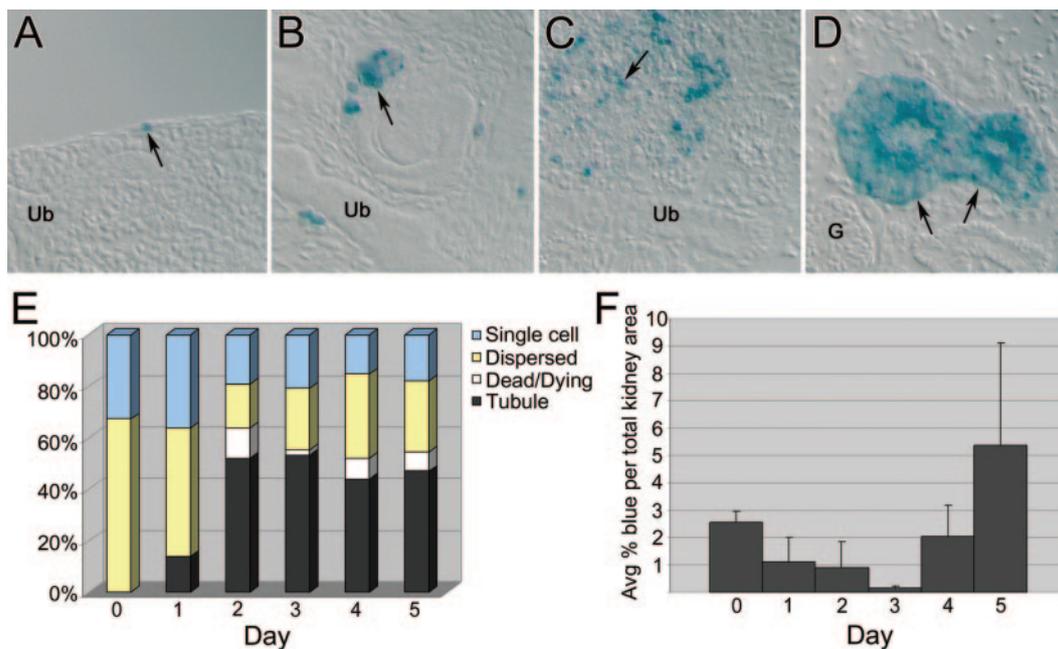


Figure 2. Embryonic kidneys that had been injected with ROSA26 ES cells and cultured for 1 h (0) to 5 d were developed for β -galactosidase activity. Four phenotypes of the resulting blue structures were observed: single cells (A), dispersed or clustered blue cells (B), areas that appeared dead or dying (C), or epithelial tubules (D). Three kidneys from each time point were serially sectioned and each blue structure was classified and counted, then expressed as a percentage of total blue structures (E). Total area occupied by β -galactosidase–positive ES cells was determined for three individual kidneys from each time point and expressed as a percentage of total kidney area (F).

Morphology of Resulting ES Structures

To define the frequency of ES cell–derived tubule formation within cultured kidneys, a time course experiment was performed in which kidneys were sampled each day from 1 h postinjection (day 0) to day 5. Three kidneys from each time point were serially sectioned and developed for β -galactosidase. Four main phenotypes containing β -galactosidase–positive areas were found: single cells (individual cells completely surrounded by host cells) (Figure 2A), dispersed cells (blue cells in loose groups, apparently nonpolarized) (Figure 2B), dead or dying cells (often bright blue, rough appearance) (Figure 2C), or epithelial tubules (polarized, often with lumen) (Figure 2D). The type and number of blue structures were assessed in every third serial slide (Figure 2E). One hour after injection, only single cells or clusters of dispersed cells were observed (Figure 2E). One day after injection, however, tubules were first observed and 13.9% of all blue structures were classified as tubules. By day 2, 51.9% of all β -galactosidase–positive structures were tubules, and this proportion remained constant throughout the remaining time in culture (day 5 = 47.0%). In addition, the area occupied by ES cells was found to decrease during the initial 3-d culture period, but increased dramatically thereafter to constitute approximately 5% of the total kidney area in 5-d cultures (Figure 2F). Thus, there was an expansion of the β -galactosidase–positive ES cells during the culturing period, with a tubule morphology representing the most prominent phenotype.

