Staged *in vitro* reconstitution and implantation of engineered rat kidney tissue

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A major hurdle for current xenogenic-based and other approaches aimed at engineering kidney tissues is reproducing the complex three-dimensional structure of the kidney. Here, a stepwise, *in vitro* method of engineering rat kidney-like tissue capable of being implanted is described. Based on the fact that the stages of kidney development are separable into *in vitro* modules, an approach was devised that sequentially induces an epithelial tubule (the Wolffian duct) to undergo *in vitro* budding, followed by branching of a single isolated bud and its recombination with metanephric mesenchyme. Implantation of the recombined tissue results in apparent early vascularization. Thus, in principle, an unbranched epithelial tubular structure (potentially constructed from cultured cells) can be induced to form kidney tissue such that this *in vitro* engineered tissue is capable of being implanted in host rats and developing glomeruli with evidence of early vascularization. Optimization studies (of growth factor and matrix) indicate multiple suitable combinations and suggest both a most robust and a minimal system. A whole-genome microarray analysis suggested that recombined tissue recapitulated gene expression changes that occur *in vivo* during later stages of kidney development, and a functional assay demonstrated that the recombined tissue was capable of transport characteristic of the differentiating nephron. The approach includes several points where tissue can be propagated. The data also show how functional, 3D kidney tissue can assemble by means of interactions of independent modules separable *in vitro*, potentially facilitating systems-level analyses of kidney development.

kidney development | systems biology | tissue engineering

Given the prevalence of chronic kidney disease and the shortage of donor organs (1), a variety of tissue-engineering strategies are being pursued. For example, the extracorporeal renal tubule assist device utilizes a synthetic hemofilter connected in series with a bioreactor cartridge containing human proximal tubule cells (2). In addition, renal primordia obtained at a sufficiently early developmental stage have been demonstrated to undergo differentiation and grow when transplanted into animal hosts (3–8). *In vitro* propagation of the branching ureteric bud or subdivided progenitor tissue has been proposed as a method for generating a number of kidneys from a single progenitor tissue (9). Cellular components and scaffolding have been combined to create cell-based filtering devices and nephron components (10, 11). Additional cell-based strategies have included the use of adult renal stem-like cells (12–21). Certain populations of these cells are capable of forming tubules in culture and migrating to developing tubules in organ culture (14). Injection of stem-like cells into diseased kidneys may promote healing (20–23). However, engineering a kidney-like tissue from cells with appropriate 3D spatial relationships of nephrons has yet to be achieved (24).

Here, we have devised a method that [based on the stages of metanephric kidney development (25, 26)] utilizes elements of kidney primordia, the Wolffian duct (WD) and metanephric mesenchyme (MM), to engineer *in vitro* kidney-like tissue containing functional tubular transporters and glomeruli with apparent early vascularization. Moreover, this *in vitro* stepwise approach provides not only the potential for propagation at several levels but also the potential for introduction of immunomodulatory or other genes. Perhaps most importantly, if a tubular structure can be formed from adult, amniotic, embryonic stem cells or other cell types, this approach provides a potential strategy for engineering a 3D vascularized kidney-like tissue from cells *in vitro*.

Results

WD Budding. The initiating event in metanephric kidney development is the outgrowth of the ureteric bud (UB) from the WD (27). Using three separate culture systems, this developmental event was replicated *in vitro* [Fig. 1 and supporting information (SI) Table 3]. First, the whole mesonephros was isolated and cultured in the presence of 10 ng/ml glial cell-derived neutrophic factor (GDNF) as described (28). After 3 days in culture, numerous budding events occurred at multiple foci along the WD (Fig. 1 *A* and *B*). In the second method, the mesonephric tubules, along with most of the nonepithelial mesoderm, were removed from the WD before *in vitro* culture. Although the GDNF concentration had to be increased from ≈10 ng/ml to ≈125 ng/ml, and an additional soluble growth factor was required [either fibroblast growth factor-1 (FGF1), ≈250 ng/ml, or fibroblast growth factor-7 (FGF7), ≈50 ng/ml], similar impressive budding of multiple UB-like structures was observed (Fig. 1 *C* and *D*). In the third method, the WD was cleared of all surrounding mesoderm before *in vitro* culture, leaving essentially a "naked" epithelial tube. In this "minimal" system, the WD was able to bud in the presence of soluble growth factors only when suspended in a 3D extracellular matrix gel (Fig. 1 *E* and *F*). Under all three culture conditions, although the overall surface area of the WD increases because of budding at multiple foci, the WD did not appear to lengthen significantly.

