

Development of embryonic stem cells in recombinant kidneys

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Embryonic stem cells (ESC) are self-renewing and can generate all cell types during normal development. Previous studies have begun to explore fates of ESCs and their mesodermal derivatives after injection into explanted intact metanephric kidneys and neonatal kidneys maturing in vivo. Here, we exploited a recently described recombinant organ culture model, mixing fluorescent quantum dot labeled mouse exogenous cells with host metanephric cells. We compared abilities of undifferentiated ESCs with ESC-derived mesodermal or non-mesodermal cells to contribute to tissue compartments within recombinant, chimeric metanephroi. ESC-derived mesodermal cells downregulated Oct4, a marker of undifferentiated cells, and, as assessed by locations of quantum dots, contributed to Wilms' tumor 1-expressing forming nephrons, synaptopodin-expressing glomeruli, and organic ion-transporting tubular epithelia. Similar results were observed when labeled native metanephric cells were recombined with host cells. In striking contrast, non-mesodermal ESC-derived cells strongly inhibited growth of embryonic kidneys, while undifferentiated ESCs predominantly formed Oct4 expressing colonies between forming nephrons and glomeruli. These findings clarify the conclusion that ESC-derived mesodermal cells have functional nephrogenic potential, supporting the idea that they could potentially replace damaged epithelia in diseased kidneys. On the other hand, undifferentiated ESCs and non-mesodermal precursors derived from ESCs would appear to be less suitable materials for use in kidney cell therapies.

Introduction

Pluripotent embryonic stem cells (ESCs) exist within, and can be isolated from, the inner cell mass of the mouse and human blastocyst.^{1,2} They normally generate endoderm, mesoderm and ectoderm, from which all cells in the mature organism derive. ESCs express octamer-binding transcription factor 4 (Oct4), a pluripotency marker downregulated during differentiation.³ In vitro, ESCs can undergo limitless self-renewal, particularly when cultured under adherent conditions.¹ In suspension culture, however, they spontaneously form aggregates called embryoid bodies (EBs), so-called because their initial development resembles that of inner cell masses.⁴ For instance, EBs differentiate to form the three embryonic germ layers, including *brachyury*-expressing (*Bra*⁺) nascent mesoderm^{5,6} from which kidneys derive.⁷ The ability of ESCs to generate any cell type⁸ has led to the suggestion that they could be used to replace diseased or injured cells. In preclinical studies, ESC-derived retinal pigmented epithelial cells have treated macular degeneration,⁹ and the first human Phase I trial using ESC derivatives to treat spinal cord injury began in 2010.¹⁰

Previous studies have begun to explore the fates of LacZ or green fluorescent protein (GFP) labeled ESCs or their immediate derivatives after injection into murine metanephric kidneys

grown in culture¹¹⁻¹³ and neonatal kidneys maturing in vivo.¹² In the former context, although ESCs generated tubule-like structures, the therapeutic potential of ESCs may be limited by their propensity to form tumors.^{2,14} Others have first partially differentiated ESCs into a mesodermal lineage before injecting them into intact kidneys,^{11,12} reporting that implanted cells differentiated into proximal tubules (PTs), as assessed by morphology and segment-specific markers. However, although PTs derive from the metanephric mesenchyme (MM)/nephron lineage, labeled cells were hardly ever detected in glomeruli, even though podocytes also derive from this lineage. Furthermore, any potential adverse effect of the exogenous implanted cells on the development of host precursor cells was not specifically investigated in these studies,¹¹⁻¹³ nor was the physiological functionality of implanted cells.

Here, we exploited a recently described recombinant kidney organ culture model, mixing labeled mouse ESCs or their immediate derivatives with host metanephric cells. In the resulting recombinant, chimeric organs, we compared abilities of undifferentiated ESCs with ESC-derived mesodermal or non-mesodermal cells to integrate with, and contribute to, nascent kidney structures. Our strategy used four key techniques. First, we exploited an ESC line, *Bra-GFP*,¹⁵ in which GFP is expressed from within the *Brachyury* locus, enabling mesodermal cells to be

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selected from ESC-derived EBs using fluorescence activated cell sorting (FACS). Second, to visualize fates of exogenous cells, they were first labeled with fluorescent nanocrystals called quantum dots (QDs), a technique previously used to ascertain fates of mesenchymal stem cells within hearts.¹⁶ QDs can label cells rapidly and efficiently without affecting their differentiation potential,¹⁶ and an advantage of QDs over traditional fluorescent markers is that they resist photobleaching. We have recently shown that the specific QD technology we are using, as assessed in kidney-derived stem cells over several days observation, affects neither proliferation nor expression of lineage-specific renal markers. Moreover, labeled cells do not excrete QDs, and transfer of QDs to adjacent kidney cells is negligible.¹⁷ On the other hand, QDs do not replicate upon cell division, so that not all progeny of initially labeled cells will be found to contain dots.¹⁸ Third, these labeled cells were intimately mixed with disaggregated cells from “host” metanephric rudiments, potentially facilitating the integration of exogenous cells into nascent structures.^{19,20} Last, we tested the ability of labeled cells to participate in organic ion transport.^{21–24}

Results

Gene expression in *Bra-GFP*⁺ cells. To investigate whether the nephrogenic potential of mouse ESCs could be enhanced by differentiating them to mesoderm, a *Bra-GFP* mouse ESC line was used to isolate an enriched population of mesodermal cells.⁵ As described,¹⁵ ESCs were seeded onto non-adherent dishes to generate EBs that gave rise to GFP⁺ cells after 4 d of culture (results not shown). To confirm that GFP⁺ cells were mesodermal, GFP⁺ and GFP⁻ cells were separated by FACS, and reverse transcription polymerase chain reaction (RT-PCR) performed. As expected, GFP⁺ cells expressed the nascent mesodermal marker, *Bra*, as well as *Forkhead box protein c1* (*Foxc1*), normally expressed by paraxial mesoderm²⁵ and later by the nephrogenic cord,²⁶ and the intermediate mesodermal marker, *Odd-skipped-related 1* (*Osr1*), essential for metanephrogenesis.^{27,28} The GFP⁺ population appeared to express lower levels of these genes but showed more prominent expression of the ectodermal marker, *Paired box protein 6* (*Pax6*) (Fig. 1A).