ES Cell–Derived Tubules Are Polarized Epithelia

To characterize the ultrastructure of the ES cell derivatives within the embryonic kidney, cultured metanephroi were reacted with the Bluo-gal substrate for electron microscopic detection of β -galactosidase activity. Bluo-gal–positive cells contained numerous intracellular and paracellular electron-dense precipitates (Figure 3, arrowheads), and a survey view revealed that many of these cells had adopted an epithelial morphology resembling tubules (Figure 3A). These ES cell–derived tubules had developed typical epithelial specializations on their luminal surfaces, including apical microvilli and junctional complexes (Figure 3, B, D, and E). Basal bodies were also observed in the apical region of Bluo-gal–positive cells (Figure 3, D and E), and an axoneme of a primary cilium was observed projecting from the luminal surface of a Bluo-gal–positive ES cell (Figure 3E, arrow). In addition, many of the ES cell–derived tubule-like structures were surrounded by basement membranes (Figure 3C). The presence of basement membrane was also shown by light microscopy as positive laminin and type IV collagen immunofluorescence on the margin of areas containing blue ES cells (Figure 4, B and D).

ES Cell–Derivatives Express WT1, LTA, and Na^+/K^+ ATPase

Given that the ES cell–derived structures exhibited epithelial characteristics, certain markers of various segments of the mature nephron were used to investigate how closely these epithelia mimicked terminally differentiated kidney structures. Glomerular tufts developed normally within metanephroi that

received ES cell injections (Figure 1C); however, ES cell derivatives were rarely seen integrating into glomerular structures. On occasion, single isolated cells were localized to areas resembling glomerular epithelial tufts, but given the rarity of this, we were unable to stain these isolated cells for markers of glomerular epithelial differentiation (Figure 5, A and B). Undifferentiated ROSA26 ES cells did not stain with the glomerular epithelial marker, WT1 (data not shown). However, some ES cell–derived tubules were shown to express WT1, similar to the low levels normally expressed in metanephric mesenchyme (Figure 5, C and D). Colocalization of β -galactosidase and WT1 was also shown using antibody labeling (Figure 5, E and F).

Initial studies revealed a subset of ES cell–derived tubules that labeled with the lectin LTA, a marker of proximal tubules. By day 5, tubules that were positive for both LTA and β -galactosidase were seen (Figure 6, A and B). The inhibitory sugar L-fucose completely blocked staining of LTA–FITC in both normal proximal tubules (data not shown) and ES cell–derived tubules (Figure 6C). ES cell–injected, organ-cultured kidneys were also labeled with antibodies against the α_1 subunit of the Na^+/K^+ ATPase (Figure 6, D and E). In host tissue, nephron segments with robust labeling were seen (Figure 6D, arrow), along with other segments with weaker labeling (Figure 6D, arrowhead). We also observed areas of cultured embryonic kidney with no detectable signal (Figure 6D, double arrows). In ES cell–derived tubules, we found lateral membrane staining of Na^+/K^+ ATPase, similar to levels found in adjacent host kidney nephron segments (Figure 6, E and F). We also labeled, with the distal tubule marker, anti-Tamm Horsfall protein and the collecting duct lectin, *Dolichos biflorus*. Despite binding of both markers to endogenous nephron segments, ES cell–derived structures did not bind Tamm Horsfall protein or *Dolichos biflorus*.

ES Cells Can Be Genetically Modified before Metanephric Organ Culture

An adenovirus construct containing the cytomegalovirus promoter driving GFP was used to transiently infect undifferentiated ES cells. GFP expression was seen in the cells after 18 h of culture (Figure 7, A and B). When these infected cells were microinjected into E13 mouse kidneys and cultured for 24 to 48 h, GFP-positive ES cells were seen in aggregates (Figure 7, C and D). Control kidneys that were microinjected with ES cells not infected with virus did not show GFP fluorescence (Figure 7, E and F). The GFP signal persisted on the second day of culture in serial sections either developed for β -galactosidase or labeled with an anti-GFP antibody (Figure 7, G and H). GFP fluorescence was difficult to detect beyond 48 h in cultured kidneys.

Discussion

We sought to determine whether ES cells could differentiate into kidney structures when placed into the embryonic mouse kidney microenvironment. Our work describes the following important findings. First, undifferentiated ES cells, when injected into the E13 mouse kidney, survived and 5 d after injection

