Engineering Complex Kidney Structures for Disease Modelling, Drug Testing, and Studying Kidney Development

Thesis

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ENGINEERING COMPLEX KIDNEY STRUCTURES
FOR DISEASE MODELLING, DRUG TESTING,
AND STUDYING KIDNEY DEVELOPMENT

Thesis submitted by
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For the degree of
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ABSTRACT

Although existing kidney tissue engineering systems and cell-based strategies favoured significant advances in the field, they cannot reproduce the organ’s complex architecture. This prevented the use of these tissues in studying kidney development realistically, modelling diseases, and establishing therapeutic approaches.

To fill these gaps, we devised a 3D engineering system for rapid generation of custom-made geometrically predefined kidney units that more faithfully resemble their counterparts in vivo.

Combining 3D printing and PDMS prototyping, we fabricated differently sized and shaped scaffolds into which MDCK cells were seeded and cultured under tubulogenic conditions. Cells grew and self-assembled into branched tubules with single lumen delimited by a polarised monolayered epithelium, exhibiting kidney-specific functions.

To model polycystic kidney disease (PKD), we pharmacologically induced cyst formation within engineered tubules. Next, we tested and quantified different compounds’ effect on cyst regression, identifying new potential pharmacological treatment; we showed that 2-deoxy-D-glucose is more effective than other compounds and discovered that berberine possesses high therapeutic potential for PKD treatment.

Optimising the protocol and using different human iPSC lines, we successfully engineered functional human ureteric bud (UB)-like tubules capable of recapitulating early steps of UB morphogenesis. Exploiting these developmental capacities, we used tubules to identify a novel growth factor combination that induces budding events in a way comparable to mouse embryonic kidneys and that may therefore be involved in human UB development. Observing a marked reduction of ramified buds in tubules derived from a patient with heterozygous PAX2 mutation affected by focal segmental glomerulosclerosis, we speculated that such developmental disorder might have contributed to the patient’s condition.

Overall, these findings document that our innovative and robust technology for controlled tubule engineering provides a valuable and reliable platform for kidney disease modelling, drug discovery and developmental studies, and may lay the groundwork for creating anatomically correct kidney tissue in vitro.
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CHAPTER 1 - INTRODUCTION
1.1 Chronic kidney disease: a public health problem

Chronic kidney disease (CKD) is a leading cause of morbidity and mortality in modern society. Currently, CKD affects hundreds of million people worldwide and has a consistent estimated global prevalence of between 11 to 13% (Hill et al., 2016). Its global incidence and prevalence are rapidly increasing, particularly in developing countries, thus making CKD, without a doubt, a major global public health issue.

Major causes of CKD are diabetes, hypertension, infectious diseases and acute kidney injury as consequence of several drug exposure, and polycystic kidney disease (PKD), the most common genetic renal disease affecting all ethnic groups (Ong et al., 2015; Brunelli et al., 2015; Mao et al., 2016).

In most patients, CKD eventually progress towards kidney failure, also called end-stage renal disease (ESRD) that, being characterised by irreversible loss of nephrons and deterioration of renal function, is incurable.

The only treatment for ESRD is renal replacement therapy (RRT), which aims to substitute the critical kidney functions and sustain life in affected people. RRT consists of hemodialysis, peritoneal dialysis and kidney transplantation.

Although dialysis keeps patients alive by replacing exclusively the non-endocrine kidney functions such as removal of molecular waste products from the blood, the patients’ quality of life is severely impaired by life-threatening complications (Rodrigues-Diez et al., 2017).

The global healthcare costs for dialysis patients are between $150,000 and $200,000 per year and will exceed $1 trillion this decade. This significant economic burden, combined with the ongoing 7% annual increase in patients who need dialysis, will make this treatment impractical and unaffordable even in developed countries (Perico and Remuzzi, 2012).
The alternative to dialysis is kidney transplantation, which, at present, represents the most effective treatment for patients with ESRD. In fact, both quality of life and life expectancy in people who underwent kidney transplantation are better than in long-term dialysis patients (Wolfe et al., 1999). Unfortunately, almost every patient who receives kidney transplant undergoes non-specific immunosuppressant treatments, with increased risk of developing malignancies, opportunistic infections, cardiovascular and metabolic diseases, all of which negatively influence graft function and outcome. In addition to these problems, the critical shortage of compatible donor organs for transplantation makes it extremely hard to meet the existing demand for renal replacement, so that the vast majority of patients die on the waiting list before receiving a kidney (Rodrigues-Diez et al., 2017).

The high costs associated with RRT, combined with the rising imbalance between the number of patients waiting for kidney transplantation and the availability of donor organs, make indispensable the development of alternative therapeutic approaches. The establishment of effective therapies for CKD is also hindered by the lack of experimental models systems that faithfully reproduce the disease and the kidney’s extremely complex cellular composition, intricate anatomy and physiology.

1.2 Early kidney development

The posterior primitive streak, a linear band of cells that forms at the caudal edge of the embryo epiblast, gives rise to the intermediate mesoderm (IM), a narrow cell population comprised between paraxial and lateral plate mesoderm, from which mammalian kidney originates. The IM differentiates, in turn, into the nephric duct (ND) epithelium and the adjacent metanephric mesenchyme (MM), the two key renal precursor tissues that will form the metanephric kidney through reciprocal and finely orchestrated interactions (Saxén, 1987; Little et al., 2016; Takasato et al., 2016) (Figure 1).
In mice, at 10.5 embryonic days (E10.5), and E35 in humans, morphogenetic signals from the MM induce the outgrowth of the ureteric bud (UB) as a lateral branch at the caudal end of the ND epithelium. The UB is a tubular structure endowed with a lumen delimited by a polarised epithelium and a basement membrane (Meyer et al., 2004; Chi et al., 2009). UB formation occurs in correspondence of a single area, so that only one UB arises from the ND, thus guaranteeing the development of a single and unique collecting duct system. The UB stalk between the MM and the ND epithelium develops into the ureter that joins the kidney with the bladder.

At E11, the UB invades the adjacent MM and, through direct cell-cell contact and secretion of morphogens by its cells, induces the condensation of a subset of MM cells surrounding the bud tip. These condensed mesenchymal cells form the cap mesenchyme (CM), which comprises nephron progenitor cells morphologically distinguishable from the surrounding un-induced metanephric blastema (Davidson, 2008) (Figure 1).

At E11.5, the CM induces the UB epithelium to branch for the first time and generate a T-shaped bifurcation consisting of two UB tips, each at the end of a stalk. Concomitantly, the newly formed UB tips induce condensation of surrounding un-induced MM cells, leading to the formation of a CM in correspondence of each bud tip (Vainio and Lin, 2002; Hendry et al., 2011; Krause et al., 2015). From this time onwards, each CM will induce the UB tips to branch and, simultaneously, undergo mesenchymal-to-epithelial transition (MET) to form a primitive polarised epithelium, the renal vesicle (RV). The RV grows, elongates and forms a comma-shaped body, which then develops into an S-shaped body that undergoes segmentation and eventually gives rise to the nephron (Figure 1). In particular, the S-shaped body’s distal portion, which fuses to a UB branch, becomes the distal tubule of the nephron, the middle region gives rise to both the loop of Henle and the proximal
tubule of the nephron, and the other end develops its own vascularisation to form the glomerulus (Davidson, 2008).

As kidney development proceeds, the UB epithelium continues to grow, elongate and branch, with each new UB tip acting as an inductive centre for nephrogenesis (Vainio and Lin, 2002). Through its developmental program, named branching morphogenesis, which consists of iterative budding and branching events, the UB eventually forms the tree-like collecting duct system of the kidney with the typical cortical-medullary organisation.

These MM and UB derivatives will develop a network of interconnected epithelial tubules consisting of polarised monolayered epithelia of aligned and tightly adhering cells surrounding a single central lumen. This complex structural organisation arises through the formation of specialised junctions between cells, which establish apical domains oriented toward the lumen, and basal and lateral domains in contact with the basement membrane and adjacent cells, respectively (Andrew and Ewald, 2010). In order to assemble into appropriately polarised tissue, cells must also interact with the extracellular matrix (ECM) through different types of receptors, such as integrins, and with neighbouring cells through diffusible molecules, such as morphogens, chemoattractants and chemorepellants (Bryant and Mostov, 2008; Bryant et al., 2010). Therefore, the combination of cell-cell and cell-ECM interactions drives the apical-basal polarity establishment by segregating membrane components into different cell domains.

As every epithelial tubular structure, kidney epithelial tubules can form through the mechanisms of cord hollowing, cell hollowing and cavitation (Andrew and Ewald, 2010; Lubarsky and Krasnow, 2003).

In cord hollowing, small multiple lumens form at the apical membrane contact sites following cell polarity establishment within tissue primordia and then fuse together to create a single and continuous lumen.
In cell hollowing, luminal spaces originate within individual cells as large cytoplasmic vesicles, which then fuse together and, ultimately, with the plasma membrane to connect with lumens formed within the adjacent cells of the developing tubular epithelium.

The mechanism of cavitation begins with polarisation of unpolarised primordia’s peripheral cells that will constitute the mature tubular epithelium, followed by death for apoptosis of the cells in the central region that are not in contact with the ECM, leading to the generation of a single lumen.

Kidney tubules are surrounded by supporting cells, also known as stromal cells, which originate from the MM and constitute a population of cells distinct from that of the nephrogenic precursors (Figure 1). Shortly after UB invasion, stromal cells can be identified in the MM as spindle-shaped mesenchymal cells (Hatini et al., 1996). Stromal progenitor cells can differentiate into various non-epithelial cells in the adult kidney, including interstitial fibroblasts responsible for synthesizing collagen and producing erythropoietin (Bachmann et al., 1993; Li et al., 2014; Maxwell et al., 1993), endothelial cells that form the peritubular capillaries (Sims-Lucas et al., 2013), vascular smooth muscle cells, pericytes and glomerular mesangial cells (Humphreys et al., 2010). Furthermore, by secreting ECM proteins and signalling molecules, stromal cells play pivotal roles for the appropriate nephrogenesis and collecting duct system formation. In particular, stromal cells are involved in regulating MM epithelialisation, UB growth and branching morphogenesis, and, by antagonising inductive signals from MM, in inhibiting budding from inappropriate or multiple sites of the ND epithelium (Al-Awqati and Oliver, 2002; Cullen-McEwen et al., 2005).

Overall, cell populations and tissues described above interact in a spatiotemporally regulated manner to develop an intricate architecture, which supports the functional and physiologically active adult kidney.
1.3 Overview of kidney anatomy and functions

The kidney is a vital organ with an exceptionally complex morphology and highly specialised in maintaining body fluid homeostasis. Indeed, by filtering blood, kidney regulates the body’s water balance and chemical composition, controls levels of electrolytes and metabolites, and removes metabolic waste products to finally produce and excrete urine. Additional major kidney functions are reabsorption of essential nutrients, regulation of blood volume and pressure, and production of hormones indispensable for regulating erythropoiesis and the bones’ density.

1.3.1 Kidney anatomy

In vertebrates, kidneys are two bean-shaped organs located on the left and right sides of the body retroperitoneal space. Each kidney shows a convex and a concave side. The narrow opening on the concave side, through which the renal artery enters the kidney and the renal vein and the ureter leave, is called renal hilum. A thin layer of connective tissue, known as the renal capsule, envelopes the whole kidney’s surface (Rodrigues-Diez et al., 2017) (Figure 2). Beneath the renal capsule, the kidney can be divided in two main regions easily recognisable by observing the sagittal section of the organ: (i) the renal cortex on the outside, and (ii) the deeper medullary region (Taal et al., 2011). In humans, the medulla is organised into 8 to 18 cone-shaped structures, the renal pyramids, spaced out by projections of the cortex, called renal columns. The base of each renal pyramid resides within the renal cortex, while its tip, the papilla, extends inward towards the renal pelvis, and empties the urine formed by each pyramid into a minor calyx (Figure 2). The minor calyces coalesce to form 2 to 3 major calyces, which in turn fuse to form the renal pelvis - a funnel-like structure that exits the kidney through the renal hilum and drains the urine away into the ureter (Taal et al., 2011). Renal pelvis, calyces and hilum are contained into
a fatty compartment, the renal sinus, which separates these structures from the renal medulla (Figure 2). This structural organisation is crucial to the appropriate kidney function since it establishes an osmotic gradient from the cortex to the medulla that drives urine concentration.

1.3.2 The nephron

The nephron is the key structural and functional unit of the kidney. It filters blood and produces urine. Structurally, it can be subdivided in two main sections: (i) the renal corpuscle, needed for blood filtration, and (ii) the renal tubule, needed for reabsorption and secretion (Rodrigues-Díez et al., 2017).

The renal corpuscle is composed of glomerulus, mesangium and Bowman’s capsule (Figure 3). The glomerulus is a tuft of capillaries (or capillary loops) surrounded by mesangial cells, and is located between an afferent arteriole, which supplies blood to the glomerulus, and an efferent arteriole, which drains blood away from the glomerulus. The region where renal arterioles enter and leave the glomerulus is called vascular pole. The Bowman’s capsule envelops the glomerulus and consists of an inner layer of specialised epithelial cells, called podocytes, and an outer epithelial layer of parietal cells. The narrow cavity between these two epithelial layers is the Bowman’s space, also known as urinary space. Podocytes are extremely specialised cells characterised by interdigitating foot processes wrapped around the glomerular capillary loops, and forming cell-cell junctions termed slit diaphragms. The latter are crossed by pores with heterogeneous size and shape, which are responsible for the selective passage of macromolecules (Gagliardini et al., 2010). The Bowman’s capsule connects to the renal tubule, which can be subdivided in three segments: (i) the proximal convoluted tubule, lined by a simple cuboidal epithelium of cells with brush borders that strongly increase the absorption surface; (ii) the loop of
Henle, a U-shaped epithelial conduit consisting of two portions: the descending limb and the ascending limb; (iii) the distal convoluted tubule that drains urine into the collecting duct via the connecting tubule (Figure 3). The human kidney contains up to 1.5 million nephrons, each of them spans the renal cortex and medulla. Specifically, the renal cortex contains glomeruli, proximal and distal convoluted tubules, and the first trait of the collecting ducts; the renal medulla contains both the loops of Henle and the collecting ducts. Since the collecting duct system’s initial portion is located in the renal cortex, whereas the rest extends inwards, towards the renal medulla, it is characterised by cortical-medullary orientation (Figure 3).

The fact that both nephron and collecting duct epithelia consist of cells with well-defined morphological and functional properties, explains why they are competent at exerting diverse and specific physiological roles.

1.3.3 Kidney functions

The kidney is responsible for regulating body fluids’ composition and volume by filtering the blood to eliminate metabolic waste products, such as creatinine, ammonia, urea, uric acid, and toxins, and by reabsorbing water and essential nutrients. Blood filtration begins in the glomerulus and leads to the production of urine (Rodrigues-Díez et al., 2017). The filtration barrier that divides blood and Bowman’s space consists of the fenestrated monolayered endothelium lining glomerular capillaries, the glomerular basement membrane, and the slit diaphragm’s pores between the interdigitated foot processes of the podocytes enveloping the capillary tuft (Haraldsson et al., 2008).

In physiological conditions, only molecules of lower molecular size than albumin (68 kDa), for instance water and small molecules, can pass through this filter and reach the Bowman’s space, forming the glomerular ultrafiltrate. The latter, flows through the
sequential segments of the renal tubule where it is modified by the highly regulated
processes of reabsorption and secretion. In fact, together with waste and toxic products, the
ultrafiltrate also contains metabolically useful solutes that require reabsorption.
Most of the ultrafiltrate reuptake (65-70%) occurs in the proximal convoluted tubule and
allows water, electrolytes, amino acids, glucose, vitamins, and other essential
macromolecules to come back to the bloodstream through peritubular capillaries.
Additional ultrafiltrate concentration takes place along the loop of Henle’s descending and
ascending limbs via water, sodium and other ions reabsorption. This process results from
the osmotic difference existing between the hypotonic ultrafiltrate and the hypertonic
medullary interstitium (Rodrigues-Diez et al., 2017).
As the ultrafiltrate passes through the distal convoluted tubule, reabsorption of sodium
chloride, calcium and magnesium, and secretion of potassium takes place (Subramanya
and Ellison, 2014). This is followed by further acidification of the ultrafiltrate since water
and bicarbonate are reabsorbed as it passes from the distal convoluted tubule to the
medullary collecting duct. Once at the end of the collecting duct, in the renal papilla, the
modified ultrafiltrate, now called urine, is composed exclusively of a minimum amount of
water and non-reabsorbed compounds, reaching the renal pelvis to be finally drained away
to the bladder through the ureter.
In virtue of these sophisticated mechanisms, kidneys play a central role in the regulation of
water and electrolyte homeostasis, and blood pH level and pressure. Kidneys can also
regulate blood pressure through the production of the proteolytic enzyme renin secreted by
the juxtaglomerular cells of the juxtaglomerular apparatus located between the glomerular
vascular pole and the distal convoluted tubule of the same nephron. By triggering the
renin-angiotensin system, a hormonal cascade that exerts the homeostatic control of arterial
In virtue of its ability to synthesize and secrete hormones, the kidney also exerts multiple endocrine functions. The two main hormones produced by kidney are erythropoietin and calcitriol. The first is a glycoprotein secreted by the peritubular cortical interstitial fibroblasts that stimulates red blood cell production in response to anemia. The second is the biologically active form of vitamin D produced by the proximal convoluted tubular cells. Calcitriol has been shown to be involved both in suppression of renin production and in maintenance of calcium homeostasis by regulating calcium reabsorption in distal convoluted tubules and medullary collecting ducts (Freundlich et al., 2008; Wang et al., 2012).

1.4 Engineering approaches to regenerate the kidney

Many investigation groups aimed at creating 3D kidney tissue ex vivo by exploiting the MM and UB cells’ innate ability to reciprocally interact and instruct each other, as well as the mouse embryonic kidney’s capacity to reconstruct itself after single-cell dissociation and reaggregation. Specifically, these studies - overviewed in the following paragraphs and summarised schematically in Figure 4 - employed embryonic kidney fragments and renal progenitor cells obtained either from rodent embryonic kidneys or human pluripotent stem cell (hPSC) directed in vitro differentiation.

1.4.1 Tissue-based approaches

The pioneering studies by Grobstein, dated early 1950s, demonstrated that MM and UB tissues could be grown ex vivo and partially recapitulate the kidney’s developmental program, giving rise to nephron formation and collecting duct branching morphogenesis,
respectively (Grobstein, 1953). Based on such discoveries, other groups co-cultured isolated intact MM with various inductors in order to attain nephrogenesis in vitro (Ekblom et al., 1981; Barasch et al., 1996). Specifically, isolated intact MM grew and matured into glomerular and renal tubular epithelia when cocultured with exogenous embryonic spinal cord explants used as nephrogenesis inductors (Ekblom et al., 1981). In another study, Barasch et al. showed that isolated MMs could be induced to undergo nephrogenesis when cultured in direct contact with UB cell pellets, without the need of spinal cord or any other exogenous tissues (Barasch et al., 1996).

