Comparing the differentiation potential of \textit{Brachyury}^{+} mesodermal cells generated from 3-D and 2-D culture systems

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Abstract

Mesodermal populations can be generated \textit{in vitro} from mouse embryonic stem cells (mESCs) using three-dimensional (3-D) aggregates called embryoid bodies or two-dimensional (2-D) monolayer culture systems. Here, we investigated whether \textit{Brachyury}-expressing mesodermal cells generated using 3-D or 2-D culture systems are equivalent, or instead, have different properties. Using a \textit{Brachyury}-GFP/E2-Crimson reporter mESC line, we isolated \textit{Brachyury}-GFP$^{+}$ mesoderm cells using flow-activated cell sorting and compared their gene expression profiles and \textit{ex vivo} differentiation patterns. Quantitative RT-PCR analysis showed significant up-regulation of \textit{Cdx2}, \textit{Foxf1} and \textit{Hoxb1} in the \textit{Brachyury}-GFP$^{+}$ cells isolated from the 3-D system compared with those isolated from the 2-D system. Furthermore, using an \textit{ex vivo} mouse kidney rudiment assay, we found that irrespective of their source, \textit{Brachyury}-GFP$^{+}$ cells failed to integrate into developing nephrons, which are derived from the intermediate mesoderm. However, \textit{Brachyury}-GFP$^{+}$ cells isolated under 3-D conditions appeared to differentiate into endothelial-like cells within the kidney rudiments, whereas the \textit{Brachyury}-GFP$^{+}$ isolated from the 2-D conditions only did so to a limited degree. The high expression of \textit{Foxf1} in the 3-D \textit{Brachyury}-GFP$^{+}$ cells combined with their tendency to differentiate into endothelial-like cells suggests these mesodermal cells may represent lateral plate mesoderm.
1. Introduction

The formation of the primitive streak (PS) marks the onset of antero-posterior axis determination in the developing mouse embryo (Stern, 2004; Rodriguez et al., 2005). The epiblast cells egress through the PS to generate the nascent mesoderm in-between the primitive ectoderm and the overlying visceral endoderm. *Brachyury* (*Bra*, also known as *T*) is the key marker of the entire PS and is a pan mesodermal marker that is expressed in the posterior epiblast, PS, node, notochord, allantois and tail bud (Wilkinson et al., 1990; Kispert and Herrmann, 1994; Conlon et al., 1995; Kispert et al., 1995; King et al., 1998; Showell et al., 2004; Papaioannou, 2014; Concepcion and Papaioannou, 2014).

Following gastrulation, the *Bra*⁺ nascent mesoderm generates (i) paraxial mesoderm, which gives rise to the somites; (ii) lateral plate mesoderm, which gives rise to the heart, vessels, haematopoietic stem cells and endothelial cells; and (iii) intermediate mesoderm, which gives rise to the urogenital system (Gilbert, 2010; Wolpert et al., 2015). The intermediate mesoderm then becomes further specified to anterior intermediate mesoderm that gives rise to the ureteric bud (UB), and posterior intermediate mesoderm that gives rise to the metanephric mesenchyme (MM) (Little et al., 2016). The UB and MM generate the collecting ducts and nephrons, respectively, of the mature kidney (Pietilä and Vainio, 2014; Little et al., 2016).

The small size and inaccessibility of the peri-implantation mouse embryo makes it difficult to study. However, the isolation of embryonic stem cells (ESCs) from mouse blastocysts in the 1980s (Evans and Kaufman, 1981; Martin, 1981) has provided an alternative model for studying the early development of the mouse embryo.

When cultured in suspension, mESCs spontaneously form spheroid multicellular aggregates called embryoid bodies (EBs) (Wobus et al., 1984; Doetschman et al., 1985; Robertson, 1987; Murray and Edgar, 2004). A typical EB has an outer layer of primitive endoderm, an inner layer of primitive ectoderm, a basement membrane separating them, as well as a central cavity that resembles the proamniotic cavity (Shen and Leder, 1992). The primitive ectoderm differentiates to generate derivatives of definitive ectoderm, endoderm and mesoderm (Wobus et al., 1984; Doetschman et al., 1985; Keller et al., 1993). Therefore, EBs can recapitulate some aspects of peri-implantation mouse development and provide an excellent model system for studying these early events (Wobus et al.,...
However, the heterogeneous nature of the EBs means that the extent of differentiation towards any specific cell type can vary considerably depending on culture conditions, and can even vary between EBs cultured under the same culture conditions. The complex 3-D structure also hinders the visualisation of the differentiation process at an individual cell level. For this reason, various 2-D differentiation protocols have been developed to direct differentiation to specific cell-types more efficiently. Several studies have demonstrated in vitro derivation of monolayer mESCs into lineages of neural progenitors, endothelial cells, osteochondrogenic and myogenic cells using chemically defined media (Ying and Smith, 2003; Sakurai et al., 2009; Blancas et al., 2011; Blancas et al., 2013). Recently, Turner et al showed that Activin/Nodal and Wnt signalling pathways promote mesoderm formation in monolayer mESC culture, with the mesodermal cells differentiated from mESCs displaying Bra expression, similarly to the nascent mesoderm that develops in the primitive streak of developing mouse embryos and of ‘gastrulating’ EBs. By using a combination of Activin A (Activin/Nodal agonist) and Chiron (Wnt3a agonist), this group developed a highly efficient strategy for inducing E14 mESCs to differentiate into nascent mesoderm. After 2-day culture in neural differentiation medium and a further 2-day culture in medium supplemented with Activin A and Chiron, robust Bra expression was observed in over 90% of the population (David Turner, University of Cambridge, personal communication) (Turner et al., 2014a,b).

Although mesoderm differentiation occurs within both the 3-D EB and 2-D mESC culture systems, it is not clear whether the differentiated cells (e.g. mesodermal cells) that are generated by the 2-D protocols are equivalent to those that form in EBs. In the mouse embryo, the fate of the Bra+ cells is determined by the microenvironment that the cells find themselves in following their migration from the primitive streak (Gilbert, 2010). This cannot be replicated using in vitro culture systems, which raises the question of whether the Bra+ cells generated in vitro are equivalent to nascent mesoderm, or instead, are partially committed to a specific mesodermal lineage. For instance, the Little group have previously reported that BRA+ cells derived from human ESCs have a tendency to spontaneously differentiate into FOXF1+ lateral plate mesoderm when cultured in the absence of exogenous growth factors (Takasato et al., 2014). This observation highlights the fact that the differentiation potential of Bra+ cells generated in vitro is likely to be influenced by the specific culture conditions used.
We have previously shown that Bra+ mesodermal cells isolated from mESC-derived EBs were able to integrate into the developing UB and MM of mouse kidney rudiments and generate specialised renal cells (Rak-Raszewska et al., 2012). However, in this previous study, the EBs from which the Bra+ mesodermal cells were isolated did not mimic early embryo development, in that they did not form a primitive ectoderm epithelium, nor a proamniotic cavity. In the present study, we aimed to investigate whether Bra+ cells generated using the recently described 2-D culture system, and those derived from cavitating EBs, express similar lineage-specific genes, and have similar developmental potential to those derived from non-cavitating EBs. In order to do this, we have generated a Bra-GFP/Rosa26-E2C mESC reporter line (Zhou et al., in press) that will allow us to isolate the GFP-expressing nascent mesodermal cells from both systems so that their gene expression can be analysed using RT-PCR and their developmental potential can be assessed by investigating their fate following incorporation into mouse kidney rudiments ex vivo (Unbekandt and Davies, 2010; Kuzma-Kuzniarska et al., 2012; Rak-Raszewska et al., 2012; Ranghini et al., 2013; Dauleh et al., 2016).