Exogenous cell effects on rudiment growth and differentiation. Chimeric organs were generated using host cells from dissociated embryonic day (E) 13.5 mouse kidneys mixed in an 8/1 ratio (total of 18×10^4 cells) with Bra⁺, Bra⁻, undifferentiated ESCs, or native kidney rudiment cells (KRCs), the latter representing a positive control (Fig. 1B–D). Mouse metanephroi originate at E10.5 when they comprise a ureteric bud (UB) growing into MM.⁷ By E13.5, the UB has branched several times, forming a collecting duct tree, and MM has begun to undergo mesenchymal/epithelial transition to generate nephrons. At this time point, these first layers of nascent nephrons comprise vesicles and comma-shaped bodies and have yet to differentiate into either glomerular podocytes, which express both Wilms’ tumor 1 (Wt1) and synaptopodin, or PTs.⁷ Following 3 d of culture, chimeras made with ESCs or Bra⁺ cells tended to be smaller, as assessed by explant areas, than those made with KRCs. Furthermore, examining individual explants, it was obvious that some Bra⁻ recombinant organs

were very growth-retarded (Fig. 2A and B). We defined “developing nephrons” as being discrete, Wt1⁺ structures on whole-mount immunostaining (Fig. 2C). KRC and Bra⁺ chimeric metanephroi contained on average approximately 25–30 developing nephrons/mm², ESC chimeras contained on average around 50/mm², while no Bra⁻ chimeric organ contained over 8/mm² (Fig. 2D). Average cross-sectional areas of developing nephrons in KRC and Bra⁺ chimeras were similar, whereas those in both ESC and Bra⁻ chimeras were approximately 3–4 times smaller (Fig. 2E). Given that the Bra⁻ chimeric organs appeared both tiny and nephron-deplete, we elected not to analyze them further.

Next, we undertook confocal microscopy of KRC, ESC and Bra⁺ chimeras probed with a panel of metanephric cell and tubule markers (Table 1). In all three varieties of chimeric organ, after 3 d culture, we detected calbindin-28-expressing UB branch tips capped by cells expressing sine oculis homeobox homolog 2 (Six2), a transcription factor marking induced, condensing MM beginning to form nephrons.^{29,30} A range of structures expressed Wt1, including nephron vesicles and also crescents of podocyte precursors in S-shaped bodies.³¹ After 5 d culture of KRC, ESC and Bra⁺ recombinant organs, we detected cell clusters expressing synaptopodin, representing podocytes,³² and tubules binding *Lotus tetragonolobus* agglutinin (LTA) on their luminal/apical zones, as do PTs in vivo.³³ These patterns resembled those in cultured intact E13.5 kidneys (Fig. 3).

Oct4 expression. Having established that the addition of ESCs and Bra⁺ cells was compatible with chimeric metanephric maturation, we determined the detailed fates of exogenous cells. We found that both ESCs and freshly sorted Bra⁺ cells expressed *Oct4* transcripts (Fig. 4A). Upon immunostaining, Oct4 was undetectable in intact (non-recombinant) metanephroi (data not shown), and *Oct4* transcripts were undetectable in just-dissociated native KRCs (Fig. 4A). Oct4⁺ cells were plentiful in both ESC and Bra⁺ chimeras on the day each recombinant organ was created (“day 0” in Fig. 4B). Subsequently, in ESC chimeric organs, prominent Oct4⁺ colonies were present for up to 8 d, the limit of the observation period.

In marked contrast, Bra⁺ recombinant organs displayed only sparse, tiny Oct4⁺ colonies on day 3, and hardly any Oct4⁺ positive cells were detected at day 8, suggesting that cells derived from Bra⁺ exogenous cells had switched off Oct4 expression and begun to differentiate.

Locations of QDs in explants. We measured the extent to which exogenous cells (or their progeny) appeared in different zones of chimeric organs, as assessed by detection of QD⁺ cells. Following 3 d culture, rudiments were immunostained for Wt1 and laminin. For these analyses, we defined three zones: (1) “developing nephrons” contained Wt1⁺ cells surrounded by laminin⁺ BM, (2) “other tubules” contained Wt1⁻ cells surrounded by BM (note that, in ESC recombinants, these would comprise both normal kidney tubules and Oct4⁺ aggregates, as described above) and (3) “stroma” where cells neither expressed Wt1 nor were surrounded by BM (Fig. 2C). In KRC, ESC and Bra⁺ recombinant organs, we detected QD⁺ cells in each of the three zones. The numbers of QD⁺ cells within developing nephrons per section area (0.84 mm²) were similar in chimeric organs made with

