Aberrant Differentiation of Human Pluripotent Stem Cell-Derived Kidney Precursor Cells inside Mouse Vascularized Bioreactors

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Keywords

Artery · Dysplasia · Flow-through · Glomerulus · Muscle · Tubule · Vein · Vessel

Abstract

Background: Numerous studies have documented the *in vitro* differentiation of human pluripotent stem cells (hPSCs) into kidney cells. Fewer studies have followed the fates of such kidney precursor cells (KPCs) inside animals, a more life-like setting. Here, we tested the hypothesis that implanting hPSC-derived KPCs into an *in vivo* milieu surgically engineered to be highly vascular would enhance their maturation into kidney tissues. **Methods:** 3D printed chambers containing KPCs were implanted into the thighs of adult immunodeficient mice. In some chambers, an arterial and venous flow-through (AVFT) was surgically fashioned. After 3 weeks and 3 months, implants were studied by histology, using qualitative and quantitative methods. **Results:** After 3 weeks, chambers containing AVFTs were richer in small vessels than contralateral chambers without AVFTs. Glomeruli with capillary loops and diverse types of tubules were detected in all chambers. At 3 months, chambers contained only rudimentary tubules

and glomeruli that appeared avascular. In chambers with AVFTs, prominent areas of muscle-like cells were also detected near tubules and the abnormal tissues immunostained for transforming growth factor β 1. These features have similarities to renal dysplasia, a typical histological signature of human congenital kidney malformations. **Conclusions:** This study urges a note of caution regarding the *in vivo* fates of hPSC-derived kidney precursors, with pathological differentiation appearing to follow a period of increased vascularity.

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Introduction

The potential to generate kidney tissues from human pluripotent stem cells (hPSCs) promises to help understand the biology of healthy and diseased organs and may eventually lead to regenerative medicine therapies [1–4]. It is now well established that PSC-derived tissues can be maintained for several weeks in 3D cultures as “organoids” that contain nephron components, including immature glomeruli and diverse kidney tubules [5–10]. Far fewer studies have followed the fates of hPSC-derived kidney precursor cells

(KPCs) inside animals, a more life-like setting, where longer follow-up may be feasible compared with *in vitro* experiments. As examples, it was reported that more mature glomerular differentiation, including formation of glomerular capillary loops, can be achieved by implanting hPSC-derived kidney tissues either subcutaneously [8, 11] or under the kidney capsule [12, 13] in immunocompromised mice. Unfortunately, there are drawbacks [3, 14] to current *in vitro* and *in vivo* technologies, including the limited size and vascularity attained by PSC-derived kidney tissues.

Using tissue engineering, so-called “vascularized bioreactors” can be created by surgically fashioning either an arteriovenous loop, in which an artery is directly connected with a vein, or an arterial and venous flow-through (AVFT) [15–18]. In the latter technique, a space is cleared between an artery and its adjacent vein, and these 2 vessels are laid inside a chamber, perforated to allow their ingress and egress. When chambers containing AVFTs were filled with Matrigel™, an extracellular matrix gel, they were reported to become populated by adipose tissue rich in blood vessels [18]. Furthermore, immature non-renal cells, such as fetal hepatocytes [15, 16], have been reported to differentiate in a realistic manner when placed inside such vascularized bioreactors *in vivo*.

Here, we designed and undertook experiments to answer 2 questions. First, could an AVFT be fashioned in the thigh of an immunocompromised mouse and could it be maintained in this location inside a perforated 3D chamber? We show that this technical approach is feasible and that, assessed at 3 weeks, it results in a highly vascular environment. Second, would this scenario enhance the growth and differentiation of hPSC-derived KPCs placed inside such a chamber containing an AVFT? We found that implanted cells had formed glomeruli and tubules when assessed at 3 weeks but, unexpectedly, they subsequently came to resemble malformed kidney tissues when assessed at 3 months.

Results

Establishing AVFTs in Thighs of Immunodeficient Mice

In the first set of experiments, 3D printed perforated plastic chambers filled with Matrigel™, but not containing human cells, were surgically placed into the thighs of 6 adult female *severe combined immunodeficiency beige* (*SCID/beige*) mice, as detailed in the Materials and Methods. In 1 thigh of each mouse, an AVFT was fashioned to run through the gel filled chamber, and a gel filled chamber that lacked an AVFT was implanted into the opposite thigh of each mouse (Fig. 1a–c). Harvested after 3 weeks, the chambers were recovered from all 12 thighs (Fig. 1d, e). Histology revealed patent arteries and veins inside all 6 chambers with AVFTs, and the linings of these arteries (Fig. 1f) and veins (Fig. 1g) immunostained for the endothelial marker cluster of differentiation 31 (CD31), also known as platelet endothelial cell adhesion molecule. Small-calibre vessels expressing CD31 were detected inside all chambers, in both those with (Fig. 1h) and without (Fig. 1i) AVFTs, but they were not quantified. Some of these small-calibre vessels were detected close to arteries and veins (Fig. 1f, g). As expected, no kidney-like tissues, such as glomeruli or tubule-like structures, were detected inside any of the chambers.

