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Dissecting nephron morphogenesis using kidney organoids from human pluripotent stem cells

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Abstract

During kidney development the emergence of complex multicellular shapes such as the nephron (the functional unit of the kidney) rely on spatiotemporally coordinated developmental programs. These involve gene regulatory networks, signaling pathways and mechanical forces, that work in concert to shape and form the nephron(s). The generation of kidney organoids from human pluripotent stem cells now represent an unprecedented experimental set up to study these processes. Here we discuss the potential applications of kidney organoids to advance our knowledge of how mechanical forces and cell fate specification spatiotemporally interact to orchestrate nephron patterning and morphogenesis in humans. Progress in innovative techniques for quantifying and perturbing these processes in a controlled manner will be crucial. A mechanistic understanding of the multicellular dynamic processes occurring during nephrogenesis will pave the way to unveil new mechanisms of human kidney disease.

Keywords

Human pluripotent stem cells; kidney organoids; kidney development; nephron patterning; nephron morphogenesis; disease modelling.

1. Introduction

The morphogenetic mechanisms of vertebrate kidney development have been studied for almost a century now. Early studies using mice and avian embryonic kidneys showed that ex vivo dissociation and reaggregation of embryonic kidneys resulted in the formation of proper renal epithelial tubules surrounded by stroma-like cells when cultured in vitro [1]. These pioneering studies were followed by Clifford Grobstein's investigations, who established the fundamentals of mouse kidney organ culture. By recombining the isolated components of day 11 mouse embryonic kidneys (E 11.0), those studies described that inductive signaling events between the metanephric mesenchyme (MM) and the ureteric bud (UB) epithelium were necessary for the formation of epithelial nephrons in mammals [2]. Importantly, those observations provided for the first time an *ex vivo* culture platform for studying kidney organogenesis [2], also supporting the hypothesis that MM and UB components secreted molecules reciprocally stimulated each other. This idea was further developed by Lauri Saxén and colleagues (1987) who discovered that isolated mouse MM was competent to respond to inductive signals from several embryonic tissues (i.e. the embryonic spinal cord) giving rise to the formation of proper renal epithelial tubules [3]. Overall, these and other early studies [4, 5] set the foundation for the current understanding of kidney development (**Box 1**) [6–8].

In parallel with all these findings, the need to develop culture platforms sustaining ex vivo organ culture advanced together with the generation of genetic mouse models that were key for the identification of genes and signaling pathways determinant for the induction of nephrogenesis in mice [8–10] (**Figure 1A**). Although accumulative knowledge from these studies have provided a significant mechanistic understanding for mammalian kidney organogenesis, still many questions remain on how these developmental processes (i.e. nephron epithelization and segmentation) are coordinated to shape the human kidney. In this regard, the pioneering studies of Edith Potter over six decades ago described the anatomy of human renal tract development [11]. Nowadays, the field has been able to investigate human embryonic kidney samples using single-cell RNA-sequencing (scRNA seq) to start performing a thorough characterization of the multiple cell types encountered in the human kidney as well as the proportion of specific cell populations with respect to the others [12–16]. Importantly, these works have started to shed light on differences between human and mouse kidneys [16]. In the same manner intense research has been dedicated towards the isolation of nephron progenitor cells (NPCs) from both mouse and human embryonic kidneys in an attempt to identify proper culture conditions for their in vitro expansion while preserving their multipotent potential [17–19]. However, access to human NPCs from embryonic kidneys is still not affordable for many laboratories and rises important ethical concerns. Alternatively, human pluripotent stem cells (hPSCs) - both hESCs [20] and human induced pluripotent stem cells (hiPSCs) [21]- have represented an ideal cell source to start exploring the possibility to generate NPC-like cells in the Petri dish. Profiting from the inherent properties of hPSCs numerous research groups have worked on identifying cell culture conditions that support the generation of renal progenitor cells including intermediate mesoderm progenitors (IM), NPCs, MM and UB [22, 23]. Indeed, it has

been remarkable how these pioneering works made use of optimized versions of the Grobstein assay to assess the differentiation potential of the hPSCs-renal derivatives in vitro and ex vivo. As an early example, Xia and colleagues re-aggregated hPSC-derived UB-committed progenitor cells with dissociated kidney cells from E11.5 mouse kidneys resulting in the generation of chimeric kidney organoids [24]. Further understanding of the biochemical cues guiding hPSCs towards the renal lineage together with the application of 3D organ culture techniques have led to the definition of procedures to generate kidney organoids from hPSCs (**Figure 1B**) [23, 25, 26].