With regard to UB branching morphogenesis, it was demonstrated that uninduced intact rat UB isolated from surrounding MM could undergo branching morphogenesis in a 3D culture system (Qiao et al., 1999a). Specifically, UB was embedded in ECM-mimicking gel, and cultured in conditioned medium from an E11.5 mouse MM-derived cell line (BSN cells), which was supplemented with glial cell line-derived neurotrophic factor (GDNF) (Qiao et al., 1999a). This molecule is expressed by the MM during the early stage of kidney development and is essential for UB formation, growth and morphogenesis (Sainio et al., 1997). Under these culture conditions, the UB developed into 3D polarised and extensively branched epithelial tubular structures endowed with a single lumen, without the need of direct contact with MM tissue. These branched tubular structures were capable of inducing nephrogenesis when recombined with E13.5 rat MMs in vitro. Remarkably, MM-derived nascent nephrons were connected to the UB tubular structures to form a continuous lumen, and, in turn, UB responded to the morphogenetic inductive effects of the adjacent MM by further growing and extending branches into the MM tissue. These data demonstrated that (i) UB branching morphogenesis does not require direct contact with MM, (ii) the in vitro-derived branched tubular structures retain their intrinsic ability to induce nephrogenesis as occurs in vivo, and (iii) the regulation of branch elongation, as
well as the appropriate branching pattern establishment, take place only when UB was physically combined in vitro with MM (Qiao et al., 1999a). This 3D culture system was also used to propagate isolated rat UBs in vitro (Steer et al., 2002). The isolated intact UBs were cultured and induced to undergo branching morphogenesis, then after 8 days of culture were subdivided into thirds and each portion cultured independently for additional 8 days. This process was repeated for several UB generations. Of note, propagated UBs recombined with freshly isolated MMs were able to induce nephrogenesis and retained the ability to connect with the MM-derived tubules, leading to the formation of ‘neokidneys’ containing tubular structures comparable to those of whole rat embryonic kidney cultures (Steer et al., 2002). Building on this strategy, Rosines et al. devised an approach that sequentially induced an isolated ND to bud and branch in vitro, demonstrating that the capacity to undergo branching morphogenesis in vitro is not retained exclusively by the UB (Rosines et al., 2007). When freshly isolated MM was recombined with a ND-derived branched structure, the 3D nephron formation occurred, similarly to that obtained in UB-derived branched tubular structures and MM recombination experiments. The MM derivatives appeared phenotypically normal, expressed tubular transporters and were capable of organic ion transport, all features demonstrating maturation and functional capacity of the recombined tissue. Moreover, when the recombined kidney-like tissue was implanted beneath the renal capsule of a rat host, it developed multiple glomeruli positive for endothelial markers and containing erythrocytes. Finally, through global gene expression pattern analyses, it was demonstrated that the transcriptome of the nephron structures that had developed within the recombined tissue bore a resemblance to the transcriptome of the E18 rat kidney (Rosines et al., 2007).
1.4.2 Creating 3D kidney tissue using embryonic kidney cells and cell lines

One of the most promising approaches in the field of tissue engineering is the generation of ‘foetal’ organs by exploiting the progenitor cells’ intrinsic capacity to organise themselves into 3D structures typical of early organogenesis (Xinaris et al., 2015). Traditional reaggregation experiments revealed that embryonic kidney cells are able to differentiate and self-organise to form 3D tubular structures. In these pioneering studies, Auerbach and Grobstein developed a method for *in vitro* generation of a 3D tissue containing rudimental nephron-like structures through MM dissociation into single cell suspensions followed by reaggregation and co-culture onto embryonic dorsal spinal cord cell layer (Auerbach and Grobstein, 1958). Other early studies focused on kidney tissue generation via cell-based strategies demonstrated that adult renal epithelial cell lines, cultured in ECM gel-based 3D culture systems, could undergo tubulogenesis in the presence of conditioned media or embryonic kidneys (Montesano et al., 1991a, 1991b; Steer and Nigam, 2004)

These studies showed that when Madin-Darby canine kidney (MDCK) cells and murine inner medullary collecting duct (mIMCD3) cells were embedded in ECM gel and cultured with fibroblast-conditioned medium (Montesano et al., 1991a, 1991b) or with embryonic kidneys in the absence of direct contact (Santos et al., 1994; Barros et al., 1995) underwent branching tubulogenesis.

Using a similar approach, Machiguchi and Nakamura implanted individual tubular epithelial cells and mesenchymal stem cells into the subcutaneous spaces of immunodeficient rats, and stimulated them with a combination of vascular endothelial- and tubular cell-derived conditioned media to form glomerular and renal tubular structures (Machiguchi and Nakamura, 2013).
A step forward was made through the derivation and \textit{in vitro} propagation of immortalised cell lines from uninduced MMs (BSN cell line) and unbranched UBs, which were isolated from E11.5 mouse embryonic kidneys (Sakurai \textit{et al.}, 1997). Using these cells, Sakurai \textit{et al.} developed a method for establishing a tubulogenesis system \textit{in vitro}. In particular, the authors reported that, when cultured in a 3D ECM-gel with BSN cell-conditioned medium, UB cells formed cell processes and then multicellular cord-like structures - morphogenetic changes peculiar of early \textit{in vitro} branching tubulogenesis. The multicellular cord-like structures eventually branched and gave rise to ramified and polarised epithelial tubular structures endowed with lumen comparable to those observed in cultured intact embryonic kidneys.

A more recent \textit{in vivo} study showed that MM and UB cell lines cocultured in 3D matrices and implanted in immunodeficient mice, organised into spheroid and tubuloid structures that matured to finally form epithelia with lumens surrounded by capillary-like structures (Velagapudi \textit{et al.}, 2012). In particular, UB cells formed almost exclusively the tubuloid structures and expressed UB/collecting duct markers, whereas MM cells differentiated mainly into capillary-like cells. Although the tubuloid structures exhibited specialised epithelial features such as apical vacuoles, microvilli, junctional complexes and linear basement membranes, the formation of brush borders and the expression of aquaporin 1 - features of mature proximal tubular epithelium - were not observed (Velagapudi \textit{et al.}, 2012).

In the last decade, several groups showed that kidney tissue could be generated using single cells freshly isolated from embryonic kidneys as starting material (Osafune \textit{et al.}, 2006; Unbekandt and Davies, 2010; Xinaris \textit{et al.}, 2012; Ganeva \textit{et al.}, 2011; Xinaris and Yokoo, 2014).
By modifying previous culture protocols (Grobstein, 1953; Kispert et al., 1998), Osafune et al. demonstrated that murine E11.5 MM progenitor cells strongly expressing Sall1 - a zinc-finger nuclear transcription factor expressed in the MM and essential for kidney development (Nishinakamura et al., 2001) - generated 3D colonies when cultured onto a feeder layer of a mouse embryonic fibroblast cell line stably expressing Wnt4, a glycoprotein required to trigger nephrogenesis in isolated MM (Kispert et al., 1998; Osafune et al., 2006). The colonies reconstituted a 3D kidney tissue consisting of glomerular- and tubular-like structures positive for the podocyte marker Wilm’s tumor 1 (WT1) and the proximal tubule marker Lotus tetragonolobus lectin (LTL), respectively (Osafune et al., 2006). Although functional features of these structures were not investigated in vivo, this bioengineering approach suggested that rudimental 3D kidney tissues could be obtained from single cell suspensions.

Building on Grobstein’s group studies (Grobstein, 1955; Auerbach and Grobstein, 1958), Unbekandt and Davies set up a novel method to generate 3D embryonic kidney tissue through E11.5 mouse kidneys dissociation into single cell suspensions followed by reaggregation, without the need for any exogenous tissue (Unbekandt and Davies, 2010). A key innovation of this method was the transient culture (for the first 24 hours) of the cell reaggregates with a Rho-associated kinase inhibitor in order to enhance cell self-organisation and UB branching reactivation, and prevent cell death for apoptosis, which is due to the dissociation process (Schmidt-Ott, 2010; Unbekandt and Davies, 2010). Even though this protocol allowed the engineering of embryonic kidney tissue containing nephrons at different developmental stages and with normal anatomies, the UB developed as a multitude of small individual collecting duct trees, rather than a single highly branched UB/collecting duct tree, as normally occurs during kidney organogenesis. Furthermore, this would mean that, assuming that the tissue were vascularised and physiologically
active, the multiple individual collecting ducts would not be effective for draining urine away towards a common exit. To overcome the above limitation, the authors refined the system by culturing, for 3-4 days, MM cell reaggregates in combination with a single reformed UB dissected from a reaggregated tissue of a previous round of dissociation-reaggregation. This manoeuvre led to the engineering of 3D embryonic kidney tissue containing immature nephrons directed outwards and each distally connected to a branch of the single UB-derived collecting duct tree. Such configuration resembled the normal in vivo kidney tissue patterning (Ganeva et al., 2011).

Although the dissociated and reaggregated MM (drMM) maintains its nephrogenic potential, if nephrogenesis is induced following the dissociation step, drMM degenerates and undergoes apoptosis (Koseki et al., 1992; Saxén, 1987). To address this problem, a novel protocol that allows drMM to maintain its nephrogenesis competence ex vivo has been recently developed (Junttila et al., 2015). In particular, drMM was cultured in the presence of bone morphogenetic protein 7 (BMP7) and fibroblast growth factor 2 (FGF2) - both showed to promote the survival of isolated intact MM - for 24 hours before nephric tubule induction (Junttila et al., 2015). As a result, when cultured onto the dorsal piece of an E11.5 embryonic spinal cord as inducer of tubulogenesis, the uninduced drMM cells survived and assembled into segmented nephrons expressing markers of glomerular podocytes, proximal and distal tubules, and ascending loop of Henle. Furthermore, to evaluate potential for kidney organogenesis in vitro, the BMP7/FGF2-treated MM cell reaggregates were combined with one freshly isolated intact UB, which was previously stimulated with GDNF, to form explants. After 9 days, explants displayed a unique branching UB, multiple developing nephrons around the UB tips, and renal corpuscle-like structures, as occurs during normal kidney organogenesis. Consequently, the major advantage of this novel protocol for kidney tissue engineering was the extension of the in
vitro culture time of the uninduced drMM, which in the above-mentioned culture conditions survived and maintained its nephrogenic potential with no sign of apoptosis (Junttila et al., 2015).

Despite the advances in the field, none of these cell-based strategies has favoured the development of vascularised glomeruli with appropriately structured glomerular filtration barrier, due to the avascular in vitro environment. Hence, this important impediment to the generation of mature and functional kidney tissue from renal progenitor cell suspensions persisted.

In order to overcome this obstacle, our group, building on previously reported technologies (Auerbach and Grobstein, 1958; Unbekandt and Davies, 2010), generated kidney organoids in vitro and implanted them into living recipients. Once in vivo, organoids further grew and matured, developing vascularised glomeruli and functional nephrons (Xinaris et al., 2012). Specifically, organoids were constructed using single cell suspensions of fully dissociated E11.5 mouse kidneys and, after growing in vitro for 5 days - the time they develop elongating tubular profiles with distal poles connected to the adjacent branching UB/collection duct epithelia - they were implanted below the renal capsule of unilaterally nephrectomised athymic rats. The unilateral nephrectomy - a surgical intervention known to enhance the expression of several mitogenic, prosurvival, and morphogenic genes in rodents (Siegel et al., 1996; D’Agati, 2012) - was performed to promote growth and maturation of the grafted tissue.

Two pivotal steps introduced with this optimised reaggregation method were the generation of kidney organoids as large cell aggregates to allow survival and growth of the implanted tissues in vivo and, to promote endogenous vascular development, their pretreatment with vascular endothelial growth factor (VEGF) before implantation, followed by systemic VEGF injections into the host animals. This second manoeuvre
restored the podocyte-endothelial cell stimulation mediated by VEGF, which drives the formation of glomerular capillary loops (Kitamoto et al., 1997, 2002; Tufro et al., 1999; Guan et al., 2006). Indeed, during kidney development, endothelial progenitor cells residing in the renal stroma express VEGF receptors and migrate into the vascular cleft of developing glomeruli - where they will form the glomerular capillary loops - in response to podocyte secretion of VEGF (Dressler, 2006; Jeffrey and Miner D.R.A., 2012).

As a result, the implanted organoids grew, became vascularised, and developed glomeruli with fenestrated capillaries and fully differentiated podocytes having foot processes spaced by slit diaphragms (Xinaris et al., 2012). Furthermore, implanted tissues exhibited basic kidney-specific functions, including capacity for proximal tubular reabsorption of systemically injected labelled macromolecules that reached tubular lumen by transglomerular passage, and production of erythropoietin by stromal cells when recipient animals were made anaemic (Xinaris et al., 2012). More in-depth investigations combining electron microscopy analysis and macromolecular tracing experiments revealed that, in vivo, the organoids recapitulated the extremely complex 3D structural framework of the glomerular slit diaphragm, and performed selective glomerular filtration and tubular reabsorption (Xinaris et al., 2015).

The organoid system is a reliable tool to validate the renal differentiation potential of human stem cells from different sources. By using this technology, several groups determined the human stem cells’ capacity to follow renal developmental program and their contribution to developing kidney compartments (Siegel et al., 2010; Papadimou et al., 2015; Xinaris et al., 2016). Siegel et al. showed that, when mixed with suspension of mouse embryonic kidney cells and reaggregated to form 3D chimeric organoids, human amniotic fluid stem cells (hAFSCs) integrated into developing kidney structures positive for PAX2 - a marker expressed in UB and developing nephrons (Rothenpieler and
Dressler, 1993) - and calbindin - a specific marker for UB and distal tubules (Siegel et al., 2010) - wherein they also started to express both markers.

More recently, our group applied the optimised technology described above (Xinaris et al., 2012) to generate 3D chimeric organoids from E11.5 mouse kidney cells and hAFSCs that were genetically modified to express GDNF (Xinaris et al., 2016; Benedetti* et al., 2016) in order to enhance their contribution to developing nephrons (Yokoo et al., 2005). This manipulation promoted hAFSCs integration in the caps of condensing MM positive for neural cell adhesion molecule (NCAM) - a specific marker of condensed MM (Bard et al., 2001) - and PAX2 (Xinaris et al., 2016). When implanted beneath the renal capsule of athymic rats, chimeric organoids grew, developed tubular structures and glomeruli that contained red blood cells, thus revealing the establishment of vascular connection between host and graft. In the implanted tissues, the majority of hAFSCs incorporated in glomerular structures, and differentiated into mature podocytes endowed with interdigitating foot processes separated by well-formed slit diaphragms (Xinaris et al., 2016). Remarkably, the hAFSC-derived podocytes displayed active endocytosis of systemically injected fluorescent bovine serum albumin (BSA), a functional feature typical of normal podocytes in vivo (Eyre et al., 2007).

This chimeric organoid system have been also applied by our group to validate renal differentiation and integration potentials of various cell types, such as the HK2 renal proximal tubular epithelial cell line, human bone-marrow mesenchymal stem cells (hBM-MSCs), and hBM-MSC-derived proximal tubular-like epithelial cells (Papadimou et al., 2015; Benedetti* et al., 2016). The latter cell type (CL17) was generated by direct hBM-MSC reprogramming using HK2 cell extracts. In 1-day chimeric aggregates, CL17 cells incorporated into the condensing MM. At 5 days, both CL17 and HK2 cells formed chimeric elongating tubular structures adjacent to early glomerular-like structures.
Otherwise, hBM-MSCs did not form or contribute to renal structures, thus indicating neither nephrogenic nor integration potential (Papadimou et al., 2015).

1.4.3 Creating 3D kidney tissue from pluripotent stem cells

Pluripotent stem cells (PSCs) are able to self-renew indefinitely in vitro while maintaining the capacity of differentiating into specialised derivatives of all the three primary germ layers (Yu and Thomson, 2008). Due to these characteristics, human PSCs (hPSCs) emerged as an extremely valuable source for studying human development, modelling disease, testing drugs, and establishing strategies aimed at engineering human tissues in vitro. The hPSCs are classed as human embryonic stem cells (hESCs), which derive from the inner cell mass of a blastocyst (Thomson et al., 1998), and human induced pluripotent stem cells (hiPSCs). The latter have been generated by the Shinya Yamanaka’s group, first from mouse (Takahashi and Yamanaka, 2006) and then from human (Takahashi et al., 2007) somatic cell reprogramming. Specifically, the authors induced adult terminally differentiated donor cells to revert to a pluripotency state by transfection of four selected transcription factors (OCT4, KLF4, SOX2 and c-myc). The hiPSCs possess morphology, proliferation capacities and differentiation potential highly comparable to hESCs and, as their generation does not entail destruction of human embryos, they allow circumventing all ethical issues associated with the use of hESCs. Importantly, hiPSCs, which can be derived from both healthy people and patients, are, accordingly, immunocompatible with the original donor, thus avoiding the need for immunosuppressants when transplanted back into the donor organism. For all these reasons, the discovery of hiPSCs gave way to a new era in stem cell biology and regenerative medicine, creating significant prospects for the in vitro generation of renal progenitor cells as source for engineering immunocompatible kidney tissue applicable to transplantation.
In the last few years, intense research has been dedicated to develop protocols for differentiating hPSC towards specific renal progenitor cell types. Mae et al. differentiated hPSCs (both hESCs and hiPSCs) towards IM cells (Mae et al., 2013) in a stepwise fashion. Initially, hPSCs were stimulated with Activin A and the Wnt agonist CHIR99021 for 3 days to differentiate into mesoderm, and then were treated with BMP7 and CHIR99021 for an additional 20 days to generate IM cells highly expressing OSR1, a specific marker of IM. The authors documented that these cells also expressed several markers specific to cell types of both embryonic (ND, UB, MM and metanephric stromal cells) and mature kidney. However, when hiPSCs-derived IM cells were cocultured with E11.5 mouse metanephric cells to generate 3D chimeric aggregates, they formed polarised proximal tubular-like structures with low efficiency and exhibited scarce differentiation capacity into UB cells, indicating a limited developmental potential.

In view of these limitations, the same group improved the efficiency of the protocol by skipping the stage of mesoderm induction and stimulating the hiPSCs exclusively with small molecules - CHIR99021 and retinoic acid receptor agonists - for 5 days. This modification significantly increased the capacity of hESC/hiPSC-derived IM cells to form proximal tubular-like structures within chimeric aggregates (Araoka et al., 2014).

Following a similar line of research, Lam et al. reported a robust protocol for hPSC differentiation into IM cells that spontaneously formed tubule-like structures (Lam et al., 2014). Initial treatment of hPSCs with CHIR99021 for 1-2 days induced mesendoderm differentiation. Cells were then treated with a combination of FGF2 and retinoic acid for 3 days to generate PAX2- and LHX1-positive IM cells with high efficiency. When the authors withdrew growth factors from culture media, differentiated cells proliferated and formed polarised tubular epithelial structures positive for the proximal tubule markers LTL and N-cadherin, and endowed with primary cilia on the apical surface. Nevertheless, when
recombined with E12.5 mouse kidney cells to form chimeric kidney explants, these cells partially contributed to laminin-bounded tubular structures, indicating a low integration potential into developing kidney tissue. In another set of experiments, PAX2- and LHX1-positive IM cells were stimulated with FGF9 - a growth factor known to induce and maintain nephron progenitors in vitro (Barak et al., 2012) - and Activin A to differentiate into CM cells expressing SIX2, SALL1 and WT1. In chimeric kidney explants, these hPSC-derived CM cells were found in organising clusters of LTL-positive cells (Lam et al., 2014).

Xia et al. established a rapid and efficient two-step protocol for hESCs and hiPSCs directed differentiation into UB progenitor-like cells (Xia et al., 2013). In the first step, hPSCs were committed to mesodermal fate by exposure to BMP4 and FGF2 for 2 days. In the second step, combined treatment with BMP2, Activin A and retinoic acid drove cells to acquire a late IM/UB-like phenotype. After 4 days of differentiation, cells expressed high levels of IM and ND/ureteric epithelium markers (OSR1, LHX1, PAX2 and GATA3), and UB-specific markers (HOXB7, RET and GFRA1), rather than MM markers. To investigate the hPSC-derived UB-like progenitor cells for their propensity to contribute to complex 3D renal structures, the authors cocultured them with E11.5 mouse kidney cells to form 3D chimeric organoids. Under these culture conditions, the human cells exclusively integrated into the murine developing UB structures, indicating efficient in vitro UB-lineage commitment and ex vivo maturation capacity in response to renal developmental cues from mouse embryonic kidney cells (Xia et al., 2013, 2014). Moreover, the chimeric UB epithelia became polarised along the apical-basal axis and induced MM condensation and nephrogenesis, as occurs in vivo. In agreement with the absence of MM marker expression, differentiated human cells did not integrate at all in the MM derivatives.
In the past few years, significant advances towards the generation of multilineage and/or functional kidney organoids have been made by exposing hPSCs to chemically defined culture conditions that more accurately mimic early kidney developmental cues in vitro.

Working on this approach, Takasato et al. established a protocol for the stepwise simultaneous differentiation of hESC monolayers towards UB and MM cells under chemically defined culture conditions (Takasato et al., 2014). In the first step, hESCs were induced to posterior primitive streak by exposure to either BMP4 and Activin A or CHIR99021 alone. Next, these cells were differentiated into IM cells using FGF9 and heparin. Finally, IM cells were stimulated with a combination of FGF9, heparin, BMP7 and retinoic acid to derive MM cells. Otherwise, CHIR99021-treated cell lines were deprived of any growth factors for 12 days to generate a mixed population of both UB and MM progenitor cells. When reaggregated following monolayers’ dissociation into single cell suspensions, UB and MM progenitor cells self-organised to form human 3D kidney tubular structures in the absence of any other supporting cell type or tissue. Furthermore, upon coculture with E12.5 murine kidney cells to form chimeric aggregates, the hESC-derived kidney progenitor cells integrated into all cellular compartments and epithelia of the developing kidney tissue (Takasato et al., 2014).