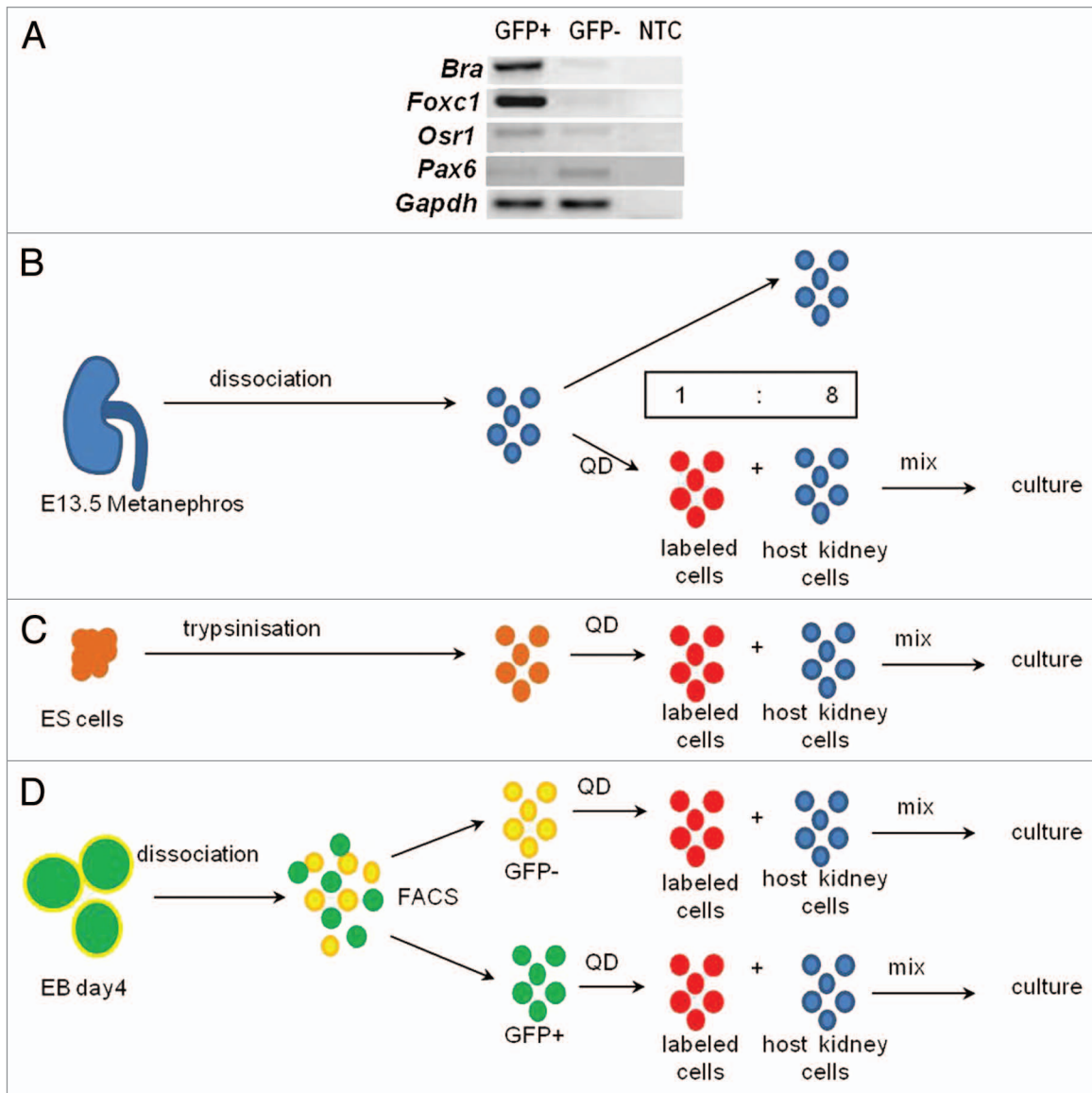


Figure 1. Chimera formation. (A) RT-PCR confirmed that GFP⁺ cells prominently expressed mesodermal genes, *Bra*, *Foxc1* and *Osr1* whereas the ectodermal marker, *Pax6*, was more prominent in GFP⁻ cells; NTC, no template control. (B–D) KRCs (blue) from E13.5 metanephroi (B), ESCs (orange) (C) and FACS-sorted Bra⁺ (green) or Bra⁻ (yellow) cells (D) were dissociated to single cells and labeled with QDs (thus becoming red cells) and mixed with unlabeled KRCs (blue) in a ratio of 1 to 8 prior to re-aggregation.

Bra⁺ or KRCs, whereas QDs were rarely detected in this compartment in ESC recombinant organs (Fig. 5A). The proportion of Wt1⁺QD⁺ cells within the developing nephrons of Bra⁺ chimeras averaged 12–13%, and was not significantly different from that of KRC chimeras. In contrast, Wt1⁺QD⁺ cells comprised only 4% of cells within the developing nephrons of ESC chimeras, indicating that in comparison to KRCs and Bra⁺ cells, ESCs have a limited ability to integrate into these structures (Fig. 5B). With respect to “other tubules,” numbers of QD⁺ cells per section area were similar in the KRC, ESC and Bra⁺ groups (Fig. 5A). With the same panel of markers used in Figure 3, we ascertained more precisely which kidney cell types contained QDs. At 3–5 d of culture, in both KRC and Bra⁺ chimeric organs, subsets of cells expressing either Six2, Wt1, synaptopodin or calbindin-28 proteins contained

QDs, and the fluorescent marker was also found in some tubule cells binding LTA at their apical surfaces, marking them as PT epithelia. In ESC chimeric organs, QDs were found in calbindin-28⁺ cells, but were only rarely detected in LTA-binding PTs and synaptopodin⁺ structures (Fig. 6).

ESC-derived mesodermal cells generate functional tubular cells. We used an organic anion transporter (OAT) assay to investigate whether rudiment cells manifested OAT-dependent uptake of the organic anion mimic, 6-carboxyfluorescein (6-CF).^{21–24} In the absence of the OAT inhibitor, probenecid, 6-CF is transported from the interstitium into the cells before being secreted into the tubular lumen of intact rudiments. Peanut agglutinin (PNA)-rhodamine was also used to label outer surfaces of tubules (Fig. 7). QD⁺ tubule cells in both KRC control and Bra⁺ chimeras