These methodologies have been assessed using different hPSC cell lines and by different laboratories around the world demonstrating that the generated kidney organoids transcriptionally match the human fetal kidney of the first or second trimester of gestation [27–29]. Moreover, a side-by-side comparison of different procedures for the generation of kidney organoids from hPSCs by scRNA seq demonstrated a high level of congruence in the relative proportions of nephron, stromal, and endothelial cell types and in the expression of key cell type markers between organoid and human fetal datasets [27, 30–34].

All in all, these findings highlight the relevance of kidney organoids as an in vitro model system to understand the spatiotemporal regulation of human nephrogenesis [35]. Major kidney organoid limitations, including the lack of maturity and organized vasculature, among others, are expected to be solved in the future through the application of engineering techniques (i.e. microfabrication techniques, microfluidics, 3D bioprinting, among others) [36]. Importantly, bioengineering approaches have already been proved promising for creating controlled tissue geometries, applying of fluid flow and producing high-throughput organoid platforms for drug screening which

now can be exploited to externally control kidney organoid generation and differentiation (**Figure 1C**).

2. Studying human nephrogenesis with hPSC-derived kidney organoids

Nephron induction and epithelization

During embryonic development, the MM comprises NPCs and stromal progenitors that give rise to nephron structures from the glomerulus to the distal tubule. In this process the mesenchymal cells convert into highly organized epithelia under the influence of signals from the UB. In turn, the MM induces the UB to extend and branch. The morphological sequence of this conversion includes the formation of a corona (or cap) of mesenchymal cells surrounding the tips of the UB followed by the development of pre-tubular aggregates (PTAs), which evolve into epithelial vesicles. These polarized spheres, also called renal vesicles (RVs) arise via a hollowing process and constitute the preliminary forms of the segmented nephron(s). At this stage progenitor stromal cells are interspersed with vascular progenitors surrounding the cap mesenchyme. To date our comprehension of these processes have largely relayed on histological and morphological criteria and the expression of marker molecules orchestrating MM conversion into polarized epithelia.

Interestingly, early polarity during renal morphogenesis can already be demonstrated at the coronal cell stage when the MM at the tip of the UB reveal a columnar appearance. At this moment, the epithelial structures appear *de novo* during mesenchyme to epithelial transition (MET). This differs from most epithelia in the developing embryo (including airway, most of the urogenital system, exocrine glands, pancreas), which arise by the branching or the folding of pre-existing epithelial sheets or tubules, rather

than from non-epithelial cells [37]. Thus, renal morphogenesis represents a unique experimental scenario to identify the sequence of polarization in mammalian epithelia. In this regard, nephron formation from RVs is driven by elongation to form a primordial tubule(s) called Comma-shape bodies (CSB) that develop into S-shape bodies (SSB) which then fuse with the tip of adjacent UB tubules forming a continuous lumen (**Figure 2A**). The SSB subsequently undergoes extensive morphogenesis to form the epithelial portion of the nephron from the glomerulus to the distal tubule, whereas the UB gives rise to the collecting duct system. Collectively, kidney epithelia arise during development from both the UB and CM through at least two cellular processes including budding and hollowing [38]. In this complex and coordinated processes the lumen from the UB arises from a pre-existing lumen whereas in nascent nephron(s) the lumen arises *de novo* during MET [39, 40]. Importantly, these disparate tubule types then undergo a unique process of tubular (and consequently luminal) fusion. The mechanisms that regulate the lumen interconnection between these two epithelial tissues are yet to be fully understood [41].

Given the central role of the UB in kidney organogenesis, it is widely stated that defects in UB and collecting duct development can lead to malformation of the kidney, low nephrons numbers at birth, and congenital anomalies of kidney and urinary tract (CAKUT) [42, 43]. Now, the possibility to develop kidney organoids form hPSCs opens an unprecedented experimental setting to decipher early stages of nephron induction and epithelization in the human setting. However, a better understanding of kidney branching morphogenesis is needed for in vitro efforts toward rebuilding the human embryonic kidney. While early studies on the generation of hPSCs-kidney organoids lead to the derivation of 3D culture systems merely composed of nephron-like structures lacking a proper UB interconnected system [28, 29, 44–46], current accumulative

efforts are guided towards the generation of nephron-containing kidney organoids also presenting distal nephron epithelium and UB. Recently, pioneering studies have proved the possibility of generating distal nephron-like cells which upon culture and conversion into ureteric epithelial tip-like cultures and re-aggregation with hPSCs-NPCs can give rise to hybrid organoids for further studies in disease modeling [47]. Interestingly, the work from Zeng and colleagues has now shown the possibility to generate expandable, 3D branching UB organoid culture models from hPSCs which differentiated into principal and intercalated cells adopting spatial assemblies reflective of the adult kidney's collecting system [48]. Moreover, in their hands the authors also demonstrate that aggregating 3D-cultured NPCs with UB organoids in vitro results in a reiterative process of branching morphogenesis and nephron induction, similar to kidney development [48].