In parallel, Taguchi et al. optimised a differentiation protocol to derive MM cells expressing WT1, PAX2, SALL1 and SIX2 from both mouse ESCs and hiPSCs, by first defining MM developmental origins through in vivo lineage-tracing studies, and then by accurately mimicking them in vitro (Taguchi et al., 2014). The authors demonstrated that MM progenitor cells derive from the posterior nascent mesoderm expressing Brachyury - a primitive streak and posterior nascent mesoderm marker (Herrmann et al., 1990) - and they established a combination of factors for inducing posterior mesoderm and its subsequent differentiation into MM. To induce posterior mesoderm differentiation, embryoid bodies...
EBs from PSCs were initially treated with Activin A alone, and then with the combination of BMP4 and CHIR99021. Subsequently, IM identity was achieved by combined treatment with Activin A, BMP4, CHIR99021 and retinoic acid. Finally, differentiation into MM cells was accomplished using CHIR99021 and FGF9. Coculture of MM cell-aggregates with mouse embryonic dorsal spinal cords resulted in MM cell maturation and reconstitution of contiguous 3D glomerular structures, and proximal and distal tubules. When co-transplanted with dorsal spinal cords below the kidney capsule of immunodeficient animals, the EB-derived MM cell aggregates underwent massive tubulogenesis and developed vascularised glomeruli containing erythrocytes, indicating the graft’s connection to host circulation (Taguchi et al., 2014). Although vascularised glomeruli are required for blood filtration, neither urine production nor any other kidney functions were shown.

Later, the same group, generated hiPSC lines expressing green fluorescent protein (GFP) in the *NPHS1* locus - which encodes the podocyte slit diaphragm protein nephrin - and, differentiating these cells according to previous protocol, induced formation of 3D GFP-glomerular structures containing nephrin-positive podocytes with primary processes and immature slit diaphragm-like structures (Sharmin et al., 2016). Next, the authors transplanted the sorted 3D GFP-positive glomerular structures beneath the host kidney capsule. Here, the glomerular structures further matured, were vascularised by host endothelial cells, and developed podocytes with foot processes wrapping the fenestrated endothelium (Sharmin et al., 2016). Importantly, the hiPSC-derived podocytes displayed both gene expression pattern and structural features typical of *in vivo* podocytes.

By recapitulating metanephric kidney development *in vitro*, Morizane et al. have recently published a differentiation protocol to obtain MM cells expressing *SIX2, SALL1* and *WT1* from hPSCs. Moreover, the authors demonstrated that the hPSC-derived nephron
progenitor cells could spontaneously assemble into human kidney organoids in both 2D and 3D culture conditions (Morizane et al., 2015). Specifically, to stimulate consecutive differentiation into primitive streak and posterior IM, hiPSCs were cultured in the presence of CHIR99021 and Noggin - a BMP4 signaling antagonist - for 4 days, and then with Activin A alone for additional 3 days, respectively. To establish MM identity and induce cell self-organisation, cells were stimulated with FGF9 for another 7 days, with transient 2-day treatment with CHIR99021 starting on day 9. Finally, cell culture in the absence of growth factors for additional 14 days led to the spontaneous formation of hPSC-derived ‘nephron organoids’ containing appropriately segmented nephrons, but completely lacking of UB derivatives. Remarkably, by exposing organoids to commonly used antibiotics and anticancer drugs, the authors demonstrated that they could be used as a tool to investigate mechanisms of proximal and/or distal tubular toxicity in vitro (Morizane et al., 2015).

Almost simultaneously, the same group showed that hPSC-derived kidney cells could self-organise to generate tubular organoids, recapitulate kidney epithelial transport, and, following genome editing, could model human kidney disease (Freedman et al., 2015). The authors developed a 3D culture system for sequential generation of hPSC-derived epiblast spheroids and kidney tubular organoids. In this system, hPSCs were first embedded in Matrigel to spontaneously form spheroids consisting of a polarised epithelium surrounding a hollow lumen in 3 days. Subsequent exposure to CHIR99021 for 1.5 days followed by incubation in B27-supplemented media for 11.5 days induced epiblast spheroid differentiation into kidney tubular organoids in which tubules positive for podocyte, vascular, proximal and distal tubular markers developed, and exhibited transport characteristics typical of proximal tubules. Interestingly, when biallelic truncating mutations in *PKD1* (polycystin-1) or *PKD2* (polycystin-2) genes were introduced in hPSCs through the CRISPR/Cas9 genome editing technology, kidney tubular organoids formed
large LTL-positive cyst-like structures alongside proximal tubular structures. Although this represents the first report of 3D kidney organoids capable of modelling human kidney disease in vitro, cysts formation was detected in only 6% of the self-forming organoids.

In parallel, Takasato et al. modified their previous protocol (Takasato et al., 2014) in order to induce hiPSC differentiation and self-organisation into 3D kidney organoids composed of UB epithelia and MM-derived segmented nephrons, endothelial cells and renal stroma (Takasato et al., 2015). The in vitro self-forming organoids resulted transcriptionally similar to first trimester human foetal kidneys. Moreover, from the bottom to the top regions, organoids showed multiple individual collecting ducts linked to the distal and proximal tubules, and glomeruli containing podocytes with primary and secondary foot processes. The proximal tubular cells were capable of absorbing labelled macromolecules and underwent specific apoptosis in response to cisplatin, indicating tubular functional maturation (Takasato et al., 2015).

Very recently, by recapitulating kidney developmental stages in vitro, our group set up a robust three-step protocol for hiPSC differentiation into functional podocytes (Ciampi et al., 2016). The hiPSCs were first specified into IM, then committed to nephron progenitor cells and, finally, differentiated into mature podocytes with typically low proliferation rate. The hiPSC-derived podocytes resulted able to endocytose and accumulate albumin in the perinuclear regions, and displayed cytoskeletal reorganisation when treated with Angiotensin II, a known inducer of podocyte damage. Moreover, when human podocytes from differentiation day 13 were reaggregated with E12.5 mouse kidney cells to form chimeric organoids, they incorporated into WT1-positive developing structures, where they also started to express WT1 (Ciampi et al., 2016).

The technologies described in the paragraphs above strongly fostered the creation of kidney epithelial tubular structures that, in part, resemble their counterparts in vivo, and
hPSC-derived kidney organoids displaying considerable structural complexity and functional maturation. However, they cannot accurately replicate normal kidney tissue patterning, due to the organ’s complex architecture and the finely orchestrated interactions occurring between ECM and several cell lineages during organogenesis. Indeed, as kidney tubule and organoid formation solely relied on cell-driven self-assembly - reason why cells were not geometrically directed to acquire appropriate spatial configuration - engineered tubules were heterogeneous in size and shape and far from being anatomically realistic, whereas kidney organoids displayed serious developmental anomalies and structural malformations. First and foremost, within the organoids, the UB was either totally missing or developed as a multitude of small individual ducts (Taguchi et al., 2014; Freedman et al., 2015; Morizane et al., 2015; Takasato et al., 2015; Xia et al., 2013; Xinaris et al., 2016) instead of a unique collecting duct system of tubules that could drain urine away to a single exit if the tissue were vascularised. These anatomical deficiencies prevented development and maturation of kidney tissue arranged organotypically around one single and branched UB/collection duct system, as instead occur during normal kidney organogenesis. Another issue is the short viability (3-4 weeks) of the organoids in vivo, as they soon after begin to degenerate.

Additional technical insufficiencies of the existing tubule engineering systems are the requirement for extensive cell culturing - from several days to weeks (Montesano et al., 1991a; Sakurai et al., 1997) - as well as the need for other cell types (Montesano et al., 1991a), tissues (Steer et al., 2002; Santos et al., 1994) or conditioned media (Montesano et al., 1991b; Sakurai et al., 1997).

Overall, the constrictions described above hinder the reliability, reproducibility, cost-effectiveness, and applicability of all these technologies. Moreover, systems for
engineering pure kidney units developed so far have not been adopted when using human cell sources, thus limiting investigations concerning human kidney developmental and pathophysiological mechanisms.

To summarise, the generation of renal tubular structures and hPSC-derived kidney organoids has created considerable advances in the field, providing opportunities for disease modelling and toxicology studies. However, the usefulness of these tissues is limited because of serious technical weak points and anatomical deficiencies related to the UB development, namely the lack of collecting ducts or their random formation as multiple disconnected units within the organoids.

Although Xia et al. attempted to derive collecting duct cells from hPSCs, they obtained UB-like cells that did not demonstrate significant UB tissue formation and maturation capacities (Xia et al., 2013).

Therefore, developing 3D tissue engineering systems that could direct the generation and growth of unique and complex UB/collecting duct units from hiPSCs may be extremely helpful for creating more realistic human kidney tissue that bona fide resembles normal kidney anatomy and physiology, and that can be used for disease modelling and drug testing/discovery studies. Moreover, this kind of engineering system would allow the generation of patient-specific kidney tissue - an unresolved challenge in the field - useful for studying kidney diseases caused by abnormal developmental processes that cannot be investigated due to the shortage of suitable human samples.
1.5 Specific Aims

1) To develop and optimise a 3D culture method to engineer functional epithelial kidney tubules using micro-patterned scaffolds and a distal tubule/collecting duct renal cell line.

2) To use this method as a tool to:
   - Model kidney disease and perform drug testing/discovery studies;
   - Engineer human epithelial tubules using hiPSC-derived UB progenitor-like cells;
   - Study human UB developmental processes and defects.
CHAPTER 2 - MATERIALS AND METHODS
2.1 PDMS scaffold fabrication

Polydimethylsiloxane (PDMS) scaffolds (Sylgard 184 Silicone elastomer kit, Dow Corning, Midland, MI) were fabricated by NanoMed Labs (University of Genova, Italy) using a 3-step prototyping approach: scaffold design, 3D printing and replica moulding. First, by using Computer Aided Design (CAD) software (Autodesk 123D® Design ©2014 Autodesk Inc.), scaffolds were designed with different geometries: (i) three scaffolds with width and depth of 1 mm: a linear one with a straight cavity, a bifurcated one with terminal 80° branching and an asymmetrical one with two 30° lateral branches extending from the central trunk; (ii) two more complex ramified and tree-like scaffolds, the latter containing a fractal-like pattern, both 0.7 mm in depth, and ranging in width from 0.7 to 0.5 mm, respectively; (iii) a multichannel scaffold containing 11 linear microchannels, which were 0.4 mm in depth x 0.4 mm in width x 9 mm in length, within a miniaturised culture chamber (1 mm in depth).

3D printing technology (Professional Desktop Pico Plus39 stereolithographic 3D printer, Asiga Global Helpdesk) was applied to build plastic masters (Asiga PlasWhite photopolymer resin). The printer uses an upside-down system with nominal XY pixel resolution down to 39 μm and servo resolution of 250 nm. The masters were printed at 0.5 seconds of exposure time, with a printing speed of 0.33 cm/hour and 10 μm vertical step size. After printing, masters were cleaned by rinsing with isopropyl alcohol for 5 minutes, washed in distilled water, dried and exposed to UV light (365 nm) (Bio-Link-BLX-365 nm, 80 W, Vilber Lourmat) for 40 minutes to complete curing. To facilitate the demoulding of the polymeric replicas, masters were functionalised with an oxygen plasma treatment (Tucano plasma reactor Gambetti Kenologia) and with the deposition, from vapour phase, of an anti-sticking layer of Trichloro (1H,1H,2H,2H-perfluorooctyl) silane (FOTS, 448931-10G, Sigma-Aldrich, Saint Louis, Missouri, USA). Subsequent REplica
Moulding (REM) steps provide several polymeric replicas starting from a single master as previously described (Angeli et al., 2015). Briefly, the PDMS base and the curing mixture (10:1 w/w) were cast into 3D printed masters, degassed using a vacuum desiccator for 20 minutes, cured at 60 °C in oven for 2 hours, and then peeled off the master mould. Finally, the bottom side of the cavities was closed up with a 20 μm-thick PDMS layer. After assembling, cavities were open-topped and completely made of PDMS. In order to enable high-resolution imaging, the thickness of the PDMS scaffolds was maintained between 0.7 and 1 mm. Scaffold hydrophobicity was preserved to avoid cell attachment. PDMS scaffolds were re-used after standard sterilisation procedures.

2.2 Madin-Darby canine kidney (MDCK) cell culture

The MDCK type II cell line (MDCK II; 00062107; Lot Number 06D026; Origin: Canine Cocker Spaniel Kidney; European Collection of Authenticated Cell Cultures, ECACC, Salisbury, UK) was maintained in Minimum Essential Medium Eagle (MEM) (M5650; Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum (FBS) (10270; Invitrogen Corporation, Carlsbad, CA, USA), 1% L-glutamine (25030024; Invitrogen), 1% penicillin and streptomycin (Pen-Strep) (15140122; Invitrogen). Cells were cultured as monolayers in tissue culture-treated T75 (75 cm²) flasks (353136; Corning Falcon®, Corning, NY, USA) under standard conditions (37°C incubator, humidified atmosphere of 5% CO₂). For passaging, 70-80% confluent MDCK cells were washed once with sterile filtered pre-warmed BioWhittaker® 1x phosphate buffered saline (PBS) pH 7.2 without Ca²⁺ and Mg²⁺ (BE17-516F; Lonza, Verviers, Belgium), and then were treated with 3 ml of pre-warmed 0.25% Trypsin-Ethylene diaminetetraacetic Acid (EDTA) (25200056; Invitrogen) for 4 minutes at 37°C. Detached cells were gently pipetted 2-3 times to ensure complete dissociation of any remaining clumps and dislodgement of any cells still attached to the
bottom of the flask. Next, cells were collected in a 50 ml conical tube containing the appropriate volume of culture medium, counted and seeded in T75 flasks at a density of 1.6x10^4 cells/cm^2 in 15 ml of fresh culture medium. Cells were maintained for 3 days without culture medium change until subsequent passaging.

2.3 Human induced pluripotent stem cell (hiPSC) culture

The hiPSC lines used in our experiments were derived from healthy donor somatic cells by different technologies and characterised as previously described (Imberti et al., 2015; Ciampi et al., 2016). Specifically, hiPSC clone IV was obtained from human dermal neonatal fibroblasts via STEMCCA lentivirus-mediated reprogramming (Imberti et al., 2015), whereas hiPSC#16 cells were derived from peripheral blood mononuclear cells (PBMCs) through non-integrative Sendai virus-mediated reprogramming (Ciampi et al., 2016). For feeder-free culture conditions, cells were maintained in mTeSR1 medium enriched with mTeSR1 5X Supplement (05850; Complete Kit; StemCell Technologies, Vancouver, Canada), and cultured as monolayers on Matrigel hESC-qualified Matrix (354277; Corning)-coated 100x20 mm dishes (353003; Corning) under standard conditions. Matrigel hESC-qualified Matrix was used following the manufacturer’s instructions. Briefly, aliquots were prepared according to the dilution factor indicated on the Certificate of Analysis and frozen at -80°C. One aliquot was thawed on ice and added to 25 ml of ice-cold DMEM/F12+GlutaMAX (31331028; Invitrogen) to coat the dishes (5 ml/dish), which were then incubated at room temperature for at least 1 hour before use. The remaining coating liquid was aspirated from the dish just before hiPSC seeding on top of the thin gel layer.

When 80% confluent, hiPSCs were abundantly washed with PBS and then treated with 3 ml Accutase (a1110501; Invitrogen) for 4 minutes at 37°C for passaging. Detached cells
were gently pipetted 2-3 times to ensure complete dissociation of any remaining clumps and dislodgement of any cells still attached to the bottom of the dish. Next, cells were collected in 50 ml conical tube containing culture medium, counted, and seeded onto Matrigel hESC-qualified Matrix-coated dishes at a density of $4.5 \times 10^4$ cells/cm$^2$ in the appropriate volume of fresh mTeSR1 complete medium supplemented with 10 μM Y-27632 dihydrochloride [Rho-associated protein (ROCK) inhibitor; 07172; Sigma-Aldrich] for the first 24 hours to avoid/reduce cell death for apoptosis. The hiPSCs were maintained in 10 ml of mTeSR1 complete medium for 3-5 days, with daily medium change, until subsequent passaging.

2.4 Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs (lymphocytes, monocytes, and thrombocytes) were isolated under sterile conditions from 40 ml of whole blood taken from a patient carrying a heterozygous mutation in the $PAX2$ gene. Patient’s whole blood was collected in sterile 50 ml conical tube and 10 U/ml heparin (Pharmatex Italia, Milan, Italy) were added to prevent blood coagulation. The blood was diluted 1:1 with saline solution (Pharmatex Italia) and 20 ml of diluted sample were delicately layered over 15 ml of Ficoll-Paque™ (17-1440-02; GE Healthcare Life Sciences, Little Chalfont, UK) in a total of 4 sterile 50 ml conical tubes, which were centrifuged 800xg for 20 minutes at room temperature without brake. Next, tubes were recovered from the centrifuge taking care of not disturbing the layering. The layer containing PBMCs, which was positioned at the interphase, was gently aspirated carefully avoiding Ficoll-Paque™ aspiration, and was transferred to new 50 ml conical tube. At this point, PBS was added to reach a final volume of 50 ml, and the suspension was centrifuged at 400xg for 8 minutes at room temperature. Then, supernatant was discarded and cell pellet was washed again using PBS. After discarding the supernatant, 2
ml of RPMI medium (11875093; Thermo Fisher Scientific, MA, USA) supplemented with 20% human serum-AB were added and PBMC pellet was resuspended to obtain a uniform suspension of single cells. After cell counting, aliquots of 5-10x10^6 PBMCs were gently dropped into cryovials containing 1-1.5 ml of freezing medium [RPMI medium supplemented with 20% human serum-AB and 10% dimethyl-sulphoxide (DMSO) (D2650; Sigma-Aldrich)]. After the cells were exposed to freezing medium containing DMSO, they were frozen within 2 minutes by placing cryovials in a cryofreezing container and transferring it to -80°C overnight. This manoeuvre allows freezing the cells at 1°C per minute. After overnight storage, cryovials were transferred into a liquid nitrogen tank vapour phase for long-term storage.

2.5 Generation of patient-derived iPSCs

The patient involved in this study is affected by an autosomal dominant form of Focal Segmental Glomerulosclerosis (FSGS) linked to a disease-segregating missense heterozygous genetic mutation in PAX2 gene (c.G565A, p.G189R) (Barua et al., 2014). FSGS patient-derived iPSCs were generated from PBMCs (the written informed consent was obtained from the patient in accordance with the Declaration of Helsinki guidelines) through Sendai virus-mediated reprogramming by using CytoTune-iPS 2.0 Sendai Reprogramming kit (A16517; Thermo Fisher Scientific) according to the manufacturer’s instructions as follows:

2.5.1 PBMCs seeding. Four days before infection, cryopreserved PBMCs were thawed in 37°C water bath, gently transferred into 15 ml conical tube containing 10 ml of pre-warmed complete PBMC medium [StemPro®-34 medium (10639-011) supplemented with 2 mM L-Glutamine (25030), 100 ng/ml human recombinant
SCF (c-kit Ligand) (PHC2111), 100 ng/ml human recombinant FLT-3 Ligand (PHC9414), 20 ng/ml human recombinant IL-3 (PHC0034), and 20 ng/ml human recombinant IL-6 (PHC0065). All PBMC medium components were purchased from Thermo Fisher Scientific and centrifuged at 200xg for 10 minutes at room temperature. An aliquot of cells was taken for cell counting before centrifugation. After centrifugation, supernatant was discarded, and pellet was resuspended in complete PBMC medium to 5x10^5 cells/ml. Next, 1 ml per well was dropped in 24-well plates (3527; Corning), and PBMCs were cultured as suspension of not-proliferating cells under standard conditions for the next 3 days, with daily 500 µl (half) medium change without disturbing the cells.

2.5.2 PBMC infection. The day of infection (day 0), PBMCs were harvested, and brought to 3x10^5 cells/ml into sterile, round-bottom and screw capped centrifuge tubes (352054; Corning). At this point, PBMCs were infected by adding 5 multiplicity of infection (MOI) of hKLF4-OCT4-SOX2 (hKOS) Sendai virus vector, 5 MOI of hc-Myc Sendai virus vector, and 3 MOI of hKLF4 Sendai virus vector in a total volume of 1 ml. Both Sendai reprogramming tubes’ thawing and virus volume calculation were performed following the manufacturer’s instructions. To increase transduction and reprogramming efficiencies, capped tubes were centrifuged at 2,250 rpm for 30 minutes at room temperature using the SL-16 centrifuge (Thermo Fisher Scientific). After centrifugation, PBMC pellets were resuspended in the same virus-containing medium, and 1 ml of fresh PBMC complete medium was added to each tube. At this point, PBMCs were seeded in 12-well plates (3513; Corning) and incubated overnight under standard conditions. The next day (day 1 post-infection), both cells and medium were harvested from
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the wells and transferred into centrifuge conical tube. Each well was rinsed with 1 ml of PBMC complete medium to ensure harvesting most of the cells, and volumes were added to the centrifuge tube. PBMC suspension was centrifuged at 200xg for 10 minutes, supernatant was discarded, and cells were resuspended in 500 μl of PBMC complete medium per well of 24-well plate. Cells were seeded and cultured under standard conditions for the next 2 days (day 2 and 3 post-infection).