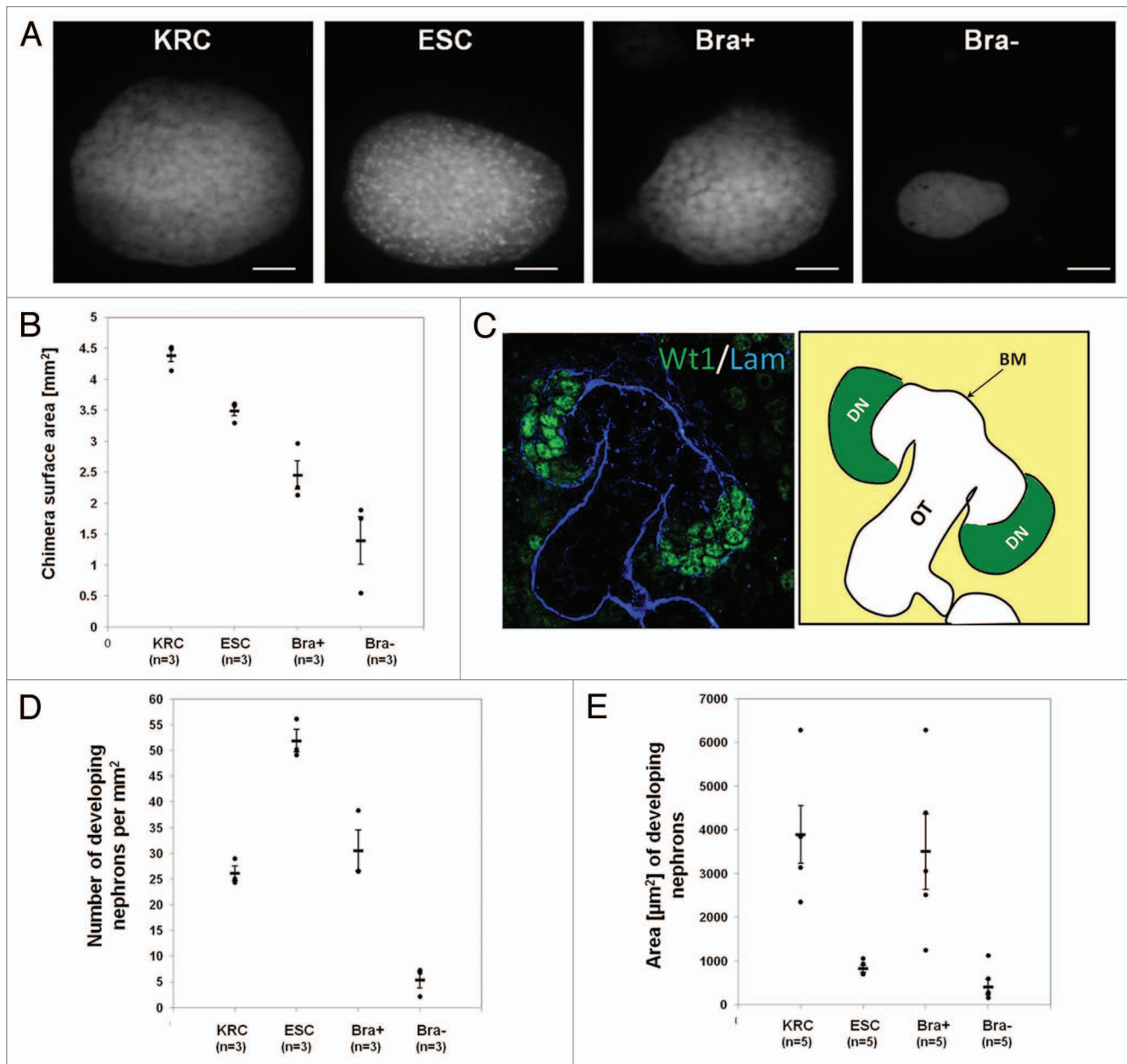


Figure 2. Effect of exogenous cells on chimera development. **(A)** Photomicrographs of Wt1 immunostained rudiments. **(B)** Graph showing explant areas. All chimeric rudiment areas are significantly different from the control (KRC chimera) ($p < 0.05$). **(C)** Left panel shows a confocal image of a day 3 KRC chimera immunostained for Wt1 (green) and laminin (blue). Schematic in right panel indicates developing nephron structures, (DN), basement membrane (BM) and other tubules (OT), which comprise nephron tubules and UB branches. **(D and E)** Graphs showing numbers of developing nephrons per unit area **(D)** and areas of these structures **(E)**. In **D and E**, while ESC and Bra⁻ are significantly different from the KRC control chimeras ($p < 0.05$), there is no significant difference between Bra⁺ chimeras and controls ($p > 0.05$). In **B, D and E**, each point represents data from a single explant, with mean and standard error shown for each group. Note that the number and size of developing nephrons in KRC and Bra⁺ chimeras were similar, whereas ESC chimeras had significantly higher numbers of developing nephrons that had a smaller cross-sectional area. When organs were made with Bra⁻ cells, they were generally very small and contained only a few, small nephrons. Scale bars in **(A)**, 500 µm.

demonstrated active transport of 6-CF (Fig. 8; Fig. S1), whereas those in ESC chimeras did not (data not shown).

Discussion

By identifying labeled cells in recombinant metanephric kidneys in organ culture, our results clarify the conclusion that

ESC-derived mesodermal cells have nephrogenic potential because they were found within glomeruli and transporting tubules, supporting the notion that they may serve as progenitors to replace damaged epithelia in diseased kidneys. On the other hand, undifferentiated ESCs and non-mesodermal precursors derived from ESCs respectively failed to downregulate Oct4 and inhibited kidney growth and nephrogenesis, and would therefore

Table 1. Markers used to identify renal cell populations

Marker	Staining pattern in developing kidney	Reference
Wt1	Expressed in MM and forming nephrons; later, intensely expressed in podocytes and downregulated in PT	31
Six2	Expressed in condensed MM	29, 30
Laminin	Found in BMs surrounding nephrons and UB branches	50
Calbindin-28	Expressed in cells of the distal tubule and ureteric bud branches	51
Synaptopodin	Expressed in glomerular podocytes	32
LTA	Binds strongly to the apical surface of PT cells; weak binding to BMs of developing nephrons	33
PNA	Binds to BMs of developing nephrons and ureteric buds	33

appear to be less suitable materials to use in kidney cell therapies. Our findings are summarized in Table 2, where they are compared with previous studies investigating fates of ESC and/or their mesodermal derivatives after injection into developing kidneys.¹¹⁻¹³

Recombined metanephroi made with KRC, ESC and Bra⁺ exogenous cells contained plentiful developing nephrons. Versus both KRC positive control and Bra⁺ chimeras, those made using undifferentiated ESCs contained a greater number of Wt1⁺ nephrons per explant area, and these nephrons were smaller in size, suggesting that undifferentiated ESCs somehow alter the normal regulated pattern of nephrogenesis. One possibility is that, following incorporation into kidney rudiments, ESCs secrete factors capable of promoting early nephrogenesis, including Wnts³⁴ or fibroblast growth factors,³⁵ or Notch2.³⁶ As mentioned in the Introduction, when ESCs differentiate into more specialized cell types they downregulate Oct4. We found that ESC chimeric rudiments contained prominent Oct4⁺ colonies located between normal-looking tubules. We also noted a lack of propensity for ESCs to appear in developing Wt1⁺ nephrons, as assessed by detection of QD⁺ cells. We speculate that this could be due to the fact that the UB secretes leukemia inhibitory factor (LIF),³⁷ a known inhibitor of mouse ESC differentiation.³⁸ An earlier study reported that mouse ESCs readily generated PT cells following injection into metanephroi ex vivo, as evidenced by the appearance of ESC-derived columnar cells binding LTA.¹³ However, these chimeric rudiments were not analyzed for Oct4 expression, and it is thus possible that at least some of the LTA⁺ cells observed were in fact ESC-derived columnar epiblast-like cells binding LTA.³⁹

In the current study, in contrast to recombined rudiments made with undifferentiated ESCs, those made with Bra⁺ cells contained very few Oct4⁺ cells from the 3rd day of culture onwards. This is consistent with the report of Vigneau and colleagues, who showed that Bra⁺ cells do not generate teratomas

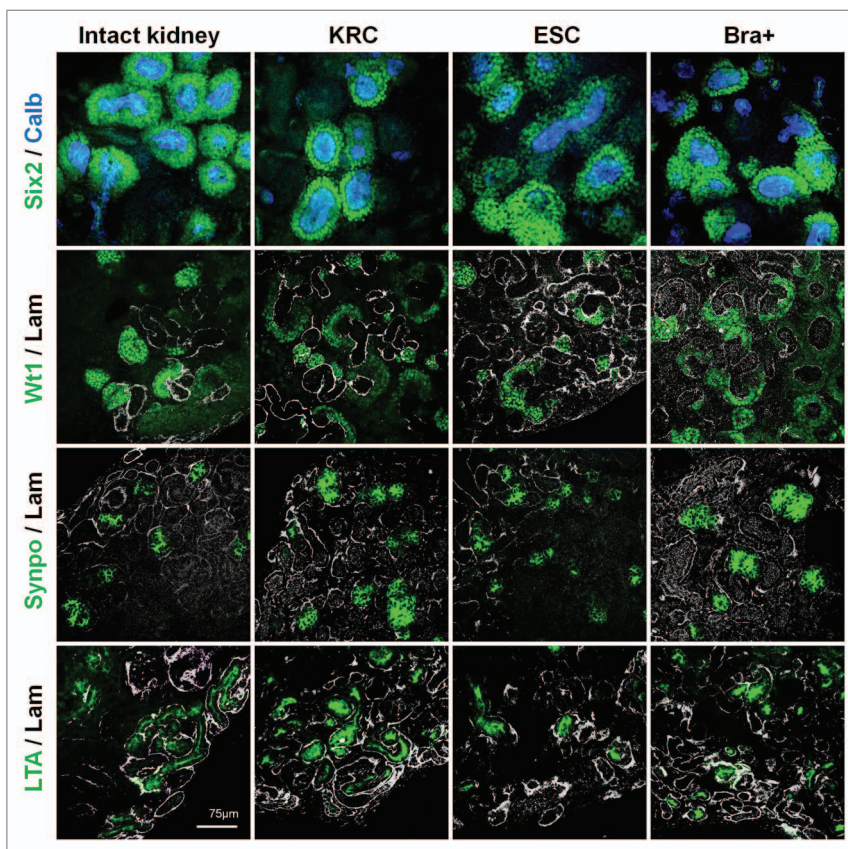


Figure 3. Segment-specific markers in intact and recombinant chimeric metanephroi. The expression patterns of Six2 (nuclear signal, green), calbindin-28 (cytoplasmic signal, blue), Wt1 (nuclear signal, green) and synaptopodin (cytoplasmic signal, green) as well as apical LTA binding (green) in intact metanephric kidneys (left hand set of four panels) and in chimeric rudiments generated by mixing host cells with either KRC, ESC or Bra⁺ exogenous cells. Laminin staining is shown in white. Rudiments in the top two panels were analyzed after 3 d culture, whereas those in the bottom two panels were analyzed after 5 d culture. Scale bars, 75 μm.