2. Results

2.1 Mesoderm development within EBs is affected by seeding density

The Bra-GFP/Rosa26-E2C mESCs were plated at different densities and cultivated for 7 days in EB medium. At densities of 2.5×10^5 and 1.25×10^5 cells mL^-1, cavitated EBs could be observed by day 4, but at the lower seeding density of 6.25×10^4 cells mL^-1, most EBs failed to cavitate, even by day 7 (Fig. 1). Mesoderm development was identified in all conditions by GFP fluorescence, but the expression patterns were different. At 6.25×10^4 cells mL^-1, GFP was expressed at an earlier stage and peaked on day 4 before decreasing. In contrast, at higher densities, GFP became visible at day 4 or later and the fluorescence signal increased from day 4 to 7, but there appeared to be more GFP+ cells in the 1.25×10^5 cells mL^-1 EBs (Fig. 1). Therefore, given that the EBs developing in the 1.25×10^5 cells mL^-1 density cultures appeared to be typical cavitating EBs that contained a high proportion of GFP+ cells, we used this plating density in all future experiments. To investigate if E2C expression affected mesoderm differentiation, immunostaining of Bra-GFP/Rosa26-E2C EB sections was performed to confirm that the GFP+ cells within the EB expressed E2C. The results showed that all cells within the Bra-GFP/Rosa26-E2C EBs continued to express E2C, including the GFP+ mesodermal cells, indicating that E2C expression did not inhibit mesoderm differentiation (Fig. 1).
2.2 Comparing the timing and extent of mesodermal cell differentiation using the 3-D and 2-D culture systems

In order to accurately monitor changes in GFP expression in the developing EBs over time, *Bra-GFP/Rosa26-E2C* mESCs were plated at a density of $1.25 \times 10^5$ cells mL$^{-1}$ and at day 3, were embedded in a sandwich-like agarose system (2% agarose bottom layer – EB – 1% agarose overlay) and imaged in real-time using the Cell-IQ instrument every hour from day 3 to day 9 post plating. GFP started to be expressed on day 4 (96 h), and reached maximum levels on day 6–7. Although expression levels began to decrease at this time point, GFP$^+$ cells were still present at day 9 (Fig. 2A). To quantify the proportion of mesodermal cells within the EBs, flow cytometry analysis was performed. EBs derived from the wild-type E14TG2a mESCs were used as a negative control. The results were consistent with the Cell-IQ data, and showed that the peak GFP expression was at day 6, at which time, approximately 39% of the EB population were GFP$^+$ (Fig. 2B).

We then determined the efficiency of the previously described 2-D culture system (Turner et al., 2014a,b). The *Bra-GFP/Rosa26-E2C* mESCs were cultured under differentiation conditions for 4 days, and were then screened for GFP expression. Analysis of fixed cells in culture showed that the vast majority of the population expressed GFP. Flow cytometry analysis showed that approximately 89% of the population was GFP$^+$, which is consistent with the efficiency reported previously with this method (Figs 2C–D).

2.3 Comparing the expression profile of key genes in GFP$^+$ mesodermal cells generated under 3-D and 2-D differentiation conditions

Before comparing the expression levels of the key target genes in the GFP$^+$ cells isolated from the 3-D and 2-D culture systems, it was first necessary to determine the purity of the GFP$^+$ cell populations isolated from each culture system. Single cell suspensions from day 6 EBs and day 4 2-D monolayer cultures were sorted by FACS and then re-analysed using the same parameters. Results showed that the proportion of GFP$^+$ cells was over 94% (Fig. 3A), confirming they were pure populations.

In order to characterize the *Bra-GFP$^+$* and *Bra-GFP$^-$* populations, quantitative real-time polymerase chain reaction (qRT-PCR) was performed to examine the expression patterns of key genes of mesodermal lineages and of early kidney development (Table S1). Relative gene expression levels were
evaluated and compared between the following groups: (i) the Bra-GFP+ and Bra-GFP− populations isolated from the EBs (3-D system); (ii) Bra-GFP+ and Bra-GFP− populations isolated from the 2-D system; and (iii) the Bra-GFP+ populations isolated from the 3-D and 2-D systems. Stemness markers Oct4 and Nanog and the primitive ectoderm marker, Fgf5, were also evaluated to assess whether undifferentiated mESCs and/or ectoderm cells were present.

Firstly, comparisons were made between gene expression levels in the Bra-GFP+ cells and the Bra-GFP− cells isolated from the 3-D and 2-D system. The results showed that the early mesoderm genes Bra, Cdx2, Tbx6, Foxf1, Foxa2, Hoxb1 and Hoxc9 were expressed by Bra-GFP+ cells isolated from both the 3-D and 2-D systems, but the relative expression levels differed in comparison to the respective Bra-GFP− populations. For instance, under the 3-D conditions, the expression levels of Bra, Cdx2, Tbx6, Foxf1, and Hoxb1 in the Bra-GFP+ population were approximately 55-, 10-, 40-, 10- and 55-fold higher than in the Bra-GFP− population, respectively, whereas under the 2-D conditions, Bra, Tbx6 and Hoxb1 levels in the Bra-GFP+ cells were only 2-, 4-, and 5-fold higher, respectively, than in the Bra-GFP− cells (Figs 3B–C).

There was a 1- to 10-fold up-regulation of Hox10 and Hox11 paralogy groups (Hoxa10, Hoxa11 and Hoxd11) in the Bra-GFP+ population compared to the Bra-GFP− population isolated from cells under 3-D conditions. In contrast, down-regulation of the same genes was observed in the Bra-GFP+ population isolated from cells under 2-D conditions compared to the Bra-GFP− population (Figs 3B–C). This suggested that the status of Bra-GFP− cells isolated from EBs may be closer to a stage resembling posterior mesoderm, as it has been shown previously that posterior mesoderm, which gives rise to the MM, expresses higher levels of Hox10 and 11 genes compared to anterior mesoderm (Taguchi et al., 2014).

Genes of intermediate mesoderm and metanephric mesenchyme, i.e., Lhx1, Osr1, Pax2 and Wt1, displayed a similar trend in the change of relative expression levels between the Bra-GFP+ and Bra-GFP− groups under 3-D and 2-D conditions. It is of note that in the cells isolated from the EBs, Lhx1 was up-regulated by approximately 10-fold in the Bra-GFP+ cells compared to the Bra-GFP− cells, whereas there was minimal up-regulation in the Bra-GFP+ cells isolated from the 2-D conditions (Figs 3B–C, Fig. S1). Oct4, Nanog and Fgf5 were also evaluated and the data showed no difference between
the Bra-GFP$^+$ cells and Bra-GFP$^-$ cells isolated from both 3-D and 2-D conditions (Fig. S1).