2.5.3 Cell seeding on mouse embryonic fibroblasts (MEFs). MEFs were isolated from CD1 mouse (Charles River Italia S.p.A., Calco, Lecco, Italy) embryonic day (E) 13.5 embryos, and used as feeder layers for hiPSC generation. MEFs were cultured in T75 flasks as monolayers under standard conditions in Dulbecco’s Modified Eagle Medium (DMEM) (11960044; Invitrogen) supplemented with 10% FBS (16000; Invitrogen), 0.1 mM non-essential amino acids (11140050; Invitrogen) and 1% Pen-Strep. When 95% confluent, MEFs were mitotically inactivated by treating them with 0.01 mg/ml mitomycin-C (M4287; Sigma-Aldrich) for 2 hours, and then seeded on 6-well plates (353046; Corning) at a density of 8x10⁴ cells/cm². At day 3 post-infection, 1-5x10⁴ live PBMCs per well were seeded on mitotically inactivated MEFs in a total volume of 2 ml StemPro®-34 medium without cytokines, and cultured under standard conditions for other 3 days (day 4-6 post-infection). From day 4 to day 6, 1 ml (half) of StemPro®-34 medium without cytokines was replaced every other day with the same amount of fresh medium, taking care of not disturbing cells.

2.5.4 Transitioning-PBMC exposure to hiPSC-medium. On day 7 post-infection, 1 ml (half) of StemPro®-34 medium without cytokines was replaced with the same
amount of hiPSC-medium [DMEM/F12+GlutaMAX supplemented with 20% knock-out serum (10828028; Invitrogen), 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptopoethanol (M6250; Sigma-Aldrich), 10 ng/ml human fibroblast growth factor 2 (FGF2) (100-18B; PeproTech, NJ, USA ), and 1% Pen-Strep]. The next day (day 8 post-infection), culture medium was completely replaced with 2 ml of hiPSC-medium, which was changed every day thereafter with the same amount of fresh medium, until the appearance of the first cell colonies (about day 10-12 post-infection), indicative of reprogrammed cells.

2.5.5 **Picking of patient-derived iPSC colonies and cell expansion.** When reprogrammed cell colonies grew to an appropriate size (~500 μm in diameter; day 21 post-infection), they were manually picked under an inverted microscope placed in a sterile cell culture hood. The colonies to be picked were cut in 4 similarly-sized pieces using a sterile 25-gauge 1 1/2-inch needle (insulin-needle) directly in the cell culture wells. Cut pieces were gently aspirated with a 200 μl micropipette (Gilson, Inc., Middleton, USA), transferred into sterile Eppendorf® Safe-Lock microcentrifuge tubes (T9661; Sigma-Aldrich), containing 500 μl of hiPSC-medium and fragmented into smaller clumps by gently pipetting. At this point, clumps were aspirated and seeded onto newly prepared MEF feeder layers in wells of 6-well plates in 2 ml of hiPSC-medium - supplemented with 10 μM ROCK inhibitor for the first 24 hours - and cultured under standard conditions. Clumps were allowed to attach and grow as large hiPSC colonies, and culture medium was changed daily. In order to be expanded, 70-80% confluent hiPSC colonies were mechanically split (manual picking) for 4 passages, and seeded on freshly prepared MEF feeder layers.
2.5.6 Patient-derived iPSCs adaptation to feeder-free culture conditions. Patient-derived iPSCs grown and expanded on MEF feeder layers were cut and manually picked as described in the paragraph 2.5.5. Cut pieces were transferred into sterile Eppendorf® Safe-Lock microcentrifuge tubes containing 500 µl of mTeSR1 complete medium and fragmented into smaller clumps by firmly pipetting. At this point, clumps were aspirated and seeded onto Matrigel hESC-qualified Matrix-coated wells of 6-well plates in 2ml of mTeSR1 complete medium - supplemented with 10 µm ROCK inhibitor for the first 24 hours - and cultured in feeder-free conditions as described in the paragraph 2.3. Patient-derived hiPSC#1 clone was selected for characterisation and additional experiments (see the paragraph 2.6 below).

2.6 Characterisation of patient-derived iPSCs

To characterise patient-derived iPSCs (hiPSC#1 clone) we: (i) performed immunofluorescence and quantitative real-time PCR (qRT-PCR) analyses to evaluate pluripotency marker expression, (ii) tested cell capacity to form embryoid bodies (EBs) as a stringent assay to screen for pluripotency, (iii) investigated stochastic differentiation of the cells forming the EBs into all three somatic germ layers, and (iv) carried out karyotype analysis.

2.6.1 Immunofluorescence staining. For immunofluorescence analysis, patient-derived iPSCs were washed three times with 2 ml of PBS and fixed in PBS containing 4% paraformaldehyde (PFA) (157-8; Electron Microscopy Sciences, Hatfield, PA, USA) for 15 minutes at room temperature. Next, cells were permeabilised with 0.3% Triton X-100 (93418; Sigma-Aldrich) prepared in PBS for 10 minutes at
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room temperature, blocked with PBS containing 5% bovine serum albumin (BSA) (A2153; Sigma-Aldrich) for 1 hour at room temperature, and incubated with the primary antibodies diluted in PBS containing 2% BSA (Sigma-Aldrich). Primary antibodies: rabbit anti-NANOG (sc-33759; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; 1:100), mouse anti-OCT4 (sc-5279; Santa Cruz Biotechnology; 1:100), mouse anti-TRA 1-60 (MAB4360; Merck-Millipore, NJ, USA; 1:200), mouse anti-TRA 1-81 (MAB4381; Merck-Millipore, 1:200), rat anti-SSEA3 (sc-21703; Santa Cruz Biotechnology; 1:100) and mouse anti-SSEA4 (sc-21704; Santa Cruz Biotechnology; 1:100) over night at 4°C.

The next day, samples were abundantly washed with PBS and incubated for 1 hour at room temperature in the dark with the appropriate secondary antibodies diluted in PBS containing 2% BSA (Sigma-Aldrich). Secondary antibodies: Donkey anti-Rabbit IgG Alexa Fluor® 546-conjugated (A10040; 1:300), Goat anti-Rat IgM Alexa Fluor® 488-conjugated (A21212; 1:300), Donkey anti-Mouse IgG Alexa Fluor® 546-conjugated (A10036; 1:300) and Goat anti-Mouse IgM Alexa Fluor® 488-conjugated (A21042; 1:300). All secondary antibodies were purchased from Thermo Fisher Scientific. After incubation with secondary antibodies, cells were washed twice with PBS for 5 minutes, and then nuclear staining was performed by incubating cells with 1 mg/ml 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI) (D9542; Sigma-Aldrich) for 10 minutes in the dark. DAPI was prepared and stored according to the manufacturer’s instructions. Images were taken with the CF40 Axiovert fluorescence microscope (Carl Zeiss, Oberkochen, Germany) and are representative of three independent experiments.
2.6.2 Embryoid body (EB) formation and patient-derived hiPSC differentiation into the three germ layers. For EB formation, sub-confluent patient-derived iPSCs were harvested as described in paragraph 2.3, and counted. Before cell seeding, AggreWell™800 plates (27965; Stem Cell Technologies), which are endowed with microwells of 800 µm in diameter, were centrifuged at 2,000xg for 5 minutes in a swinging bucket rotor with plate-holders in order to remove any air bubbles from the microwells. To obtain 3,000-4,000 cell-sized EBs, the harvested hiPSCs were first resuspended in order to have 1x10^6 hiPSCs/well in 1.5 ml/well of AggreWell™ medium (EB medium; 05893; Stem Cell Technologies) supplemented with 10 µM ROCK inhibitor, and then were seeded. Soon after seeding, cell suspension in each well was gently pipetted several times to homogeneously distribute the cells. Next, plate was centrifuged at 100xg for 3 minutes at room temperature for capturing cells in the microwells and was finally incubated under standard conditions. EBs formed within 2 days of culture. At this point, EBs were displaced and harvested by firmly pipetting 2-3 times the EB medium in each well. Then, EBs were transferred into 6-well ultra-low-adherence plates (27145; Corning) (~800 EBs/well) in a total volume of 5 ml/well of EB medium, and were cultured in suspension under standard conditions, changing EB medium every 2 days. After 8 days as floating cultures, EBs were transferred onto 0.1% gelatine (G2500; Sigma-Aldrich)-coated plates [6-well plate coating was made by adding 2 ml/well of 0.1% sterile gelatine solution prepared in PBS (Lonza) and incubating plates at 37°C for 30 minutes] and cultured for another 8 days, by changing EB medium every 2 days. Under these culture conditions, EBs gradually adhered to the gelatine-coated well bottom and grew as monolayered cells. On culture day 16, cells were fixed in PBS containing 4% PFA, and
processed for immunostaining as described in paragraph 2.6.1. In particular, differentiation into the three germ layers was evaluated by immunostaining the EB-derived cells for Alexa Fluor® 488-conjugated mouse anti-β-Tubulin III (ectoderm marker; CBL412X; Merck-Millipore; 1:100), Cy3™-conjugated mouse anti-α-smooth muscle actin (SMA) (mesoderm marker; C6198; Sigma-Aldrich; 1:100) and rabbit anti-GATA4 (endoderm marker; sc-9053; Santa Cruz Biotechnology; 1:50).

2.6.3 Gene expression analysis. Gene expression level of pluripotency markers was analysed by performing qRT-PCR assays using predesigned Taqman probes (Thermo Fisher Scientific) specific to OCT4, NANOG and SOX2 genes (Table 1) as described in the paragraph 2.8 below. Gene expression levels were normalised to the housekeeping gene hypoxanthine phosphoribosyltransferase-1 (HPRT1).

2.6.4 Karyotype analysis. When 60-70% confluent, patient-derived iPSCs were treated with 10 mg/ml Colcemid (10295892001; Roche, Basel, Switzerland) in mTeSR1 medium for 2 hours and a half, in order to induce cell cycle synchronisation and increase the yield of metaphase chromosomes. After treatment, cells were harvested as described in the paragraph 2.3 and centrifuged at 1000xg for 6 minutes at room temperature. Soon after, pellets were delivered to the Genetic Medicine Laboratory of the Azienda Socio-Sanitaria Territoriale (ASST) Papa Giovanni XXIII, Bergamo (Italy), where karyotype analysis was performed. Briefly, metaphase spreads (consisting of condensed metaphase chromosomes) were prepared onto microscope slides, and were processed for G-banding karyotype analysis. Twenty metaphases were analysed for the chromosomes’ size, shape and number.
2.7 Differentiation of hiPSCs toward ureteric bud (UB) progenitor-like cells

The hiPSCs were induced to differentiate toward UB-like cells as previously described with minor modifications (Xia et al., 2013). When 70-80% confluent, hiPSCs cultured in 6-well plates were abundantly washed with PBS and then treated with 2 ml of pre-warmed DMEM/F12+GlutaMAX (31331028; Invitrogen) containing 1 U/ml Dispase (1x Dispase; 07913; StemCell Technologies) for 4 minutes at 37°C. After treatment, 1x Dispase was removed and cells were washed twice with 2 ml of DMEM/F12+GlutaMax. Next, 2 ml of DMEM/F12+GlutaMAX were added to each well and the hiPSC sub-confluent colonies were fragmented through gentle manual scraping with a 5 ml glass pipette into small clusters (each consisting of about 200-400 cells), which were then split onto Basement Membrane Growth-Factor-Reduced (GFR) Matrigel (356231; Corning)-coated 6-well plates at a ratio of 1:4. Basement Membrane GFR Matrigel was used following the manufacturer’s instructions. Briefly, 0.5 ml aliquots of Basement Membrane GFR Matrigel were prepared and frozen at -80°C. One aliquot was thawed on ice and added to 15 ml of cold DMEM/F12+GlutaMAX (31331028; Invitrogen) (1:30 dilution) to coat wells of 6-well plates (1 ml/well), which were then incubated at room temperature for 1 hour prior to be used. Just before hiPSC seeding and culturing on top of the thin gel layer, the unbound coating liquid was aspirated from each well.

After 24 hour-recovery in mTeSR1 complete medium supplemented with 10 μM ROCK inhibitor, cell colonies were grown in 1.5 ml of chemically defined basal differentiation medium [DMEM/F12+GlutaMAX (Invitrogen), 17.5 mg/ml BSA fraction V (126579; Merck-Millipore), 17.5 µg/ml human insulin (I9278; Sigma-Aldrich), 275 µg/ml human holo-transferrin (T0665; Sigma-Aldrich), 450 μM 1-thioglycerol (M6145; Sigma-Aldrich), 0.1 mM non-essential amino acids (Invitrogen), 1% Pen-Strep (Invitrogen)] supplemented with 50 ng/ml human fibroblast growth factor 2 (FGF2) (100-18B; PeproTech) and 30
ng/ml human bone morphogenetic protein 4 (BMP4) (120-05; PeproTech) for 2 days to induce mesoderm commitment. For the next 2 days, cells were exposed to the basal differentiation medium supplemented with 1 μM all-trans retinoic acid (R2625; Sigma-Aldrich), 10 ng/ml human Activin A (120-14E; PeproTech) and 100 ng/ml human bone morphogenetic protein 2 (BMP2) (H00000650-Q01; Abnova Corporation, Taiwan) for UB fate induction. 1.5 ml of appropriate differentiation medium was added daily. Activin A, all-trans retinoic acid, and all growth factors were reconstituted and stored in accordance with the manufacturer’s instructions.

2.8 RNA isolation and gene expression analysis with quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Both undifferentiated (d0) and differentiated (d1-d4) hiPSCs were harvested with Accutase as described in the paragraph 2.3, and separately collected in 50 ml conical tubes. After cell counting, aliquots of 2-3x10^6 cells were transferred to 15 ml conical tubes, and centrifuged at 200xg for 5 minutes at room temperature. After centrifugation, supernatant was discarded, pellet was resuspended in 8 ml of PBS, and cell suspension was centrifuged again. Next, supernatant was carefully aspirated without disturbing the pellet, and dry pellets were stored at -80°C overnight or until RNA isolation procedure. Total cellular RNA was isolated with Trizol® Reagent (15596026; Invitrogen) according to the manufacturer’s instructions using the appropriate precautions to avoid RNase contamination. The total RNA yields and purity were determined by measuring the absorbance at 260 nm and 280 nm (A_{260/280} > 1.8), respectively, with NANODROP 1000 UV/VIS spectrophotometer (Thermo Fisher Scientific) following dilution of the isolated RNA samples in RNase-free water on ice. To avoid DNA contamination, 10 μg of total RNA were treated using the RQ1 RNase-free DNase kit (M6101; Promega Corporation,
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Madison, WI, USA) [DNase treatment for 1 hour at 37°C in the presence of 1 U/µl of SUPERaseInh™ RNase Inhibitor (AM2694; Invitrogen), followed by the addition of DNase STOP solution for 10 minutes at 65°C]. Purified RNA suspension volume was made up to 200 µl with RNase-free water, and was precipitated by adding 20 µl of 3M sodium acetate buffer pH 5.4 and 600 µl of ice-cold 100% ethanol, overnight at -80°C. The next day, samples were centrifuged at 13,200 rpm for 30 minutes at 4°C in the Eppendorf® 5415-R refrigerated microcentrifuge. After centrifugation, supernatant was discarded, pellets were washed by gently adding 500 µl of ice-cold 75% ethanol, and were centrifuged at 13,200 rpm for 5 minutes at 4°C. Subsequently, supernatant was aspirated, pellets were resuspended with RNase free-water on ice, and the absorbance at 260 nm and 280 nm was measured. At this point, 2.5 µg of purified RNA were reverse-transcribed using SuperScript VILO cDNA synthesis kit (11754050; Invitrogen) adhering to the manufacturer’s instructions. No enzyme was added to reverse transcriptase-negative controls. Expression of pluripotency, early renal and UB-related markers was evaluated by TaqMan gene expression assay on ViiA 7 Real Time PCR system (Applied Biosystems, CA, USA) using predesigned TaqMan probes (Thermo Fisher Scientific) specific to NANOG, T, LHX1, PAX2, GATA3 and HOXB7 genes (Table 1) according to the supplier’s instructions. Gene expression levels were normalised to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The ∆∆Ct technique was adopted to calculate cDNA content in each sample using cDNA expression of the pluripotent state (d0) as a calibrator. Three independent experiments for each hiPSC line were performed, and samples were analysed in triplicate.

2.9 Kidney tubule engineering

Sub-confluent MDCK type II cells and in vitro differentiated hiPSCs were harvested, as
described in the paragraphs 2.2 and 2.3, using pre-warmed 0.25% Trypsin–EDTA and Accutase, respectively. Once detached, cells were counted, collected in sterile Eppendorf® Safe-Lock microcentrifuge tubes containing the appropriate culture medium, and centrifuged at 300xg for 3 (hiPSCs) or 4 (MDCK cells) minutes in the CL17-R Microcentrifuge (75002455; Thermo Fisher Scientific). Subsequently, supernatant was completely discarded, pellets were resuspended in 2.4 mg/ml rat-tail collagen type I (354236; Corning) on ice taking care to avoid air bubble formation and obtain a homogeneous cell suspension, which was finally seeded into the PDMS scaffold cavities. Prior to cell seeding, PDMS macro- and microscaffolds were placed into sterile 60x15 mm (353004; Corning) and 35x10 mm (DTC035; Sterilin® Ltd, British Plastics Federation, Parkway, UK) tissue culture dishes, respectively. Rat-tail collagen type I was stored according to the manufacturer’s instructions and was gelled by bringing its pH to alkalinity following the gelation procedure described in the Certificate of Analysis provided by the producer. Optimal cell densities were set-up at 1.2x10^5 cells/µl collagen and 2x10^5 cells/µl collagen for macro- and microscaffolds, respectively. The total volume of seeded cell-collagen mixture vary according to the size of the PDMS scaffold cavities. After collagen polymerisation, which for macroscaffolds occurs after 30 minutes at 37°C and for microscaffolds occurs after 2 minutes at room temperature, the appropriate culture medium (6 ml for macroscaffolds and 200-250 µl for microscaffolds) was gently dropped to cover the scaffolds.

Culture media: DMEM/F12+GlutaMAX supplemented with 1% FBS, 1% Pen-Strep and 40 ng/ml hepatocyte growth factor (HGF) (100-39; PeproTech) for MDCK tubules; basal differentiation medium supplemented with 1% FBS, 40 ng/ml HGF and 100 ng/ml glial cell-derived neurotrophic factor (GDNF) (ab73450; Abcam, Cambridge, UK) for hiPSC-derived tubules.
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Bright-field images of cultured tubules were obtained using Leica ZOOM 200 stereomicroscope (Leica Microsystems, Wetzlar, Germany) and Primo Vert inverted microscope (Carl Zeiss). Both HGF and GDNF were reconstituted and stored following the manufacturer’s instructions. Tubules were cultured for up to 2 days in standard conditions without changing culture medium.

2.10 Kidney tubule histological staining

For cross-sectional histological analysis, 2-day cultured macrotubules were gently harvested with a 1000 µl micropipette (Gilson), transferred into sterile 60x15 mm dish, washed with PBS for 10 minutes, and then fixed in PBS containing 4% PFA for 15 minutes. After abundant washing with PBS, tubules were embedded in Optimal Cutting Temperature (OCT) compound (4583; Tissue-Tek, Sakura Finetek, Japan) and frozen at -80°C. After freezing, 3-µm serial tubule cryosections were cut using Leica CM1950 cryostat (Leica), mounted on Polysine® slides (J2800AMNZ; Thermo Fisher Scientific) and immediately placed at -80°C. Subsequently, slides were submerged in Harris’s haematoxylin (05-M06004; Bio-optica) for 2 minutes, rapidly washed with distilled water, mounted with Ready-to-use Dako Faramount Aqueous Mounting Medium (S3025; DAKO Corporation, Carpinteria, CA, USA) and observed by light microscopy.