following injection into neonatal kidneys in vivo, whereas ESCs do.¹² Indeed, it is well established that maintained expression of the Oct4 pluripotency marker indicates a tumorigenic potential.^{40,41} Kim and Dressler¹¹ found that if ESCs were first cultured as EBs in mesoderm-inducing culture conditions, they could generate PTs after injection into explanted kidney rudiments. Likewise, Vigneau and coworkers¹² showed that Bra⁺ cells isolated

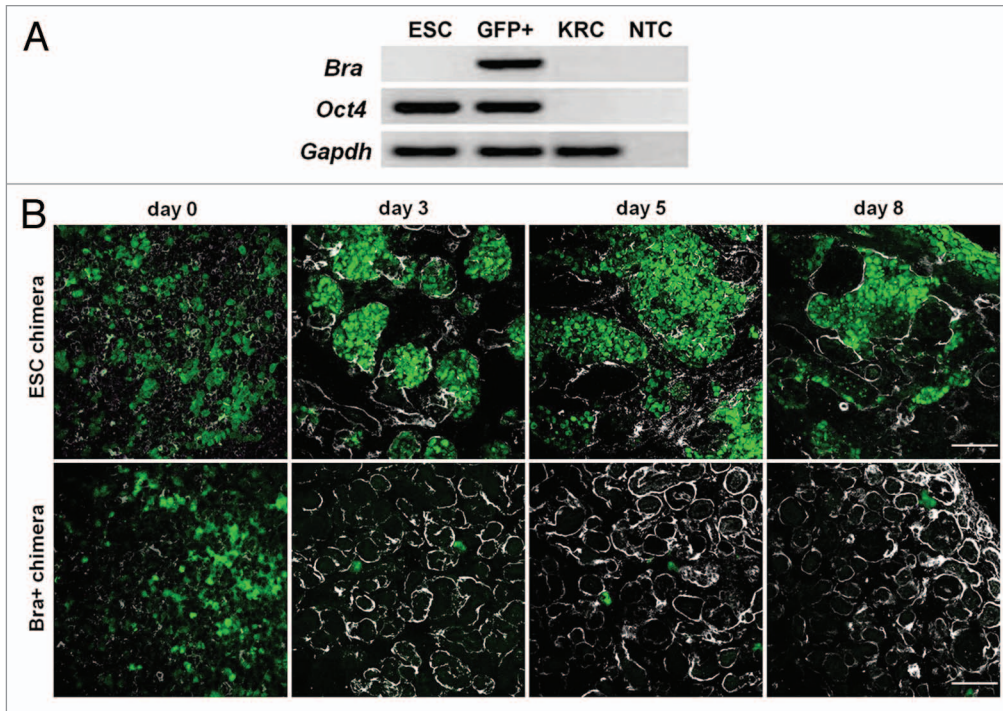


Figure 4. Expression of Oct4. **(A)** RT-PCR for *Oct4* in undifferentiated ESCs, Bra⁺ (GFP⁺) cells and KRCs. Note that undifferentiated ESC and Bra⁺ cells express *Oct4* just before being introduced into the chimera, whereas KRCs do not; NTC, no template control. **(B)** Oct4 (green nuclei) and laminin (white BMs) immunostaining within chimeras containing ESCs or Bra⁺ cells. Chimeric organs at day 0 were fixed 2–3 h following re-aggregation. Note plentiful large Oct4⁺ colonies in ESC chimeras. In contrast, there is a paucity of Oct4⁺ cells in the Bra⁺ chimeras. Scale bars, 60 μ m.

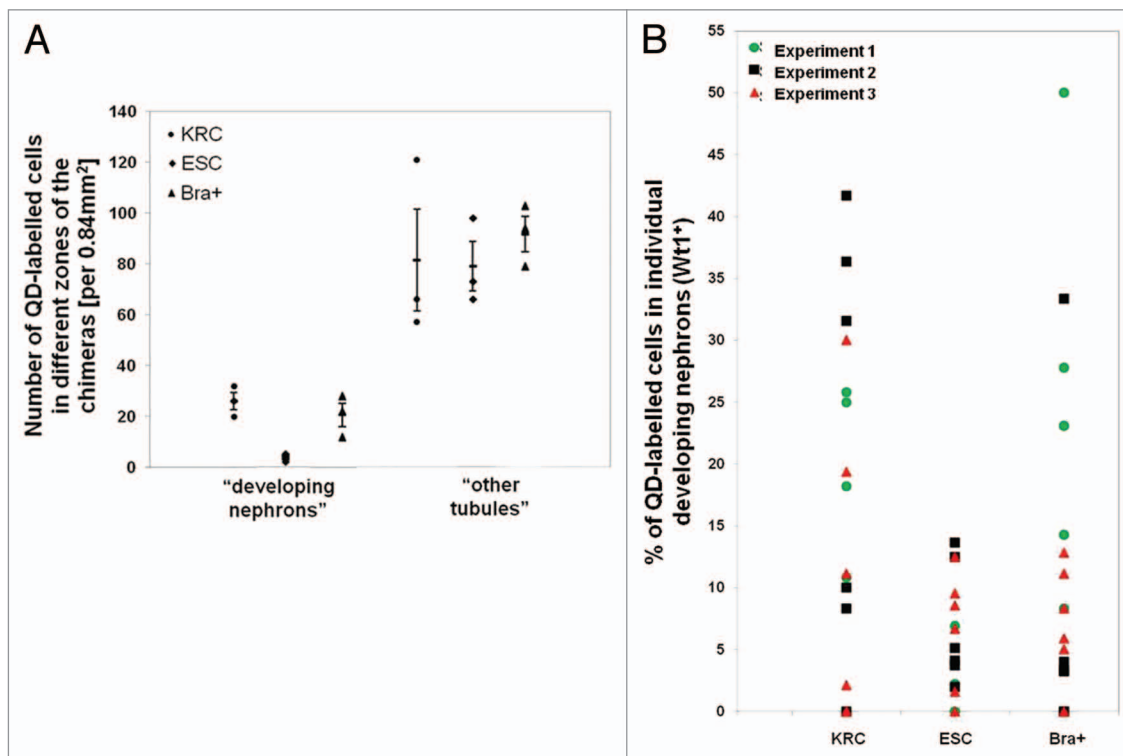


Figure 5. QD⁺ cell quantification. **(A)** Graph presenting the extent of integration of labeled cells into developing nephrons and other tubules per unit area (0.84 mm²). Numbers of QD⁺ cells in KRC and Bra⁺ chimeras (n = 3 organs for each) within developing nephrons was not significantly different, but the numbers in ESC chimeras was significantly less (p < 0.05). **(B)** Percentages of QD⁺ cells contributing to developing nephrons. Note that significantly fewer labeled cells contributed to developing nephrons (p < 0.05) in ESC chimeras vs. the other groups (n = 3 rudiments analyzed for each group, and for each rudiment, 6 nephrons were analyzed).