Generating Human KPCs in Culture for Implantation into Mice

Having established the feasibility of fashioning AVFTs in *SCID/beige* mice, this technique was modified to study the effects on human hPSC-derived KPCs. In these mice, the *SCID* mutation causes immunodeficiency affecting B and T lymphocytes, while the *beige* mutation results in defective natural killer cells. Our previous studies of hPSC-derived KPCs implanted subcutaneously in the backs of similar mice [8, 11] found no rejection of the hu-

Fig. 1. Implantation of 3D printed chambers without human KPCs. **a** In each mouse, an AVFT (red, artery; blue, vein) was fashioned in 1 thigh, while the other thigh served as a control with no AVFT. **b** An AVFT being fashioned. **c** The 2 halves of the chamber in situ before being closed; note the lower half contains the AVFT and the central part of each half chamber contains a half pillar (small circle) that will form a single pillar when the chamber is closed. **d–i** Histology from chambers harvested after 3 weeks. **d** Cross section of a chamber with an AVFT stained with hematoxylin and eosin. Note that a indicates artery; v, vein; and p, the central pillar. Asterisks indicate sections of the wall (white) of the chamber; note the wall appears discontinuous because of the perforations. **e** Cross section of a chamber that did not receive an

AVFT stained with hematoxylin and eosin. Asterisks indicate the wall of the chamber. **f** High-power view of artery in chamber with an AVFT immunostained with CD31 antibody (brown colour) and counterstained with hematoxylin. **g** High-power view of vein in chamber with an AVFT immunostained with CD31 antibody (brown colour) and counterstained with hematoxylin. **h** High-power of small CD31 immunostained (brown) vessels in a chamber containing an AVFT. **i** High power of small CD31 immunostained (brown) vessels in a chamber that did not receive an AVFT. Bars in histology sections are 500 µm in **d, e**; 100 µm in **f, g**; and 200 µm in **h, i**. KPC, kidney precursor cells; AVFT, arterial and venous flow-through.

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Fig. 2. *In vitro* differentiation of hPSCs to KPCs. **a–c** Phase contrast views of cultures at 1, 12, and 22 days of the kidney differentiation protocol. Note that the lawns of cells (day 1) acquired increasing complex morphology at 12 days (at which point, some of these KPC cultures were dissociated and implanted) and 22 days. **d–i** Immunocytochemistry at 32 days after start of *in vitro* differentiation with DAPI (blue) nuclear counterstain; all bars are 500 μ m. **d** A CDH1 (red) immunostained tubule-like structure. **e** A tubule-like structure double immunostained for CDH1 (red) and cubilin (green). **f** A tubule-like structure immunostained for

CDH1 (red) surrounded by condensations of cells positive for WT1 (green). **g** Rudimentary capillary-like structures immunostained with anti-human CD31 (red). **h** This field shows a tubule-like structure immunostained for CDH1 (red) near a collection of cells that immunostained for podocin (green); note that the latter did not have an overt glomerular-like structure. **i** Zones of cells immunostained for α -SMA (green). hPSCs, human pluripotent stem cells; KPC, kidney precursor cells; CDH1, cadherin 1; WT1, Wilms tumour 1; α -SMA, α -smooth muscle actin.

man cells. In the current study, in each of 12 mice, we created an AVFT in 1 thigh and placed the flow-through in a chamber containing KPCs that had been embedded in MatrigelTM. The fates of these cells were compared with those implanted in contralateral thighs inside chambers that lacked AVFTs. KPCs to be implanted were generated *in vitro* by differentiating the MAN13 hPSC line (Fig. 2a) by sequential application of CHIR99021, a GSK-3 α/β inhibitor, and fibroblast growth factor 9/heparin, as first described by Takasato et al. [5, 6]. The kidney differentiation culture protocol used here was identical to the one we had used in other studies of the same hPSC line [8, 11], with the exception that commercial STEMdiffTM APELTM2

Medium was used instead of the now discontinued STEMdiffTM APELTM Medium. Similar to our published observations using the former media [8], cultures at day 12 of the current protocol contained aggregates of cells (Fig. 2b), resembling cell clusters that we reported to express markers of nephron, stromal and ureteric bud lineages [8]. At this point, some cultures were enzymatically dissociated into single cells, and a pellet of 2×10^6 of these KPCs was embedded in MatrigelTM inside each chamber to be implanted into *SCID/beige* mice. Other parallel cultures were maintained in 2D for another 3 weeks. As expected [8], they began to form tubule-like structures (Fig. 2c). Cultures were examined by immunocytochem-