Tubules 3D-cultured in Transwell inserts (see paragraphs 2.13 and 2.14 for details) were stained with haematoxylin and eosin as follows: firstly, they were washed with 2 ml PBS for 15 minutes, fixed in PBS containing 4% PFA for 45 minutes at room temperature, and washed again in PBS (at this point samples can be stored at 4°C in 3 ml PBS). Secondly, tubules were harvested from collagen (see the paragraph 2.13 for details on this manoeuvre), submerged in 3 ml of haematoxylin (05-M06012; Bio-Optica, Milan, Italy) diluted 1:6 in distilled water for 5 minutes, washed abundantly in distilled water and then
soaked in 3 ml of eosin (05-M10002; Bio-optica) diluted 1:9 in distilled water for 1 minute. After washing, samples were placed on Polysine® slides, completely covered with Ready-to-use Dako Faramount Aqueous Mounting Medium, and observed by light microscopy.

All digital images were acquired and analysed using AxioImager Z2 microscope and AxioVision 4.8 imaging software (Carl Zeiss), respectively.

2.11 Kidney tubule immunofluorescence staining

For immunofluorescence analysis, tubules were harvested from the scaffolds, fixed in PBS containing 4% PFA as described in the paragraph 2.10, washed in PBS for 10 minutes, and permeabilised in 100% ice-cold methanol (414814; Carlo Erba Reagents, Cornaredo, Milan, Italy) for 10 minutes. Next, samples were washed again and incubated with the following primary antibodies diluted in PBS: mouse anti-E-cadherin (610182; BD Biosciences, CA, USA; 1:100), rabbit anti-podocalyxin (NB110-41503; Novus Biologicals, Littleton, CO, USA; 1:80), rabbit anti-PKC-ζ (C-20) (sc-216; Santa Cruz; 1:100) and rabbit anti-cleaved caspase-3 (D175-5A1E; Cell Signaling Technology, MA, USA; 1:200) overnight at 4°C. After washing, tubules were incubated with the appropriate secondary antibodies diluted in PBS: Cy™3-conjugated AffiniPure Donkey Anti-Mouse IgG (715-165-151; 1:50), Cy™5-conjugated AffiniPure F(ab’)2 Fragment Donkey Anti-Mouse IgG (715-176-151; 1:50), Cy™3-conjugated AffiniPure Donkey Anti-Rabbit IgG (711-165-152; 1:50) and Fluorescein (FITC)-conjugated AffiniPure Donkey Anti-Rabbit IgG (711-095-152; 1:50) overnight at 4°C in the dark. All secondary antibodies were purchased from Jackson ImmunoResearch Labs, PA, USA. After washing, samples were soaked in DAPI for 10 minutes to label cell nuclei, washed again, placed on 24x60 mm coverslips (Knittel Gleaser, Germany), and completely covered with Dako Fluorescence
Mounting Medium (S3023; DAKO Corporation).

For the immunostaining of 3-µm cross-sections, sample sections were permeabilised with PBS containing 0.3% Triton X-100 for 5 minutes, washed, blocked with 1% BSA solution in PBS for 30 minutes, washed again, and incubated overnight at 4°C with the indicated primary antibodies. After washing, sections were incubated with the appropriate secondary antibodies (Jackson ImmunoResearch Labs; 1:50) for 2 hours at room temperature in the dark, followed by washing and nuclear staining with DAPI for 10 minutes. Negative controls were obtained by omitting primary antibodies on adjacent sections on the same slide. Finally, slides were mounted with Dako Fluorescence Mounting Medium and examined.

In some cases, samples were further labelled with wheat germ agglutinin-lectin (WGA-lectin) (FL-1021; Vector Labs, Burlingame, CA, USA; 1:400) for 15 minutes or peanut agglutinin-lectin (PNA-lectin) (RL-1072; Vector Labs; 1:80) for 1 hour and 30 minutes, both diluted in PBS. Digital images were acquired using the LSM 510 Meta inverted confocal laser scanning microscope and LSM Image browser (Carl Zeiss). All images were analysed using the free open-source image processing ImageJ software (1.49v, National Institutes of Health).

2.12 Functional studies

After 2 days of culture in PDMS scaffolds, engineered tubules were harvested, transferred in transparent plastic tubes with screw cap, washed with 1 ml of PBS for 10 minutes, and then soaked in 500 µl of PBS containing 25 mg/ml 10-KDa FITC-conjugated dextran (FD10S; Sigma-Aldrich) or 2 mM 6-Carboxyfluorescein (6CF) (C0662; Sigma-Aldrich) alone or in combination with 2 mM probenecid (P8761; Sigma-Aldrich) for 1 hour at 37°C in the dark. Subsequently, tubules were washed 4-5 times with ice-cold PBS, were fixed
with PBS containing 4% PFA for 10 minutes, and processed for immunofluorescence analysis as described in the paragraph 2.11.

2.13 Cyst formation and drug testing in 3D culture system

After 2 days of culture, MDCK cell-derived tubules were harvested from PDMS microscaffold cavities through gentle suction with a 200 µl micropipette (Gilson), washed in PBS for 10 minutes, embedded within 200 µl of 2.4 mg/ml rat-tail collagen type I, and finally transferred onto polyester Transwell membranes (0.4 µm pore size) in 12-well plates (CC3460; Corning-Costar, MA, USA). Following collagen polymerisation (35-40 minutes at 37°C), 1.5 ml of culture medium, composed of MEM supplemented with 10% FBS, 1% L-glutamine and 1% Pen Strep, was added. To induce cyst formation, samples were cultured in culture medium supplemented with 10 µM forskolin (F6886; Sigma-Aldrich) for 7 days. Next, ‘polycystic’ tubules were cultured in the presence of 10² µM Octreotide acetate (OCTR) (0239950; Toronto Research Chemicals, Brisbane Rd, North York, Canada), 10² µM Pasireotide diaspartate (PAS) (kind gift from Novartis Farma S.p.A., Varese, Italy), 10⁴ µM 2-Deoxy-D-glucose (2DG) (D6134; Sigma-Aldrich), 10 µg/ml berberine chloride (B3251; Sigma-Aldrich), 0.5 µM Rapamycin (R0395; Sigma-Aldrich), or with 10⁻² µM Arginine Vasopressin (AVP) (V9879; Sigma-Aldrich) alone or in combination with 10⁻² µM Tolvaptan (T7455; Sigma-Aldrich) for another 7 days. Control samples were maintained in culture medium alone for all 14 days. In each culture condition, culture medium was changed every 2 days. After fixing, tubules were harvested by gently cutting the surrounding collagen gel with insulin-needles under a stereomicroscope, and were processed for immunofluorescence and histological analysis as described in the paragraphs 2.10 and 2.11. The number of cysts was quantified in different fields of haematoxylin and eosin-stained tubules using ImageJ software. Haematoxylin and
eosin staining images were acquired with AxioImager Z2 microscope (Carl Zeiss). The area of each field was calculated using AxioVision 4.8 software (Carl Zeiss). Data are expressed as number of cysts/mm$^2$. Two independent scientists performed quantification analyses in blind. According to the manufacturer’s indications, both forskolin, rapamycin, Tolvaptan and berberine were reconstituted in DMSO; OCTR and PAS were reconstituted in sterile saline solution; and AVP and 2DG were resuspended in sterile distilled water. Once in solution, compounds were diluted in culture medium to achieve the desired final concentration. The final DMSO concentration in the culture media never exceeded 0.1%.

2.14 3D culture experiments for human developmental studies

Fragments of about 0.6 mm in width x 4.0 mm in length of 2-day cultured hiPSC-derived macrotubules were transferred to polyester Transwell membranes as described in the paragraph 2.13. To obtain fragments of the appropriate size, tubules harvested from PDMS scaffolds were manually cut using insulin-needles. E13.5 CD1 mouse kidneys were freshly isolated as previously described (Xinaris et al., 2012, 2016). For 3D co-culture experiments, four E13.5 kidneys were placed within collagen in close proximity to each tubule and, after collagen polymerisation, culture medium [Advanced DMEM (12491023; Invitrogen) supplemented with 2% embryonic stem cell FBS (16141079; Invitrogen), 1% L-glutamine and 1% Pen-Strep] was added. Other samples were cultured in Advanced DMEM supplemented with 1 μg/ml heparin (VERACER, Medici Italia S.r.l., Roma, Italy) and with different combinations of the following growth factors: 40 ng/ml HGF, 100 ng/ml GDNF, 200 ng/ml FGF1 (100-17A; PeproTech) and 100 ng/ml FGF7 (100-19; PeproTech). Both heparin and growth factors were reconstituted and stored following the manufacturer’s instructions, and diluted in culture medium to achieve the desired final concentration indicated above. Cultures were monitored by light microscopy for up to 2
days without changing the medium, and eventually fixed and processed for immunofluorescence analysis as described in the paragraph 2.11. Bright-field images of tubules cultured in the different aforementioned conditions were acquired with Primo Vert inverted microscope (Carl Zeiss). The total budding events of the human UB-like tubules were quantified using ImageJ software. Two independent scientists performed quantification analyses in blind. Data are expressed as percentage of ramified buds over total buds that emerged from human tubules.


2.15 Statistical analyses

Samples were randomly allocated in every experimental groups/conditions and no inclusion/exclusion criteria were used. Statistical analysis was performed using GraphPad Prism software, version 7 (GraphPad, San Diego, CA, USA). When two conditions were compared, two-tailed Student’s t-test was used. When more than two conditions were compared, one-way ANOVA with the Tukey’s multiple comparisons test
(for cyst quantification) and with the Holm-Sidak’s multiple comparisons test (for ramified over total buds quantification) was used. Differences were considered to be significant when $P < 0.05$. Data are expressed as means ± s.e.m. See figure legends for details on number of replicates and $n$ values used.
### Table 1. List of TaqMan probes used in qRT-PCR experiments

<table>
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<tr>
<th>Catalogue number</th>
<th>Gene symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hs00742896_s1</td>
<td>OCT4</td>
<td><em>H. sapiens</em> POU class 5 homeobox 1</td>
</tr>
<tr>
<td>Hs00602736_s1</td>
<td>SOX2</td>
<td><em>H. sapiens</em> SRY (sex determining region Y)-box 2</td>
</tr>
<tr>
<td>Hs02387400_g1</td>
<td>NANOG</td>
<td><em>H. sapiens</em> NANOG homeobox</td>
</tr>
<tr>
<td>Hs00610080_ml</td>
<td>T</td>
<td><em>H. sapiens</em> T, brachyury homolog</td>
</tr>
<tr>
<td>Hs00232144_m1</td>
<td>LHX1</td>
<td><em>H. sapiens</em> LIM homeobox 1</td>
</tr>
<tr>
<td>Hs01057416_m1</td>
<td>PAX2</td>
<td><em>H. sapiens</em> paired box 2</td>
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<td><em>H. sapiens</em> GATA binding protein 3</td>
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<td>HOXB7</td>
<td><em>H. sapiens</em> homeobox B7</td>
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<tr>
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<td><em>H. sapiens</em> hypoxanthine phosphoribosyltransferase 1</td>
</tr>
<tr>
<td>Hs02758991_g1</td>
<td>GAPDH</td>
<td><em>H. sapiens</em> glyceraldehyde-3-phosphate dehydrogenase</td>
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CHAPTER 3 - RESULTS
3.1 Design and fabrication of 3D-printed PDMS scaffolds

In collaboration with NanoMed Labs (University of Genova, Italy) and relying on the physiological structure of the UB tree during early kidney development (Lin et al., 2003; Watanabe and Costantini, 2004; Lindström et al., 2015), we designed three basic patterns shaped to resemble some UB segments. These patterns consisted of: a straight line (Supplementary Figure 1a) for UB trunks, a terminal 80° bifid branch (Supplementary Figure 1b) and asymmetric lateral 30° branches (Supplementary Figure 1c), both for UB ramifications. To mimic advanced stages of UB branching morphogenesis, we merged the 80° and 30° basic schemes into a ramified pattern (Supplementary Figure 1d). Moreover, to quickly and routinely design fractal-like ramifications similar to those observed in early kidney development (Lin et al., 2003), we developed a ‘golden fractal tree’ (GFT) formula (Supplementary Figure 1e). Unlike the previously described GFTs with a constant amplitude (Taylor, 2007; Pons, 2013), our formula allows progressive narrowing of bifurcations for an infinite number of times, preventing branch overlapping (Supplementary Figure 1f). Finally, with the aim of engineering numerous individual tubules at the same time, we designed a linear multichannel pattern (Supplementary Figure 1g). Thereafter, we converted the custom-designed patterns into plastic masters through rapid prototyping technologies and 3D printing, and used them as moulds to produce polydimethylsiloxane (PDMS) replicas (Figure 5a). PDMS is a silicon-based organic polymer with numerous desirable properties that make it suitable for cell biology studies. In particular, PDMS is biocompatible, non-toxic to cells, chemically inert, gas permeable, and optically transparent for imaging applications (Briganti et al., 2006; Adler et al., 2010; Young and Beebe, 2010; Markov et al., 2014).

Given their distinct geometries, we refer to the replicas with bigger, linear and ramified cavities as ‘macro-scaffolds’, and those with multiple micro-patterned linear cavities as
‘micro-scaffolds’. Differently from typical PDMS devices made for cell cultures, which are pre-treated to possess hydrophilic properties and adhesive surfaces (Halldorsson et al., 2015), our scaffolds were kept hydrophobic to facilitate detachment of engineered tissues without compromising cell-ECM seeding, and to enable standard sterilisation processes so that the scaffolds could be reused.

3.2 Engineering kidney tubular structures with different geometries by seeding epithelial cell suspensions into PDMS scaffolds

To develop and optimise our system for tubule engineering, we used the MDCK epithelial cell line, which is composed of both distal tubule and collecting duct cells (Rindler et al., 1979; Herzlinger et al., 1982), and has been widely used in studies examining cyst and tubule formation. Collagen type I was chosen as ECM-mimicking gel in which to resuspend the cells as it is the most abundant ECM component, can store and support diffusion of nutrients and growth factors, permits 3D intercellular interactions, and provides structural support for cell movement and organisation into tissues (Davies, 2001). With the aim of engineering epithelial kidney tubules, a range of 0.7-6.5x10⁵ MDCK cells/µl collagen were seeded into PDMS scaffold cavities, and cultured for up to 2 days (Figure 5b). In accordance with previous studies, tubulogenesis was induced by supplementing culture medium with 40 ng/ml hepatocyte growth factor (HGF), a molecule capable of strongly inducing in vitro branching tubulogenesis (Montesano et al., 1991b, 1991a; Sakurai et al., 1997; Pollack et al., 1998; Liu et al., 2007). The seeding conditions with the highest rate of tubule formation (94% of total seeded scaffolds) were 1.2x10⁵ and 2x10⁵ cells/µl collagen for macro- and micro-scaffolds, respectively (Figure 5c-e and f). It is important to know that, in the remaining cell concentrations tested, only cell clustering
was observed within the scaffolds’ cavities and no tubules developed (Supplementary Figure 2a and b).

Under the aforementioned conditions, three hours post-seeding, dispersed cells organised to give rise to tubular structures with analogous size and shape to the scaffold’s cavity (Supplementary Figure 2c). At day 1, single and branched structures noticeably shrunk and began detaching from the moulds (Figure 5c). At day 2, the shrinkage increased and whole intact branches emerged autonomously from the cavity. Strikingly, at bifid branching points, a presumptive luminal space was observed through bright-field microscopy and appeared as a translucent region delimited by darker edges. This suggested that a common cavity between tubular structures was forming (Figure 5c). Using PDMS scaffolds with more complex geometries, we successfully applied our method to engineer increasingly branched and tree-like tubular structures (Figure 5d and e). By seeding cells into multichannel scaffolds with micro-patterned linear cavities, we concomitantly obtained multiple individual linear microtubules within 2 days of culture (Figure 5f), thus demonstrating the applicability of our system on a micro-scale.

Depending on scaffold size, we refer to the tubular structures generated and depicted in Figure 5c-e and Figure 5f as ‘macrotubules’ (both single and in networks) and ‘microtubules’, respectively.

3.3 Engineered tubules undergo rapid lumen formation and epithelial polarisation

Development of mature epithelial tubules involves cell rearrangements that lead to the formation of a lumen enclosed by an epithelium with appropriate polarisation (Andrew and Ewald, 2010). Kidney tubules consist of aligned epithelial cells, which adhere by means of specialised intercellular junctions and lie on a basement membrane, giving rise to a monolayered epithelium enclosing a central lumen. This configuration entails that cells
acquire a well-established apical-basal polarity, with apical membranes facing the lumen, and lateral and basal membranes in contact with neighbouring cells and basement membrane, respectively (Bryant and Mostov, 2008; Andrew and Ewald, 2010).

To examine the presence of a lumen and evaluate the establishment of a polarised epithelium, we immunostained the engineered microtubules and analysed them from the surface to the inner region through confocal Z-stack imaging. Remarkably, this analysis revealed the presence of a single lumen surrounded by a monolayer of epithelial cells positive for E-cadherin (Figure 6a). This transmembrane glycoprotein is abundantly expressed in distal tubule, collecting duct and most medullary segments, where it plays an important role in the establishment of intercellular adherens junctions and renal epithelial polarity (Prozialeck et al., 2004). Identically to microtubules, branches from macrotubular systems possessed well-established epithelial structures with a coalesced lumen. In particular, the lumen was lined by tubular walls consisting of a monolayer of cells showing aligned nuclei, positivity for E-cadherin in the lateral membranes and wheat germ agglutinin (WGA)-lectin in the basement membranes, (Figure 6b). Some cells of the tubular walls were positive for peanut agglutinin (PNA)-lectin - a known MDCK cell luminal surface marker (Schumacher et al., 2008) - and podocalyxin - an integral membrane protein expressed in the apical domain of terminally polarised MDCK cells – in the apical membranes (Meder et al., 2005) (Supplementary Figure 3a).

Overall, these findings proved that we were successful in engineering tubules consisting of a properly apical-basal polarised monolayered epithelium lining a continous lumen.

Apoptosis is one crucial step underlying lumen formation during tubulogenesis. In tubule primordia, cells that are not in direct contact with ECM undergo programmed cell death generating an empty space, namely the lumen. Hence, this process gives rise to a tubule. Considering the timely occurrence of this phenomenon, engineered tubules were examined
at an earlier stage of development, providing insight into the mechanism of lumen formation in our system. Haematoxylin staining of cross-sectioned 1-day macrotubules showed that these had a major lumen enclosed by a peripheral layer of cells with nuclei aligned along the tubular walls. In macrotubules, centrally located cells featured chromatin condensation and immunoreactivity for cleaved caspase 3 (Supplementary Figure 3b and c respectively), both clear signs of apoptosis. By contrast, peripheral cells constituting tubular walls revealed normal cell morphology and no signs of apoptosis (Supplementary Figure 3c). These findings implied that, in our system, cells in the central region of the tubular aggregate die by apoptosis, creating a hollow, eventually giving rise to the lumen by a process known as cavitation (Schumacher et al., 2008; Sigurbjörnsdóttir et al., 2014).

In control experiments, in which cells were cultured in the absence of HGF, phenotypically compact tubules did indeed form, but could not develop a lumen (Supplementary Figure 3d).

3.4 Engineered tubules exhibit macromolecule absorption and organic anion transport capacities

The mature renal tubular epithelia reabsorb macromolecules, and control the excretion of endogenous compounds, xenobiotics and organic anions. It has been previously described that both medullary and cortical collecting duct cells are capable of dextran macromolecule internalisation from their basolateral surfaces (Schwartz and Al-Awqati, 1985; Raghavan et al., 2014). Organic anion transepithelial transport and excretion into the lumen are mediated by specific organic anion transporters (OATs) widely distributed across kidney tubules. Specifically, these localise to both the basolateral and apical membranes of proximal tubule as well as collecting duct epithelial cells (Kojima et al., 2002; Sweet et al., 2006; Yokoyama et al., 2008; Burckhardt, 2012).
Therefore, to test whether the engineered microtubules possessed such functional properties, we soaked them in solutions containing either fluorescein isothiocyanate-conjugated (FITC)-dextran (molecular weight 10 kDa) or the tracer anion 6-carboxyfluorescein (6CF) (Sweet et al., 2006). The latter has better kinetic properties than fluorescein for investigating cellular OAT-mediated transport, due to its additional negative charge that, in comparison, causes increased cell retention (De Clerck et al., 1994).