from mouse ESC-derived EBs could generate PT cells following injection into neonatal mouse kidneys which then matured in vivo. The present study supports the notion that Bra⁺ cells can integrate into nascent PTs. Furthermore, for the first time, we show that such labeled tubule epithelial cells are physiologically active, as evidenced by their ability to transport organic anions. Notably, in neither previous study^{11,12} did ESC-derived mesodermal cells appear to generate glomerular podocytes. This is in contrast with our results, which show that in chimeras made with Bra⁺ cells, some cells located in clusters expressing synaptopodin contained QDs, suggesting that Bra⁺ cells are indeed capable of generating podocytes. The reasons for the observed differences could be due to the fact that in the current study we use a disaggregation/re-aggregation method²⁰ to introduce exogenous cells, whereas in the other studies, cells were introduced by bolus injections.^{11,12} Another possible advantage of the current strategy is that any inhibitory effect of intact epithelia or basement membranes in restricting the integration of exogenous cells would be minimised, permitting improved cell integration.

A potential concern for all such studies is that it might be possible for labeled exogenous cells to fuse with host tubules, leading to false positives.⁴² However, using an elegant genetic strategy in which reporter R26R-EYFP ESCs were injected into Ksp-Cre host metanephroi, Kim and Dressler¹¹ found no evidence for fusing of exogenous cells with host tubules. A second concern with respect to using QDs as a label is that the fluorescent dots might theoretically be released from labeled exogenous cells and taken up by host cells. However, we have recently shown that the extent of QD transfer in rudiment chimeras is negligible.¹⁷ Moreover, we noted that the percentage of QD⁺ cells within Wt1⁺ developing nephrons in KRC and Bra⁺ chimeras was several-fold greater than that found in chimeric rudiments made from undifferentiated ESCs. Similarly, synaptopodin⁺ glomeruli containing subsets of QD⁺ cells were detected in rudiments made with KRC and Bra⁺ cells but not with those made with ESCs. Collectively, these results support the conclusions that the differentiation/integration behavior of exogenous cells is cell-type specific and that our results cannot be explained simply by invoking an unspecific transfer of QDs between exogenous and host cells. It is worth noting that because QDs do not replicate along with the cells, the proliferation of QD⁺ cells is associated with attenuation of the QD signal. Thus, the percentage of QD⁺ cells within developing nephrons in KRC and Bra⁺ chimeras probably represents a conservative estimate of integrated stem cells and/or their progeny.

A striking finding in our study was that Bra⁻ cells had a negative effect on metanephros development, resulting in very small recombinant organs which contained only sparse Wt1⁺ nephrons. Using the same chimeric rudiment culture assay, we have recently shown that mouse and human bone marrow-derived mesenchymal stem cells (MSCs) were unable to integrate into developing renal structures, and similar to Bra⁻ cells, had a negative effect on rudiment development.⁴³ Conditioned medium derived from the MSCs had a similar negative effect,⁴³ suggesting that secreted molecules were likely to be responsible. A previous study has shown that human bone marrow-derived CD34⁺ cells, which consist mainly

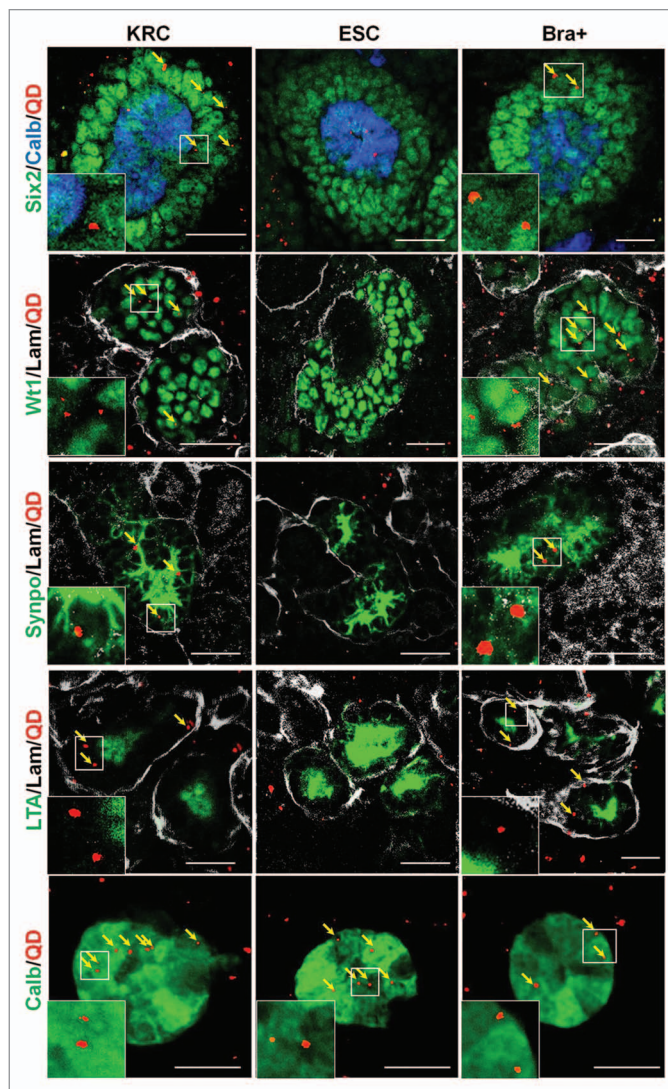


Figure 6. Expression of kidney specific markers by QD⁺ cells in chimeras. Representative confocal photomicrographs showing the expression of different markers in chimeras made with KRC, ESC and Bra⁺ cells. Yellow arrows point to QDs within specific cells. Boxed areas outlined in white are presented as enlargements within the main frame of some of the images. Note that QD⁺ cells are not seen in Six2, Wt1, synaptopodin and LTA (all in green) positive regions in the ESC chimera vs. the presence of QDs in these regions within rudiments made with Bra⁺ and positive control KRC cells. Basement membrane structures are stained with laminin (white). Scale bar, 20 μm.

of hematopoietic stem cells, were also unable to generate renal structures in both kidney rudiments and damaged adult kidneys, and instead contributed to hematopoietic lineages, and to a lesser extent, endothelial lineages.⁴⁴ In the current study, the mechanisms whereby the Bra⁻ cells inhibit kidney rudiment development have not been explored, but it is noteworthy that compared with the Bra⁺ mesodermal cells, the Bra⁻ population expressed higher levels of the ectodermal marker, *Pax6*, whose encoded protein directly upregulates *transforming growth factor b2*,⁴⁵ which codes for a known inhibitor of metanephrogenesis.⁴⁶ Vigneau et al.¹² did not report any obvious negative effect of