Isolated *in Vitro*-Formed UB Branching. After its emergence from the WD, the UB penetrates the MM where it is induced to undergo extensive branching morphogenesis, possibly due to establishment of an autocatalytic network (25). We have shown that embryonic rat isolated UBs suspended in an extracellular matrix gel undergo robust branching in the presence of GDNF and a conditioned medium (CM) secreted by a MM-derived cell line (BSN cells) (29). Here, we demonstrated that a single isolated bud induced from a WD as described above (an *in vitro*-formed UB) retains the ability to branch in 3D culture (Fig. 2) by using conditions similar to those described for the "T-shaped" UB dissected from E13 rat kidneys (29). Thus, *in vitro*-formed UBs, although not achieving a charac-

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The authors declare no conflict of interest.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE9570).

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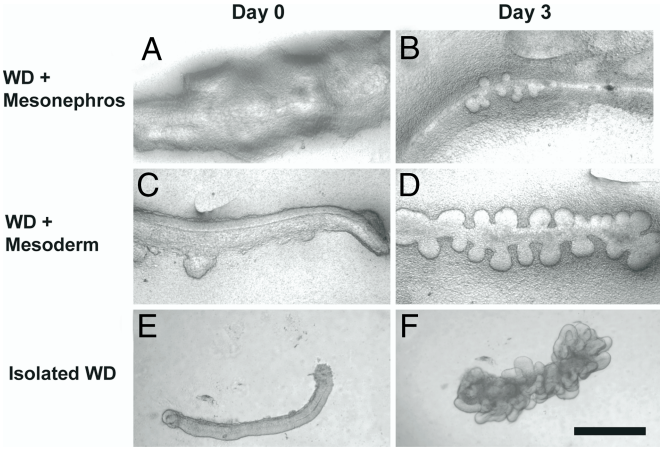

Fig. 1. WD budding systems. The whole mesonephros (*A* and *B*), the WD with a thin layer of intermediate mesoderm (*C* and *D*), and the WD devoid of other cell layers (*E* and *F*) can be induced to bud according to the conditions outlined in the text and SI Table 3. (Scale bar: 500 μm.)

teristic T-shaped structure assumed to be necessary for normal development of the kidney based on knockout studies (25, 30, 31), can grow and branch *in vitro* in a fashion similar to the excised T-shaped bud. This suggests that the T-shaped bud stage can be bypassed in our developmental strategy for tissue engineering.

Extracellular Matrix Requirements for Isolated 3D UB Culture. Given that the extracellular matrix plays a significant role as a scaffold for isolated UB growth and branching as well as modulation of growth factor effects, we attempted to optimize the growth conditions for 3D UB branching. Although previous studies of isolated UB branching used a matrix of "growth factor-reduced" Matrigel and type I collagen (50:50 vol/vol) (29), we found that type I collagen alone did not support branching morphogenesis

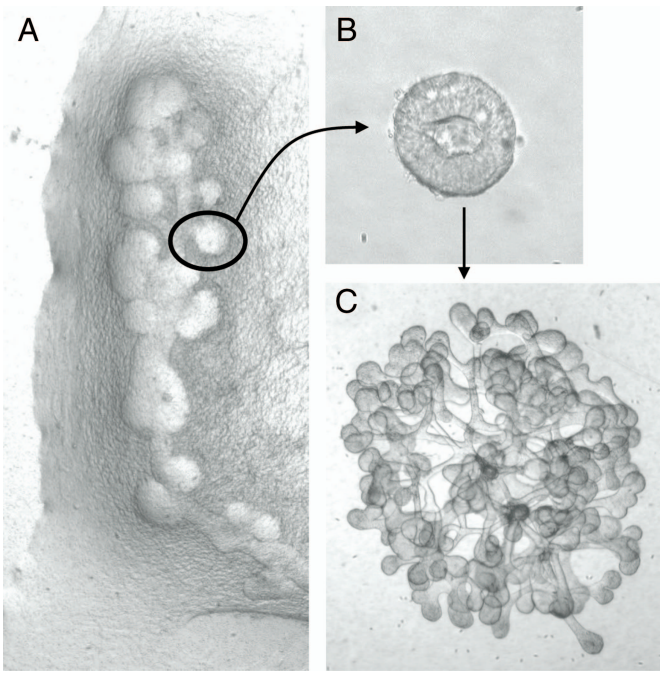

Fig. 2. WD budding to isolated *in vitro*-formed UB branching. One bud from a budded WD after 4 days in culture (*A*) can be excised, suspended in a 3D extracellular matrix gel (*B*), and induced to branch (*C*). Please also see Table 1.