Since kidney organoid systems allow for a direct access to human kidney developing tissue, manipulations can be studied in real-time and time-lapse modes. Thus, it is expected that soon the field will be able to refine more kidney organoid culture systems to further understand how imbalances in tissue-scale (nephron) morphogenetic process during the in vitro development and self-organization of human kidney organoids can be related to CAKUT manifestation in vitro. At the same time, many of the 40 established monogenic causes of human CAKUT have been initially identified as candidate genes from observations in mouse models of CAKUT and subsequently screened for their prevalence in human disease cohorts [42]. However, the insights from mouse models do not always directly translate to human genetics. Explanations for this discrepancy include potential species-specific differences in kidney and urinary tract development, alterations in the required gene dosage, functional compensation by redundant genes, incomplete penetrance, among others [49]. In this regard, the

transcription factor PAX2 which is essential for MET of NPC and UB lineage development in mice causes renal coloboma syndrome when mutated in humans. Interestingly, when PAX2-null human NPCs were generated from human iPSCs via transcription activator-like effector nucleases (TALENs), PAX2 was shown to be dispensable for NPCs generation from human iPSCs [50]. This early example by the Nishinakamura laboratory highlights the potential use of kidney organoid technology to unveil the precise role and expression patterns of genes driving nephron induction and epithelization. In the future, it will be interesting to continue exploiting kidney organoid technology and genome editing tools to assess how the different components of epithelial polarity appear in these model systems and how these processes are related to kidney development and disease. A recent example stands in the early study from the Li laboratory employing the CRISPR/Cas9 system to knock out (KO) Ret/RET in mouse and human UB organoids to dissect the critical role of GDNF signaling to maintain the UB progenitor cells and stimulate UB branching morphogenesis in hPSCs derived UB organoids [48]. In this manner, the authors demonstrated the critical role of RET KO in the arrest of branching morphogenesis in both mouse and human UB organoids, while differences in the expression of progenitor markers in these model systems suggest that species-specific regulatory network downstream of Ret/RET are responsible for the UB progenitor fate, consistent with previous observations of convergent and divergent mechanisms of nephrogenesis between mouse and human [51, 52].

Nephron patterning and segmentation

The morphological transformations occurring during the process of nephrogenesis are tightly controlled by specific patterns of gene expression and signaling pathways. The RV stage is characterized by the acquisition of proximal to distal polarized domains, the distal domain being adjacent to the UB tip. Next, in the SSB stage an additional medial

domain becomes evident in between the proximal and distal areas. This process of regionalization, so-called nephron patterning, is essential for proper specification of the different nephron fate segments (**Figure 2A**).

Studies performed in the mouse have shown that the acquisition of proximal-distal polarity requires Rho-kinase signaling [53], whereas Wnt/β-catenin and Notch signaling pathways are key for driving nephron patterning and segmentation [54]. For example, in coordination with the Notch signaling pathway, WT1 directs the specification of the podocyte fate by antagonizing PAX2. At the same time, HNF1b guides proximal and medial fate commitment by regulating Notch ligand expression whereas medial and distal fates are regulated by BRN1 [6]. Importantly, we and others have recently found that these same signaling pathways also guide nephron formation in hPSCs-derived kidney organoids through self-organization and self-patterning events (**Figure 2B**) [28, 55]. Alongside these findings, a recent study by the Nishinakamura group showed that modulation of the Wnt signaling pathway during hPSC-derived kidney organoid generation promoted selective podocyte induction in high yields [56].

Nonetheless, the full comprehension of the genetic programs that guide nephron segmentation and differentiation in the human context is yet sparse, partially due to the limited access and further culture of human embryonic kidney cells. Interestingly, a recent study by the McMahon laboratory has developed an approach to unveil spatial organization, diversity, and gene expression profiles during human nephron patterning from NPCs towards SSB stage. The authors performed confocal analysis and single cell RNAseq of human kidney samples from 14 to 17 weeks of gestation, and they related transcriptomic data to the developing nephron anatomy. With this approach authors could also predict cell-type specific functional gene networks [15]. The possibility to

spatiotemporally resolve the human nephrogenic program will provide new insights in kidney development and disease.