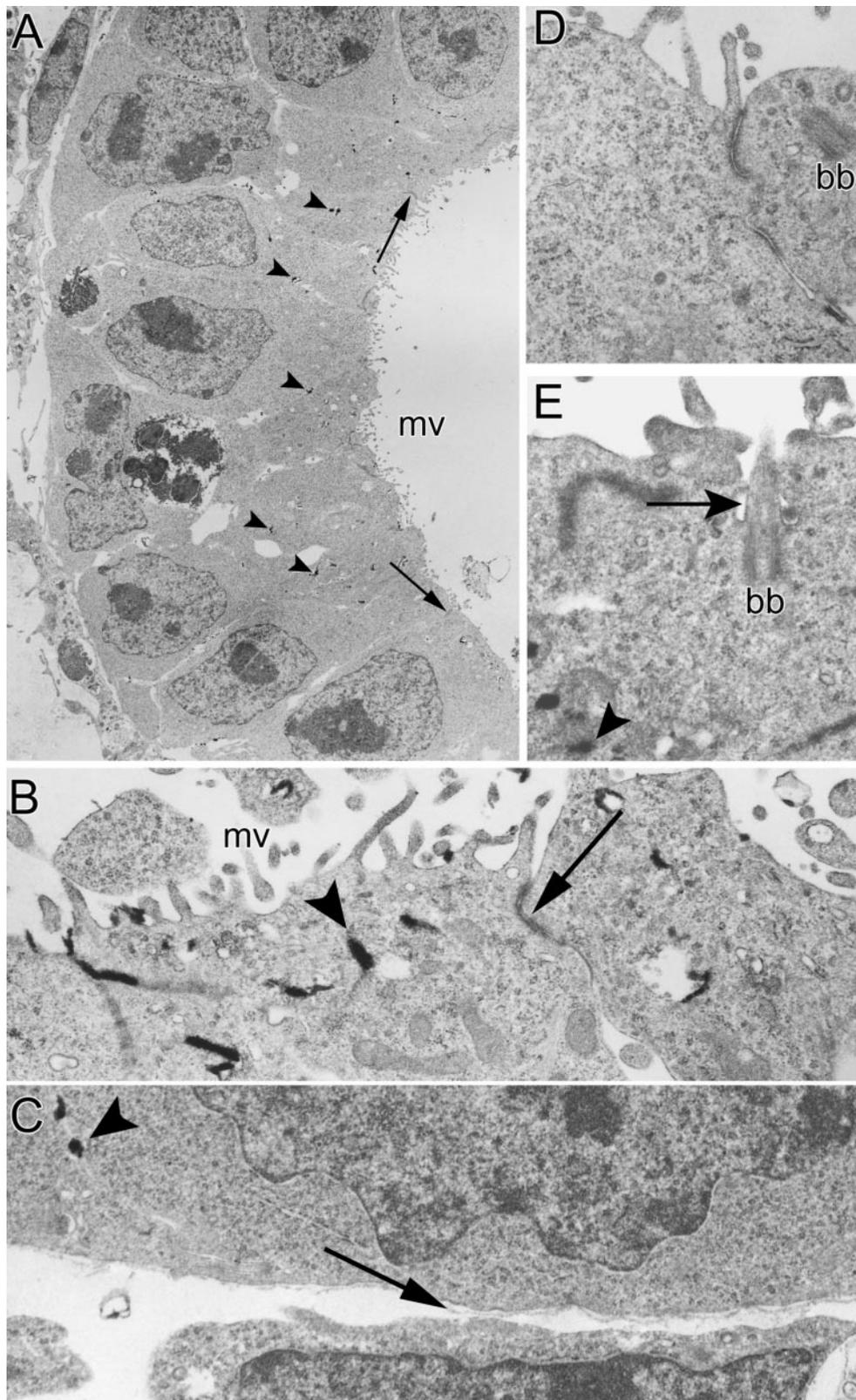


Figure 3. Embryonic kidneys that had been injected with ROSA26 ES cells and cultured for 5 d were developed for β -galactosidase activity with the substrate Blueo-gal. Blueo-gal-positive areas were seen in the electron micrographs as small, electron-dense precipitates (arrowheads), confirming that these cells are *lacZ*-positive ES cell derivatives. (A) A portion of a Blueo-gal-positive tubule is shown. Microvilli were seen on the apical surface (mv), as were junctional complexes (arrows). (B) Higher magnification of Blueo-gal-positive cells at their apical surface, showing microvilli and junctional complexes. The basal surfaces of the Blueo-gal-positive areas were surrounded by basement membrane (arrow, C). Basal bodies were also positioned near the apical membrane (D, bb). A primary ciliary axoneme was observed extending from a basal body of a Blueo-gal-positive cell (arrow, E).