Table 1. Extracellular matrix effects on ureteric bud branching, network-forming matrices

Matrices	Branching effect
Matrigel (15–100%)	Supports
Type IV Coll (0.75 mg/ml)	Supports
Type I Coll (0.4–4.0 mg/ml)	Does not support
Alginate (0.3–1.5%)	Does not support
Puramatrix (50–100%)	Does not support

and was, in fact, inhibitory, whereas Matrigel supported branching, although diminished branching was observed at extreme concentrations (Tables 1 and 2). In addition to these two original components, a 1% alginate solution, cross-linked with 100 mM $CaCl_2$, and Puramatrix, an inert self-assembling peptide matrix, were also tested in the isolated UB system; neither supported UB branching (Table 1). Pure type IV collagen [the network-forming component of Matrigel is type IV collagen (32)] also supported UB branching, whereas additional basement membrane components such as laminin I had little apparent effect on branching (Table 2). However, UBs cultured in type IV collagen did not grow as well as those in Matrigel (data not shown), possibly because Matrigel contains a concentration of type IV collagen (≈3.3 mg/ml) (32), more than triple that of the pure type IV collagen commercially available (≈1 mg/ml).

Taken together with our previous studies (29, 33–36), a minimal set of conditions can be defined for UB growth/branching as pleiotrophin (PTN) plus GDNF (35) in a type IV collagen matrix. The most robust system for both tip and stalk formation, as well as growth, of the *in vivo*-isolated bud, however, appears to be BSN-CM supplemented with GDNF and FGF1 (36, 37) in a 50% Matrigel solution, which were the culture conditions used for the *in vitro*-formed UB described below.

***In Vitro*-Formed UB and MM Recombination.** Once the WD has formed buds *in vitro*, and the dissected *in vitro*-formed UB had branched, we sought to determine whether 3D nephron formation would occur; our approach was to recombine the branched structure with fresh MM. These experiments differ from previous studies (29) in that the branched UB was derived from an *in vitro* budded WD [that does not form a T-shaped structure, considered a key developmental stage (25, 30, 31)]. The recombined tissue was cultured on a Transwell filter without additional growth factors (Fig. 3*B*). Several days after recombination, branched structures had grown and elongated (Fig. 3*E*), and induction was evident by both phase-contrast and fluorescent lectin staining (Fig. 3 *C–F*). Connections between the collecting system and the more proximal portions of the tubule derived from the MM were evident (Fig. 3*F*).

Recombinations between MM and the branched *in vitro*-formed UB, the branched isolated T-shaped UB, or the induced WD (containing multiple nonbranched *in vitro*-formed UBs) were highly similar; conceivably, any of these recombination systems could be used to engineer kidney-like tissue. For convenience, many subsequent experiments were carried out in the latter two systems.

Table 2. Extracellular matrix effects on ureteric bud branching, Matrigel/Coll IV additions

Addition	Branching effect
Laminin (0.2–0.75 mg/ml)	No effect
Alginate (0.1–0.9%)	Diminished
Type I Coll. (0.4–3.0 mg/ml)	Diminished
Type IV Coll. (0.25–0.75 mg/ml)	No effect