Next, the relative expression levels of the various genes in Bra-GFP$^+$ cells isolated from 3-D and 2-D system was compared. There was no significant difference in the expression levels of Bra and Tbx6, whereas Cdx2, Foxf1 and Hoxb1 were significantly up-regulated by 9-, 30-, 5-fold, respectively, in the Bra-GFP$^+$ cells isolated under 3-D conditions. Another early mesoderm gene Hoxc9 as well as posterior mesoderm genes Hox10 and Hox11 were also up-regulated but not significantly. The expression levels of Lhx1, Osr1, Pax2, Wt1 and Gdnf were comparable between the two populations. On the other hand, Foxd1, which, is expressed in MM and stroma, showed a slight 2-fold up-regulation in the 3-D Bra-GFP$^+$ cells, but this was not statistically significant (Fig. 3D).

### 2.4 Ex vivo development of intact and re-aggregated non-chimeric mouse kidney rudiments

In order to evaluate how the Bra-GFP$^+$ cells behave in the rudiment culture, it was first necessary to establish the typical staining pattern of various renal cell-specific antibodies in intact kidney rudiments cultured ex vivo. Following 5 days of ex vivo culture, the rudiments were fixed and immunofluorescence was performed to detect the following markers: megalin, which is expressed on the apical surfaces of proximal tubule cells (Ranghini et al., 2013; Taguchi et al., 2014); Wt1, which is expressed in MM and developing nephrons, and expressed at very high levels in nascent and mature podocytes (Moore et al., 1999; Ranghini et al., 2013; Taguchi et al., 2014); synaptopodin, which is expressed in mature podocytes (Mundel et al., 1997; Shankland et al., 2007). The rudiments were also stained with rhodamine-labeled peanut agglutinin (PNA), which mainly binds to the basement membranes of UBs, and more weakly to those of the developing nephrons (Laitinen et al., 1987). PNA staining showed an intact UB tree, and immunostaining for megalin showed typical staining of the apical surfaces of proximal tubule cells (Fig. 4A). As expected, immunostaining for Wt1 showed weaker expression in MM and developing nephrons and intense expression in nascent and mature podocytes, whereas synaptopodin was exclusively expressed in mature podocytes (Fig. 4A).

To confirm that re-aggregated kidney rudiments could develop nephron and UB structures as previously reported (Unbekandt and Davies, 2010; Rak-Raszewska et al., 2012; Ranghini et al., 2013), dissociated kidney rudiment cells were pelleted and cultured ex vivo prior to staining with the aforementioned markers. Firstly, it was important to confirm that the disaggregation process was
effective and that no non-dissociated renal structures were present at the start of the culture period. Therefore, at day 0, rudiments were stained for megalin and PNA. The results showed that no staining was present at day 0, whereas multiple tubular structures were present by day 5 (Fig. 4B). More detailed analysis of the re-aggregated rudiments showed that the pattern of tubular structures and nascent glomeruli appeared similar to that of the intact rudiments, which was consistent with previous studies (Kuzma-Kuzniarska et al., 2012; Rak-Raszewska et al., 2012; Ranghini et al., 2013). Although UB tubules formed, they did not form a contiguous UB tree (Fig. 4C).

2.5 The behaviour of mESC-derived Bra-GFP+ cells within chimeric kidney rudiments cultured ex vivo

Before assessing the differentiation potential of the mESC-derived Bra- cells in the chimeric rudiment assay, it was first necessary to confirm that chimeric rudiments comprising a positive control cell population developed as expected. To this end, chimeric rudiments containing GFP+ mouse neonatal kidney-derived stem cells (KSCs) were generated, as we have previously shown that KSCs can generate proximal tubule cells and podocytes within rudiments (Ranghini, 2011; Ranghini et al., 2013). The chimeric rudiments were cultured for 5 days ex vivo and analysed as previously using the renal cell-specific markers. On day 0, the KSCs were evenly distributed in the chimeric rudiments (Fig. S2). After 5 days of culture, the chimeric rudiments had developed proximal tubule-like structures that stained positively for megalin, as well as nascent glomeruli that contained podocytes, as evidenced by positive staining for Wt1 and synaptopodin. KSCs showed integration into the tubules and glomeruli of the developing nephrons (Figs 5–7).

To investigate the behaviour of mESC-derived Bra-GFP+ cells within chimeric kidney rudiments cultured ex vivo, Firstly, the behaviour of E2-Crimson-expressing (E2C+) Bra-GFP+ cells isolated from mESC-derived EBs (3-D culture system) were investigated in the ex vivo rudiment assay. Staining for PNA, megalin, Wt1 and synaptopodin showed that similarly to the positive control chimeras comprising KSCs, the re-aggregated metanephric cells were able to develop tubular structures and nascent glomeruli (Figs 5–7). However, immunostaining for E2C showed that the EB-derived cells did not integrate into tubules or glomeruli, and instead, appeared to elongate and form interconnected cell networks throughout the rudiment. In many cases, the EB-derived cells appeared to align against the outer surface of developing glomeruli (Figs 5–7).
Next, the behaviour of E2C\(^+\) Bra-GFP\(^+\) cells isolated from the 2-D culture system was investigated using the chimeric rudiment assay. As with the EB-derived Bra-GFP\(^+\) chimeras, staining for PNA, megalin, Wt1 and synaptopodin showed that re-aggregated metanephric cells in chimeras comprising Bra-GFP\(^+\) cells isolated from the 2-D culture system were able to generate tubular structures and nascent glomeruli (Figs 5–7). Similarly to the E2C\(^+\) EB-derived Bra-GFP\(^+\) cells, the cells isolated from the 2-D culture system did not appear to integrate into tubules or glomeruli. However, in contrast to the EB-derived cells, those isolated from 2-D culture tended not to form connections with each other. Although elongated cells were occasionally observed in close proximity to developing glomeruli, the majority of the cells were not elongated and did not from interconnected cell networks (Figs 5–7). Furthermore, there appeared to be fewer E2C cells present in these chimeras compared to those generated from mESC-derived Bra-GFP\(^+\) isolated from EBs.

The morphology of E2C\(^+\) Bra-GFP\(^+\) cells within the chimeras generated from EB-isolated cells appeared similar to that of endothelial cells within ex vivo kidney rudiments (Halt et al., 2016). To investigate if the E2C\(^+\) cells had differentiated into endothelial cells, the rudiments were immunostained for the endothelial marker, PECAM-1 (platelet and endothelial cell adhesion molecule 1) (Kondo et al., 2007). It was found that the metanephric cells generated PECAM-1\(^+\) interconnected cell networks in both types of chimeric rudiment, indicating that endothelial cells had differentiated. Analysis of E2C\(^+\) cells within the chimeric rudiments generated from EB-derived Bra-GFP\(^+\) cells showed that the majority of these cells appeared to stain positively for PECAM-1, suggesting that they had differentiated into endothelial cells. In contrast, most of the E2C\(^+\) cells within the chimeric rudiments generated from 2-D culture-derived Bra-GFP\(^+\) cells did not stain positively for PECAM-1. Instead, only the elongated cells which were occasionally observed within these chimeras were found to stain for PECAM-1 (Fig. 8, Movies S1–2).