During nephron patterning and morphogenesis, multicellular behaviors (i.e. cell polarity, proliferation, morphological changes, cell rearrangements, among others) are coordinated across space and time allowing the emergence of complex and organized structures. This is possible thanks to numerous molecular and mechanical cues which guide proper cell-to-cell and cell-to-ECM interactions via feedback mechanisms. In this regard, renal epithelial tubulogenesis is thought to occur thanks to polarization of epithelial cells along apical-basal and planar polarized axes [53]. Moreover, cell force generation within the cells and from the extracellular matrix (ECM) may act guiding cell polarization during the shaping of the nephron. While apical-basal cellular polarity is critical for de novo lumen formation in the early nephron [39], the planar cell polarity (PCP) pathway has a known role in kidney tubule elongation through convergent extension [57, 58]. However, these mechanisms are yet to be fully dissected in the human context. In the same manner, these processes involve a tight control over the generation of local internal forces and the dynamics of the ECM stiffness and viscosity, allowing the development of complex tissue shapes through deformation, local growth, and remodelling [59]. Changes in ECM composition and distribution over time also regulate diverse cellular processes including cell shape, cell motility, cell growth and the establishment of cell polarity [59, 60]. Nonetheless, how nephron formation and folding occurs with respect to changes in ECM remains elusive. In this regard, our recent findings show that tightly controlling cell-cell and cell-ECM interactions can be exploited as a new approach to generate higher-grade kidney organoids [28]. Furthermore, the derivation of hPSCs-NPCs in soft substrates mirroring the embryonic milieu resulted in similar results leading to the generation of hPSCs-kidney organoids in

which nephron-like structures were generated in higher amounts and in shorter time compared to control conditions (i.e., plastic) [28].

3. Future perspectives

Feedback interactions between cellular polarity, mechanical forces and cell fate specification are known to drive tissue morphogenesis. However, how these processes are coordinated during nephron patterning and morphogenesis remains unknown [59]. In this regard, hPSC-derived kidney organoids may represent an affordable model system for studying such dynamic processes in the human context. To this end, the combination of advanced imaging techniques together with hPSC reporter lines for the expression of key marker genes [61–63] and/or reporter systems for tracking of single cells and their progeny in an unbiased manner [64] will be crucial to monitor cellular behaviors at high spatiotemporal resolution. Additionally, techniques for spatiotemporal perturbation of relevant genetic, biochemical and mechanical effectors will be key to systematically test the interaction between these parameters [41, 68]. Furthermore, techniques to measure cell and tissue mechanics including traction force microscopy, optical tweezers and atomic force microscopy, among others are now being adapted to quantify cell generated forces during organoid development [65–67]. All this information can be further integrated to computational models for prediction of multicellular behaviours [68–71].

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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BOX 1. Kidney development

The mammalian kidney development is characterized by the formation of three successive pair of kidneys, the pronephros, mesonephros, and metanephros. The pronephros are rudimentary structures that are temporary, whereas the mesonephros contain primitive nephrons that further degenerate and are incorporated into the developing genital system. The metanephros, located at the most caudal region of the nephric duct will constitute the permanent kidney, so called metanephric kidney. In humans the metanephric kidney develops at 5 weeks of gestation and becomes functional at the end of the first trimester [72, 73].

The mammalian kidney develops from the intermediate mesoderm (IM), an embryonic mesoderm region that is located between the lateral and paraxial mesoderm(s). In humans the IM appears soon after gastrulation and primitive streak (PS) induction by embryonic day (E) 22 (~E 8.0 in mice). The IM further specifies into two different embryonic tissues: the ureteric bud (UB) and the metanephric mesenchyme (MM). Reciprocal inductive interactions between the UB and MM will further give rise to the collecting duct (CD) system and the nephrons, respectively [2]. The UB extends into the MM and branches repeatedly to give rise to the collecting duct (CD) system, whereas the MM specifies into the renal stroma and the cap mesenchyme (CM). The CM undergoes mesenchymal to epithelial transition (MET) to generate epithelial vesicles, also called renal vesicles (RVs), that further develop into nephrons through patterning and segmentation (nephron induction). After birth the mammalian kidney is characterized by high cellular complexity with more than 30 different cell types, including epithelial, endothelial, and stromal components [74]