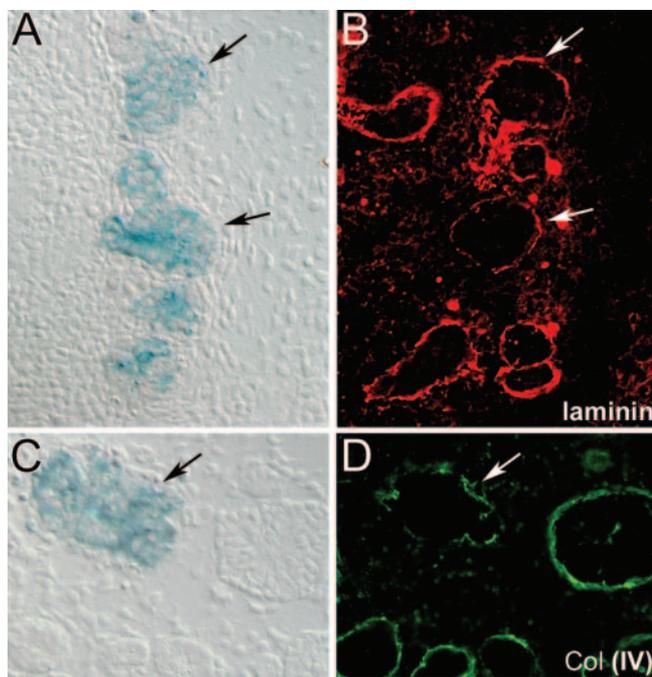


Figure 4. Serial frozen sections (A to D) were developed for β -galactosidase activity (A, C) or were reacted with sheep anti-laminin antibody (B), or with goat anti-type IV collagen antibody (D) to reveal areas of basement membrane. Many ES cell-derived structures were surrounded by a linear basement membrane (arrows).

tion ES cell derivatives constituted approximately 5% of the cultured kidney. Second, the dominant morphology expressed by the ES cell derivatives resembled epithelial tubules. Third, we describe adenoviral infection of ES cells before injection into E13 mouse kidneys and showed transgene expression after 2 d of culture. Thus, we believe direct injection of ES cells into embryonic mouse kidneys will allow the study of aspects of epithelialization, basement membrane formation, and tubulogenesis in a simplified *in vitro* model. Finally, on rare occasions, a small number of ES cells integrated into structures resembling glomerular tufts.

Most models of *in vitro* ES cell differentiation begin with the formation of embryoid bodies, which are structures containing all three germ layers and display a complex gene expression profile (28). Our study is one of a few that begins by using undifferentiated ES cells. Markers of terminally differentiated tissues, such as liver, often take several days, even weeks, to become expressed in embryoid bodies (8,18,29). We have not observed the appearance of an endodermal or mesodermal layer surrounding the epithelial structures formed by the ES cells within the embryonic kidney. Thus, we do not believe that the injected ES cells were merely forming embryoid-like bodies within the developing kidney. Instead, the epithelialization we observed in our experiments closely resembles structures formed when embryonic germ cells are cocultured with urogenital ridge aggregates (30), when monkey ES cells are grown suspended in collagen (31), or when human ES cells are transplanted into chick embryos (32).

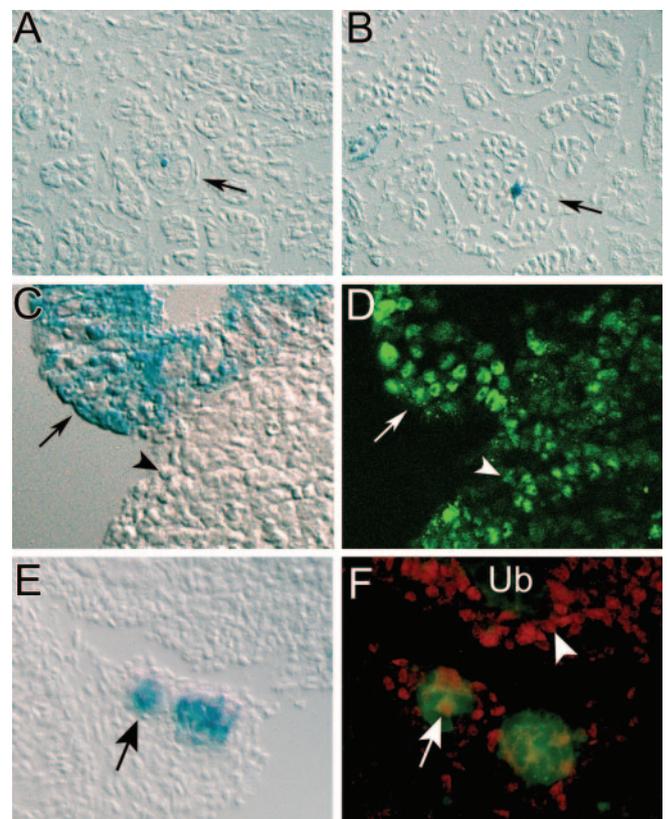


Figure 5. Frozen sections developed for β -galactosidase showing two examples of single ES cells within areas believed to be glomerular epithelial tufts (A, B). After ES cell microinjection into metanephroi, β -galactosidase-positive tubules (C) expressed low levels of WT1 (arrow, D), similar to the levels seen in the mesenchyme of host kidney (arrowhead, D). WT1 expression by a β -galactosidase-positive ES cell (arrow) was also shown by double-labeling with WT1 and β -galactosidase antibodies (F, WT1, red; β -galactosidase, green). Metanephric mesenchymal cells surrounding the ureteric bud of the host kidney tissue also expressed WT1 (arrowhead, F). A serial slide developed for β -galactosidase activity showed the corresponding ES cell-derived tubules (arrow, E).