3. Discussion

In this study, we generated mesoderm populations from a Bra-GFP/Rosa26-E2C mESC reporter line using 3-D and 2-D culture systems. The dynamics of GFP expression during EB culture was similar to what has been previously observed in our group (Rak-Raszewska, 2010); i.e., at low seeding density, GFP appeared to peak earlier than at
higher seeding densities. A possible explanation is that mESCs might express inhibitors of mesoderm differentiation, such as noggin, which would be present at higher levels in higher density cultures, and might therefore delay mesoderm differentiation (GFP expression) (Tonegawa and Takahashi, 1998; Gratsch and O'Shea, 2002). Also, GFP expression was detected in EBs generated at low density that had not cavitated. This is similar to our lab’s previous findings using the same E14-Bra-GFP mESC line, but with a different culture protocol developed by Fehling et al (Fehling et al., 2003; Rak-Raszewska et al., 2012). In that study, GFP was only expressed within the EBs during days 3 to 4 with about 60% of the population expressing GFP at day 4 (Rak-Raszewska, 2010). This is much higher than the proportion we observed in the current study (less than 40%). However, EBs generated using Fehling’s method did not form a proamniotic-like cavity, extra-embryonic endoderm or basement membranes. It is therefore envisaged that the properties of Bra+ mesoderm cells generated from the two types of EBs (i.e., cavitating or non-cavitating), might have different properties and differentiation potential.

An interesting finding from the qRT-PCR analysis was that the expression levels of Bra in the GFP+ cells isolated from the 3-D system were approximately 50 times higher than in the GFP− cells, but Bra levels in GFP+ cells isolated from the 2-D system were only approximately three times higher than in the corresponding GFP− cells. Yet despite this, when Bra levels in the GFP+ cells from the 3-D system were directly compared with levels in GFP+ cells from the 2-D system, there was no significant difference. A possible explanation for this is that the GFP− cells in the EBs are likely to be endoderm or ectoderm cells that do not express Bra, whereas in the 2-D system, it is possible that the GFP− cells might be committed to the mesodermal lineage and have started to up-regulate Bra, but due to the time-lag between transcription and translation, might not have yet started to produce GFP. If this were the case, such cells would be Bra+ but GFP−, and would thus have been sorted into the GFP-negative fraction by FACS.

When comparing the expression levels of key genes between the GFP+ cells from the 3-D and 2-D systems, there were only three genes that were significantly up-regulated in the cells from 3-D system, namely, Foxf1, Cdx2 and Hoxb1. The high expression levels of Foxf1 might suggest that the GFP+ cells from the 3-D system might be lateral plate mesoderm cells. It is known that high levels of BMPs promote the differentiation of lateral plate mesoderm, whereas low levels of BMPs promote
intermediate mesoderm (Tonegawa and Takahashi, 1998). It is therefore possible that in the larger
cavitating EBs, there might be higher levels of BMPs which would then drive the differentiation of
lateral plate mesoderm. However, the cells also had significantly higher levels of the nascent mesoderm
gene, Cdx2, and the posterior mesoderm gene, Hoxb1. Furthermore, although not significant, there was
a clear trend that the Hox genes tested, which are expressed in intermediate mesoderm, were
up-regulated in the cells from the 3-D system.

By introducing the E2C-expressing mesodermal cells into the chimeric rudiments ex vivo, we showed
that neither the Bra-GFP+ cells derived from the 3-D nor 2-D culture systems appeared to integrate into
the developing nephrons. The results are strikingly different from our lab’s previous studies that
investigated the nephrogenic potential of Bra-GFP+ cells isolated from non-cavitating EBs in the same
rudiment culture assay (Rak-Raszewska et al., 2012). In these earlier studies, it was found that
Bra-GFP+ mESCs derived from non-cavitating EBs were able to integrate into both the developing
nephrons and UBs, and could form functional proximal tubule cells and podocytes (Rak-Raszewska et
al., 2012). Another study by Vigneau et al showed that Bra+ cells derived from mouse EBs contributed
to the proximal tubules when injected into the neonatal mouse kidney in vivo (Vigneau et al., 2007).
The results we obtained with the Bra-GFP+ cells obtained from cavitating EBs were surprising. We had
expected that as these cells were isolated at a later time point than the Bra-GFP+ cells in the
non-cavitating EBs, they might more closely resemble posterior mesoderm, which has recently been
shown to generate the MM but not the UB (Taguchi et al., 2014). We therefore thought that these cells
might integrate into developing nephrons, but not the UBs. However, they did not integrate into either
of these structures and instead appeared to differentiate into endothelial cells. There have been
contrasting reports concerning the presence of endothelial cells in mouse kidney rudiments cultured ex
vivo, with some studies suggesting endothelial cells cannot survive in ex vivo rudiments (Loughna et
al., 1997) and others suggesting they do (Halt et al., 2016). Our findings are consistent with the Halt et
al study that indicates endothelial cells are present in rudiments, and similarly to that study, we found that
although the endothelial cells formed interconnected networks, they did not form capillaries with
lumen, nor did they invest the developing glomeruli.

The key differences in the gene expression profile of the Bra-GFP+ cells isolated from cavitating EBs
(current study) and non-cavitating EBs (previous study) (Rak-Raszewska, 2010) is that in comparison
to GFP− cells, the former expressed much higher levels of Foxf1, which is highly expressed in lateral
plate mesoderm, and lower levels of the MM genes, Gdnf and Osr1 (Rak-Raszewska, 2010). The high
expression levels of Foxf1 might explain why the EB-derived Bra-GFP+ cells in the current study had a
tendency to generate endothelial cells, because it is known that Foxf1 is essential for vasculogenesis in
the developing embryo and is expressed in endothelial cells (Mahlapuu et al., 2001; Ren et al., 2014).

High levels of BMP signals and their receptors ALK3/6 have been shown to promote a lateral plate
mesoderm fate (James and Schultheiss, 2005). Due to the heterogeneous nature of the EBs, it is
possible that mesoderm niches that resemble dynamic microenvironments of the in vivo primitive
streak have been formed. Cells residing in the niches that are exposed to high concentrations of BMP
signals might, therefore, adopt a lateral plate mesoderm fate. Retinoic acid, FGF and Wnt signals might
also affect the cell commitment of lateral plate mesoderm but their effects may be stochastic within the
EBs. Nevertheless, we cannot exclude the possibility that the timing might have been another factor;
for instance, Bra-GFP+ cells isolated at slightly earlier or later time-points might have expressed genes
of other mesodermal lineages.