Table 1. Antibodies used in this study

Antibody	Host	Commercial source and catalogue number	Application and dilution
α -SMA (reacts with mouse and human tissue)	Mouse	Sigma A2547	IHC 1:16,000 ICC 1:16,000
CD31 (reacts with human but not mouse tissue)	Mouse	Cell Signalling 3528	IHC 1:100 ICC 1:100
CD31 (reacts with mouse and human tissue)	Goat	R&D Systems AF3628	IHC 1:200
CDH1 (reacts with mouse and human tissue)	Mouse	Abcam 76055	IHC 1:5,000 ICC 1:400
Cubilin (reacts with human tissue)	Goat	Santa Cruz Biotechnology sc-20607	IHC 1:100 ICC 1:200
HNF1B (reacts with mouse and human tissue)	Rabbit	Santa Cruz Biotechnology sc-22840	IHC 1:300
Human mitochondria (reacts with human but not mouse tissue)	Mouse	Millipore MAB1273	IHC 1:200
Podocin (reacts with mouse and human tissue)	Rabbit	Sigma P0372	IHC 1:1,000 ICC 1:200
Synaptopodin (reacts with mouse and human tissue)	Mouse	Santa Cruz Biotechnology sc-515842	IHC 1:200
TGF- β 1 (reacts with mouse and human tissue)	Mouse	Santa Cruz Biotechnology sc-130348	IHC 1:20
UMOD (reacts with mouse and human tissue)	Rabbit	Santa Cruz Biotechnology sc-20631	IHC 1:100
WT1 (reacts with mouse and human tissue)	Rabbit	Calbiochem CA1026	IHC 1:1,000
WT1 (reacts with mouse and human tissue)	Rabbit	Santa Cruz Biotechnology sc-192	ICC 1:100

α -SMA, α -smooth muscle actin; CDH1, cadherin 1; HNF1B, hepatocyte nuclear factor 1B; TGF- β 1, transforming growth factor- β 1; UMOD, uromodulin; WT1, Wilms tumour 1; ICC, immunocytochemistry; IHC, immunohistochemistry.

istry at 32 days after the start of the differentiation protocol at the limit of the culture period. Subsets of tubules were positive for cadherin 1 (CDH1) (Fig. 2d), also known as E-cadherin, a collecting duct and distal tubule marker, or for cubilin (Fig. 2e), a proximal tubule marker. Subsets of nearby cells were reactive with antibodies to the nephron lineage protein Wilms tumour 1 (WT1) (Fig. 2f); CD31 (Fig. 2g), using an antibody that detects human protein (Table 1); podocin, a podocyte protein (Fig. 2h); or α -smooth muscle actin (α -SMA) (Fig. 2i). Neither the WT1- nor the podocin-expressing cells, however, formed morphologically recognizable glomeruli in this culture setting, a similar result to our previous experiments with this hPSC line [8].

Human KPC Implants Harvested 3 Weeks after Surgery

All KPC implant sites (i.e., in the 12 thighs of the 6 mice) harvested 3 weeks after surgery contained cham-

bers (Fig. 3a, b), and a patent artery and vein were detected by histology inside each chamber in which an AVFT had been fashioned (Fig. 3a). Small-calibre CD31 expressing vessels were detected inside all chambers, and they appeared more prominent in chambers with AVFTs (low power views, Fig. 3a, b, and high power view, Fig. 3c, d). Quantification of vessels, expressed as the percentages of cross-sectional areas occupied by CD31-positive structures, was undertaken. For each chamber, the quantified area comprised that enclosed by the chamber wall plus areas within the perforations of each chamber; tissues external to the chamber wall were excluded from analysis. For each chamber, a section from every 15th serial slide was quantified in this manner, and all areas were summated to generate 1 consolidated result per chamber. Typically, using this method, around 35 mm² was assessed per chamber, a summation of around 10 individual sections. Vessel density was statistically significantly ($p = 0.031$) higher in the chambers with AVFTs than in

Fig. 3. Identification and quantification of vessels in chambers implanted with human KPCs and harvested after 3 weeks. **a–d** Histology sections immunostained for CD31 (brown) of chambers harvested 3 weeks after surgery. Bars are 500 μ m in (**a, b**) and 50 μ m in (**c, d**). **a** Low power of cross section of chamber with an AVFT. The dotted line is drawn around the outside of the chamber and the asterisks indicate chamber walls. v and a indicate a vein and an artery, respectively. Note that the brown CD31 signals are discerned. **b** Low power of cross section of chamber that lacks an AVFT. Note the lack of an artery and a vein and the fact that CD31 signals are hardly discerned at this low power. The dotted line is drawn around the outside of the chamber and the asterisks indicate walls and the central pillar of the chamber. **c** High power of dense

small vessels from chamber with an AVFT. **d** High power of apparently more sparse small vessels in chamber lacking an AVFT. **e** Similar total areas used for CD31 quantification in chambers with AVFTs (left column) and those lacking AVFTs (right column); note that each mouse has a designated colour with the AVFT chamber shown as a circle and the opposite non-AVFT chamber shown as a triangle. **f** A significantly greater proportion of the chamber area was occupied by vessels, as assessed by CD31 immunostaining, in chambers with AVFTs compared with chambers lacking AVFTs. Of note, we did not observe direct continuity between the AVFT vessels and the small CD31 immunostaining vessels around them. KPC, kidney precursor cells; AVFT, arterial and venous flow-through.

chambers without AVFTs (Fig. 3e, f). The total areas assessed for these analyses were similar ($p = 0.345$) for chambers with or without AVFTs. Of note, we did not observe direct continuity between the AVFT vessels and the small CD31 immunostaining vessels around them.