Although the ES cells developed into structures resembling tubular epithelium and acquired certain kidney-specific markers expressed by native kidney cells, their developmental program appeared to differ from normal nephrogenesis. Initially, the ES cells appeared round; they did not have a spindle-shaped morphology akin to metanephric mesenchymal cells. Once they developed into tubules, however, the ES cells expressed low levels of WT1, a tumor suppressor found in metanephric mesenchyme and important for the induction of the kidney (33). This low level of WT1 expression in the ES cell-derived tubules may represent the acquisition of mesenchymal characteristics, and tubules on day 5 of culture may have been downregulating WT1 as a consequence of the epithelial transition. Further studies on WT1 expression by ES cells on various days of culture are needed to clarify this question. However, we rarely saw individual ES cells participating in the development of glomerular tufts, and we were unable to assess if ES cells

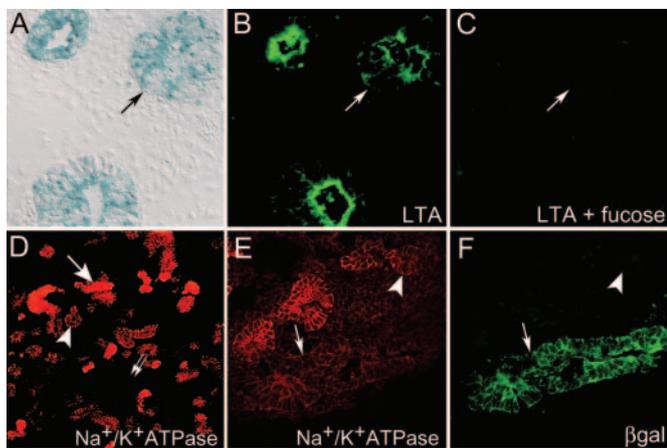


Figure 6. ROSA26 ES cells were microinjected into embryonic kidney, which were then organ cultured 5 d. Serial frozen sections were processed for β -galactosidase activity (A) or labeled with the proximal tubule marker, *Lotus tetragonolobus* (LTA) (B, C). LTA-FITC labeling was blocked by previous treatment with inhibitory sugar, 0.5 M fucose (C), demonstrating specificity of binding. (D) Frozen section of cultured embryonic kidney was labeled with anti- α_1 Na^+/K^+ ATPase. Some nephron segments labeled strongly (arrow), whereas other segments showed weaker labeling (arrowhead). In other areas, no signal was detected above background (double arrows). ES cell-injected kidneys were double-labeled with anti- Na^+/K^+ ATPase (E) and anti- β -galactosidase (F). ES cell-derived, β -galactosidase-positive tubules expressed membrane labeling of Na^+/K^+ ATPase (arrow, E, F) at levels similar to that seen by other host nephron segments (arrowhead, E, F).

within glomerular tufts were expressing WT1, indicative of terminally differentiating podocytes.

The majority of injected ES cells quickly formed tightly compacted spheres, which developed lumens during the culturing period. The ES cell-derived tubules were often larger in diameter than typical tubules found in the host kidney, and our ultrastructural analysis suggested that each ES cell-derived tubule contained only Bluo-gal-positive ES cells. In other words, we did not observe tubules containing a mixed population of cells (ES cell derivatives together with native host cells). We also have not yet examined whether the tubules were a product of a single dividing ES cell or if they occurred through the adhesion of several ES cells, although the appearance of an aggregate of ES cells immediately after injection suggests the latter case. Given that hybrid cells are capable of forming by cell fusion when ES cells are cocultured with bone marrow or embryonic brain cells (34,35), the possibility exists that *lacZ*-positive cells expressing kidney markers in our experiments represent ES cells fused with host metanephric cells. However, we did not see evidence for multinucleated Bluo-gal-positive cells in our electron microscopic analysis. In addition, if ES cells were fusing with host cells, we would expect to see integration of ES cells into glomerular and interstitial compartments of the developing metanephros, as well as forming tubules. Although we obtained clear binding of the proximal