Regarding the Bra-GFP+ isolated from the 2-D system, it was found that these also did not integrate
into developing nephrons or UBs. Furthermore, only a small proportion of these cells appeared to
differentiate into endothelial cells. The majority of the cells did not form interconnected cell networks
and appeared to be randomly dispersed throughout the stroma. Similarly to the Bra-GFP+ cells from the
cavitating EBs, the Bra-GFP+ cells from the 2-D system did not show any noticeable up-regulation of
Gdnf or Osr1 in comparison with the Bra-GFP− cells. However, in contrast to the EB-derived cells,
those isolated from the 2-D system did not show up-regulation of Foxf1, which is consistent with their
limited tendency to generate endothelial cells. It is possible that the Bra-GFP+ cells from the 2-D
system might have differentiated into stromal cells, but it was not possible to test this due to the lack of
a stroma-specific antibody. It is interesting to note that the Bra-GFP+ cells from the 2-D system
expressed higher levels of the stromal gene, Foxd1 (Mugford et al., 2008) compared to those from the
3-D system, but the results were not statistically significant.

4. Materials and methods

4.1 Routine cell culture
Bra-GFP/Rosa26-E2C mESCs (Zhou et al., in press) were maintained in 0.1% gelatinised 6-well tissue culture plates with mitomycin-C (Sigma-Aldrich, M4287) inactivated STO (ATCC, SCRC-1049) feeder cells at 37°C in a humidified incubator with 5% CO₂ in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma-Aldrich, D6546) supplemented with 150 mL L⁻¹ FBS (Sigma-Aldrich, F2442), 10 mL L⁻¹ MEM non-essential amino acid (Sigma-Aldrich, M7145), 10 mL L⁻¹ L-glutamine (Sigma-Aldrich, G7513), 0.1 mmol L⁻¹ β-mercaptoethanol (Gibco, 31350) and 1,000 U mL⁻¹ mouse leukemia inhibitory factor (mLIF) (Merck Millipore, ESG1107). Cells were passaged every other day and those at passage 13–22 were used for experiments.

GFP-expressing mouse neonatal kidney-derived stem cells (GFP-KSCs) (Ranghini, 2011) were maintained in 60 mm tissue culture dishes at 37°C in a humidified incubator with 5% CO₂ in DMEM supplemented with 100 mL L⁻¹ FBS (Gibco, 10270), 10 mL L⁻¹ MEM non-essential amino acid (Sigma-Aldrich, M7145), 10 mL L⁻¹ L-glutamine (Sigma-Aldrich, G7513) and 0.1 mmol L⁻¹ β-mercaptoethanol (Gibco, 31350). Cells were passaged 2–3 times per week and those at passage 17–20 were used for experiments.

4.2 3-D EB system
mESCs were sub-cultured in gelatinised 6-well tissue culture plates for 48 h to deplete feeder cells. Cells were then collected and plated in 90 mm bacterial petri dishes (Sterilin, 101VR20) at the densities of 6.25×10⁴, 1.25×10⁵ and 2.5×10⁵ cells mL⁻¹ to form aggregates. The EBs were maintained at 37°C in a humidified incubator with 5% CO₂ in DMEM supplemented with 100 mL L⁻¹ FBS (Sigma-Aldrich, F2442), 10 mL L⁻¹ MEM non-essential amino acid, 10 mL L⁻¹ L-glutamine and 0.1 mmol L⁻¹ β-mercaptoethanol for up to 9 days with a medium change every other day. Each dish was split 1:2 on day 3 and EB morphology was examined on days 4 and 7. Experiments were carried out in 3 independent biological replicates.

4.3 2-D system
mESCs were sub-cultured in gelatinised 6-well tissue culture plates for 48 h to deplete feeder cells. Cells were collected and plated into gelatinised 6-well plates at 1×10⁵ cells per cm² for 24 h. 2-D induction culture was based on the protocols previously described (Turner et al., 2014a,b). Briefly, cells were then harvested and re-plated into 60 mm tissue culture dishes at a density of 4.7×10³ cells per cm².
with overnight incubation in mESC culture medium. The following morning, medium was changed to NDiff® 227 (Clontech, Y40002) for 48 h and then to NDiff® 227 supplemented with Activin-A (R&D Systems, 338-AC) and CHIR 99021 (Tocris, 4423) to a final concentration of 100 ng mL⁻¹ and 3 µmol L⁻¹, respectively, for a further 48 h incubation. Medium was changed on a daily basis. Experiments were carried out in 3 independent biological replicates.

4.4 Cell-IQ real-time imaging

On day 3, EBs that were formed from mESCs at the plating density of 1.25×10⁵ cells mL⁻¹ were harvested and plated onto solidified 2% agarose gel (Sigma-Aldrich, A9045) in glass bottom 6-well plates (MatTek, P06G-0-20-F). They were then embedded in a thin overlay of 1% agarose. Each well was filled with 3 mL EB medium once the overlaid gels were set. Plates were maintained in Cell-IQ (Chip-Man Technologies Ltd) imaging facility. EBs were imaged by the Cell-IQ Imagen (Chip-Man Technologies Ltd) software on days 3 to 9 on an hourly basis. Imaging data from both bright field and 488 nm laser for the GFP fluorescence signal were documented from 3 independent biological replicates. Raw data were analysed by the Cell-IQ Analyser (Chip-Man Technologies Ltd) and ImageJ (NIH) softwares.

4.5 EB fixation and cryo-sectioning

EBs were harvested on day 7 and fixed with 4% paraformaldehyde (PFA). They were then soaked in 15% sucrose followed by embedding in the 7.5% molten gelatin. Samples were mounted onto cork disks with Shandon™ Cryomatrix™ embedding resin (Thermo Fisher Scientific, 6769006) and cut with a cryostat at 20 µm.

4.6 Flow cytometry analysis

Single cell suspensions of 1×10⁶ cells mL⁻¹ were obtained from 3-D or 2-D culture systems and examined by a BD FACScalibur (BD Biosciences) flow cytometer according to manufacturer’s instructions, using a 488 nm laser to detect the GFP signal. For analysis of the GFP expression window in the EBs, wild-type E14TG2a-derived EBs were used as a negative control. For analysis of GFP expression in the 2-D system, undifferentiated Bra-GFP/Rosa26-E2C mESCs sub-cultured in gelatinised dishes in mESC medium for 24 h prior to induction were used as a negative control. Data were acquired from two biological replicates by the BD CellQuest (BD Biosciences) software based on
10^5 events and analysed using the Cyflogic (CyFlo Ltd, version 1.2.1) software.

4.7 Fluorescence-activated cell sorting (FACS)

Single cell suspensions of 1×10^7 cells mL^-1 were obtained from day-6 3-D EBs or day-4 2-D monolayer cultures. Sorting was performed to isolate Bra-GFP^+ cells using the BD FACS Aria (BD Biosciences) flow sorter with the 530/30 bandpass filter and 502 longpass mirror. Day-6 EBs derived from wild-type E14TG2a mESCs and undifferentiated Bra-GFP/Rosa26-E2C mESCs sub-cultured in gelatinised dishes for 24 h prior to induced differentiation were used as negative controls for 3-D and 2-D systems, respectively. Data output was performed using BD FACSDiva (version 6.1.3) software. Experiments were performed in 3 independent biological replicates.