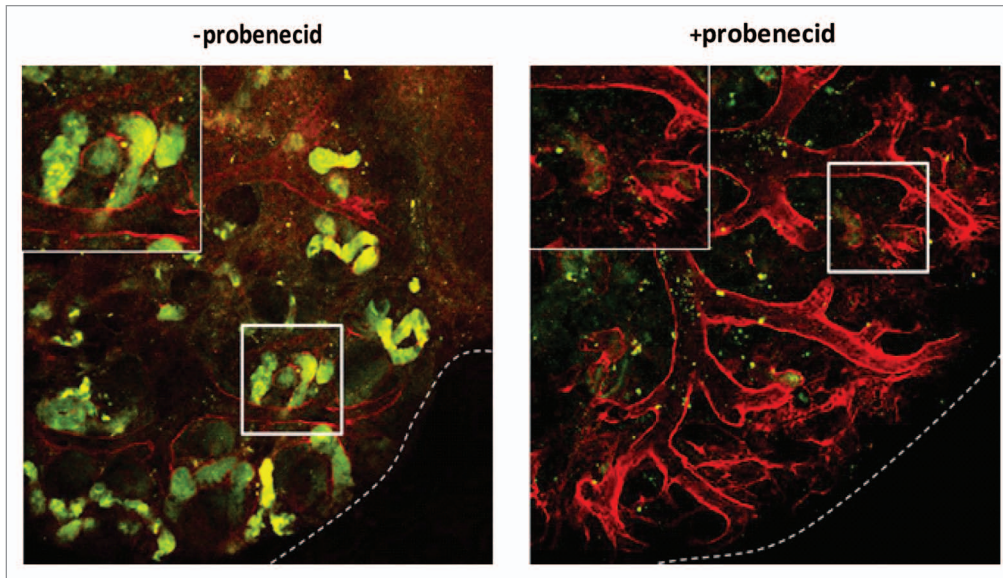


Figure 7. Uptake of organic ions by E13.5 intact kidney rudiments. In the absence of the OAT inhibitor, probenecid, vital staining of intact E13.5 kidney rudiments with 6-CF (green), indicates uptake by tubular epithelial cells. Uptake of 6-CF is blocked in the presence of probenecid. Vital staining with PNA (red) was used to identify tubule BMs. The rudiments had been cultured for 5 d prior to staining. Scale bars, 100 μm .

Bra⁺ cells on kidney development in culture, although this was not formally quantified by measuring explant growth and nephrogenesis.

Materials and Methods

Cell culture. The mouse Bra-GFP ESC line^{15,47} was subcultured every 2–3 d on tissue culture dishes (Nunc) in medium comprising high glucose DMEM (Sigma) supplemented with 10% ESC-grade FCS (PAA Laboratories), 2 mM L-glutamine, 1% penicillin/streptomycin, 1.5 $\times 10^{-4}$ M monothio glycerol (MTG) (all from Sigma) and 1,000 U ml⁻¹ LIF (Millipore). For differentiation as EBs, cells were seeded on non-adherent dishes (Sarsted) at 3 $\times 10^4$ cells ml⁻¹ in differentiating medium comprising 15% FCS IMDM supplemented with 2 mM L-glutamine, 4.5 $\times 10^{-4}$ M MTG, 1 \times ITS (Sigma), 0.5 M ascorbic acid, 1% penicillin/streptomycin (Sigma) in the absence of LIF. EBs at day 4 of development were disaggregated using 1 \times trypsin/EDTA (Sigma). For FACS, cells were used at a final density of 10⁶ cells ml⁻¹. Sorting was performed with a FACS Vantage SE (BD Biosciences) and the autofluorescence level of the cells was set using wild-type mouse E14 ESCs. FACS data were analyzed with Cell Quest PRO software (BD Biosciences).

Recombinant organ culture. CD1 mouse (Charles River) metanephroi were isolated and transferred onto isopore (1.2 μm) membrane filters (Millipore), placed on a metal grid and grown in kidney culture medium containing 10% FCS MEME (Sigma) supplemented with 2 mM L-glutamine and 1% penicillin/streptomycin (Sigma). Chimeric rudiments were generated

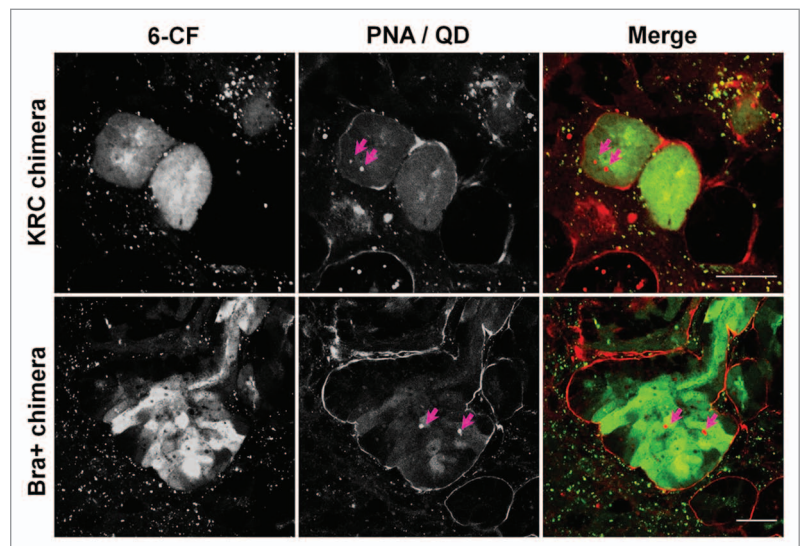


Figure 8. QD⁺ ESC-derived mesodermal cells take up organic ions. KRC and Bra⁺ chimeric organs contain QD⁺ (arrowed) cells which take up 6-CF. For each type of chimeric organ, the following images are shown of the same field: from left to right, the first image shows 6-CF, the second image shows PNA/QDs, and the third frame is a merged, color image (6-CF in green and PNA/QDs in red). Scale bars, 20 μm .

as described,²⁰ except that E13.5 instead of E11.5 rudiments were used. Metanephroi were trypsinised in 1 \times trypsin/EDTA solution for 5–7 min at 37°C generating single cells. The trypsin reaction was stopped using 10% FCS-DMEM medium and cells were centrifuged at 1400 \times g for 1 min. The supernatant was discarded and pelleted cells resuspended in kidney culture medium. These embryonic kidney-derived cells, obtained by disaggregating E13.5 mouse kidney rudiments to give a single

Table 2. Studies investigating the contribution of ESC and their derivatives to developing renal structures

