Organoids — Preclinical Models of Human Disease

Organoid Technology Development for Preclinical Models of Human Disease

Mo Li[1], PhD and Juan Carlos Izpisua Belmonte[2,*], PhD

[1]King Abdullah University of Science and Technology
[2]The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037
*Corresponding Author: belmonte@salk.edu

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Introduction

Currently, an organoid is commonly understood to be a three-dimensional (3D) construct composed of multiple cell types, originating from stem cells through self-organization, and capable of simulating the architecture and functionality of native organs. Organoids have recently emerged as a versatile model that spans the crossroads of *in vivo* and *in vitro* investigation. The technique represents an innovation in the long quest (see historical notes in Supplementary Appendix) for an *in vitro* model that faithfully recapitulates physiological processes of whole organisms. Organoids have many advantages over traditional 2D cultures. They display near-physiological cellular composition and behaviors. Many organoid cultures can undergo extensive expansion in culture and maintain genome stability,[1-4] which makes them suitable for biobanking and high-throughput screens.[5] Compared to animal models, organoids can reduce experimental complexity, facilitate precision genetic and imaging techniques, and, more importantly, enable the study of human development and diseases that is not feasible in animals (Fig. 1).[6-9] To date, organoid technologies have been successfully implemented to address a wide range of questions–from brain gyrification to personalized cancer therapeutics.[6,7] Here we review the common platforms of organoid technology, highlight recent progress in their application, and discuss ongoing challenges and future possibilities.

Organoids from Various Stem and Other Cell Sources

Organoids can be generated using somatic cells, adult tissue-resident stem cells (including progenitor cells) or pluripotent stem cells. Because of limited tissue availability, expandability, and throughput, somatic cell organoids (recently reviewed[8]) are less widely used than stem cell organoids and so will not be further discussed.

Organoids from Adult Stem Cells

An important breakthrough in intestinal organoid technology occurred in 2009 when the group led by Hans Clevers showed that intestinal adult tissue-resident stem cells have an uncanny ability to proliferate and self-organize *in vitro*.[9] Intestinal stem cells are characterized by the expression of the leucine-rich repeat-containing G-protein coupled receptor 5 (*LGR5*) gene–a receptor for the Wnt agonist R-spondin.[10,11] Intestinal stem cells niche factors include Wnt, epidermal growth factor (EGF), and Noggin, a bone morphogenetic protein (BMP) inhibitor.[9] Extracellular matrix is another important constituent of the niche, as dissociated intestinal cells undergo anoikis.[12] Based on such knowledge, Sato et al.[9] developed a 3D-embedded culture (often called the R-spondin method, see Fig. 2) to reconstitute an *in vitro* niche-like milieu for intestinal stem cells. The cultures that developed with this method allow single Lgr5+ stem cells to generate a crypt-villus architecture with all differentiated cell types in a self-renewing fashion.[9] These organoids can expand more than three months and remain genomically stable, which facilitates the purification of large quantities of organ-specific cell types.

The R-spondin method has since been adapted to generate organoids from epithelial tissues from all three germ layers (Supplementary Appendix Table 1). Details about

different types of organoids can be found in a recent review[13] and will not be discussed here. It is worth noting that the presence of Lgr5+ stem cells is not a prerequisite for organoid generation.[14-16] The liver and pancreas do not have appreciable Lgr5 expression under homeostatic conditions. Interestingly, Lgr5+ ductal cells are induced during regenerative responses following liver or pancreatic injury. Such Lgr5+ cells can form clonal organoids composed of bipotential progenitors (hepatocyte and bile duct potential for the liver, and ductal and endocrine lineages for the pancreas).[17,18] Bipotent human liver and pancreas organoids have also been generated.[1,17,19] Thus, the R-spondin method appears to be applicable to long-term maintenance of adult stem cells of many epithelial tissues in organoids.

Several organoid cultures of the components of the genitourinary system have been recently reported—female reproductive and male reproductive tracts, as well as kidney.

In the female reproductive tract, the human endometrium provides the microenvironment for implantation and nutritional support for the early conceptus. Because in vivo study is impractical, long-term culture models are needed to study the role of endometrial secretion and endometrium-placenta interactions during early pregnancy. To culture isolated endometrial epithelia, Turco et al. started with the R-spondin method, and supplemented the medium with growth factors to mimic the *in vivo* niche of glandular progenitor cells (Supplementary Appendix Table 1). Endometrial organoids were established from human non-pregnant endometrium and decidua. They are genetically stable, mount an appropriate transcriptional response to sex hormones, and recapitulate characteristics of gestational endometrium when stimulated with early pregnancy signals.[20] Organoids have also been obtained from malignant endometrium, although clonogenic and xenotransplantation assays need to be performed to substantiate their value as a model for endometrial cancer. To realize their potential in basic research of human pregnancy, and in developing therapies for endometriosis and endometrial cancer, these organoids need to be further characterized for their secretory function and their ability to model implantation of *in vitro* cultured blastocysts.