4.8 qRT-PCR and statistical analysis

Cell lysis of FACS-sorted Bra-GFP^+ populations, reverse transcription and qPCR amplification was performed using the Fast SYBR® Green Cells-to-CT™ Kit (Thermo Fisher Scientific, 4405659) in accordance with the manufacturer’s instructions. Gene transcription was detected by the Bio-Rad CFX Connect Real-time PCR Detection System (Bio-Rad) using specific primers validated in-house (Table S2). The reaction was set up with the following steps: 95°C for 20 s initial DNA polymerase activation followed by 40 cycles of denaturation at 95°C for 3 s and annealing/extension at 60°C for 30 s. qPCR specificity was assessed by melt curves and then verified by agarose gel electrophoresis. Non-template control was performed for each analysed gene and the non-reverse transcriptase control was also included to verify the elimination of genomic DNA. Three biological replicates for the Bra-GFP^+ populations isolated from 3-D and 2-D systems, and two biological replicates for Bra-GFP^- populations derived from the 3-D and 2-D systems were assessed. For each reaction product analysed, two technical replicates were prepared. Data were acquired using the incorporated Bio-Rad CFX Manager (version 3.1) software. Relative gene expression levels normalised to two endogenous reference genes Gapdh and β-actin (ΔΔC_t) and statistical analysis were also performed using two-tailed Student's t-test by the same software, where P<0.05 was considered statistically significant.

4.9 Mouse embryonic kidney rudiment ex vivo culture

The Mouse embryonic kidney rudiment ex vivo culture was based on the protocols previously described (Unbekandt and Davies, 2010). Briefly, kidneys were dissected out from embryonic day (E)
13.5 CD1 mouse (Charles River) and dissociated into single cells following an incubation of 15 min in 0.25% trypsin/PBS (Sigma-Aldrich, T4174) with intermittent gentle agitation. Cells were pelleted at 800 ×g for 2 min and re-suspended in kidney rudiment medium comprising MEME (Sigma-Aldrich, M5650) and 100 mL L⁻¹ FBS. In the meantime, FACS-sorted Bra-GFP⁺ cells derived from mESC 3-D or 2-D systems were collected in rudiment medium and counted. A total of 2×10⁵ cells were used in each rudiment, wherein kidney rudiment cells and Bra-GFP⁺ cells were mixed at a ratio of 1:9.

Rudiments were cultured with Rho-associated, coiled-coil containing protein kinase inhibitor (ROCKi, Y-27632, Merck Millipore, 688001) for 24 h followed by a further 4-day in the absence of ROCKi. Controls were also set up, including kidney rudiments comprising GFP-KSCs (1:9 ratio of KSC: kidney rudiment cells), reaggregated kidney rudiments (formed by kidney rudiment cells only), and intact kidney rudiments. Experiments were performed in 3 independent biological replicates.

4.10 Immunofluorescence staining

For EB frozen section assay, sections were blocked in 10% serum solution and incubated with E2C primary and secondary antibodies followed by nuclear counter-staining of 4′,6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific, D1306, 1/100 000). Slides were mounted with DAKO fluorescent mounting medium (Agilent Technologies, S3023) and sealed for viewing on the Leica DM2500 (Leica) fluorescence microscope with a 40× objective and appropriate excitation and emission filter sets. Data were acquired using the Leica Application Suite (LAS, Leica) integrated software and analysed by the ImageJ (NIH, version 1.50i) software.

For mouse embryonic kidney rudiments assay, immunofluorescence and image analysis were carried out based on the protocols described previously (Rak-Raszewska et al., 2012; Ranghini et al., 2013). Briefly, rudiments of days 0 and 5 were fixed with 4% PFA and blocked with 10% serum solution containing 0.1% Triton-X 100, followed by incubation with primary antibodies for E2C, megalin, Wt1, synaptopodin and PECAM-1, where necessary. They were then incubated with secondary antibodies followed by counter-staining of 10 µg µL⁻¹ PNA (Vector, RL–1072). Controls were also included as above to check for non-specific binding of secondary antibodies. Samples were mounted with DAKO fluorescent mounting medium (Agilent Technologies, S3023) and sealed. Data were acquired using the Zeiss LSM 510 META (Zeiss) multiphoton confocal laser scanning microscope with a 40× oil immersion, 20× or 10× lens and appropriate excitation and emission filter sets. Image data analysis was
performed by the ImageJ (NIH) and Imaris (Bitplane, version 9.0.2) softwares.

The following primary antibodies were used: rabbit polyclonal IgG E2C (Clontech, 632496, 1/1 000), mouse monoclonal megalin IgG1 (Acris, DM3613P, 1/200), mouse monoclonal Wt1 (Millipore, 05–753, 1/100), mouse monoclonal synaptopodin IgG1 (Progen, 65194, 1/2), rat monoclonal PECAM (BD Pharmingen, 550274, 1/100). Secondary antibodies used were: Alexa Fluor (AF) 488-conjugated chicken anti-rabbit IgG (Thermo Fisher Scientific, AF A21441, 1/1 000), AF594 goat anti-rabbit (Thermo Fisher Scientific, AF A11012, 1/1 000), AF488 goat anti-mouse IgG1 (Thermo Fisher Scientific, AF A21121, 1/1 000), AF647 donkey anti-mouse IgG (H+L) (Thermo Fisher Scientific, AF A31571, 1/1 000), AF488 donkey anti-rat IgG (H+L) (Thermo Fisher Scientific, AF A21208, 1/1 000).

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Competing interests

The authors declare no conflict of interests.