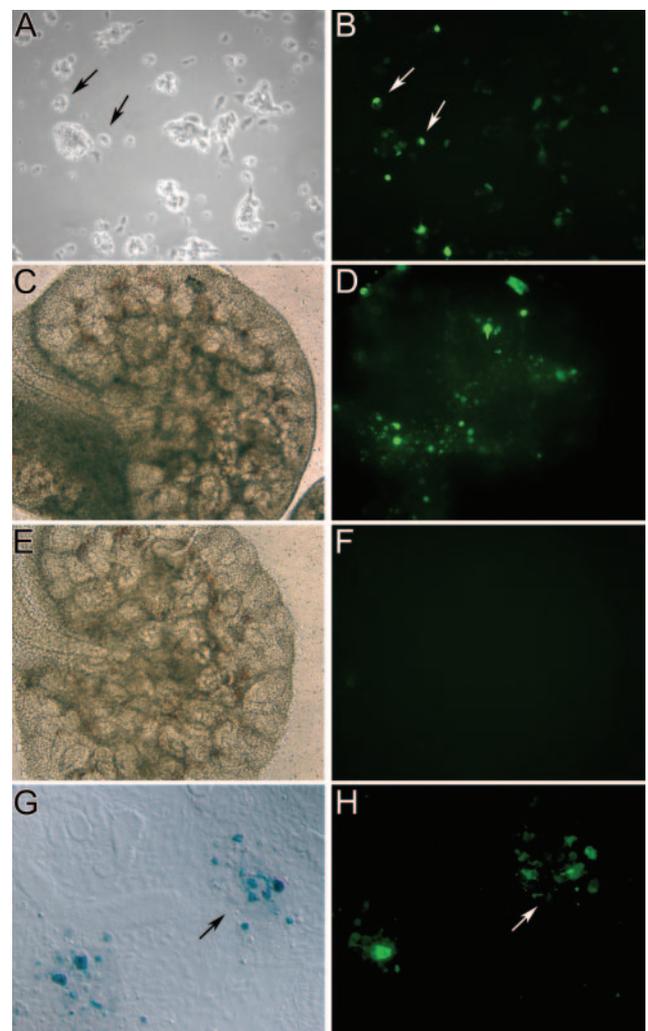


Figure 7. A cytomegalovirus–green fluorescence protein (CMV–GFP) adenovirus (Ad5.CMV5–GFP) was used to infect ROSA26 ES cells 1 d before injection into E13 mouse kidneys. GFP fluorescence was observed in many of the cells at 18 h postinfection (A, brightfield; B, GFP). The infected cells were then microinjected into E13 mouse kidneys and were imaged on subsequent days of organ culture. After 24 h of organ culture, GFP expression was seen as clusters of green cells distributed throughout the kidney (C, brightfield; D, GFP). Kidneys microinjected with ES cells not infected with virus did not fluoresce (E, brightfield; F, GFP). Serial sections of kidneys injected with virus-infected ES cells and cultured for 48 h were developed for β -galactosidase (G) or were reacted with anti-GFP (H). GFP expression was seen specifically in areas containing blue cells (arrows, G, H).

tubule differentiation marker (LTA) to some of the ES cell-derived tubules, marker analyses for collecting duct and distal tubules were negative. Additionally, many of the ES cell-derived tubules expressed membrane Na^+/K^+ ATPase in patterns similar to that seen by native kidney tubules *in vitro*. Although many kidney morphogenic events are replicated in metanephric organ culture, this culture system is nevertheless an imperfect model of normal kidney organogenesis. For ex-

ample, the cultures lack a blood supply and consequently the hemodynamic events associated with filtration and peritubular blood flow are totally absent. Laminin isotype switching is altered in cultured embryonic kidneys as well (37). The significance of expression (or not) of any segment-specific marker by ES cells or, for that matter, cells of the cultured host kidney *in vitro* is therefore uncertain. However, the speed and frequency in which the ES cells underwent acquisition of epithelial characteristics was striking. All of the ES cell–derived tubule cells displayed a polarized phenotype and contained apical junctional complexes and most were surrounded by basement membranes. By these criteria at least, the metanephric organ culture environment appears to have induced and supported the development of undifferentiated ES cells into structures resembling kidney tubules that bound LTA and expressed WT1 and Na⁺/K⁺ ATPase.

We do not know why the ES cell–derived structures were predominantly tubule-like and why so few of the ES cells appeared in glomerular tufts. Perhaps the injected ES cells were so comparatively underdeveloped that they competed poorly with native host cells for glomerular compartments. Transfection of ES cells with WT1 or other genes involved in podocyte differentiation before injection may divert more of these cells into adopting a glomerular epithelial program, and we are currently testing this possibility.

To fully exploit this system further, we wanted to verify whether transgenes introduced into the ES cells before injection could be expressed in the developing tubules. Adenovirus-infected ES cells had been previously shown to retain reporter gene expression after *in vitro* differentiation (36). ES cells infected with a cytomegalovirus–GFP adenoviral construct before injection into metanephric kidney showed robust GFP expression after 18 h of organ culture. We also colocalized β -galactosidase activity in the same cells that were expressing GFP on day 2 of culture. The GFP signal was attenuated on the second day of culture, possibly caused by a combination of the increased autofluorescence of the host kidney and the transient nature of the viral infection. Cell lines selected for stable integration of transgenes could circumvent any attenuation because of transient expression. Regardless, this transfection experiment strongly suggests that ES cells can be manipulated in a variety of ways before microinjection into metanephroi.