As visualized after hematoxylin and eosin staining, kidney-like tissues were apparent in all 12 chambers (Fig. 4a, b); no such tissues were detected outside the chamber walls. On closer inspection, these kidney-like tissues contained tubule-like and glomerular-like struc-

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tures (Figs. 4c, d, 5). Next, we measured the amount of “kidney tissue” in each chamber, as assessed by the summed areas occupied by tubules and glomeruli factored for the total areas assessed. There was no significant ($p = 0.174$) difference between inside the chambers with AVFTs compared with chambers without AVFTs (Fig. 4e). Considering all 12 chambers, there was no statistically significant ($p = 0.610$) correlation between the amount of CD31-positive vessels and the amount of kidney tissues (Fig. 4f).

Further inspection and immunohistochemical analyses revealed similar types of renal structures in chambers with or without AVFTs. These included glomeruli with red blood cells in their tufts (Fig. 5a). Cells in the Bowman capsule and peripheral glomerular tuft cells were of human origin as assessed by immunostaining for human mitochondrial protein (HuMt) (Fig. 5b and its inset). CD31-positive cells were detected inside glomerular tufts (Fig. 5c). Subsets of glomerular cells immunostained for the podocyte markers WT1 (Fig. 5d), synaptopodin (Fig. 5e), and podocin (Fig. 5f). Also detected were tubules (Fig. 5g) immunostaining for HuMt (Fig. 5h); as we had noted in subcutaneous implant experiments [8], tu-

bular immunostaining was more intense than in other cells such as presumed podocytes, most likely because tubules are enriched for mitochondria. Subsets of tubules immunostained for cubilin (Fig. 5i); uromodulin (Fig. 5j), a marker of the thick ascending limb of the loop of Henle; or CDH1, a marker of distal tubules and collecting ducts (Fig. 5k). No signals were detected upon omitting the primary antibodies (Fig. 5l). The CD31 antibody used in the above experiments reacts with both mouse and human protein, so we also probed sections with a CD31 antibody (see Table 1 for details) that is reactive with human but not mouse protein. From these experiments, we concluded that CD31 expressing vessels in glomerular tufts within implant chambers were likely to be of mouse origin (online suppl. Fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000509425).

Human KPC Implants Harvested at 3 Months after Surgery

The other 6 mice were followed for 3 months after implantation of the same batches of human KPCs. All 12 chambers were recovered and analysed by histology (Fig. 6a–f), and all 6 chambers in which an AVFT had

Fig. 4. Identification and quantification of kidney-like tissues within chambers implanted with human KPCs and harvested after 3 weeks. **a–d** Histology sections stained with hematoxylin and eosin of chambers harvested 3 weeks after surgery. Bars are 500 μ m in (**a**, **b**) and 200 μ m in (**c**, **d**). **a** Low power of cross section of chamber with an AVFT. The dotted line is drawn around the outside of the chamber and the asterisks indicate walls of the chamber. **a** and **v** indicate an artery and a vein, respectively. The kidney-like structures appear blue and an enlargement of the boxed area is depicted in **c**, where tubule-like (**t**) and glomerular-like (**g**) structures separated by loose stromal-like tissues are noted. **b**, **d** respectively show an overview and enlarged section of a chamber lacking an AVFT. Note that this chamber also contains tubules and glomer-

uli. **e** Quantification of “kidney tissues” as assessed by the areas occupied by both tubules and glomeruli but excluding intervening stroma. The graph shows a non-significant tendency for more “kidney tissues” in chambers with AVFTs (left column) compared with those without AVFTs (right column). **f** Considering all 12 chambers with KPC implants harvested at 3 weeks, there was no significant correlation between percentage areas occupied by “kidney tissues” and CD31 structures. In the 2 graphs, note that each mouse has a designated colour with the AVFT chamber shown as a circle and the opposite non-AVFT chamber shown as a triangle. KPC, kidney precursor cells; AVFT, arterial and venous flow-through.