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Fig. 1. Representative fluorescence and phase contrast photomicrographs of mesoderm development within EBs derived from Bra-GFP/Rosa26-E2C mESCs at different seeding densities cultured for up to 7 days. (A) EB morphology was examined on days 4 and 7. The majority of EBs derived from mESCs plated at densities of $2.5\times10^3$ and $1.25\times10^5$ cells mL$^{-1}$ showed evidence of cavitation, whereas cavitated EBs were less abundant in the lower density culture ($6.25\times10^4$ cells mL$^{-1}$). Maximal levels of GFP expression were observed in day 7 EBs derived from the $1.25\times10^5$ density cultures. (B) Immunostaining of cryo-sections of day 7 EBs for E2C, counterstained with DAPI. Representative photomicrographs of lower density culture showed that all cells within the EBs derived
from the E2C-expressing mESCs stained positively for E2C (red), including the GFP+ (green) mesodermal cells. Data were collected from three biological replicates. Scale bars, 100 µm.
Fig. 2. Timing and extent of GFP expression in *Bra-GFP/Rosa26-E2C* mESCs following mesodermal differentiation in 3-D and 2-D culture systems. (A) Fluorescence and phase contrast photomicrographs of EBs derived from mESCs plated at 1.25×10⁵ cells mL⁻¹. EBs were cultured for up to 9 days and imaged in real-time every hour from day 3 to day 9 post plating. GFP expression could still be detected at day 9. (B) Flow cytometry analysis of disaggregated *Bra-GFP/Rosa26-E2C* EBs at different time points revealed that GFP started to be expressed on day 4, and reached maximum levels on days 6–7. At the peak of expression (day 6), GFP⁺ cells comprised 39% of the population. (C) Representative fluorescence and phase contrast photomicrographs of mESCs following directed...
differentiation to mesoderm using a 2-D culture system. Four days following induction, cells no longer formed colonies, appeared differentiated, and the majority expressed GFP. (D) Flow cytometry analysis showed that ~89% of cells expressed GFP under 2-D culture conditions. Undifferentiated Bra-GFP/Rosa26-E2C mESCs sub-cultured in gelatinised dishes in mESC medium for 24 h prior to induction were used as a negative control. Data were collected from at least 2 biological replicates. Scale bars, 200 µm (A) and 100 µm (C).
Fig. 3. Isolation and analysis of gene expression profiles of the mesodermal and non-mesodermal populations from Bra-GFP/Rosa26-E2C mESCs cultured in 3-D and 2-D systems. (A) Day-6 EBs or mESCs cultured under 2D differentiating conditions for 4 days were harvested for FACS. Untransfected day-6 E14-Bra-GFP EBs or mESCs maintained undifferentiated in gelatinised dishes in mESC medium for 24 h prior to induction were used as negative controls. Flow cytometry was used to confirm the purity of the populations isolated using FACS. (B) Relative expression levels of mesoderm and early kidney development genes were compared between Bra-GFP$^+$ and Bra-GFP$^-$ populations isolated from the 3-D system (n=2 biological replicates), presented as mean±s.e.m. Data were not statistically assessed on significance due to 2 biological replicates however they gave an indication of the difference between Bra-GFP$^+$ and Bra-GFP$^-$ populations. (C) Relative expression levels of mesoderm and early kidney development genes were compared between Bra-GFP$^+$ and Bra-GFP$^-$ populations isolated from the 2-D system (n=2 biological replicates), presented as mean±s.e.m. Data were not statistically assessed on significance due to 2 biological replicates however they gave an indication of the difference between Bra-GFP$^+$ and Bra-GFP$^-$ populations. (D) Relative gene expression levels of mesoderm and early kidney development genes were compared between Bra-GFP$^+$ and Bra-GFP$^-$ populations.
populations isolated from 3-D system (n=3 biological replicates) and 2-D system (n=3 biological replicates), presented as mean±s.e.m. $P<0.05$ (asterisks) was considered as statistically significant ($t$-test).
Fig. 4. Development of intact E13.5 mouse embryonic kidney and re-aggregated kidney rudiments cultured ex vivo for 5 days. (A) Representative confocal photomicrographs of intact kidney showed that proximal tubules were positively stained for megalin (Meg, green) and PNA (magenta). Developing glomeruli were immunostained for Wt1 (green) and synaptopodin (Synap, green) positive staining. Arrows point to developing podocytes and arrowheads point to MM. (B) E13.5 mouse embryonic kidneys were dissociated and pelleted as aggregates comprising $2 \times 10^5$ cells for each rudiment. Representative confocal photomicrographs of the re-aggregated rudiments cultured ex vivo at days 0 (B1) and 5 (B2–B3) showed that tubule-like structures formed during the 5-day culture. (C) The re-aggregated rudiments contain tubules and nascent glomerular-like structures that are similar to those of the intact rudiments cultured for 5 days. Boxed regions outlined are enlarged in the magnified image. Data were collected from three biological replicates. Scale bars: 200 µm (A1, A3, A5, B1-B2, C1 and C3); 100 µm (A2, A4 and A6); 50 µm (B3, C2 and C4)
Fig. 5. Potential of Bra-GFP/Rosa26-E2C mESCs isolated from 3-D and 2-D systems to integrate in megalin-expressing renal tubules. Rudiments were cultured ex vivo for 5 days. GFP-KSCs (green) were used as positive controls and showed integration into the tubules of the developing nephrons. Arrows point to the GFP+ KSCs that had integrated into developing tubules that were dual stained by PNA (red) and megalin (Meg, blue) (G, J and M). In the day-5 chimeric rudiments comprising Bra-GFP+ cells derived from mESC 3-D system, E2C+ Bra-GFP+ cells (blue) appeared to be elongated and formed an interconnected network within the rudiments. They were often found surrounding the
tubules but did not integrate into them (B, E, H, K and N). In the day-5 chimeric rudiments comprising 
Bra-GFP⁺ cells isolated from mESC 2-D system, fewer Bra-GFP⁺ cells (blue) were observed, and, 
unlike those from the 3-D system, most did not appear to be elongated (C, F, I, L and O). Boxed 
regions outlined are enlarged in the magnified images. Data were collected from three biological 
replicates. Scale bars, 200 µm (A–C) and 50 µm (D–F).
Fig. 6. Potential of Bra-GFP/Rosa26-E2C mESCs isolated from 3-D and 2-D systems to integrate into Wt1-expressing nascent glomeruli. Rudiments were cultured ex vivo for 5 days. Arrows point to the integrated KSCs (positive controls) that were GFP-labelled and dual stained by PNA (red) and Wt1 (blue) (D, G, J and, M). E2C+ Bra-GFP+ cells (blue) in the day-5 chimeric rudiments comprising Bra-GFP+ cells derived from mESC 3-D system were often found surrounding the tubules (red) and glomerular structures (green) but did not integrate into them (B, E, H, K and N). Bra-GFP+ cells isolated from mESC 2-D system also did not appear to integrate into any renal structures (C, F, I, L and...
O). Boxed regions outlined are enlarged in the magnified images. Data were collected from three biological replicates. Scale bars, 200 µm (A–C) and 50 µm (D–F).
Fig. 7. Potential of *Bra-GFP/Rosa26-E2C* mESCs isolated from 3-D and 2-D systems to differentiate into synaptopodin-expressing podocytes. Rudiments were cultured *ex vivo* for 5 days. Arrows point to the integrated KSCs (positive controls) that were GFP-labelled and dual stained with PNA (red) and synaptopodin (Synap, blue) (D, G, J and M). In the day-5 chimeric rudiments comprising *Bra-GFP*+ cells derived from mESC 3-D system, E2C+ *Bra-GFP*+ cells (blue) did not generate synaptopodin+ cells (B, E, H, K and N). *Bra-GFP*+ cells isolated from mESC 2-D system (blue) also failed to generate synaptopodin+ cells (C, F, I, L and O). Boxed regions outlined are enlarged in the
magnified images. Data were collected from three biological replicates. Scale bars, 200 µm (A–C) and 50 µm (D–F).
Fig. 8. Confocal photomicrographs showing PECAM-1 immunostaining within day-5 ex vivo mouse embryonic kidney rudiments comprising Brα-GFP⁺ derived from Brα-GFP/Rosa26-E2C mESCs cultured in 3-D and 2-D systems. Immunostaining for E2C was undertaken to identify the mesodermal cells, and PECAM-1 immunostaining was performed to identify endothelial-like cells. (A, D, G, J, M) Re-aggregated rudiments without exogenous cells; (B, E, H, K, N) Re-aggregated chimeric rudiments containing E2C⁺ Brα-GFP⁺ cells isolated from the 3-D culture system; (C, F, I, L, O) Re-aggregated chimeric rudiments containing E2C⁺ Brα-GFP⁺ cells isolated from the 2-D culture.
system. Boxed regions outlined are enlarged in the magnified images. Data were collected from three
biological replicates. Scale bars, 200 µm (A–C) and 50 µm (D–F).
**Supplementary Figures**