In sum, we report the establishment of a new model system in which to study aspects of epithelialization within the developing kidney. Direct injection of ES cells provides a rapid tool for the evaluation of steps required for epithelial differentiation and tubulogenesis, including junctional complex development and basement membrane assembly. In addition, grafting techniques are available that will allow studies of vascularization of the embryonic kidney and the effect endothelial cells may have on the differentiating ES cells. This experimental system offers much versatility in that the genotype of the host tissue and the injected ES cells can be modified by various methods. We hope that continued exploration of epithelial development from ES cells will provide new insights on renal organogenesis.

Acknowledgments

We thank Dr. Philippe Soriano for providing ROSA26 ES cells and Eileen Roach for technical assistance. Funds came from National Institutes of Health grants DK052483 and DK065123 (D.R.A.). Portions of this work were presented previously in abstract form: Steenhard BM, *et al.*: Integration of embryonic stem (ES) cells in metanephric kidney *in vitro* and *in vivo* [Abstract]. *J Am Soc Nephrol* 14: 571A, 2003.

References

1. Dressler GR: Development of the excretory system. In *Mouse Development: Patterning, Morphogenesis, and Organogenesis*, edited by Rossant J, Tam PPL, San Diego, London, Academic, 2002, pp 395–420
2. Abrahamson DR, Wang R: Development of the glomerular capillary and its basement membrane. In *The Kidney: From Normal Development to Congenital Disease*, edited by Vize PD, Woolf AS, Bard JB, Boston, Academic Press, 2003, pp 221–249
3. Saxen L: *Organogenesis of the Kidney*. Cambridge, Cambridge University Press, 1987
4. Robert B, St John PL, Abrahamson DR: Direct visualization of renal vascular morphogenesis in Flk1 heterozygous mutant mice. *Am J Physiol* 275: F164–F172, 1998
5. Sequeira Lopez ML, Pentz ES, Robert B, Abrahamson DR, Gomez RA: Embryonic origin and lineage of juxtaglomerular cells. *Am J Physiol Renal Physiol* 281: F345–F356, 2001
6. Robert B, St John PL, Hyink DP, Abrahamson DR: Evidence that embryonic kidney cells expressing flk-1 are intrinsic, vasculogenic angioblasts. *Am J Physiol* 271: F744–F753, 1996
7. Loebel DA, Watson CM, De Young RA, Tam PP: Lineage choice and differentiation in mouse embryos and embryonic stem cells. *Dev Biol* 264: 1–14, 2003
8. Schuldiner M, Yanuka O, Itskovitz-Eldor J, Melton DA, Benvenisty N: Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc Natl Acad Sci U S A* 97: 11307–11312, 2000
9. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM: Embryonic stem cell lines derived from human blastocysts. *Science* 282: 1145–1147, 1998
10. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A: Embryonic stem cell lines from human blastocysts: Somatic differentiation *in vitro*. *Nat Biotechnol* 18: 399–404, 2000
11. Lin F, Cordes K, Li L, Hood L, Couser WG, Shankland SJ, Igarashi P: Hematopoietic stem cells contribute to the regeneration of renal tubules after renal ischemia-reperfusion injury in mice. *J Am Soc Nephrol* 14: 1188–1199, 2003
12. Morigi M, Imberti B, Zoja C, Corna D, Tomasoni S, Abbate M, Rottoli D, Angioletti S, Benigni A, Perico N, Alison M, Remuzzi G: Mesenchymal stem cells are renotropic, helping to repair the kidney and improve function in acute renal failure. *J Am Soc Nephrol* 15: 1794–1804, 2004
13. Cascalho M, Ogle BM, Platt JL: Xenotransplantation and the future of renal replacement. *J Am Soc Nephrol* 15: 1106–1112, 2004
14. Hanss B, Bruggeman LA: Applications of gene therapy to kidney disease. *Curr Opin Nephrol Hypertens* 12: 439–445, 2003
15. Strom TB, Field LJ, Ruediger M: Allogeneic stem cell-