Fig. 5. Glomeruli and tubules within chambers implanted with human KPCs and harvested after 3 weeks. Histology high-power views for “kidney-like tissues.” These frames show sections from chambers with AVFTs but similar staining results were found in chambers without AVFTs that received KPC implants (data not shown). All sections were counterstained with hematoxylin, and were also counterstained with eosin (**a**, **g**). **a–f** Glomeruli. **g–k** Tubules. **l** An example of each. All bars are 50 μ m. **a** A glomerulus with red blood cells in its tuft. **b** Immunostaining (brown) with HuMt antibody. In the inset, enlargement **p** is a presumed podocyte and the red arrow indicates the Bowman capsule; both types of cell appear to have brown cytoplasm. **c** CD31 vessels (brown) in a glomerular tuft. **d** WT1 nuclear immunostaining (brown), most

prominent in presumed podocytes. **e** Synaptopodin immunostaining (brown). **f** Podocin immunostaining, with a linear pattern at the base of presumed podocytes; with this antibody, there is some background staining in surrounding tissues. **g** Tubule-like structures. **h** HuMt immunostaining (brown) in cytoplasm of tubules. **i** A subset of tubules (red arrows) react (brown) with the cubilin antibody. **j** Rare tubules (red arrows) react (brown) with the UMOD antibody. **k** Tubules (red arrows) reacting (brown) with the CDH1 antibody. **l** No brown colour when the primary antibody is omitted. KPC, kidney precursor cells; HuMt, human mitochondrial protein; WT1, Wilms tumour 1; UMOD, uromodulin; CDH1, cadherin 1.

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been fashioned contained a large artery and vein. The histological appearances of all 6 chambers that contained AVFTs (Fig. 6a, c, e) were similar but were strikingly different when compared with chambers lacking AVFTs (Fig. 6b, d, f). As visualized after hematoxylin and eosin staining, chambers with AVFTs were dominated by areas

of elongated cells, which appeared muscle-like, and there were also primitive appearing tubules and avascular glomeruli. Chambers lacking AVFTs also contained primitive tubules, avascular glomeruli, and stromal-like cells but were not dominated by the muscle-like cells. As noted below, the muscle-like areas were of human origin,

as were the kidney-like tissues. Quantification of histology (Fig. 6g–i) showed that chambers with AVFTs contained significantly ($p = 0.045$) larger areas of human tissues (i.e., tubules plus glomeruli plus muscle-like cells)

than chambers without AVFTs. There was, however, no significant ($p = 0.135$) difference in amounts of “kidney tissues” (i.e., tubules plus glomeruli) between the 2 types of chamber.

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We then probed sections with a panel of antibodies, comparing chambers containing AVFTs with those lacking AVFTs. Inside all chambers, tubules were detected, and they were positive for HuMt (Fig. 7a, b; see online suppl. Fig. 2 for higher resolution images). Muscle-like cells in chambers with AVFTs were also reactive to this antibody (Fig. 7a; online suppl. Fig. 2 for higher resolution image). Sparse small-calibre CD31-positive vessels of mouse but not human origin were noted in all chambers (Fig. 7c, d) but were not quantified. Tubules immunostained for CDH1 (Fig. 7e, f) and for hepatocyte nuclear factor 1B (Fig. 6g, h), a transcription factor expressed human fetal kidney tubules [19]. Some of these tubules appeared to be branched (Fig. 7e), a collecting duct characteristic. Tubules did not stain with cubilin or uromodulin antibodies, and only sparse rudimentary glomerular structures were seen (data not shown). In all chambers, loosely arranged stromal-like cells around tubules were positive for α -SMA (Fig. 7i, j), as were the prominent muscle-like cells that were located further away from the tubules in chambers containing AVFTs (Fig. 7i). These muscle-like cells immunostained prominently for transforming growth factor- β 1 (TGF- β 1) (Fig. 7k), while TGF- β 1 immunostaining appeared less prominent in chambers without AVFTs (Fig. 7l). No signal was obtained upon omission of primary antibodies (Fig. 7m, n). Picrosirius red (PSR), a dye that binds to various matrix proteins including collagens [20], stained both the dense blocks of tissues around tubules in chambers with AVFTs as well as the loosely packed cells immediately surrounding tubules (Fig. 7o, p). Inspecting the same PSR-stained slides under polarized light, a technique more specific for collagens [20], revealed apparently more prominent signals around tubules in chambers with AVFTs versus those without AVFTs (online suppl. Fig. 3).

Fig. 6. Quantification of tissues within chambers implanted with human KPCs and harvested after 3 months. Histology sections stained with hematoxylin and eosin of chambers harvested 3 months after surgery. Bars are 500 μ m in (a, b), 200 μ m in (c, d), and 50 μ m in (e, f). **a** Low power of cross section of chamber with an AVFT. The dotted line is drawn around the outside of the chamber and the asterisks indicate walls of the chamber. The inside of the chamber is dominated by pink tissues that on serial enlargements in (c, e) resolve into areas occupied by muscle-like cells. Tubule-like (t) and glomerular-like (g) structures are apparent in **e, b, d, f** are a similar series of views of a chamber that had not received an AVFT. Note that some tubule-like structures are present but the zones of eosin staining muscle-like cells are not