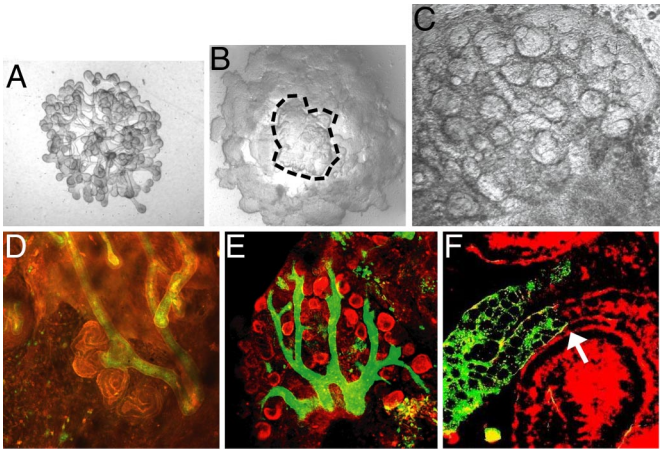

Fig. 3. Recombination of branched *in vitro*-formed UB with MM. (*A* and *B*) The branched *in vitro*-formed UB from Fig. 2*C* (*A*) was mechanically separated from the matrix (delineated by the dotted line) and recombined with freshly dissected undifferentiated MM (*B*). (*C–F*) Recombined tissues were grown for approximately 4–6 days (*C*). A ×10 dual fluorescent micrograph of the recombined tissue stained with FITC-labeled *D. biflorus* (green) and rhodamine-conjugated peanut agglutinin (PNA) lectin (red) shows the mesenchymal-to-epithelial transition occurring around the UB branches (green) (*D* and *E* ×4). A higher-magnification (×40) at the fusion (arrow) of the WD-derived (green) and MM-derived (red) epithelial cells demonstrates a contiguous tubule lumen (*F*).

Although the recombined tissues comprised nephron structures resembling late stages of renal development, it was important to verify that the induced MM was not only undergoing mesenchymal-to-epithelial transformation (MET), but also that the MM-derived tubules were expressing functional transporters. The anion 6-carboxyfluorescein (6-CF) is a fluorescent organic anion taken up by specific tubular transporters.** When the recombined tissue (branched T-shaped UB and MM) was incubated with 6-CF, cellular uptake was observed in the cells of mesenchymally derived tubules, suggesting both differentiation and function of the MM-derived nascent proximal tubules (Fig. 4*A* and *B*). This uptake was blocked by 2 mM probenecid, an organic anion transport competitive inhibitor (38) (Fig. 4*C*).

In addition to demonstrating the functional capacity of the recombined tissues, the global gene expression of recombined tissue (nonbranched *in vitro*-formed UBs and MM) was analyzed and compared with the gene expression of early, late, and postdevelopmental kidneys to determine whether normal developmental pathways were being followed. The methods and analysis are available in *SI Text*. Briefly, genes with at least a 3-fold difference in expression among any of the four conditions [embryonic day (E)13, E18, Wk4, recombined tissue] were analyzed and grouped into one of 10 expression patterns (SI Fig. 7). The expression of each group of genes was analyzed in the recombined tissue to compare the recombined tissue gene expression levels to the three *in vivo* time points (SI Table 4). The comparison suggested that developmental pathways are similarly modulated/regulated in tissue engineered from *in vitro*-induced WD recombined for 4 days with fresh MM. Almost 50% of the genes that were up-regulated at E18 *in vivo* were up-regulated in the recombined tissue. In addition, 72% of the down-regulated genes also down-regulated in the recombined tissue to at least E18 levels. Nevertheless, there were a few genes expressed at much higher or lower levels in the recombined tissue that did not change in the stages compared; these genes represented <5% of the total number of genes in the analysis.

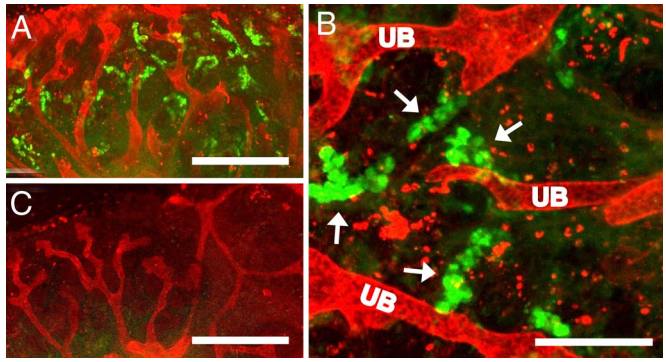

Fig. 4. Differentiated tubules of the recombined tissue are functionally capable of organic anion transport. (*A*) Accumulation of 6-CF (green), a fluorescent organic anion, in the MM-derived tubules (UB-derived tubules stained with TRITC-conjugated *D. biflorus*, red) of the recombined tissue (branched T-shaped UB, MM) suggests both differentiation and function of mesenchymal tubules. (*B*) The accumulation is seen only in the cells of non-UB-derived tubules (arrows). (*C*) The accumulation is probenecid- (a competitive inhibitor of organic anion transport) sensitive, confirming that the accumulation was transporter mediated. (Scale bars: *A* and *C*, 500 μm; *B*, 200 μm.)