Study	Cell type	Experimental procedure	Effect on nephron number	Presence of Oct4+ colonies	Integration of exogenous cells into glomeruli	Exogenous cells form physiologically active tubule cells
Steenhard et al. ¹³	ESC	Injection into E12/E13 metanephroi ex vivo	Not measured	Not determined	Not detected	Not investigated
Kim and Dressler ¹¹	EB cells cultured with nephrogenic factors	Injection into E11.5/E12.5 metanephroi ex vivo	Not measured	Not determined	Not detected	Not investigated
	EB cells cultured without nephrogenic factors		Not measured	Not determined	Not detected	Not investigated
Vigneau et al. ¹²	ESCs	Injection into neonatal kidneys in vivo	Not measured	Not investigated but teratoma formation noted	Not detected	Not investigated
	<i>Bra</i> ⁺ cells isolated from EBs	Injection into E11.5 metanephroi ex vivo	Not measured	Not investigated	Not detected	Not investigated
		Injection into neonatal kidneys in vivo	Not measured	Not investigated	Not detected	Not investigated
	<i>Bra</i> ⁻ cells isolated from EBs	Injection into E11.5 metanephroi ex vivo	Not measured	Not investigated	Not detected	Not investigated
Injection into neonatal kidneys in vivo		Not measured	Not investigated but teratoma formation noted	Not detected	Not investigated	
Present study	ESCs		Increased numbers of nephrons which were smaller than normal	Oct4 ⁺ colonies detected up to 8 d	No	No
	<i>Bra</i> ⁺ cells isolated from EBs	Creation of recombinant metanephroi by mixing exogenous with host E13.5 cells ex vivo	No effect (i.e., similar to positive control metanephric cells)	Oct4 ⁺ cells present at start but rarely detected at 8 d	Yes	Yes
	<i>Bra</i> ⁻ cells isolated from EBs		Strong adverse effect on organ size	Not investigated	Not investigated	Not investigated

cell suspension, are a heterogeneous population of kidney rudiment cells (KRCs). Our rationale for using KRCs as our positive control is that we know if we allow these cells to re-aggregate, they are capable of generating nascent nephrons and ureteric buds. We expect that the heterogeneous population of KRCs would initially include MM, UB cells, stromal cells and endothelial cells, but that after a few days of in vitro culture, the endothelial cells would die, as previously shown.⁴⁸ *Bra*⁺ and *Bra*⁻ cells sorted from day 4 EBs, mouse ESC, and KRCs were labeled with QDs (Invitrogen, Qtracker[®] Cell Labeling Kit, Q25021MP). In brief, QDs (10 nM) in 200 μ l complete kidney culture medium were applied to 1×10^6 cells in suspension and incubated for 60 min at 37°C and 5% CO₂, then washed 4 \times with complete growth medium and used for chimera formation.

We have recently shown that over 90% of cells are labeled with QDs following this procedure.¹⁷ Pellets comprising a 1:8 ratio of exogenous:KRCs (2×10^4 exogenous cells: 16×10^4 KRCs) were transferred onto isopore membrane filters (Millipore) on metal grids, and cultured for up to 8 d. For the first 24 h of incubation 5 μ M Y27632 Rho kinase inhibitor (Chemicon Int.) was applied. Samples were fixed in cold methanol for subsequent immunostaining.

Quantitative analysis of developing nephrons in chimeric metanephroi. The number of *Wt1*⁺ developing nephrons was counted in six randomly selected fields of view of day 3 KRC, ESC, *Bra*⁺ and *Bra*⁻ rudiment chimeras ($n = 3$ of each). The surface area of the different types of explants ($n = 3$ of each) and cross-sectional area of individual *Wt1*⁺ developing nephrons

Table 3. List of antibodies used in the study

Antibody	Supplier	Product	Dilution
Calbindin	Abcam	ab9481	1:500
Laminin	Sigma-Aldrich	L9393	1:1000
Oct4	Santa Cruz	sc-9081	1:500
Six2	Proteintech Europe	11562-1-AB	1:200
Synaptopodin	Acris	BM 5086	1:4
Wt1	Upstate	05-753	1:500

Table 4. List of primers used in the study

Gene	Primer sequence	Product size	Reference
<i>Bra</i>	F: AAC TTT CCT CCA TGT GCT GAG AC	532 bp	21
	R: TGA CTT CCC AAC ACA AAA AGC T		
<i>Osr1</i>	F: GCA GCG ACC CTC ACA GAC	169 bp	In-house ^a
	R: GCC ATT CAC TGC CCT GAA GGA		
<i>Foxc1</i>	F: TCA GAG CGG AAA TTG TAG GA	226 bp	In-house ^a
	R: GTA TTT GTT CAT GTG CCA ACT C		
<i>Pax6</i>	F: GAG AAG AGA AGA GAA ACT GAG GAA CCA GA	201 bp	12
	R: ATG GGT TGG CAA AGC ACT GTA CG		

^aPrimers were sequenced at University of Dundee Sequencing Lab.

(n = 5 for each rudiment type) was determined using the equation for calculating the surface area of an ellipse ($S = \pi r_1 r_2$).

Lectin- and immuno-staining. Following methanol fixation, samples were incubated for 2 h at room temperature in the dark with 10 $\mu\text{g ml}^{-1}$ LTA (Vector Laboratories). Samples were blocked for 1 h in goat serum, before overnight incubation at 4°C with the primary antibodies at concentrations shown in Table 3. Alexa fluor secondary antibodies (Invitrogen) were incubated overnight at 4°C and imaged using a confocal microscope (Leica, AOBSP2).

Quantification of QD-labeled cells in metanephroi. Numbers of QD⁺ cells in developing nephrons, other tubules and stroma were determined following immunostaining of day 3 KRC, ESC and Bra⁺ rudiment chimeras (n = 3 of each) for Wt1 and laminin. For each rudiment, the total number of QD⁺ cells within six random high power confocal fields (representing a total area of 0.84 mm²) was determined. To determine the percentages of QD⁺ cells within developing nephrons, 6 randomly selected nephrons (defined as Wt1⁺ cells bounded by laminin⁺ basement membrane) were examined from day 3 KRC, ESC and Bra⁺ chimeras (n = 3 of each type).

OAT assay. A modification of the method described by Sweet et al.²³ and Rosines et al.⁴⁹ was used. Chimeras grown for 5 d were washed twice in PBS for 5 min, then transferred into PBS

containing 1 μM 6-CF (Sigma) and 20 $\mu\text{g ml}^{-1}$ PNA-rhodamine (PNArh, Vector Laboratories) prior to incubation at 25°C for 1h in the dark. Control samples were incubated in OAT blocking solution, comprising 2 mM probenecid (Sigma) in PBS, 1 μM 6-CF and PNArh. Following incubation, samples were washed twice in ice-cold PBS and then incubated with 6-CF for 1h, followed by incubation in 8 mM probenecid in PBS for 15 min to prevent the secretion of 6-CF by tubular cells. After blocking, samples were transferred onto a glass coverslip and mounted with 80% (v/v) glycerol. The samples were immediately imaged by confocal microscopy.

RT-PCR. Total RNA was extracted from cells using Trizol (Invitrogen) and reverse transcribed using random hexamers (ThermoScientific) and Superscript III (Invitrogen). Cycling parameters were as follows: 1 cycle of 95°C/5 min, 33 cycles of 95°C/6 sec, 58°C/30 sec, 72°C/30 sec, and 1 cycle of 72°C/5 min. Primers used

are presented in Table 4.

Statistical analyses. Quantitative data are shown as a mean \pm standard error. Sets were compared using Student's t-test with $p < 0.05$ considered as significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/organogenesis/article/22597

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