Immunofluorescence analysis showed that dextran was internalised abundantly in the central areas of the microtubules (Figure 6c) and in the cytoplasm of cells of the tubular surface (Figure 6c, inset). With regards to 6CF, it was transported through the tubular epithelium and then secreted into the peripheral regions of the lumen (Figure 6d). Moreover, some E-cadherin-positive cells confined to the tubules’ wall, internalised 6CF and retained it within their cytoplasm (Figure 6d, inset). When we soaked tubules in the presence of both 6CF and probenecid - a potent and broad inhibitor of the OAT system (Nagle et al., 2011; Nigam et al., 2015) - fluorescent signal was no longer detectable, neither in the cells nor in the lumen (Figure 6e). These findings demonstrated that engineered tubules displayed absorption and transport characteristics typical of collecting duct epithelia, as they appear capable of macromolecule internalisation and organic anion transport through the OAT-mediated process.

3.5 Using engineered tubules as a tool to model polycystic kidney disease (PKD)

PKD is one of the most frequent genetic disorders (the fourth leading cause of CKD resulting in ESRD in the USA), affecting about 7 million people worldwide and accounting for 7%-15% of patients on RRT (Akoh, 2015; Saigusa and Bell, 2015). It is characterised by the development of fluid-filled epithelial cysts within kidney tubules in a cAMP-
dependent fashion. Unfortunately, at present, there is no effective therapy for PKD (Ong et al., 2015).

When cultured in a 3D ECM environment in the presence of cAMP elevating agents, MDCK cells can form individual epithelial cysts (Mangoo-Karim et al., 1989; Li et al., 2004), as the in vivo models of PKD (Gattone et al., 2003; Torres et al., 2004; Wang et al., 2005). For these reasons, they have been extensively used to model PKD and explore the effects of drugs on cyst development. However, in vitro cultures of individual cysts do not replicate the gradual expansion of cysts that occurs in PKD, and substantially differ from the physical and biochemical conditions of the tightly packed cysts within the polycystic tubules.

We decided to test whether our system, allowing the engineering of kidney epithelial tubules, could provide an efficient tool to generate polycystic tubules that better replicate pathological tissue phenotype in vitro. To this aim, we transferred microtubules to a 3D collagen culture system and stimulated them with forskolin for 7 days. Forskolin is a compound that has previously been shown to increase intracellular cAMP levels and induce cyst development in vitro and in vivo (Balkovetz, 1998; Sullivan et al., 1998; Li et al., 2004) (Figure 7a). At day 14, both macroscopic and histological examination showed that forskolin-treated tubules developed numerous translucent spheroidal cysts along the whole length of the tubule (Figure 7b). Immunofluorescence analysis confirmed that numerous round-shaped cysts formed homogeneously throughout the tubule (Figure 7c). Cysts were circumscribed by a monolayer of cells positive for E-cadherin in the basolateral membranes, and podocalyxin in the apical membranes, demonstrating the presence of polarised cysts within tubules (Figure 7d). Remarkably, cyst formation disrupted tubular epithelial organisation and caused lumen loss. Overall, these features appear similar to in
In vivo PKD pathophysiology (Martinez and Grantham, 1995; Grantham, 1996; Torres and Harris, 2006), indicating that engineered tubules efficiently model PKD.

3.6 Using engineered polycystic tubules for drug testing and discovery studies

One major aspiration in designing this system was to provide a novel tool for drug testing and discovery studies in vitro. To this purpose, we examined whether forskolin-stimulated tubules would respond to pharmacological treatment to inhibit cyst formation by comparing the effect of compounds with different mechanisms of action on cyst regression.

We, therefore, treated forskolin-stimulated tubules for 7 days with: (i) rapamycin - an inhibitor of the mammalian target of rapamycin (mTOR) pathway, which is aberrantly activated in cystic epithelia leading to cystic cell hyperproliferation and marked cystogenesis (Shillingford et al., 2006; Serra et al., 2010); (ii) tolvaptan - a highly selective arginine vasopressin (AVP) V2 receptor antagonist, which inhibits the overactivated AVP signalling for intracellular cAMP production, cell proliferation, transepithelial fluid secretion, and cyst growth in PKD collecting ducts and distal nephrons (Reif et al., 2011; Blair and Keating, 2015); (iii) octreotide (OCTR) and pasireotide (PAS) - somatostatin analogues that reduce intracellular cAMP levels, cell proliferation and cystogenesis in in vitro and in vivo experimental models, with different specificity and binding affinity to somatostatin receptors (Masyuk et al., 2007, 2013); (iv) 2-deoxy-D-glucose (2DG) - a glucose analogue that ameliorates disease progression in vivo by competing with glucose thereby blocking aerobic glycolysis in PKD cells (Rowe et al., 2013; Priolo and Henske, 2013; Chiaravalli et al., 2016); and (v) berberine - an isoquinoline quaternary alkaloid isolated from Chinese medicinal herbs, with anti-proliferative effect, mainly linked to cell cycle arrest at G0/G1 phase (Bonon et al., 2013), and able to inhibit the mitochondrial
oxidative phosphorylation resulting in ATP depletion (Fan et al., 2013). This compound has never been studied in a PKD model.

We performed immunofluorescence staining to evaluate the effect of tested compounds on cyst regression; in parallel, we carried out cyst quantification on haematoxylin and eosin stained tubules. These analyses demonstrated that rapamycin treatment had no significant effect on reducing cyst number compared to forskolin-stimulated tubules (controls) (Figure 7e and k). To test the effect of Tolvaptan, we treated forskolin-stimulated tubules with AVP alone, or in combination with Tolvaptan (Reif et al., 2011). As expected, treatment with AVP alone increased cyst number, while administration of AVP in combination with Tolvaptan significantly decreased cyst size and number (63%) (Figure 7f and l) (Reif et al., 2011). Moreover, cyst number significantly diminished when tubules were treated with OCTR (58%) (Figure 7g and k) and PAS (43%) (Figure 7h and k) compared to controls. Notably, 2DG and berberine were the most effective compounds for cyst regression, reducing the number of cysts by 71% and 72%, respectively (Figure 7i-k), and limiting cysts to the peripheral areas of tubules. An outstanding difference observed between the treatments was the drugs’ ability to restore both the tubular central lumen and epithelial organisation. Indeed, only 2DG and berberine possessed this ability (Figure 7i and j), whereas Tolvaptan, OCTR and PAS did not, even though they reduced cyst numbers to various extents, (Figure 7f-h). Together these data demonstrate that engineered ‘polycystic’ tubules responded to drug treatments in a quantifiable manner, making this system a valuable tool for drug testing and discovery studies. Furthermore, we revealed the anti-cystogenic properties of berberine in polycystic kidney tissue, making it a promising new drug with therapeutic potential for PKD treatment.
3.7 Engineering functional human epithelial tubules starting with hiPSC-derived UB-like progenitor cells

The next goal was to test our system’s ability to efficiently engineer human tubules, using hiPSCs previously generated in our laboratory through lentiviral-mediated reprogramming of somatic cells (Imberti et al., 2015). First, we directed the differentiation of the hiPSCs into UB-committed renal progenitor cells through 4-day exposure to chemically defined media as previously described by Belmonte group (Xia et al., 2013). This differentiation protocol consists of a first step of mesoderm commitment and a second step of UB fate induction (Xia et al., 2014). As expected, differentiating cells displayed a temporal gene expression pattern similar to that obtained by Xia and colleagues, which corresponded to mesoderm and IM commitment, and eventually UB-like progenitor cell differentiation (Supplementary Figure 4). Once the differentiation protocol had been optimised, we resuspended cells obtained from 2 to 4 days of differentiation in collagen, and seeded them into PDMS scaffolds according to the 3D culture settings and tubulogenic conditions established for MDCK cells (Figure 8a). Although 3- and 4-day differentiated cells generated compact tubular structures, the exposure to HGF alone was not sufficient to induce formation of monolayered epithelia surrounding a single lumen (Supplementary Figure 5a). To overcome this problem, we modified culture conditions by enriching the medium with glial cell-derived neurotrophic factor (GDNF), a molecule secreted by the MM that is essential for UB formation, growth and branching during kidney development (Shakya et al., 2005). After 2 days in these culture conditions, immunofluorescence analysis of tubules derived from 3- and 4-day differentiated hiPSCs showed the formation of a continuous lumen (Figure 8b and c) delimited by a monolayer of cells positive for WGA-lectin and E-cadherin in the basolateral membranes (Figure 8b). A fraction of the cells expressed the apical epithelial marker protein kinase C-ζ (PKC-ζ) (Yang et al., 2013)
in the apical membranes (Supplementary Figure 5b). Neither undifferentiated hiPSCs, used as controls, nor 2-day differentiated cells were able to form tubular structures (Supplementary Figure 5c and d).

Finally, to test whether this maturation degree would reflect UB-associated functions, we soaked engineered human microtubules in a solution containing FITC-dextran or 6CF. Confocal microscopy analysis revealed massive dextran internalisation throughout the tubular lumen (Figure 8d) and 6CF transport visible as a patchy signal in the cytoplasm of peripheral cells (Figure 8e), both functional features typical of in vitro-cultured UB.

Similarly to MDCK tubules, at day 1, human tubules had many apoptotic cells, positive for cleaved caspase 3 and displaying condensed chromatin, at the centre of cross-sectioned macrotubules, confirming that cavitation is the mechanism of tubular lumen formation (Supplementary Figure 5e and f).

To assess the reproducibility of our system, we engineered tubules from a hiPSC line obtained in our laboratory through a different technology, the Sendai virus-mediated expression of reprogramming factors in healthy donor-derived peripheral blood mononuclear cells (Ciampi et al., 2016). Both hiPSC differentiation towards UB progenitor-like cells and UB-like tubule engineering were successful (Supplementary Figure 6). Altogether, these data demonstrate that our innovative system is strongly robust and reproducible in that it allows successful production of human UB-like tubules from several hiPSC lines.

3.8 Using human tubules as a tool for studying UB developmental processes and defects

One of the greatest limitations in studying the developmental processes and disorders of human kidney is the scarcity of human embryonic samples and the lack of methods for
engineering kidney tissue or units from patients with genetic mutations. In virtue of our establishing a technology able to provide human UB-like tubules, we decided to investigate first whether they exhibited developmental capacities. Next, we evaluated the possibility of using the engineered tubules to examine both human UB morphogenetic mechanisms and possible developmental anomalies associated with genetic disorders.

3.8.1 Engineered human tubules recapitulate early steps of UB morphogenesis following mouse embryonic kidney induction or by exposure to selected growth factors

During the early developmental stages, the UB undergoes rapid budding and branching events, which mainly consist of iterative lateral ramifications and terminal bifid bifurcations (al-Awqati and Goldberg, 1998; Shah et al., 2004; Nigam and Shah, 2009; Costantini and Kopan, 2010). Although during kidney development the MM is involved in inducing and regulating UB branching pattern through direct contact, the ability to elongate and branch is intrinsic to the UB epithelium. Indeed, these processes can also take place without direct contact with MM, in the presence of soluble growth factors and ECM components (Qiao et al., 1999a).

Thus, we tested whether engineered human tubules possessed UB developmental properties and would respond to kidney developmental cues. Firstly, we co-cultured macrotubules into a 3D collagen culture system with mouse embryonic kidneys as sources of soluble factors to stimulate UB morphogenesis (Figure 9a). At day 1, bright-field microscopy showed primary buds emerging from the main body of the tubule. At day 2, lateral ramifications emerged from elongated primary buds, leading to the formation of secondary buds (Figure 9b). Immunofluorescence analysis revealed that these buds consisted of multicellular cords of epithelial cells with aligned nuclei and expressing E-
cadherin (Figure 9c). This configuration represents an early step of tubulogenesis in vitro (Sakurai et al., 1997).

Secondly, we assessed the effect of different combinations of selected growth factors known to be involved in rodent UB morphogenesis (Qiao et al., 1999a, 1999b, 2001; Shakya et al., 2005) on inducing human tubule budding. The basal culture condition with HGF and GDNF induced 35.05 ± 1.02% of ramified buds in the total budding events (Figure 9d). Addition of FGF1 - a strong inducer of UB branching and stalk formation (Qiao et al., 2001) - or FGF7 - a stimulator of UB survival and growth in vitro (Qiao et al., 1999b, 2001) - to the basal culture condition did not significantly increase the percentage of ramified buds (41.11 ± 10.60% and 52.38 ± 6.24%, respectively) (Figure 9d). Conversely, when all growth factors were used together, the number of ramified buds markedly increased (71.96 ± 3.22%) (Figure 9d-f) and was comparable to that observed in co-culture with mouse embryonic kidneys (74.81 ± 4.12%) (Figure 9b and d). Remarkably, when all growth factors were added in combination, a fraction of ramified buds displayed terminal bifid bifurcations (Figure 9e).

Overall, these data indicate that engineered human tubules are capable of recapitulating early steps of UB morphogenesis, thus providing a valuable tool for studying the mechanisms of human UB development.

3.8.2 Investigating UB developmental defects in tubules engineered using hiPSCs derived from a patient with a heterozygous PAX2 mutation

Taking advantage of engineered human tubules’ developmental capacities, we next applied our system to study possible defects in UB morphogenesis using Paired box 2 (PAX2) mutated cells. PAX2 gene is highly expressed during early kidney developmental stages in both condensing MM and branching UB (Dressler et al., 1990; Eccles et al., 1995; Dressler
and Woolf, 1999). It has been previously described that heterozygous PAX2 mutations cause reductions in UB branching and nephron number \textit{in vivo} (Harshman and Brophy, 2012), indicating that a direct relationship exists between UB branching and nephron induction.

We therefore derived hiPSCs from a patient with a heterozygous PAX2 mutation (Barua \textit{et al.}, 2014) through Sendai virus-mediated reprogramming of somatic cells (Supplementary Figure 7). Pluripotency was confirmed by evaluating the expression of pluripotency transcription factors (OCT4, NANOG and SOX2) and hESC surface markers (SSEA-3, SSEA4, TRA-1-60 and TRA-1-81), and the specification into the three germ layers (Supplementary Figure 7a-c). Moreover, reprogrammed cells showed a normal karyotype of 46,XY (Supplementary Fig. 7d). Having confirmed the pluripotent state, we successfully differentiated patient-derived hiPSCs towards UB progenitor-like cells, and engineered patient-derived UB-like macrotubules (Supplementary Figure 8). The latter were cultured in the presence of all selected growth factors as mentioned above (Figure 9h and i), and alongside healthy donor-derived tubules, used as controls (Figure 9e and f).

Strikingly, under these culture conditions, patient-derived tubules were capable of developing almost exclusively primary buds (Figure 9h), as opposed to the numerous ramified buds seen in healthy donor-derived tubules (Figure 9e). It is also worth noting that quantification analysis disclosed a marked decrease in ramified buds in these tubules compared with controls (Figure 9g). These results are in agreement with previous \textit{in vivo} data indicating that heterozygous mutation in the \textit{PAX}2 gene caused paucity in UB growth and significant reduction in UB branching (Porteous \textit{et al.}, 2000; Eccles \textit{et al.}, 2002). Overall, this proves the effectiveness of our system for studying developmental defects in tubules derived from patients carrying genetic mutations.
CHAPTER 4 - DISCUSSION
In the present study, we have developed a novel technology for engineering functional custom-made complex kidney epithelial tubules by 3D-printed PDMS scaffolds using different cell types. We first began by creating tubules of varying sizes and shapes with the MDCK cell line, which is derived from adult distal tubule/collecting duct epithelia. The engineered tubules were endowed with a single lumen enclosed by a monolayer of oriented and polarised cells exhibiting transport properties typical of mature renal tubular epithelia. We successfully employed engineered tubules to model PKD and screen for several anticystogenic drugs. Then, we refined our system in order to engineer functional hiPSC-derived UB-like tubules and used them as a tool for investigating human kidney developmental mechanisms and patient-specific defects.

Previous studies documented the development of engineering systems that, by exploiting the renal progenitor cells’ self-assembly potential in vitro, were able to yield 3D tubular structures. However, these systems have important limitations that made them inappropriate to generate kidney tubular units suitable for engineering a functional and anatomically correct kidney tissue. Relying on cell-driven assembly, these technologies lack control over the tubular structure formation, as tubulogenesis occurred through chaotic and geometrically uncontrolled cell organisation in 3D ECM-based cell cultures (Barros et al., 1995; Sakurai et al., 1997; Yuri et al., 2017). Consequently, instead of developing with precise architectural frameworks, engineered tubules were highly heterogeneous in size and shape.

Other kidney tubule engineering systems were based on cell seeding into 3D-printed scaffolds with pre-defined geometries (MacKay et al., 1998; Humes et al., 1999; Schumacher et al., 2008; Shen et al., 2013, 2015, Jansen et al., 2015, 2016; Homan et al., 2016). In particular, tubule formation arose from cell seeding into hollow cylindrical-shaped scaffolds with pre-coated inner surfaces to which cells adhered and grew up to form
a confluent monolayer. Thus, no cell self-assembly into tubular epithelia took place (MacKay et al., 1998; Humes et al., 1999; Schumacher et al., 2008; Shen et al., 2013, 2015, Jansen et al., 2015, 2016; Homan et al., 2016), as instead occurs in vivo when cells are embedded in a 3D ECM environment in the presence of tubulogenic conditions. In spite of their unnatural formation, these bio-artificial renal tubules were still exploited for functional and toxicological studies (MacKay et al., 1998; Humes et al., 1999; Shen et al., 2013, 2015, Jansen et al., 2015, 2016; Homan et al., 2016). The shared inability in reproducing the in vivo epithelial tubulogenesis is a limitation we have overcome by developing a system that, relying on cell self-assembly into epithelial tubules, yields renal tissues more fitting for functional and pharmacological investigations.

Most of these systems share an additional drawback: the epithelial tubular structures cannot be physically removed from the surrounding ECM or from the pre-formed 3D-printed scaffolds in which they formed. This prevents tubules from being used for further investigations, such as morphogenetic studies and kidney tissue engineering experiments. On the other hand, our technology allows engineering tubules with lumen, predefined geometries, resembling foetal UB/collecting duct epithelia’s architecture and functions, which can be easily removed from the scaffolds. We engineered kidney tubular units in a controlled fashion by seeding cells, which were previously resuspended in collagen gel, into the 3D-printed PDMS scaffolds’ cavities and exposing them to appropriate tubulogenic stimuli. Under these conditions, similar to those in vivo, cells are directed to self-assemble into geometrically defined epithelial tubules within a 3D ECM-mimicking environment. The fact that our engineered tubules can be harvested, handled and transferred to different experimental conditions makes our system a valuable platform for several applications, such as kidney disease modelling, drug testing and discovery, as well as developmental studies.
In tissue engineering, the nature of the biomaterials’ surface and the biostructures’ complexity play a pivotal role for establishing realistic diagnostic and therapeutic solutions. In our system, we decided to fabricate hydrophobic PDMS scaffolds. This technical expedient was key to enable the development of complex tubular structures. Indeed, the scaffold cavities’ hydrophobicity allowed cell-collagen mixture gathering and immobilisation, thus limiting the risk of cell and ECM dispersal, and precluded irreversible cell adhesion and collagen attachment. Hence, PDMS hydrophobicity indirectly endorsed cell movement and spatial organisation in order to generate functional complex kidney tubules for studying development, modelling disease and investigating therapeutic approaches.

To rapidly prototype more complex structures with truly biomimetic features (Díaz Lantada et al., 2013) we developed a mathematical model that can provide fractal-like geometries. This ‘Golden Fractal Tree’ model maximised the amount of matter that can suit a limited surface, such as that of the scaffolds’ cavities, and allow progressive narrowing of bifurcations an infinite number of times. This avoids branch overlapping, a clear advantage for the serial production of reproducible complex tissues. Furthermore, our PDMS scaffolds can be reused upon conventional sterilisation techniques without requiring any other kind of treatment, thus providing an additional technical advantage.

Since kidney tubulogenesis is a biological process modulated by interactions between epithelial cells, soluble factors and the surrounding ECM components (Santos and Nigam, 1993), we first determined the precise ECM concentration that will allow for cell viability, growth, movement, establishment of cell-ECM and cell-cell adhesion, and organisation into tissue. Based on data published by Hauser et al., (Hauser et al., 2016) we selected the collagen type I concentration of 2.4 mg/ml. This concentration was described as the most efficient for tubule formation compared to lower ones, whereas higher concentrations
caused collagen gel to become too much rigid, thus preventing cell organisation and, concomitantly, tubulogenesis. Even in our system, the use of collagen at a concentration of 2.4 mg/ml has proved to be effective for tubule engineering; hence, we adopted this condition for our protocol.

Although tubular structures seemingly formed at a given cell number per collagen volume, a polarised epithelium and lumen were established only in the presence of HGF, a growth factor previously used to modulate cell growth and motility, and induce kidney morphogenetic processes in various epithelial cell lines (Santos and Nigam, 1993; Nakamura et al., 1997; Maeshima et al., 2000). Unlike previous methods that require long-term cultures - often ranging from 7 to 15 days (Sakurai et al., 1997; Schumacher et al., 2008; Hauser et al., 2016) - our highly efficient method produced epithelial tubules with lumen and predetermined geometries in only 2 days of culture.