Implantation and Apparent Vascularization of the Recombined Kidney-Like Tissue. In order for a kidney tissue to be functional *in vivo*, it must contain a vasculature and glomeruli. Although the microarray analysis demonstrated that the recombined tissue recapitulates many of the gene expression patterns of renal development, few vascular genes were up-regulated. However, this does not predict whether the recombined tissue will be able to successfully integrate into a host animal. Previous studies have demonstrated that early avascular embryonic kidneys can be implanted into a host animal to recruit a vasculature with functional glomeruli (3–8). Therefore, we implanted the recombined kidney-like tissue (from a recombination of branched T-shaped UB and MM) under the renal capsule of a host rat. After 14 days, the host animal was euthanized, and the implanted tissue was analyzed (Fig. 5). The implanted recombined tissue had multiple glomeruli and expressed the endothelial marker PECAM-1 in the cells of the glomerulus (Fig. 5*D*). Erythrocytes could be seen in the glomeruli of the recombined tissue, suggesting blood flow to the implanted tissue (Fig. 5*C*, arrows). The long-term viability and *in vivo* functionality, as well as the optimal location and procedure for implantation, remains to be determined.

Discussion

We present a strategy for tissue-engineering a propagatable kidney-like tissue by following key kidney developmental events *in vitro* in a stepwise fashion beginning with a WD. We describe the optimization and elaborate details on a variety of conditions that can be used at several *in vitro* steps, potentially offering multiple approaches and points of propagation within this strategy. The strategy results in tissues with nephrons, glomeruli, and an apparent early vasculature in three dimensions. Fig. 6 illustrates how these systems can be assembled in order to create *in vitro*-engineered renal structures from the initial components.

Our optimization studies revealed that the WD requires only the presence of GDNF for budding if the whole mesonephros is cultured *in vitro* but requires additional growth factors like FGF1 or FGF7 if the mesonephric tubules and surrounding mesoderm are removed (Fig. 1). The recent detection of a GDNF bypass pathway, however, raises the possibility that even GDNF may not

**This work performed by David Truong is currently unpublished.

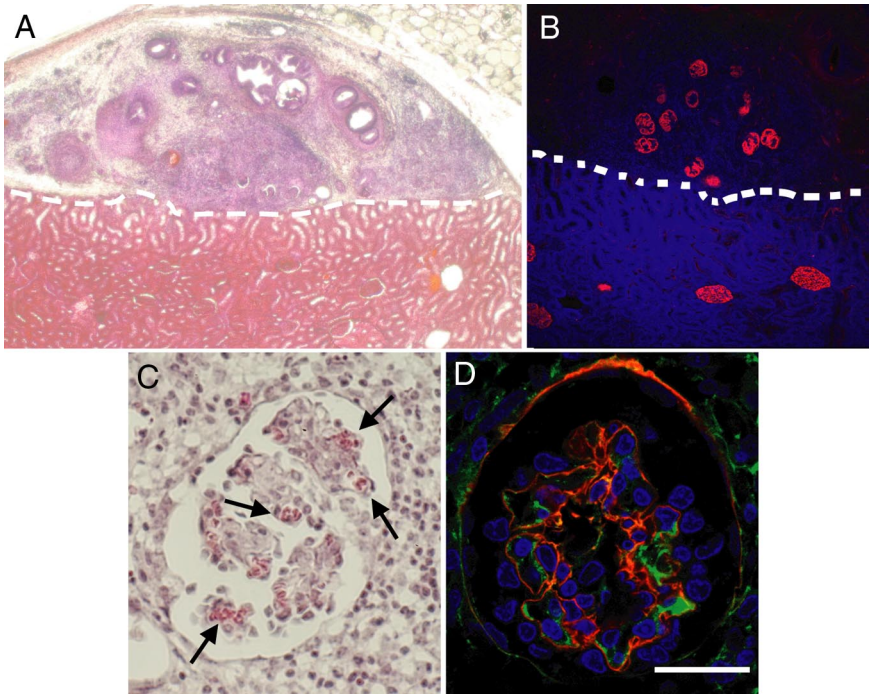

Fig. 5. Recombined tissue 14 days after implantation into a host rat. (*A* and *B*) The dashed line separates recombined tissue (above) from host tissue (below); glomeruli are stained with PNA (red). (*C* and *D*) The presence of erythrocytes (arrows) in the glomeruli suggests blood flow in the recombined tissue. The cells of the glomerulus express the endothelial marker PECAM-1 (green) and type IV collagen (red) along its basement membrane. DAPI nuclear stain is blue. (Scale bar: 50 μm.) (Magnification: *A*, ×4; *B*, ×10; *C*, ×40; *D*, ×60.)