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Figure Captions

Figure 1. In vitro 3D model systems for studying kidney development. A, Ex vivo mouse kidney models. **a)** Bright field image of a whole embryonic kidney rudiment under organotypic culture. **b)** Confocal image of a whole embryonic kidney rudiment for the expression of the nephron marker Jagged1 and the UB marker cytokeratin-8 (CK8). Optimized versions of the Grobstein assay have allowed the generation of: **c)** mouse UB reaggregates in 3D culture showing branching capacity, and **d)** mouse kidney reaggregates alike to the normal mouse embryonic kidneys. Confocal image of a mouse kidney reaggregate for the expression of the nephron marker WT1 and the UB marker GATA3. Scale bars, 100 µm. **B,** hPSC-derived kidney organoid models. **e)** Confocal image of a chimeric kidney organoid composed by reaggregation of E11.5 mouse kidney cells and NPCs derived from hPSCs following our recent methodology [28]. PAX8 demarks nascent nephron-like structures and human nuclear antigen (HuNu) stains for cells of human origin. PAX8+Hunu+ cells integrate into nascent nephron structures forming a chimeric kidney organoid. Scale bar, 250 µm. **f)** Higher magnification of e). **g)** Bright field image and **h)** immunofluorescence analysis of a hPSC-derived kidney organoid generated by our recent methodology [28]. The presence of segmented nephron-like structures is shown by the expression of PODXL and WT1 in the glomerular compartment, and E-cadherin (ECAD) in the distal tubule compartment. Scale bar, 50 µm. **C,** Next-generation bioengineered hPSC-derived kidney organoid models. Bioengineering approaches may help produce enhanced kidney organoids. **i)** and **j)** hPSC-derived kidney organoids of controlled size can be produced using microfabricated wells. Scale bar, 50 µm. **k)** Microfluidic devices can be used to provide fluid flow, which may be relevant for kidney organoid vascularization. Scale bar, 1 cm. **l)** hPSC-derived kidney organoid under fluid flow. Scale bar, 50 µm.

m) Decellularization technology allows fabrication of decellularized human kidney matrices (dECM). Confocal image of human kidney dECM showing preservation of major ECM components including collagen-I (in green), laminin (in red) and elastin (in magenta). Scale bar, 50 μm. **n)** 3D bioprinting can precisely deposit biomaterials and cells to create defined architectures, as in **o)**. Scale bar, 1 cm. In the future kidney dECM-derived can be combined with hPSC-derived renal cells for bioprinting of kidney analogues.

Figure 2. Understanding nephron patterning and morphogenesis using hPSC-derived kidney organoids. A, Schematic depicting nephron induction and patterning. Nephron progenitor cells undergo mesenchyme to epithelial transition to form the renal vesicle. This early epithelial structure acquires proximal to distal polarity and further morphologically progress towards the comma-shape body and the S-shape body. Expected proximal, medial and distal region marker genes are indicated. **B,** hPSC-derived kidney organoids recapitulate nephron development. hPSC-kidney organoids have been generated following our recent methodology [28]. Representative hematoxylin and eosin images of hPSC-kidney organoids during renal vesicle emergence (day 8), elongation (day 10) and differentiation into nephron-like structures (day 16). Immunofluorescence analysis of consecutive kidney organoid sections for the expression of the proximal (WT1), medial (JAG1) and distal (E-cadherin; ECAD) segment fates. Scale bars, 50 μm.

CURRENT OPINION IN GENETICS AND DEVELOPMENT

Author declaration

[Instructions: Please check all applicable boxes and provide additional information as requested.]

1. Conflict of Interest

Potential conflict of interest exists:

We wish to draw the attention of the Editor to the following facts, which may be considered as potential conflicts of interest, and to significant financial contributions to this work:

The nature of potential conflict of interest is described below:

X☐No conflict of interest exists.

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

2. Funding

X☐Funding was received for this work.

All of the sources of funding for the work described in this publication are acknowledged below:

[List funding sources and their role in study design, data analysis, and result interpretation]

This research has been supported by EIT Health under grant ID 20366 (R2U-Tox-Assay) to E.G. and N.M. This work has been also supported by "La Caixa" Foundation (ID 100010434), with a fellowship to Z.N. (LCF/BQ/DI20/11780019). A.M. is supported by the IBEC International PhD Programme "La Caixa" Severo Ochoa fellowships (LCF/BQ/SO16/52270019). W.S. is supported by Deutsche Forschungsgemeinschaft

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4. Research Ethics

X☐We further confirm that any aspect of the work covered in this manuscript that has involved human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

We would like to acknowledge the patients and the Fetal Tissue Bank of Vall d'Hebron University Hospital Biobank (PT13/0010/0021), part of the Spanish National Biobanks Network, for its collaboration.