Discussion

The results of the current study add a note of caution regarding the longer term *in vivo* fates of hPSC-derived KPCs and suggest that a period of increased vascularity may even enhance pathological differentiation. In the first set of experiments that we undertook, we proved that AVFTs could be reproducibly fashioned in the thighs of *SCID/beige* mice. When examined at 3 weeks after surgery, each such chamber contained an artery and vein with patent lumens. Having demonstrated the feasibility of this approach, we proceeded to undertake experiments in which hPSC-derived KPCs were placed inside AVFT chambers. When assessed at 3 weeks, KPCs implanted into chambers, either with or without AVFTs, formed glomeruli, containing capillary loops, and proximal-like tubules. Moreover, at this time point, AVFT chambers containing KPCs were more vascular than chambers in opposite thighs that contained KPCs but lacked AVFTs. This showed that the AVFT strategy had indeed created “vascularized bioreactors,” at least at this time point. There was a non-significant tendency for more renal tissues in chambers with AVFTs. Nevertheless, when correlating the amount of renal tissue with the density of blood vessels, no statistically significant relationship was found. The latter observation indicates that this currently used AVFT strategy did not overtly enhance short-term differentiation of implanted cells into kidney tissues.

We considered the origin of the CD31 expressing vessels in the chambers implanted with human KPCs. In a previous study, we implanted hPSC (MAN13)-derived KPCs subcutaneously on the backs of *SCID/beige* mice [8]. In that report, we demonstrated that blood capillaries located inside the arising glomeruli were reactive with an antibody that detected human but not mouse CD31. In the current implant study, we were therefore surprised to

prominent. **g** Similar total areas used for quantification in chambers with AVFTs (left column) and those lacking AVFTs (right column); note that each mouse has a designated colour with the AVFT chamber shown as a circle and the opposite non-AVFT chamber shown as a triangle. **h** There was a significantly greater area of “implant derived tissue” (i.e., tubules plus glomeruli plus muscle-like cells) in chambers that had received AVFTs than in those that lacked AVFTs. **i** There was no significant difference between the 2 types of chamber in the percentage of areas occupied by “kidney structures” (i.e., tubules plus glomeruli but excluding muscle-like cells). KPC, kidney precursor cells; AVFT, arterial and venous flow-through.

reach a different conclusion, namely, that vessels in glomerular tufts appeared to be of mouse origin. The reasoning behind this conclusion was that vessels within implant chambers were detectable with an antibody reacting with mouse and human CD31 but not with an antibody specific for human CD31; the latter was shown to detect glomerular capillaries in the native human developing kidney. How can we reconcile the current results with those reported in subcutaneous implants [8]? One clue could be that there had been a change in composition of culture media. Indeed, STEMdiff™ APEL™2 medium was used instead of the discontinued STEMdiff™ APEL™ medium, and the former lacks hybridoma extract. On the other hand, in the current study, we noted that CD31-positive cells were detected around tubule-like structures that formed in tissue culture. This indicates that the human culture protocol we used can indeed generate cells with at least some endothelial characteristics. Therefore, we favour a different explanation that incorporates the idea that the origin of glomerular capillaries is highly context-dependent. A subset of cells located within native metanephric mesenchyme express endothelial molecular markers [21] and appear to have the potential to form glomerular capillaries, as occurs after the mouse metanephros is implanted into the cortex of neonatal mice kidneys [21]. On the other hand, nascent metanephric vessels are labile, and they tend to regress in normoxic organ culture [22]. Moreover, when mouse metanephric kidneys are explanted onto the chorioallantoic membrane, avian vessels invade the mouse kidney explant and colonize the differentiating murine glomerular tufts, seemingly to the exclusion of mouse endothelia [21, 23]. In our current study, therefore, the “chamber implanted into thigh” scenario appears to favour mouse over hPSC-derived vessels, analogous to the chorioallantoic membrane model described above.

Some of the implants were left for 3 months before they were harvested. Having seen a tendency for more

renal tissues in implants with AVFTs at 3 weeks, we anticipated that the AVFT strategy might lead to an even more pronounced growth of differentiated kidney tissues by 3 months. At 3 months, however, chambers that had been implanted with KPCs contained tubules that, unlike the 3 week *in vivo* tubules, stained with neither cubilin nor uromodulin antibodies. Moreover, again in contrast to the 3-week implants, glomeruli appeared rudimentary and avascular. Therefore, we reason that the relatively normal kidney tissues seen at 3 weeks had de-differentiated after 3 months. In the subset of these chambers with AVFTs, prominent areas of muscle-like cells were also detected further away from tubules. The non-tubular cells were also reactive with the human mitochondrial antibody, so they must have arisen from implanted hPSC-derived KPCs.