be essential for this step (51). Furthermore, a suspended culture system using a diluted Matrigel solution, in addition to the soluble growth factors, is required for the WD devoid of surrounding mesoderm to undergo *in vitro* budding. These experiments suggest that the surrounding mesoderm plays a key role in budding by providing growth factors and/or necessary matrix components, which for tissue engineering purposes, we supply exogenously. Moreover, each of the many buds can be excised, suspended in a 3D matrix, and induced to undergo extensive branching morphogenesis. The branched *in vitro*-formed UB appears highly similar to the *ex vivo*-isolated T-shaped UB grown in culture (29). Because a single focus of budding yields a kidney *in vivo*, if each of the multiple buds were cultured individually, then many kidney-like tissues could potentially arise from a single WD cultured *in vitro*.

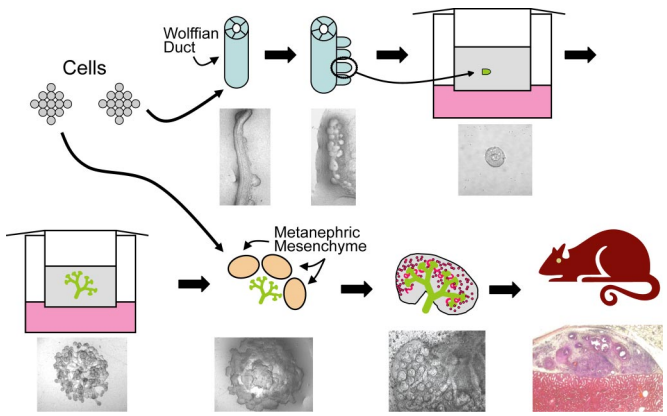

Fig. 6. Proposed *in vitro* kidney engineering strategy. First, the WD is isolated and induced to bud. Then, each bud can be isolated and induced to undergo branching. The branched *in vitro*-formed UB is then recombined with MM; after 4–6 days of mutual induction, the recombined tissue resembles a late-stage embryonic kidney. The recombined tissue is then implanted into a host animal where it is vascularized and forms glomeruli. The possibility of using cells to engineer WD and/or MM-like tissue is also indicated.

Branching growth was optimized for the T-shaped UB; optimal branching and growth occurred when we combined FGF1, a growth factor that supports branching and growth of the isolated UB culture system, with non-FGF branch-promoting growth factor combinations (i.e., GDNF plus either PTN or BSN-CM). *In vitro*, these growth factor conditions provided the most robust branch-stimulating conditions (i.e., growth plus most *in vivo*-like patterning) among the many soluble-factor combinations that we have tested (33–37). The fact that a combination of BSN-CM, FGF1, and GDNF gave more consistent branching growth than PTN, FGF1, and GDNF suggests that one or more factors within BSN-CM will need to be isolated to achieve ideal minimal conditions. Conceivably, these could include factors such as TGFβ superfamily members, including bone morphogenetic proteins 2 and 4 and activin, which can modulate branching and are involved in sculpting of the isolated UB culture system (36). Thus, it is possible that a combination of PTN plus one additional "UB-sculpting factor" could replace BSN-CM to attain an ideal minimal set of conditions for a tissue-engineering approach. Until such conditions are discovered, data indicate that the combination of BSN-CM, FGF1, and GDNF supports the most robust growth and branching of the UB *in vitro*.

Given the recent work on matrices and scaffolds for tissue engineering of bone, cartilage, and other organs (reviewed in refs. 39 and 40), both artificial and natural matrix conditions were analyzed for isolated 3D UB branching. Our data suggest that *in vitro*-isolated UB branching morphogenesis largely depends on type IV collagen and does not require additional matrix components (the UB branched only in type IV collagen or type IV collagen-based Matrigel), although one cannot rule out the

possibility that the growth of the UB is affected by other Matrigel components. Surprisingly, adding laminin I, which enhances branching of cultured cells (41), to a type IV collagen matrix did not appreciably increase branching morphogenesis (Tables 1 and 2). This suggests that the initial extracellular matrix scaffold does not require all components of the final basement membrane and that the UB itself might synthesize any necessary supplementary proteins in an isolated system. In addition, two inert ECM molecules, alginate, which has been used extensively in cartilage tissue engineering (42–44), and Puramatrix, which was successfully used to support neuronal migration (45) and to promote osteoblast differentiation (46), did not support UB branching. This may be due to the inability of the UB to remodel the artificial matrix to allow room for new branches. Nevertheless, an ideal minimal system for tissue engineering of the kidney according to our scheme ought to continue consideration of other artificial matrices as they become available.