☐IRB approval was obtained (required for studies and series of 3 or more cases)

☐Written consent to publish potentially identifying information, such as details or the case and photographs, was obtained from the patient(s) or their legal guardian(s).

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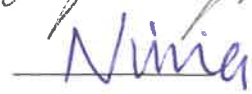
5.	__ Nuria Montserrat ___		____01/07/21___
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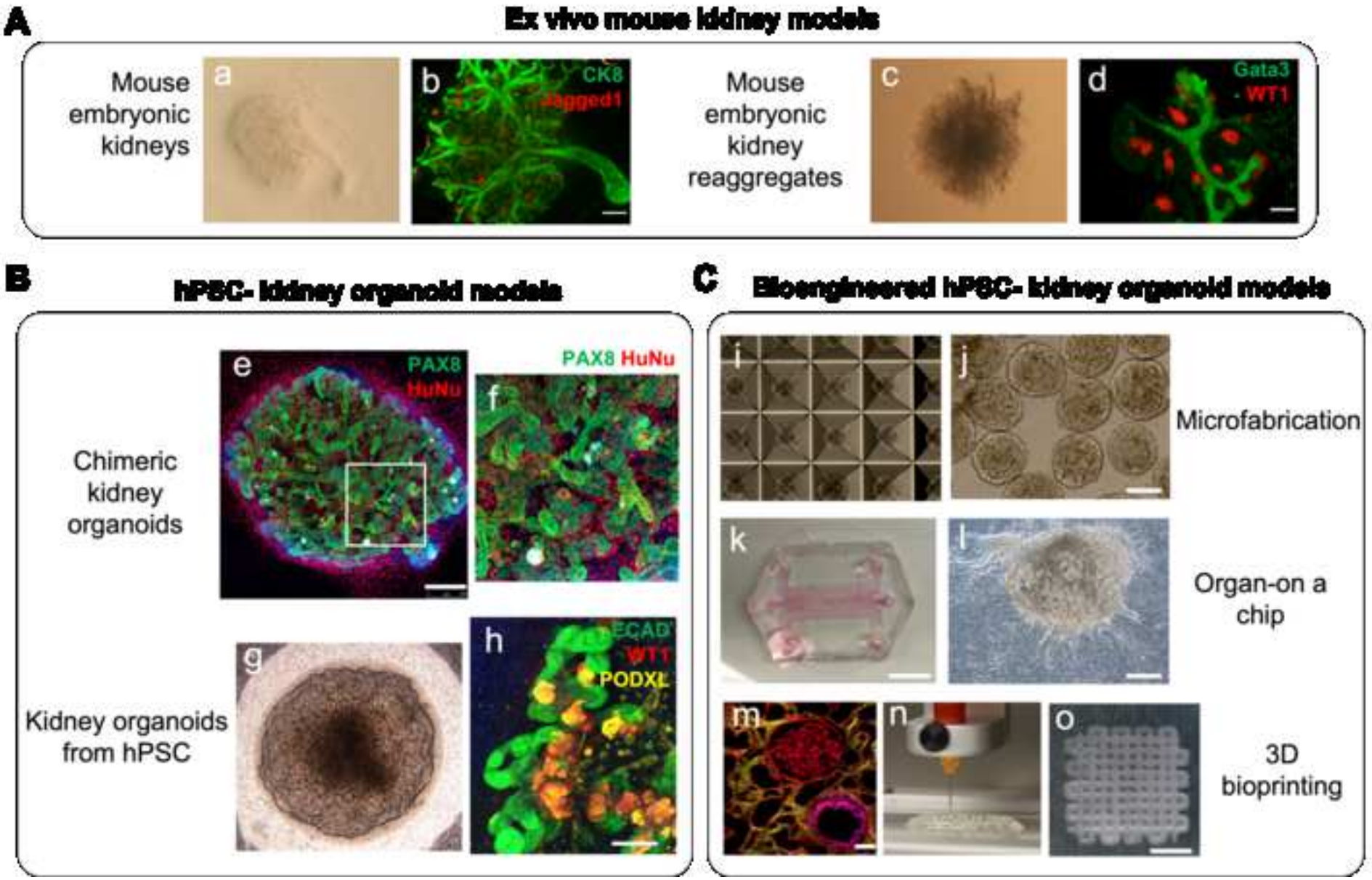
Figure 1