Congenital kidney malformations are the main cause of ESRD in children [24] and young adults [25]. A common phenotype is the dysplastic kidney, and its histology features primitive ducts surrounded by metaplastic smooth muscle-like cells, together with a scarcity of glomeruli [26–28]. It has been postulated that renal dysplasia arises from impaired induction between the ureteric bud and the metanephric mesenchyme [26], precursors that will normally form collecting ducts and nephrons, respectively. An upregulation of the TGF- β axis has been described in human renal dysplastic tissues, and application of TGF- β 1 induces trans-differentiation of human kidney dysplastic epithelia to elongated cells expressing α -SMA [29]. Moreover, exposure of explanted murine metanephric kidneys [30] and embryonic ureters [31] to TGF- β 1 elicits dysgenesis of each tissue. Thus, the phenotypes of abnormal tissues generated from human KPCs in the current study have some similarities to the histological signature of human renal dysplasia. TGF β 1, however, is just one secreted protein implicated in normal and abnormal kidney development, as well as in renal trans-differentiation and scarring. In future, it would be useful to

Fig. 7. Characterization of tissues within chambers implanted with human KPCs and harvested after 3 months. **a, c, e, g, i, k, m, o** are from chambers with AVFTs; the other frames are from chambers without AVFTs. All sections were counterstained with hematoxylin apart from **o** and **p**. All bars are 50 μ m. **a, b** HuMt immunostaining (brown); **t** indicates a tubule and the asterisk is over the elongated muscle-like cells. See online suppl. Fig. 2 for higher power images. **c, d** Sparse CD31 immunostained (brown) vessels. **e, f** CDH1 immunostained (brown) tubules. **g, h** HNF1B immunostained (brown) tubules. **i, j** α -SMA immunostaining (brown) of both the stroma directly around tubules and, in the AVFT cham-

ber, the zones of muscle-like cells further away from the tubules. **k, l** TGF- β 1 immunostaining appeared more prominent in the AVFT chamber, especially in the blocks of muscle-like cells. **m, n** No brown colour with omission of primary antibody. **o, p** PSR stained (red) the stroma directly around tubules and, in the AVFT chamber, areas where muscle-like cells were present; see online suppl. Fig. 3 for higher power bright field and also polarized light images. KPC, kidney precursor cells; AVFT, arterial and venous flow-through; HuMt, human mitochondrial protein; CDH1, cadherin 1; HNF1B, hepatocyte nuclear factor 1B; α -SMA, α -smooth muscle actin; PSR, Picrosirius red.

seek changes in a variety of other such proteins such as bone morphogenetic protein 7 and CD44/hyaluran that impact on similar processes [32, 33] in this regard, proteomic analyses of matrix-associated proteins [34] from tissues harvested from the chambers could be used.

In the current set of implants, we did not observe metaplastic cartilage, another feature of human renal dysplasia [26]. In contrast, we reported that areas of cartilage were detected in subcutaneous implants of this same hPSC on the backs of SCID mice [8], suggesting that the specific type of non-renal mesodermal differentiation may be influenced by different implant settings.

Our current study is also consistent with the conclusion that pathological differentiation may follow a period of increased vascularity. At first glance, this sounds counterintuitive because, for example, kidney fibrosis in post-natal organs is associated with a progressive depletion of vessels [35, 36]. On the other hand, some of the same molecular effectors of angiogenesis may also enhance scarring [37]. It is also possible that over-abundant externally derived vessels plus a lack of endogenous kidney vessels, as observed in the current study, may be detrimental. Another explanation for the generation of aberrant tissue could be considered apart from altered vascularity per se. Perhaps, the differentiation potential of the implanted KPCs was, in some yet-to-be-determined manner, also modified by local cellular niches conferred by multiple factors, for example, the AVFT, nearby host tissues such as thigh muscles, or the plastic 3D printed chamber. These explanations may at least in part explain why we did not detect aberrant differentiation after hPSC-derived KPCs were implanted subcutaneously into SCID/beige mice [8, 11]. In those experiments, although cells were also embedded in Matrigel™, neither chambers nor AVFTs were employed.

Materials and Methods

Chamber Production

Chambers were designed using 3D CAD software (Autodesk Inventor 2015, student edition) as hemispheres with 0.5-mm-thick walls, and 1-mm-diameter pores distributed uniformly on the surface of the chamber. The 2 parts of the chamber were joined by a central pillar. The intact chamber was kidney-shaped and was 5.0 mm long with a maximum diameter of 3.5 mm. Chambers were printed via stereolithography in a Form 2 3D printer (Formlabs, Somerville, MA, USA) with a biocompatible photopolymer resin material (Dental SG resin material; Formlabs, UK), a liquid photopolymer resin. Following print completion and curing and sterilising in UV light for 30 min, all support material was removed, and the chambers were washed in distilled sterile water and 0.1% betadine before implantation.