The branched *in vitro*-formed UB (derived from the WD) when placed adjacent to freshly isolated MM, caused a mesenchymal-to-epithelial transformation and tubule formation (as well as connections to nascent collecting ducts) resulting in nephron-containing tissues. This indicates that the *in vitro*-formed UB, after being induced to branch *in vitro*, retains the capability to form kidney-like tissues.

The recombined tissue was found to form nephron structures that not only phenotypically appeared normal but also resembled the transcriptome of the developing E18 rat kidney. Recombined tissue was also demonstrated to be functionally capable of organic anion transport (Fig. 4). The probenecid-inhibitable transport of the fluorescent organic anion, 6-CF, was observed only in cells of MM-derived structures that appear to be nascent proximal tubules.

For the recombination step, we used fresh MM tissue reported to contain pluripotent renal progenitor cells (13). It may be most ideal, however, to begin with cells alone. We have demonstrated here the ability of secreted products from the BSN cell line (derived from MM) to induce optimal branching of the *in vitro*-formed UB but, thus far, have been unable to show that the cells themselves will recombine to form nephron-containing structures (data not shown). This may require a matrix-based strategy to make cells cohere, or, alternatively, it is possible that BSN cells are too differentiated (perhaps more like mesenchymal "cap" cells) to be used for this application. Recently, it has been reported that mouse ES cells can be induced to form MM-like cells, suggesting an alternative approach (47).

Similar considerations apply to the creation of an epithelial tubule like the WD from cells. Of note, it has been shown that the UB cell line can form tubules under conditions somewhat similar to those we have shown as optimal for branching of the isolated UB (48). It has also been shown that adult "progenitor-like" cells from the injured mouse kidney can form tubular epithelial structures *in vitro* and migrate to multiple compartments of the developing kidney in organ culture (14). These types of cells, or possibly others that have recently been described (10, 49, 52), may be more suitable.

In addition to using WD-like or MM-like tissues potentially constructed from cells, the methods we describe may be suitable for xeno-based approaches. Given the fact that they are tissue/organ culture-based, and that there are at least two points for propagation (at the level of the *in vitro* cultured WD and at the level of the *in vitro*-formed UB), it may be possible to "humanize" the tissue through transfection or similar strategies or to induce expression of immunomodulatory or other genes to diminish the possibility of rejection and, potentially, improve functionality. These techniques, not currently feasible in mammalian adult organs, could provide considerable flexibility for the goal of creating immunocompatible tissues suitable for a particular genetic profile. Beginning with a single or limited *in vitro*-propagatable tissue may also help address the concern about animal viruses with xeno-based approaches by creating a single or limited set of key points in a tissue-engineering strategy where intense quality control or surveillance can be applied. Embryonic-derived tissues seem to elicit a reduced immune response in rodents (3, 5); therefore, *in vitro* manipulation of xeno-tissues or primitive cells to engineer kidney-like tissue may result in a less antigenic transplant than alternative options.

The recombined tissue seems to recruit an early vasculature, forming glomeruli that appropriately express a key endothelial marker when implanted underneath the renal capsule of a host animal, although *in vivo* functionality remains to be determined. Whether this technique will be successful as a therapy for chronic kidney disease is unknown, but it provides "proof of concept" that an *in vitro*-engineered kidney-like tissue, designed in the manner we describe, can survive over the short term and begin to recruit a vasculature when placed in a host animal. Sites other than the renal capsule may prove as, or even more, hospitable for recombined tissue (3).

Each step of this tissue-engineering procedure reflects one or more independent stages of kidney development. Therefore, in addition to being an important tool for engineering implantable kidney-like tissues, this method further demonstrates that developing kidneys consist of a number of separable but reconstitutable modules (25, 26, 36, 50). This approach may be useful for future systems-biology work on nephrogenesis (25, 26). Early vascularization of the recombined tissue appears to occur distinct from the morphogenetic processes of budding, branching, and MET.

Although many approaches are being taken toward engineering kidney substitutes, we suggest that a kidney-like tissue formed by following the normal developmental progression will likely recapitulate the 3D relationships necessary to maintain vital renal functions. Here, we have provided the guidelines for such a strategy, in rodents, to stimulate renal progenitor tissues to follow the natural developmental program resulting in an *in vitro*-engineered kidney-like tissue containing a branched collecting duct system, nephrons with functional transporters, glomeruli, and an apparent vasculature. That the strategy has strong potential for propagation of the engineered kidney-like tissue, as well as modulation of functionality and immunogenicity by transfection-type methods, adds to its possible utility.