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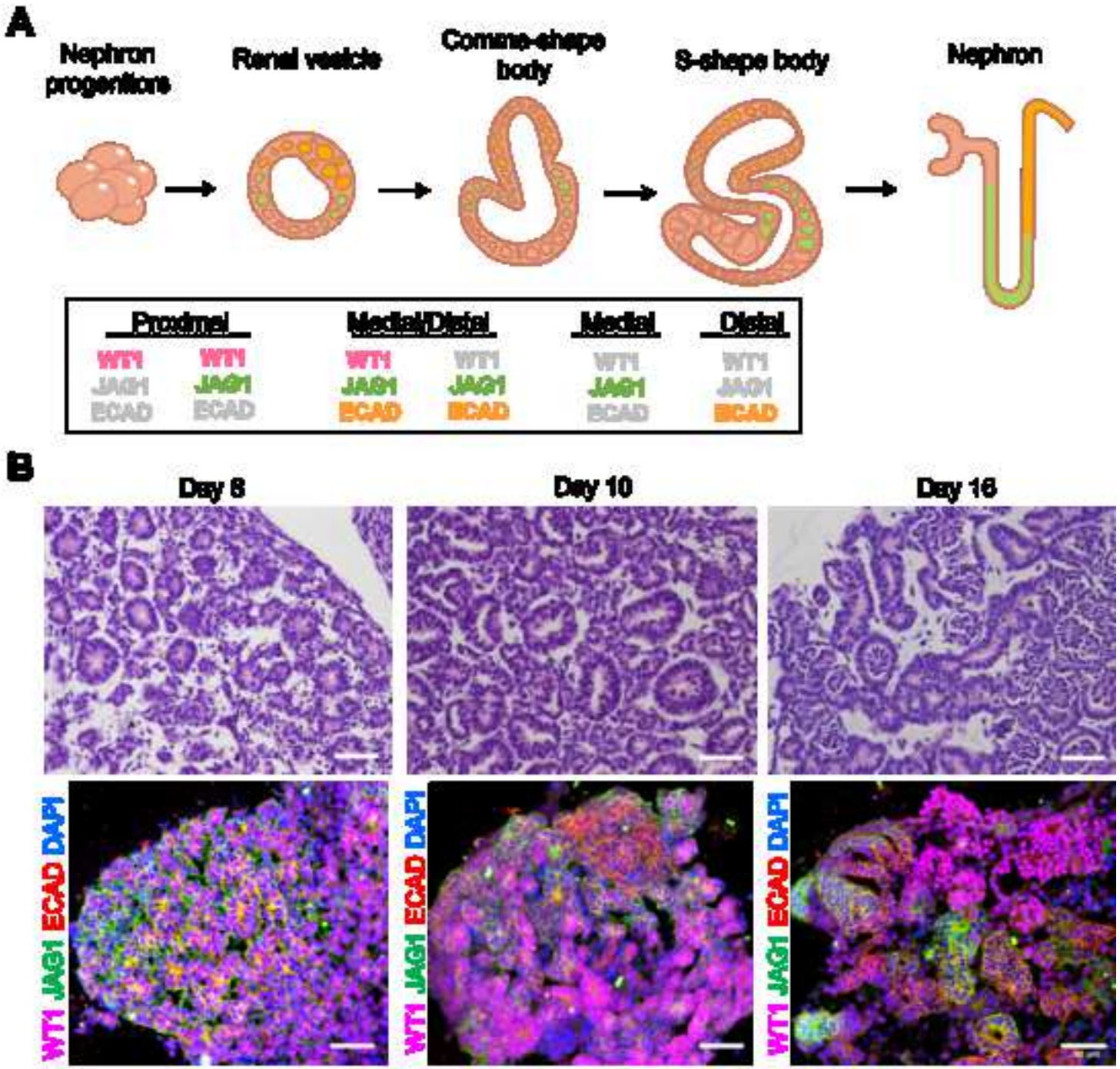