- derived “repair unit” therapy and the barriers to clinical deployment. *J Am Soc Nephrol* 15: 1133–1139, 2004
16. Bjorklund LM, Sanchez-Pernaute R, Chung S, Andersson T, Chen IY, McNaught KS, Brownell AL, Jenkins BG, Wahlstedt C, Kim KS, Isacson O: Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. *Proc Natl Acad Sci U S A* 99: 2344–2349, 2002
 17. Blyszczuk P, Czyz J, Kania G, Wagner M, Roll U, St-Onge L, Wobus AM: Expression of Pax4 in embryonic stem cells promotes differentiation of nestin-positive progenitor and insulin-producing cells. *Proc Natl Acad Sci U S A* 100: 998–1003, 2003
 18. Chinzei R, Tanaka Y, Shimizu-Saito K, Hara Y, Kakinuma S, Watanabe M, Teramoto K, Arii S, Takase K, Sato C, Terada N, Teraoka H: Embryoid-body cells derived from a mouse embryonic stem cell line show differentiation into functional hepatocytes. *Hepatology* 36: 22–29, 2002
 19. Tai YT, Svendsen CN: Stem cells as a potential treatment of neurological disorders. *Curr Opin Pharmacol* 4: 98–104, 2004
 20. Xu P, Yoshioka K, Yoshimura D, Tominaga Y, Nishioka T, Ito M, Nakabeppu Y: In vitro development of mouse embryonic stem cells lacking JNK/stress-activated protein kinase-associated protein 1 (JSAP1) scaffold protein revealed its requirement during early embryonic neurogenesis. *J Biol Chem* 278: 48422–48433, 2003
 21. Vicario I, Schimmang T: Transfer of FGF-2 via HSV-1-based amplicon vectors promotes efficient formation of neurons from embryonic stem cells. *J Neurosci Methods* 123: 55–60, 2003
 22. Kunath T, Gish G, Lickert H, Jones N, Pawson T, Rossant J: Transgenic RNA interference in ES cell-derived embryos recapitulates a genetic null phenotype. *Nat Biotechnol* 21: 559–561, 2003
 23. Friedrich G, Soriano P: Promoter traps in embryonic stem cells: A genetic screen to identify and mutate developmental genes in mice. *Genes Dev* 5: 1513–1523, 1991
 24. Tufro A, Norwood VF, Carey RM, Gomez RA: Vascular endothelial growth factor induces nephrogenesis and vasculogenesis. *J Am Soc Nephrol* 10: 2125–2134, 1999
 25. Hogan B, Beddington R, Costantini F, Lacy E: *Manipulating the Mouse Embryo: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, 1994
 26. Abrahamson DR, Caulfield JP: Proteinuria and structural alterations in rat glomerular basement membranes induced by intravenously injected anti-laminin immunoglobulin G. *J Exp Med* 156: 128–145, 1982
 27. Zambrowicz BP, Imamoto A, Fiering S, Herzenberg LA, Kerr WG, Soriano P: Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proc Natl Acad Sci U S A* 94: 3789–3794, 1997
 28. Desbaillets I, Ziegler U, Groscurth P, Gassmann M: Embryoid bodies: An in vitro model of mouse embryogenesis. *Exp Physiol* 85: 645–651, 2000
 29. Rohwedel J, Guan K, Zuschratter W, Jin S, Ahnert-Hilger G, Furst D, Fassler R, Wobus AM: Loss of beta1 integrin function results in a retardation of myogenic, but an acceleration of neuronal, differentiation of embryonic stem cells in vitro. *Dev Biol* 201: 167–184, 1998
 30. Durcova-Hills G, Wianny F, Merriman J, Zernicka-Goetz M, McLaren A: Developmental fate of embryonic germ cells (EGCs), in vivo and in vitro. *Differentiation* 71: 135–141, 2003
 31. Chen SS, Revoltella RP, Papini S, Michelini M, Fitzgerald W, Zimmerberg J, Margolis L: Multilineage differentiation of rhesus monkey embryonic stem cells in three-dimensional culture systems. *Stem Cells* 21: 281–295, 2003
 32. Goldstein RS, Drukker M, Reubinoff BE, Benvenisty N: Integration and differentiation of human embryonic stem cells transplanted to the chick embryo. *Dev Dyn* 225: 80–86, 2002
 33. Kreidberg JA, Sariola H, Loring JM, Maeda M, Pelletier J, Housman D, Jaenisch R: WT-1 is required for early kidney development. *Cell* 74: 679–691, 1993
 34. Terada N, Hamazaki T, Oka M, Hoki M, Mastalerz DM, Nakano Y, Meyer EM, Morel L, Petersen BE, Scott EW: Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. *Nature* 416: 542–545, 2002
 35. Ying QL, Nichols J, Evans EP, Smith AG: Changing potency by spontaneous fusion. *Nature* 416: 545–548, 2002
 36. Smith-Arica JR, Thomson AJ, Ansell R, Chiorini J, Davidson B, McWhir J: Infection efficiency of human and mouse embryonic stem cells using adenoviral and adeno-associated viral vectors. *Cloning Stem Cells* 5: 51–62, 2003
 37. St John PL, Wang R, Yin Y, Miner JH, Robert B, Abrahamson DR: Glomerular laminin isoform transitions: Errors in metanephric culture are corrected by grafting. *Am J Physiol Renal Physiol* 280: F695–F705, 2001