Fig. S1. qRT-PCR analysis of stemness and lineage markers expressed by the mesodermal and non-mesodermal populations from Bra-GFP/Rosa26-E2C mESCs cultured in 3-D and 2-D systems. (A) Relative expression levels of genes were compared between Bra-GFP+ and Bra-GFP− populations isolated from the 3-D system (n=2 biological replicates), presented as mean±s.e.m. Data were not statistically assessed on significance due to 2 biological replicates however they gave an indication of the difference between Bra-GFP+ and Bra-GFP− populations. (B) Relative expression levels of genes were compared between Bra-GFP+ and Bra-GFP− populations isolated from the 2-D system (n=2 biological replicates), presented as mean±s.e.m. Data were not statistically assessed on significance due to 2 biological replicates however they gave an indication of the difference between Bra-GFP+ and Bra-GFP− populations. (C) Relative gene expression levels genes were compared between Bra-GFP+ populations isolated from 3-D system (n=3 biological replicates) and 2-D system (n=3 biological replicates), presented as mean±s.e.m. P<0.05 was considered as statistically significant (t-test). No significant difference was found between the two systems.
Fig. S2. Confocal photomicrographs of the re-aggregated E13.5 mouse embryonic kidney rudiments at day 0 of ex vivo culture containing no exogenous cells or GFP-KSCs. GFP-KSCs (positive controls) showed even distribution represented by GFP (green) in the rudiments at the beginning of the culture. Data were collected from three biological replicates. Scale bar, 200 µm.
### Table S1 List of key genes investigated by qRT-PCR in this study

| Genes | Expression Regions | References | Genes | Expression Regions | References |
|-------|--------------------|------------|-------|--------------------|------------|
| Bra   | PS, TB, notocord   | a, b       | Foxa2 | Anterior PS        | m          |
| Tbx6  | PS, PM, TB         | a-c        | Foxd1 | MM stroma          | n          |
| Cdx2  | PS                 | d-f        | Foxf1 | LPM                | o          |
| Lhx1  | LPM, IM            | g          | Hoxa10| PM, MM             | e, p, q    |
| Osr1  | LPM, IM, MM        | e, g       | Hoxa11| PM, MM             | e, p, q    |
| Pax2  | IM, ND, MM         | g, h       | Hoxb1 | Posterior PS       | m, r       |
| Wt1   | IM, MM             | i          | Hoxc9 | Posterior PM       | s          |
| Gdnf  | MM                 | j-l        | Hoxd11| PM, MM             | p, q       |

Notes: PS, primitive streak; PM, paraxial mesoderm; LPM, lateral plate mesoderm; IM, intermediate mesoderm; ND, nephric duct; MM, metanephric mesenchyme; TB, tailbud.

References: a, Papaioannou, 2014; b, Herrmann et al., 1990; c, Chapman et al., 2003; d, Arnold and Robertson, 2009; e, Taguchi et al., 2014; f, Savory, et al., 2009; h, James et al., 2005; i, Little, 2015; j, Lin et al., 1993; k, Sanchez et al., 1996; l, Basson et al., 2006; m, Gadue et al., 2006; n, Mugford et al., 2008; o, Mahlapuu et al., 2001; p, Carapuço et al., 2005; q, Yallowitz et al., 2011; r, Kmita et al., 2000; s, Erselius et al., 1990.
| Genes | Forward Sequences | Reverse Sequences | Amplicons Size (bp) | References |
|-------|-------------------|-------------------|---------------------|------------|
| *Bra* | CATCGGAACAGCTCAT | GTGGGCTGGCGTTA    | 136                 | RTPrimerDB |
| β-actin | GTCACA            | GGGAGCTGG        | 145                 | Qiagen     |
| Gapdh | CAGTTCAGCAGGGATG  | TGAAGGCGGAGAT    | 150                 | Qiagen     |
| Fgf5  | AACGCAATGGCTCCA   | TCCTCAGTAATCTTA  | 88                  | Qiagen     |
| Foxd1 | CAAGAATCCGCTGG    | ACAGGTTGTGACGG   | 88                  | Qiagen     |
| Foxf1 | CCAAAACAGTCACA    | TCACACACGGCTTG   | 191                 | Qiagen     |
| Gdnf  | CGCTGACCACTGAC    | AAACGCACCCCGCA   | 222                 | In-house   |
| Nanog | AAGCAGAAGATGGCG   | GTGCTAGCCCTTC    | 232                 | Qiagen     |
| Oct4  | TGGAGACCTTTCAG    | CTTGACAGCTTGG    | 188                 | Qiagen     |
| Osr1  | GCCCCCAAAGGAGGA   | AGCCACAGTCATCG   | 161                 | Qiagen     |
| Pax2  | TCCAGGCATCGAG     | GGCCGATGAGAATA   | 104                 | Qiagen     |
| Wnt   | AATGCAGCCCTACCT   | CGTTCAGAAATGAC   | 116                 | Qiagen     |
| Cdx2  | QT00116739         |                   | 114                 |            |
| Tlx6  | QT00098861         |                   | 80,80,157           |            |
| Lhx1  | QT01660792         |                   | 87                  | Qiagen     |
| Foxa2 | QT00242809         |                   | 115                 | Qiagen     |
| Hoxa10| QT00240212         |                   | 61                  | Qiagen     |
| Hoxa11| QT00250404         |                   | 97                  | Qiagen     |
| Hoxb1 | QT00493906         |                   | 128                 | Qiagen     |
| Hoxc9 | QT00113218         |                   | 138                 | Qiagen     |
| Hoxd11| QT00267337         |                   | 97                  | Qiagen     |

* Annealing temperature (Ta) is 60°C for all primers.

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Supplementary Movies

Movie S1: Representative 360-degree horizontal 3-D construction of confocal photomicrographs showing spatial distribution of PECAM-expressing 3-D system-derived E2C^{+} Bra-GFP^{+} cells within mouse embryonic kidney rudiments. Rudiments were cultured \textit{ex vivo} for 5 days. Immunostaining for E2C (blue) and PECAM-1 (green) was performed to identify mesodermal and endothelial-like cells, respectively (attached as a separate file).

Movie S2: Representative 360-degree horizontal 3-D construction of confocal photomicrographs showing spatial distribution of PECAM-expressing 2-D system-derived E2C^{+} Bra-GFP^{+} cells within mouse embryonic kidney rudiments. Rudiments were cultured \textit{ex vivo} for 5 days. Immunostaining for E2C (blue) and PECAM-1 (green) was performed to identify mesodermal and endothelial-like cells, respectively (attached as a separate file).