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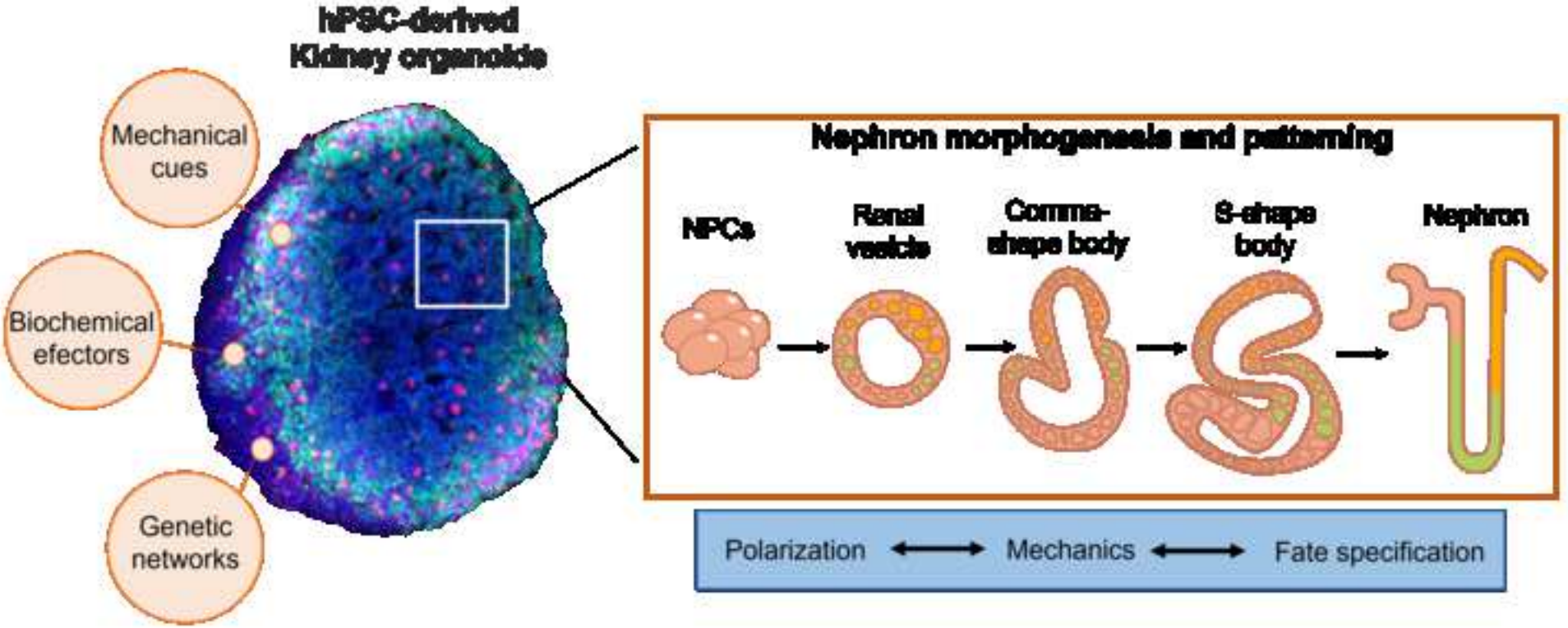


Barcelona, 4th October, 2021

Dear Editor,

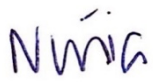
Please find enclosed the revised version of our review manuscript entitled "**Dissecting nephron morphogenesis using kidney organoids from human pluripotent stem cells**".

We would like to thank the reviewer for the positive evaluation of our work.

We have carefully revised the manuscript to address the reviewer's comments and provide an improved manuscript version.

We have highlighted the changes made in the manuscript in blue color. In addition, find bellow our response to the reviewer.

Best regards,



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Reviewer 1

This is a very informative review that spans the history of kidney development from the early days of ex vivo rudiment culture to the present day, including the elegant 2020 transcriptional mapping study of the developing human nephron by the McMahon group. The review is very clearly written and sets out what is known about kidney development, but also highlights what is still unknown. I think the review would be particularly useful to newcomers to the field.

My only minor criticism would be to undertake a careful proof-read as there are a number of small grammatical errors. For example, on the first page of the Introductions, instead of "By in vitro recombining the isolated components mouse embryonic kidneys at day 11 during embryonic development (E 11.0)...." it would be better to write as "By recombining the isolated components of day 11 mouse embryonic kidneys in vitro..."

There are a few other such instances.

Authors: We thank the reviewer for his/her positive evaluation of the manuscript. We have carefully performed proof-reading of the text and corrected the grammar errors and typos.

Highlights

- Current understanding of human nephron morphogenetic programs is limited
- Tissue mechanics and cell fate specification may act in concert to shape the nephron
- hPSC-derived kidney organoids can be used to study nephron induction and patterning
- Technologies to measure multicellular behaviors will be crucial to uncover key principles of nephron emergence
- Resulting discoveries will help unveil new mechanisms of human kidney disease