Methods

Materials. All growth factors are from R & D Systems and all other reagents are from Sigma unless otherwise noted.

Budding of WDs. The urogenital tract was isolated from timed pregnant Holtzman rats (Harlan) at E13, and WDs were dissected free of surrounding tissue (28). The mesonephric tubules and most of the surrounding intermediate mesoderm were mechanically separated from the WD, or, in some cases, the remaining intermediate mesoderm was carefully stripped away, leaving only the WD. Tissues were placed on 0.4-μm Transwell filters (Costar) and cultured at the air–media interface (except the WD devoid of intermediate mesoderm, which was suspended in a 3D extracellular matrix of growth factor-reduced Matrigel (Becton Dickinson) and DMEM/F12 (50:50 vol/vol) (GIBCO–BRL) on a Transwell filter. DMEM/F12 media supplemented with 10% FCS (BioWhittaker), 1% antibiotics, and growth factors at the noted concentrations were placed in the well beneath the filter. All cultures were carried out at 37°C in a humidified 5% CO_2 atmosphere unless otherwise noted, and all cultures were performed at least three independent times.

Isolation and 3D Branching of *in Vitro*-Formed UBs. Budded WDs were removed from the filter, lightly digested with trypsin (2 mg/ml trypsin in L-15 for 5 min at 37°), and the buds were separated from the WD and surrounding attached cells using microsurgery forceps. Microdissected *in vitro*-formed buds were suspended in a matrix of growth factor-reduced Matrigel and DMEM/F12 (50:50 vol/vol) on a Transwell filter and cultured in the presence of BSN-conditioned media [prepared as described (29)], supplemented with 10% FCS, 1% antibiotics, GDNF (125 ng/ml), and FGF1 (250 ng/ml).

Optimization of Extracellular Matrix (Natural and Artificial) Conditions for Isolated UB Branching. E13 UBs and MM were isolated from rat kidney rudiments as described (29, 34–36). UBs were cultured in the presence of BSN-CM supplemented with 10% FCS, GDNF (125 ng/ml), FGF1 (250 ng/ml), and 1% antibiotics. UBs were suspended in matrices (natural and artificial) consisting of growth factor-reduced Matrigel diluted with DMEM/F12, type I collagen, Puramatrix, 1% alginate solution (cross-linked with 100 mM $CaCl_2$), and type IV collagen at noted concentrations and combinations. All matrix solutions were supplemented with DMEM and buffered by Hepes and $NaHCO_3$ to a pH of ≈7.2.

Recombination of *in Vitro*-Formed UBs and T-Shaped UBs with MM. After 4–6 days, the branched *in vitro*-formed UBs or branched T-shaped UBs were separated from the surrounding matrix by blunt dissection. Branched buds with minimal matrix were placed on a new Transwell filter and cultured with DMEM/F12 (50:50) supplemented with 10% FCS. MM from E13 kidneys was placed next to and on the branched UB (alternatively, the nonbranched, nonisolated *in vitro*-formed UBs were directly recombined with MM). After 4–7 days of culture, the recombined kidney-like tissues were fixed in 4% paraformaldehyde, extensively washed in PBS and processed for fluorescent lectin staining as described (29). Additional details are available in *SI Text*.

Functional Organic Anion Transport Assay. Recombined tissues (branched T-shaped UB–MM) on culture day 7 were assayed as described (38), with 1 μM 6-carboxyfluorescein (6-CF) (Sigma) in place of fluorescein. Additional details are available in *SI Text*.

Implantation of Recombined Kidney-Like Tissue. Recombined tissues (branched T-shaped UB–MM) on culture day 6 were detached from the Transwell filter and suspended in cold L-15 medium. The abdominal cavity of an adult male rat was opened under inhalant anesthesia with isoflurane. A subcapsular tunnel was prepared on the right kidney by using the tip of microsurgery forceps. Between two and six recombined tissues were inserted into the subcapsular region together with a small volume of L-15 medium (≈40 μl) by micropipette. The abdominal cavity was closed by suturing muscle and skin layers. After 14 days, the kidneys with implants were excised and provided for histological analyses (described in detail in *SI Text*).

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