Engineered tubules displayed functional characteristic of kidney tubules (Meyer et al., 2004; Sweet et al., 2006; Lawrence et al., 2015), such as internalisation of the synthetic macromolecule dextran and transporter-mediated organic anion tracer transport. These functional properties may be exploited in studies focussing on the mechanisms of active and passive trafficking of endogenous substrates and metabolites across renal tubular epithelial membranes, as well as transporter-mediated absorption, distribution, and elimination of drugs and other xenobiotics. In particular, the organic anion and cation transporters are broadly involved in transporting steroids, hormones and neurotransmitters, as well as excreting antivirals, antibiotics, chemotherapeutics and statins (Roth et al., 2012). These transporters are, therefore, able to influence the pharmacokinetics of their specific drug substrates, thus playing crucial roles in the effectiveness of pharmacological therapies and removal of toxicants. Hence, our engineered tubules may potentially
constitute a powerful tool for experimental nephrology, pharmacokinetic and toxicology studies.

By optimising our protocol, we successfully engineered hiPSCs-derived-UB-like tubules that replicated the kidney tubular epithelial architecture and functions as described above and, when co-cultured with mouse embryonic kidneys, underwent budding morphogenesis. The latter phenomenon indicated that human tubules could respond to kidney developmental cues in the form of soluble factors, a key aspect that made them suitable for elucidating mechanisms of human UB early morphogenesis. Indeed, we used the engineered human tubules to identify a novel combination of growth factors that is capable of inducing human UB-like tubules budding and ramification.

Finally, we applied our system to investigate how budding morphogenesis is affected by a heterozygous mutation in the \( PAX2 \) gene through the generation of UB-like tubules from patient-derived hiPSCs.

To the best of our knowledge, no other existing technology is able to engineer custom-made, complex and functional kidney tubules with different cell sources, such as renal cell lines and \textit{in vitro}-differentiated hiPSCs. It is also remarkable that, in our tissue engineering system, kidney tubules develop into patterned and polarised epithelia enclosing a lumen through cell spatial organisation in response to tubulogenic cues, as occurs \textit{in vivo}. For these reasons, and since the engineered tubules can be transferred to different experimental conditions, we used them to model PKD, test drugs, and study human development.

PKD is characterised by the gradual formation of plenty fluid-filled cysts in the kidney. Unfortunately, there are currently no approved therapies, causing this disease to remain incurable; the existing treatments can only help manage the wide range of problems associated with this condition. PKD is an inherited disorder linked to the disturbance of multiple intracellular pathways and cell functions, and affecting, with varying incidences,
both children and adults (Grantham et al., 2011; Sun et al., 2011; Sweeney and Avner, 2011; Wallace, 2011; Willey et al., 2017). Most of children and adult patients are destined to experience ESRD, thus eventually requiring RRT. In PKD, the development and enlargement of spheroidal epithelial cysts are the consequence of aberrant cell proliferation in the renal tubular epithelia, accompanied by transepithelial fluid secretion into the cystic luminal space. The over-stimulated cell proliferation is due to increased intracellular cAMP levels, a second messenger that is responsible for up-regulating B-Raf/MEK/ERK signalling in both animal models and patients. Hence, decreasing intracellular cAMP levels is considered one of the most appealing approaches for ameliorating PKD outcome.

Given the severity of the disease, the absence of in vitro and in vivo models able to faithfully mimic human PKD and the lack of effective therapies, it is imperative to develop more realistic PKD modelling systems suitable for drug screening. To begin to address this issue, we decided to use our collecting duct-like tubules engineered with the MDCK cells. These cells are known to form individual fluid-filled cysts when cultured in 3D ECM environments and stimulated with pharmacological agents capable of elevating intracellular cAMP levels. For this reason, their use in modelling PKD and exploring drugs’ efficacy on renal cyst regression has been extensive (Mangoo-Karim et al., 1989; Li et al., 2004; Gao et al., 2011). Nonetheless, conventional PKD models present one major drawback, namely the production of individual cysts, which cannot recapitulate the gradual cyst expansion into the tubular lumen as occurs in PKD in vivo. This precluded the possibility of modelling lumen obstruction and perturbations in both tissue architecture and epithelial polarisation, as well as the induction of mechanical stress and hypoxic conditions typical of PKD tubules. No other in vitro models can recapitulate PKD appropriately enough to constitute a valid alternative to conventional 3D cell cultures. Therefore, we decided to verify whether our engineered collecting duct-like tubules could provide a tool
to more realistically model PKD and perform drug testing and discovery studies. When we transferred the engineered collecting duct-like tubules to a 3D collagen culture system and stimulated them with the diterpene forskolin - a compound that induces cAMP production by activating several adenylyl cyclases - they developed many polarised cysts closely packed along the whole length of the tubules, causing lumen loss and disrupting the overall epithelial organisation. These features resembled bona fide those of diseased tubules in vivo and indicated that our engineered tubules can be used to model PKD in vitro.

When we added Tolvaptan, OCTR, PAS, 2DG or berberine to the culture medium, cyst density within tubules decreased to different extents. This indicated that engineered tubules could be an efficient tool for quantifying the effects of different known pharmacological agents and novel compounds on cyst regression.

The AVP V2 receptor antagonist Tolvaptan has been shown able to inhibit distal tubule and collecting duct cyst progression in both humans (Torres et al., 2012) and PKD rodent model (Gattone et al., 2003; Torres et al., 2004) by decreasing intracellular cAMP levels. According to these observations, concomitant administration of AVP - the V2 receptor’s ligand and the major cAMP agonist in the collecting duct (Yasuda and Jeffries, 1998; Wallace et al., 2001) - and Tolvaptan, following forskolin stimulation, significantly decreased cyst density within our tubules, when compared to the sole AVP stimulation. This confirmed that our PKD modelling system was applicable and reliable for drug screening purposes.

The synthetic somatostatin analogues OCTR and PAS, which possess longer half-lives than somatostatin and inhibit cAMP production, have been tested in preclinical and clinical trials. It has been previously reported that OCTR, which binds to somatostatin receptor 2 (SSTR2) and SSTR3, counteracts renal cyst growth in rodent models (Masyuk et al., 2007) and humans (Ruggenenti et al., 2005; Hogan et al., 2010; Caroli et al., 2013), whereas
PAS, which has high affinity to SSTR1, SSTR2, SSTR3 and SSTR5, was more effective than OCTR in mouse and rat PKD models (Masyuk et al., 2013). Conversely, in our system, we observed that OCTR was more effective than PAS in reducing cyst density, but the difference was not statistically significant. This inconsistency may be due to the condition in which the authors evaluated and quantified the effects of the two compounds on cyst growth. Since they adopted in vivo models and kept into consideration the whole renal parenchyma, they did not exclusively regard the kidney tubular compartment. We have instead investigated the drugs’ effects in vitro and solely on the engineered ‘polycystic’ collecting duct-like tubules.

Although in vivo studies demonstrated that treatments with Tolvaptan and somatostatin analogues slow PKD progression by decreasing cell proliferation and inducing cyst regression, severe adverse effects are associated with these pharmacological treatments (Riella et al., 2014). This makes the identification of novel effective therapeutic compounds with better tolerance an imperative need, so that many research groups are currently performing intense research to achieve this goal.

Working in this direction, in the present study we showed that 2DG - a glucose analogue that cannot be metabolised - and berberine - a compound approved for clinical trials in some diseases but that has never been tested in PKD - had a robust effect on cyst regression and completely restored both the central lumen and tubular epithelium integrity. The marked cyst regression in our ‘polycystic’ tubules following 2DG treatment is in line with data from recent studies conducted in vitro and in vivo models of both aggressive (Rowe et al., 2013) and slowly progressive (Chiaravalli et al., 2016) PKD. Rowe et al. demonstrated that PKD cells and mice displayed enhanced glycolysis, whereas glucose deprivation decreased PKD cell proliferation in vitro, and 2DG treatment ameliorated kidney volume, cystic index, but also reduced cell proliferation rate in vivo (Rowe et al.,
When Chiaravalli et al. treated PKD mouse models with 2DG, slower disease progression with both kidney/body weight and tubular and collecting duct cyst number reduction was observed in the absence of obvious signs of toxicity (Chiaravalli et al., 2016). These studies indicate that, like cancer cells, PKD cells require high levels of glucose - the principal source of energy of the cells - and reprogram their metabolism to use aerobic glycolysis (known as the Warburg effect) for energy production. This metabolic switch leads to enhanced glucose metabolism and uncontrolled cell proliferation. Considering the slow progression of PKD in humans and the late onset of therapeutic treatments, the proven enhanced effect of 2DG in slowly progressive murine PKD model (Chiaravalli et al., 2016) makes this compound a compelling therapeutic agent, even though it is not currently approved for treating any human condition. Hence, our results, together with existing evidence from in vivo studies, confirm 2DG clinical potential in the treatment of PKD and encourage its use and efficacy evaluation in clinical trials.

Previous investigations indicate that berberine exerts anti-proliferative effects in different cancer cell lines (Iizuka et al., 2000) and PKD cells (Bonon et al., 2013). Iizuka et al. reported that berberine causes cancer cell accumulation in G0/G1 phase and a relative reduction of the S phase (Iizuka et al., 2000). Bonon et al. demonstrated that berberine treatment of human and mouse PKD cystic cell lines reduces cell proliferation by increasing G0/G1 phase and inhibiting both phosphorylation of the extracellular signal-regulated kinase (ERK) - which stimulates cell proliferation (Yamaguchi et al., 2003) - and activity of p70-S6 kinase, a downstream effector of mTOR (Bonon et al., 2013).

Moreover, berberine is able to increase intracellular levels of reactive oxygen species by altering mitochondrial membrane potential, leading to mitochondrial collapse and compromising oxidative phosphorylation process. Being able to interact with the adenine nucleotide translocator, berberine causes ATP depletion in cancer cells (Diogo et al.,
Moreover, because of its chemical structure that resembles that of intercalating agents, berberine can directly bind to DNA (Krey and Hahn, 1969), eliciting double-strand breaks, eventually leading to cell death for apoptosis (Zhu et al., 2014). Overall, these properties may justify the effects of berberine in our system. By demonstrating the potent effect of this compound on cyst regression, as well as on tubular lumen and epithelial architecture restoration, we highlighted the hitherto unknown therapeutic potential of berberine for the treatment of PKD.

Important issues affect the study of the mechanisms of renal epithelial tubulogenesis and UB branching in human tissues. The first is the scarcity of suitable human embryonic material. The second is the lack of systems that, starting from single cells, allow engineering UB capable of recapitulating morphogenetic events occurring during early organogenesis. These problems are also present when studying human developmental disorders, as there is lack of methods for engineering in vitro kidney tissue from patients carrying genetic mutations.

To deal with these problems, we tested our engineering system for its ability to efficiently provide human kidney tubules from hiPSCs. To this end, we differentiated hiPSCs into UB-committed renal progenitor-like cells using a robust protocol based on brief exposure to defined growth factors (Xia et al., 2013), and then directed them to grow within PDMS scaffolds under tubulogenic conditions, as we did with MDCK cells. Like MDCK cells, human cells generated seemingly compact tubular structures, but HGF stimulation alone was not enough to induce the formation of a monolayered epithelium surrounding a continuous lumen. To achieve such architectural arrangement it was necessary to add GDNF to the medium, which indicates that GDNF exerts an essential signalling role in the maturation of hiPSC-derived UB tubular structures. This reflects the utmost importance of the GDNF for UB formation and growth during early kidney development in vivo. Hence,
we successfully engineered human UB-like branched tubular structures endowed with a single lumen bounded by a monolayered epithelium. The engineered human tubules were capable of synthetic macromolecule internalisation and OAT-mediated anion tracer transport, both maturation features typical of *in vitro* cultured UBs (Sweet *et al.*, 2006; Meyer *et al.*, 2004; Lawrence *et al.*, 2015). Of note, these findings are unprecedented in stem cell-derived kidney tissues.

When engineered human tubules were co-cultured with mouse embryonic kidneys in a 3D collagen culture system, they responded to kidney developmental cues by forming primary and secondary buds. These findings clearly demonstrate that our UB-like tubules are able to recapitulate early steps of UB morphogenesis and highlight the usefulness of our system for studying mechanisms of human kidney development.

Previous *in vitro* tubulogenesis systems using rodent kidney cell lines or isolated UBs, revealed that distinct growth factors are indispensable for regulating UB growth and branching morphogenesis within a 3D ECM environment (Qiao *et al.*, 1999a, 1999b, 2001; Sakurai *et al.*, 1997; Steer and Nigam, 2004). In our 3D collagen culture system, we identified a novel combination of growth factors that allows the engineered human UB-like tubules to undergo budding morphogenesis to an extent akin to that obtained with mouse embryonic kidney co-culture.

Although a higher degree of tubule maturation needs to be reached and a number of UB functions must be still tested, our data suggest that the engineered human tubules can be employed for studying human development and modelling diseases arising from or affecting the collecting duct system.

In a similar set of experiments, we used the system to disclose and quantify possible morphogenetic defects in tubules engineered with hiPSCs derived from a patient carrying a heterozygous *PAX2* mutation, and previously diagnosed with focal segmental
glomerulosclerosis (FSGS) (Barua et al., 2014). In developing kidney, PAX2 gene is expressed in branching UB, collecting duct epithelia and early nephrons (Dressler et al., 1990; Dressler and Woolf, 1999). FSGS is a rare CKD that affects glomeruli. This condition arises when podocytes are targeted by cellular stresses to which they respond through cytoskeletal reorganisation, leading to foot-processes effacement. In case of long-lasting harmful stressors, podocytes reach critical injury levels causing their irreversible detachment from the GBM and the underlying glomerular capillaries’ sclerosis due to plentiful matrix accumulation (D'Agati et al., 2011). Therefore, pivotal in the FSGS pathogenesis is podocyte damage and loss (D’Agati et al., 2011).

However, our findings suggest that the podocytes’ attrition typical of FSGS may occur secondary to kidney developmental anomalies associated with UB morphogenesis. We observed that the patient-derived UB-like tubules’ budding ability was significantly lower than that of healthy donor-derived tubules. This suggests that PAX2 mutations, such as the one we studied here, may be contributing to the defective UB arborisation and, ultimately, to a reduction in nephron number. This is supported by data from heterozygous Pax2 mutant mice showing diminished UB branching during fetal kidney development, associated with a marked decrease in nephron number at birth (Dziarmaga et al., 2006). As the amount of UB branching events determines the definitive number of nephrons (Nigam and Shah, 2009; Nagalakshmi and Yu, 2015), the patient’s kidneys may have had a reduced UB arborisation during early developmental stages, which led to suboptimal nephron endowment. It is also conceivable that the patient’s pathological lesions may have arisen as a consequence of haemodynamic stresses, which accelerated podocyte impairment by acting on nephrons reduced in number.

Although additional studies are necessary to validate these hypotheses, our findings provide new insights in understanding the pathogenesis of FSGS and confirm the
usefulness of our system to investigate individual patient's genetic disorders, for which no models currently exist.

By directing hPSC in vitro differentiation, other groups created rudimental 3D human kidney organoids in a dish (Taguchi et al., 2014; Morizane et al., 2015; Freedman et al., 2015; Takasato et al., 2015). Yet, the complete lack of UB (Taguchi et al., 2014; Freedman et al., 2015; Morizane et al., 2015) or otherwise its formation as multiple, individual small ducts (Takasato et al., 2015; Xinaris et al., 2016) impeded establishment of the proper kidney tissue patterning and cortical-medullary orientation, which would normally be imposed by a single collecting duct tree. These developmental insufficiencies may probably account for severe anatomical malformations within the organoids, that is the establishment of nephron-nephron connections and multi-branched nephrons (Freedman et al., 2015; Morizane et al., 2015; Takasato et al., 2015). Overall, these anomalies indicate that such approaches cannot accurately replicate kidney organogenesis in vitro.

In terms of kidney organoid function, there would also be a serious limitation arising from the above-mentioned developmental insufficiencies. Indeed, as the urine produced by nephrons should be drained away via a single collecting duct tree, the absence of UB or the lack of connections among the multitude of collecting ducts would impede the accomplishment of this vital function.

Our innovative technology may prove extremely useful to overcome all these problems, as it allows engineering hiPSC-derived UB-like tubular units with developmental capacities, which could be physically combined and reciprocally interact with hiPSC-derived MM cell aggregates, giving rise to functional human kidney organoids arranged organotypically around a single collecting duct tree. Constructing functional organoids from hiPSCs, which can be directly derived from patients’ own somatic cells, will facilitate the establishment of systems for in vitro disease modelling, personalised therapies, toxicology and
developmental studies, and may offer solid foundation for regenerative medicine applications.

To summarise, we have developed a 3D culture system that produces functional epithelial tubules of predetermined geometries starting from dispersed epithelial cells. Tubules engineered using the renal epithelial cell line are able to accurately replicate the PKD phenotype and respond to pharmacological treatments in a quantifiable manner. It demonstrates that these tissues can provide a pioneering tool for studying renal pathophysiology in vitro and conducting pharmacological studies. Importantly, our system can efficiently produce human UB-like tubules able to exert kidney-specific functions, endowed with developmental capacities, and suitable to investigate mechanisms of normal kidney development as well as possible aetiological roles of genetic alterations in UB morphogenesis and disease.

Although culture conditions for other cell types have yet to be defined, our technology is applicable and can be ideally adapted, with only minor modifications, to engineer different kinds of tissue, such as branched endothelial and lung epithelia. These may in turn be used to study human vascular and pulmonary developmental and pathophysiological mechanisms, respectively.

Overall, this controlled experimental system provides an accurate, rapid, cost-effective and reproducible methodology with which to model kidney disease, screen drugs and study development. It may represent an attractive and promising platform from which the spatially and geometrically controlled engineering of human kidney tissue with realistic anatomies and functions can be optimised and applied for regenerative medicine purposes.
CHAPTER 5 – FIGURES AND LEGENDS
Figure 1
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Figure 1. Schematic diagram of kidney development and nephron formation. Metanephric kidney development begins when ureteric bud (UB) emerges as a swelling of the caudal nephric duct (ND) and invades the metanephric mesenchyme (MM) of the posterior nephric cord (NC). Reciprocal autocrine and paracrine interactions between UB and MM induce the UB to first undergo bifurcated branching to form a T-shaped bud consisting of two tips and a stalk. Hereafter, each UB tip undergoes multiple rounds of bifurcation (branching morphogenesis) giving rise to the tree-like collecting duct system, whereas the elongated UB stalk forms the ureter. In turn, the branching UB epithelium induces nephrogenesis by promoting condensation of a subset of nephron progenitor cells within the MM around each UB tip. Condensed cells form the cap mesenchyme (CM), which epithelialise to form the renal vesicle (RV), the first epithelial structure of the nephron. The RV further grows and progressively forms comma- and S-shaped bodies (SSB), which eventually develop into mature nephrons. The distal end of the SSB fuses with a UB tip to form a continuous lumen, giving rise to the distal tubule of the nephron. The MM contains another cell population, the renal interstitial (RI) cells - also known as renal stromal cells. These cells support nephrogenesis and UB branching, and are the precursors of renal interstitial fibroblasts, vascular smooth muscle cells, pericytes and mesangial cells. Kidneys are subdivided into two main regions: the cortex (C) and the medulla (M), each containing different segments of nephron and UB/collection duct tubular epithelia. Adapted from (Nagalakshmi and Yu, 2015). Copyright 2015 by the John Wiley & Sons, Inc. Reused with permission.
Figure 2
Figure 2. Kidney anatomy. Schematic cross section of the kidney showing the overall internal structure and the main anatomical components of the organ. Adapted from (Rodrigues-Diez et al., 2017). Copyright 2017 by the John Wiley & Sons, Inc. Reused with permission.
Figure 3
Auerbach & Grobstein establish a method for generating 3D rudimental nephron-like structures by culturing dissociated and reaggregated MM onto embryonic spinal cord.

Independent groups (Montesano et al., Santos et al., Barros et al.) show that MDCK and mIMCD3 cells give rise to branched tubules in 3D ECM-based cultures in the presence of fibroblast-conditioned medium or mouse embryonic kidneys.

Sakurai et al. develop a 3D tubulogenesis system in which UB-derived immortalised cells cultured with MM cell-conditioned medium or with purified growth factors form branched tubular structures with lumen.