Generation of Human KPCs in Culture

MAN13 hPSCs [38] were grown on 24-well plates coated with 5 µg/mL recombinant human vitronectin (rhVTN-N, Life Technologies, #A14700) in mTeSR1 (StemCell Technologies, #5850) with the medium changed every 2 days. The cells were passaged by treatment of the cultures with 0.5 mM EDTA solution, pH 8 (Invitrogen, #15575-038; diluted in PBS) and replating the cells in mTeSR1 medium, containing 5 nM ROCK inhibitor, Y-27632 (Tocris, #1254) for 24 h. For differentiation, hPSCs were plated on vitronectin-coated plates, at a density of 18,000 cells/cm² in mTeSR1 medium containing 10 µM Y-27632. The following day, the medium was replaced with STEMdiff™ APEL™2 Medium (STEMCELL Technologies) containing 8 µM CHIR-99021 (Tocris, #4423) for 3 days, followed by STEMdiff™ APEL™2 Medium supplemented with 200 ng/mL FGF9 (Peprotech, #100-23) and 1 µg/mL heparin (Sigma, #3149) for a further 10 days. Subsequently, the cells were cultured in basal STEMdiff™ APEL™2 Medium, which was changed daily. Immunocytochemistry is detailed in the online suppl. Materials and Methods.

Animal Surgery

Procedures were approved by the University of Manchester Ethical Committee and the UK Home Office project licence 70/8686. Animal work was undertaken under *Animal Research: Reporting of In Vivo Experiments* guidelines (<https://www.nc3rs.org.uk/arrive-guidelines>). Female SCID/beige mice (Harlan Laboratories) aged 10–12 weeks were anaesthetized by isoflurane (Abbot Laboratories Ltd., UK) as follows: induction at 4 L/min oxygen with 4% isoflurane and then maintained at 2 L/min oxygen with 2% isoflurane during the rest of the procedure. Mice were immobilized on their posterior, and the hind limb was shaved up to the arcuate line. Under an operating microscope (Leica MZ7.5; Leica Microsystems, Switzerland), the femoral artery and vein between the junction of the tibial veins to the inguinal ligament were carefully exposed from surrounding tissues with curved microscissors (SDC-15; Mercian Surgical, UK) and forceps (Dumont #5/45; Fine Science Tools, Germany). Vessels feeding into the vein were cauterized (Bovie, Clearwater, FL, USA) or ligated with 11-0 sutures (Ethilon, Ethicon, UK). The femoral artery and vein were carefully separated from adventitia to allow mobilisation to allow for placement of the 3D printed plastic chamber around the vessels. These vessels, which comprised the AVFT, passed through the chamber around the central pillar, which connected the 2 halves. A control chamber without an AVFT was secured into space created in the inguinal region of the opposite thigh. In some experiments, before they were closed and implanted, chambers were populated with 2 million human KPCs placed as a pellet in 30 µL of Matrigel™. The chamber was secured into space created in the inguinal region with 8-0 sutures (Ethilon, B. Braun, Germany), and the skin was closed with 2 layers of 8-0 sutures. Further controls contained Matrigel and no cells were also performed. Analgesia (0.1 mg/kg, Vetergesic; Alstoe Animal Health) was administered, and the animals were allowed to recover with 2 L/min oxygen. All animals underwent the procedure without complications such as chamber extrusion, infection, or haemorrhage.

Histology of Implanted Tissues

Each harvested chamber was fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned throughout its length at 5 µm. Sections were dewaxed and rehydrated and used for staining and

the quantification of areas occupied by particular cell types. For the latter analyses, every 15th slide was used and the total quantified area comprised the inside of the chamber plus the small areas in the perforations of each chamber. Tissues external to the chamber wall were excluded from these analyses. For specific stains, see online suppl. Materials and Methods.

Human Embryonic Kidneys

Human tissues, collected after consent and ethical approval (REC 08/H0906/21 + 5), were provided by the MRC and Wellcome Trust Human Developmental Biology Resource (<http://www.hdb.org/>), as described previously [31].

Statistical Methods

Analyses were performed using GraphPad Prism software (GraphPad Prism v.7) (<https://www.graphpad.com/scientific-software/prism/>). Data sets in each experimental group were subjected to the Shapiro-Wilk test to determine whether or not they were compatible with a normal distribution. In the former cases, data were shown as mean $\pm$ SD, whereas non-parametric sets were shown as the median with the interquartile and absolute ranges. We compared results from each implant with an AVFT with its counterpart lacking an AVFT in the opposite thigh of the same mouse. Thus, parametric (2-tailed *t* test) or non-parametric (2-tailed Wilcoxon) paired analyses were applied, as appropriate. Differences were considered statistically significant when $p < 0.05$ but, for clarity, exact *p* values are given in graphs of the data in the Results section.

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Statement of Ethics

Procedures were approved by the University of Manchester Ethical Committee and the UK Home Office project licence 70/8686. Animal work was undertaken under *Animal Research: Reporting of In Vivo Experiments* guidelines (<https://www.nc3rs.org.uk/arrive-guidelines>).

Conflict of Interest Statement

The authors have no conflict of interests to declare.

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