Osafo et al., generate 3D colonies containing glomerular- and proximal tubular-like structures by culturing aggregates of mouse MM cells onto a layer of Wnt4-transformed mouse embryonic fibroblasts.

Unbekannt & Davies develop a technology for creating 3D rudimental kidney tissue in vitro based on mouse embryonic kidneys’ dissociation and reaggregation. The tissue contained nephrons at different developmental stages and linked to small individual collecting ducts.

Xineras et al. generate kidney organoids by dissociating and reaggregating mouse embryonic kidneys. Following implantation in vivo, organoids mature, develop vascularised glomeruli able to filter blood, and display basic kidney-specific functions.

By differentiating PSCs under chemically defined culture conditions, independent groups develop methods to generate kidney organoids containing MM and its derivatives (Taguchi et al.), or both MM and UB progenitors (Takasato et al.).

Takasato and colleagues obtain hPSC-derived self-forming kidney organoids containing both MM derivatives and multiple individual UB epithelia.

Xineras et al. create chimeric kidney organoids with hAFSCs integrating into the condensing MM. After implantation in vivo, organoids become vascularised, and hAFSCs contribute to glomerular structures differentiating into mature podocytes.

Grobstein proves that MM and UB can form nephrons and branched collecting duct, respectively, by recapitulating early kidney developmental stages ex vivo.

Ekbloom et al. show that intact MM co-cultured with embryonic spinal cord explant forms glomerular and renal tubular structures.

Barasch and colleagues demonstrate that intact MM co-cultured with UB cell aggregates undergoes nephrogenesis.

By using a 3D culture method and MM-derived conditioned medium enriched with GDNF, Qiao and colleagues show that intact UB develops into a ramified tubular epithelium capable of inducing nephrogenesis when recombined with MM.

Rosines et al. show that intact ND can generate branched epithelial structures and induce nephron formation when recombined with MM in a 3D culture system.

By combining MM cell reaggregates with a reforming UB taken from previous dissociation-reaggregation experiment, Ganeva et al., engineer 3D immature mouse embryonic kidney with a single UB-derived branched collecting duct.

Xia et al. establish a protocol for differentiating hPSCs into UB kidney progenitors. These cells incorporated in the developing UB structures of mouse metanephric kidneys reaggregates.

Araoka et al. and Lam et al. develop protocols for hPSC directed differentiation into IM cells that when mixed with mouse embryonic kidney cells (chimeric cell aggregates) contribute to the developing proximal tubular structures.

Starting with hPSCs, Bonventre’s group (i) develops a differentiation protocol to obtain MM cells that self-assemble into “nephron organoids” (Montzane et al.), and (ii) devises a 3D culture system for generating kidney tubular organoids that contain segmented nephrons and can model kidney disease (Freedman et al.).

Figure 4
Figure 4. Historical summary of 3D kidney tissue engineering approaches. The main themes and technologies concerning kidney organ generation are listed in chronological order. Tissue-based approaches (blue); embryonic kidney cell- and cell line-based approaches (red); hPSC-based approaches (green).
Figure 5
Figure 5. Engineering tubular structures starting from MDCK cell suspensions. (a) Schematic workflow for PDMS scaffold fabrication. Based on the custom-designed patterns, plastic masters are 3D-printed using rapid prototyping technologies and then utilised to produce the scaffolds through PDMS replica moulding and polymerisation. (b) Experimental design: MDCK cells are centrifuged, resuspended in collagen I, seeded into the PDMS scaffold cavity and cultured in the presence of HGF for up to 2 days. (c) Immediately after seeding (day 0), the mixture of collagen and cells completely fills the scaffold cavity. At day 1, cells organise to form tubular aggregates corresponding to the shape of the cavity and showing marked shrinkage. At day 2, branches of the shrunk tubular structures emerge from the cavities, and a translucent area (middle panel, asterisk) delimited by darker edges (middle panel, arrows) is visible through bright-field microscopy. (d) Ramified and (e) tree-like tubular structures after 2 days of culture. (f) A multichannel scaffold containing epithelial microtubules after 2 days of culture. Scale bars: 2 mm (b-d), 500 μm (e).
Figure 6
Figure 6. Engineered tubules display lumen formation, epithelial absorption of molecules and transport of ions at 2 days. (a) Confocal Z-stack imaging from the surface to the inner region of a microtubule demonstrates the formation of a continuous lumen (L) in the central region. E-cadherin (red) is expressed by peripheral cells of the microtubule. (b) A branch from a macrotubular system showing an extended lumen (L) enclosed by a monolayered epithelium of cells with aligned nuclei and expressing E-cadherin (red) and WGA-lectin (green) in the lateral and basal membranes, respectively (inset). (c-e) Microtubules displaying (c) dextran (green) internalisation and (d) 6CF (green) anion transport in the absence or (e) presence of the organic anion transporter inhibitor probenecid. WGA-lectin, wheat-germ agglutinin-lectin; 6CF, 6-carboxyfluorescein; DAPI, blue-stained nuclei. Scale bars: 10 μm (a, c-e insets), 100 μm (b, c), 50 μm (d, e).
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Figure 7
Figure 7. MDCK-derived tubules model PKD and respond to the treatment with different compounds. (a) Schematic representation of 3D collagen culture system. After 2 days of culture, engineered microtubules are harvested from the scaffold, embedded in collagen I, stimulated with forskolin for 7 days to induce cyst formation and then treated with different compounds to evaluate cyst regression. (b) Bright-field image of a forskolin-stimulated microtubule showing many large spheroidal cysts stained with hematoxylin and eosin (insets). (c, d) Cysts are delimited by a monolayered epithelium of cells positive for E-cadherin (white) in the basolateral membranes and (d) podocalyxin (green) in the apical membranes (insets). The tubular lumen is lost. (e-j) Forskolin-stimulated tubules after a 7-day treatment with (e) rapamycin, (f) Tolvaptan in combination with AVP, (g) OCTR, (h) PAS, (i) 2DG or (j) berberine. (i, j) 2DG and berberine treatments restore tubular lumen. 

(k, l) Quantification of cyst number/mm²: No treatment (forskolin; 1205 ± 48.81); rapamycin (990 ± 174.5); OCTR (508.5 ± 39.52); PAS (688.9 ± 52.1); 2DG (348.1 ± 44.15); berberine (335.8 ± 27.93); AVP (1948 ± 283.4); AVP+Tolvaptan (716.4 ± 57). Data are expressed as means ± s.e.m. from three independent experiments. (k) Number of fields analysed: n=18 for forskolin, n=6 for rapamycin, n=6 for OCTR, n=7 for PAS, n=8 for 2DG, n=11 for berberine. *P<0.05 versus PAS, °P<0.0001 versus rapamycin, #P<0.0001 versus forskolin by one-way ANOVA with Tukey’s multiple comparisons test. (l) Number of fields analysed: n=4 for AVP, n=9 for Tolvaptan in combination with AVP. **P<0.0001 by two-tailed Student’s t-test. DAPI, blue-stained nuclei; AVP, arginine vasopressin; OCTR, octreotide; PAS, pasireotide; 2DG, 2-deoxy-D-glucose. Scale bars: 100 μm (b), 50 μm (c, e-j), 20 μm (d).
Figure 8
Figure 8. Engineering functional human UB-like tubules using iPSCs. (a) Experimental design. Human iPSCs are differentiated toward UB-like progenitor cells. Cells from differentiation days 3 and 4 are pelleted and then re-suspended in collagen I, seeded into the PDMS scaffold cavity, and cultured in the presence of HGF and GDNF for 2 days. (b) A branch from a macrotubule engineered with cells from differentiation day 3 displaying a continuous lumen (L) enclosed by a monolayer of E-cadherin (white) and WGA-lectin (green) positive cells. Inset: cells of the tubular wall show aligned nuclei (blue), and E-cadherin (white) and WGA-lectin (green) in the basolateral membranes. (c) Z-stack images of a microtubule engineered with cells from differentiation day 4 show the presence of a single central lumen (L) lined by a WGA-lectin-positive (red) epithelium. (d, e) Microtubules showing (d) dextran (green) internalisation and (e) 6CF (green) transport. UB, ureteric bud; d, day; WGA-lectin, wheat germ agglutinin-lectin; DAPI, blue-stained nuclei; 6CF, 6-carboxyfluorescein. Scale bars: 100 µm (b-d), 50 µm (e).
Figure 9
Figure 9. Engineered tubules as a tool for studying human UB developmental processes and defects. (a) Experimental design. Fragments of hiPSC-derived macrotubules are transferred from the PDMS scaffold to the 3D collagen culture system and cultured with mouse embryonic kidneys or in the presence of different combinations of growth factors for up to 2 days. (b, c) Healthy donor-derived tubule co-cultured with mouse embryonic kidneys. (b) At day 1, long primary buds emerge (left panel). At day 2, a lateral ramification (arrow) arises from the primary bud. (c) A ramified bud consists of aligned E-cadherin-positive cells with linearly oriented nuclei. (d) Percentage of the ramified buds in the total buds emerged from healthy donor-derived tubules. Data are expressed as means ± s.e.m. from three independent experiments. Number of fields analysed: $n=48$ for H+G, $n=30$ for H+G+F1 and H+G+F7, $n=22$ for H+G+F1+F7, $n=21$ for mouse embryonic kidneys. *$P<0.05$ versus H+G, °$P<0.05$ versus H+G+F1, #$P<0.01$ versus H+G by one-way ANOVA with Holm-Sidak’s multiple comparisons test. (e, f) A healthy donor-derived tubule cultured with the complete combination of growth factors displays primary and secondary buds (arrows) some of which showing terminal bifid branching (asterisks). (f) A primary bud arising from healthy donor-derived tubule cultured as that in (e). (g) Percentage of the ramified buds in the total buds emerged in healthy donor- and patient-derived tubules cultured with all growth factors. Data are expressed as means ± s.e.m. from three independent experiments. Number of fields analysed: $n=22$ for healthy donor- and $n=27$ for patient-derived tubules. **$P<0.0005$ by two-tailed Student’s t-test. (h, i) Patient-derived tubule cultured with all growth factors develops mainly primary buds. (i) A primary bud arising from patient-derived tubule cultured as that in (h). DAPI, blue-stained nuclei; H, HGF; G, GDNF; F1, FGF1; F7, FGF7. Scale bars: 100 μm (b, left panel; e, left panel), 50 μm (e, right panel; h), 20 μm (b, right panel; c, f, i).
Supplementary Figure 1

\[ \begin{align*}
\alpha_0 &= 160^\circ \\
\alpha_1 &= 130^\circ \\
\alpha_n &= \frac{\alpha_{n-2}}{\varphi} & n = 2, 3, \ldots
\end{align*} \]

\[ \begin{align*}
L_0 &= \frac{1}{\varphi} L \\
L_n &= \frac{1}{\varphi} L_{n-1} & n = 1, 3, 5, \ldots \\
L_n &= L_{n-1} & n = 2, 4, 6, \ldots
\end{align*} \]
Supplementary Figure 1. PDMS scaffold design and fabrication. (a-d) Patterns designed for PDMS scaffold fabrication. Related lengths, depths, widths and angles are indicated for each pattern. Designed geometries: (a) linear, (b) bifurcated with an 80° terminal bifid branch, (c) asymmetrical with two 30° lateral branches arising from the central channel, and (d) ramified. (e) The ‘golden fractal tree’ formula. $\alpha_0$ and $\alpha_1$ represent the amplitude of first and second branching generation, respectively; $L$ refers to central trunk length; $n$ is the order of branching generation, and $\varphi=1.618$ is the ‘golden ratio’. (f) The tree-like pattern consisting of fractal-like planar ramifications with different lengths and branching angles calculated by setting $\alpha_0=160^\circ$ and $\alpha_1=130^\circ$ and $n=3$. (g) Linear multichannel pattern for engineering multiple independent tubules at the same time.
Supplementary Figure 2
Supplementary Figure 2. Effect of cell densities on tubule formation. (a, b) Cell concentrations lower than those established as optimal completely prevent tubular structure formation both (a) in macro- and (b) in micro-scaffolds. (c) Three hours after seeding at the optimal cell concentration, MDCK cells organise to form a tube-shaped aggregate. Scale bars: 1 mm (a, c), 500 μm (b).
Supplementary Figure 3
Supplementary Figure 3. MDCK cell-derived tubules display polarised epithelium establishment and apoptosis during lumen formation. (a) At day 2, the monolayered epithelium forming the tubular wall is double positive for PNA-lectin (red) and podocalyxin (green) in the apical domains. (b) At day 1, macrotubules develop a major luminal space (L) bounded by aggregates of cells with condensed chromatin (inset, asterisks). (c) Cells forming the aggregates in the central areas of tubule display nuclear fragmentation and are positive for the apoptotic marker cleaved caspase 3 (red). (d) At day 2, tubular structures formed in the absence of HGF do not develop a single central lumen. PNA-lectin, peanut agglutinin-lectin; WGA-lectin, wheat germ agglutinin-lectin; DAPI, blue-stained nuclei. Scale bars: 10 μm (a, c), 100 μm (b, d).
Supplementary Figure 4
Supplementary Figure 4. Gene expression profile of human iPSCs during differentiation into UB-like progenitor cells. *NANOG* (pluripotency marker) expression decreases progressively. *T* (*Brachyury*) (mesendoderm marker) expression peaks at d2, while *LHX1 (LIM1)* (intermediate mesoderm marker) and *GATA3* (ureteric epithelium marker) peak at d3. *PAX2* (nephric lineage marker) and *HOXB7* (UB-related transcription factor) expression levels increase progressively up to d4. Values are relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Data shown are from one representative experiment of three independent experiments. d, day; *NANOG*, Nanog homeobox; *T*, brachyury transcription factor; *LHX1*, LIM homeobox 1; *GATA3*, GATA binding protein 3; *PAX2*, paired box 2; *HOXB7*, homeobox B7.
Supplementary Figure 5
Supplementary Figure 5. GDNF is indispensable for lumen formation in human UB-like tubules engineered with 3- and 4-day differentiated iPSCs. (a) Tubular structure engineered using 3-day differentiated hiPSCs cultured for 2 days in the presence of HGF alone fails to develop a single lumen. (b) Tubular structure engineered using 3-day differentiated hiPSCs exposed to both HGF and GDNF displays a lumen (L) and some cells in the tubular wall positive for PKC-ζ (red) in the apical domains, indicating the establishment of epithelial polarisation. (c) Undifferentiated hiPSCs proliferate massively within the scaffold cavity and appear scattered throughout the culture medium. (d) hiPSCs from differentiation day 2 form large and filamentous cell aggregates that detach from the mould and fail to form tubules. (e) At day 1, a macrotubule cultured in the presence of HGF and GDNF displays multiple lumens (L) surrounded by apoptotic cells with condensed chromatin (inset, asterisks). (f) The same cells that show chromatin condensation also express the apoptosis marker cleaved caspase 3 (red) and display nuclear fragmentation. PKC-ζ, protein kinase C isoform-zeta; DAPI, blue-stained nuclei. Scale bars: 100 mm (a, e), 10 mm (b, f), 1 mm (c), 500 mm (d).
Supplementary Figure 6
Supplementary Fig. 6. Differentiation of healthy donor-derived iPSCs toward UB-like progenitor cells and tubule engineering. (a) Gene expression profile of healthy donor-derived iPSCs during differentiation. *NANOG* expression decreases up to d4, while *T* (*Brachyury*) peaks at d2. *LHX1 (LIM1)* and *GATA3* peak at d3, while *PAX2* and *HOXB7* expression levels increase up to d4. Values are relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Data shown are from one representative experiment of three independent experiments. (b) Z-stack images of healthy donor-derived microtubule display formation of a single lumen (L) in the tubular central region. (c) Healthy donor-derived microtubule showing a continuous lumen (L) lined by a monolayered epithelium positive for (c, inset) PKC-ζ (red) in the apical membranes. d, day; *NANOG*, Nanog homeobox; *T*, brachyury transcription factor; *LHX1*, LIM homeobox 1; *PAX2*, paired box 2; *GATA3*, GATA binding protein 3; *HOXB7*, homeobox B7; WGA-lectin, wheat-germ agglutinin lectin; PKC-ζ, protein kinase C isoform-zeta; DAPI, blue-stained nuclei. Scale bars: 100 μm (b, c).
Supplementary Figure 7
Supplementary Figure 7. Characterisation of patient-derived iPSCs. (a) Patient-derived iPSCs are positive for the pluripotency markers OCT4, TRA-1-60, NANOG, TRA-1-81, SSEA3 and SSEA4. (b) The pluripotency genes’ expression level in patient-derived iPSCs is similar to that in human embryonic stem cell line H9, used as control. Data are expressed as means ± s.e.m. from three independent experiments. (c) Patient-derived iPSC in vitro differentiation into all three germ layers through EBs formation: β-Tubulin III (ectoderm marker), α-SMA (mesoderm marker) and GATA4 (endoderm marker). (d) Karyotype analysis revealing normal 46,XY chromosomal number and structure. TRA-1-60, Tumour-related antigen-1-60; NANOG, Nanog homeobox; TRA-1-81, Tumour-related antigen-1-81; SSEA3, Stage-specific embryonic antigen 3; SSEA4, Stage-specific embryonic antigen 4; SOX2, SRY-box2: α-SMA, alpha-smooth muscle actin; GATA4, GATA binding protein 4; EBs, Embryoid bodies; DAPI, blue-stained nuclei. Scale bars: 50 μm (a, c).
Supplementary Figure 8
Supplementary Figure 8. Differentiation of patient-derived iPSCs toward UB-like progenitor cells and tubule engineering. (a) Gene expression profile of patient-derived iPSCs during differentiation. *NANOG* expression decreases up to d4, while *T* (*Brachyury*) peaks at d2. *LHX1 (LIM1), PAX2, GATA3* and *HOXB7* expression levels increase up to d4. Values are relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Data shown are from one representative experiment of three independent experiments. (b) Patient-derived macrotubule after 2 days of culture shows marked shrinkage and develops a continuous lumen (L) lined by a monolayered epithelium of aligned cells positive for E-cadherin in the basolateral membranes (inset). d, day; *NANOG*, Nanog homeobox; *T*, brachyury transcription factor; *LHX1*, LIM homeobox 1; *PAX2*, paired box 2; *GATA3*, GATA binding protein 3; *HOXB7*, homeobox B7; WGA-lectin, wheat-germ agglutinin lectin; DAPI, blue-stained nuclei. Scale bars: 100 μm (b).
CHAPTER 6 - BIBLIOGRAPHY
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CHAPTER 7 - APPENDICES
7.1 Contribution to the thesis by other researchers

Part of this study was performed in collaboration with other researchers and scientists of the IRCCS - Istituto di Ricerche Farmacologiche ‘Mario Negri’, Bergamo (Italy), of the Università degli Studi di Genova, Genova (Italy), and of the Azienda Socio-Sanitaria Territoriale (ASST) Papa Giovanni XXIII, Bergamo (Italy) as follows:

- Dr. Valentina Benedetti, Laboratory of Cell Biology and Regenerative Medicine, IRCCS - Istituto di Ricerche Farmacologiche ‘Mario Negri’, helped me with most of the experiments, particularly in establishing the tubule engineering system, the engineered tubules’ 3D culture conditions, and with quantification and statistical analyses.
- PDMS scaffold design and fabrication was carried out by Dr. Patrizia Guida, Dr. Elena Angeli and Professor Ugo Valbusa, Nanomed Laboratories, Dipartimento di Fisica, Università degli Studi di Genova.
- Professor Andrea Remuzzi, Laboratory of Tissue Engineering for Regenerative Medicine, IRCCS - Istituto di Ricerche Farmacologiche ‘Mario Negri’, kindly provided MDCK type II cells.
- Dr. Marta Todeschini and Dr. Marilena Mister, Laboratory of Immunology of Organ Transplantation, IRCCS - Istituto di Ricerche Farmacologiche ‘Mario Negri’, performed the isolation of the patient’s peripheral blood mononuclear cells.
- Dr. Susanna Tomasoni, Dr. Osele Ciampi and Dr. Lorena Longaretti, Laboratory of Gene Therapy and Cellular Reprogramming, IRCCS - Istituto di Ricerche Farmacologiche ‘Mario Negri’, generated and characterised every iPSC line used in this research.
- Dr. Ursula Giussani and Dr. Paolo Fruscella, Laboratory of Genetic Medicine, ASST Papa Giovanni XXIII conducted patient-derived iPSC karyotype analysis.

The PhD Student, Valerio Brizi, carried out every other experiment by applying the technologies and procedures described in the Materials and Methods section of this thesis.
7.2 Full list of publications by the candidate


*Equal contribution.


*Equal contribution.