There also has been progress in the development of organoids of the male reproductive system. A recent study showed self-organization of dissociated human testicular cells under conditions similar to organotypic culture of neonatal mouse testis.[21,22] The dissociated cells formed a condensed spheroid that has been termed testicular organoids.[21] Despite the lack of native tissue topography, niche cells and spermatogonia persisted in testicular organoids[21] (Supplementary Appendix Table 1). However, differentiation of spermatogonia, meiosis, and sperm formation were not reported. The testicular constructs do not undergo long-term expansion, which makes them more akin to primary organ culture. Demonstration of clonogenic potential and functionality is critically needed to move the field forward.

The functional unit of the kidney composed of a renal corpuscle and a renal tubule (together termed the nephron)–depends on an intricate tissue architecture for its function. During development, nephrogenesis requires reciprocal interactions between two kidney progenitor populations in the intermediate mesoderm–the metanephric mesenchyme and

the ureteric epithelium. The spatiotemporal unfolding of mesenchyme-epithelium mutual induction, cell movement, proliferation, and cell adhesion suggests a genetically encoded self-organization program.[23] Indeed, dissociated embryonic kidney cells self-organize into their tissue of origin with high spatial fidelity.[24] The developing kidney contains transient amplifying nephron progenitor cells (NPCs) that give rise to all nephrons.[25] Such nephron progenitor cells have not been found in the adult human kidney, which cannot regenerate nephrons once these are lost.[26] However, several putative adult kidney progenitors capable of tubulogenesis in organoids have been reported,[27-29] but, there is considerable disagreement on their identity and potential fate.[27,28]

Embryonic nephron progenitor cells, on the other hand, are much better characterized and have been successfully used to create kidney organoids.[30-33] Despite such studies, many hurdles still remain—first, the loss of differentiation potential in cultured nephron progenitor cells; second, limited self-renewal of these cells; third, a lack of evidence for *in vivo* nephrogenic potential; and, finally, dependence on transgenic markers. Based on previous observations,[34-36] we developed a long-term 3D culture of genomically stable, self-renewing nephron progenitor cells[4] (Supplementary Appendix Table 1). It is worth noting that these developmental progenitors are distinct from adult stem cells, as they do not appear to exist in the adult kidney and can only self-renew under artificial culture conditions. In the classic spinal cord induction assay, clonal nephron progenitor cell lines formed nephron-like structures with proper spatial orientation, indicating an intact nephrogenic potential.[4] Cultured nephron progenitor cells can differentiate into organoids with numerous tubular structures that express major nephron segmental markers. These kidney organoids can contribute to nephrogenesis in neonatal mice and chick embryos. They generate ectopic nephron-like structures that connect with the host vasculature and filtrate urine-like fluid when transplanted into the omentum of immunodeficient mice.[4] Human nephron progenitor cell cultures with similar properties can be derived from fetal kidneys between 9 to 17 weeks of gestation. Nephron progenitor cell lines are amenable to CRISPR-based genome editing to study human organogenesis and genetic diseases (Supplementary Appendix Table 2).[4]

Pluripotent Stem Cells

Pluripotent Stem Cells can self-renew indefinitely and differentiate into any cell type in the body, thus offering an attractive alternative to the use of primary tissues to create organoids. Pluripotent stem cell-derived organoids are formed through directed differentiation of a homogeneous population, so tissue-specific cell types and their microenvironment must be created *de novo* in a dynamic process reminiscent of embryogenesis. Accordingly, pluripotent stem cell organoid culture must provide stage-appropriate niche signals during the differentiation. Because the differentiation cues are not strictly limited to the desired cell fates, pluripotent stem cell organoids often contain cell types that differ from those in a given organ and that may complicate the signaling environment and self-organization of the target tissue.[37-39]

Early work in directed differentiation established signaling requirements for germ layer formation, patterning, and induction of tissue identity in 2D cultures. Pluripotent stem cells

can also spontaneously differentiate as 3D embryoid bodies. These embryoid bodies typically differentiate further in 2D in the presence of specific cues. Pluripotent stem cells in 2D culture are known to default to a neural fate in the absence of any inductive signal.[40] The differentiated neuroepithelial cells self-organize into rosettes that resemble neural progenitor cells of the developing neuroepithelium.[41] Building on this, the Sasai group was the first to use a so-called serum-free floating culture of embryoid body-like aggregates with quick reaggregation (SFEBq, see Fig. 2) to generate neuroectodermal organoids.[42] Neurons in these organoids showed properties characteristic of neonatal cortical brain tissues.[42] Importantly, brain cortical organoids recapitulate the spatial and temporal regulation of early corticogenesis, including the organization of distinct zones along the apicobasal axis, and the birth order of layer-specific neurons in the developing cortex.[42] The neuroepithelia generated by SFEBq are often supplemented with biochemical (growth factors) and/or biophysical signals (Matrigel®) to promote specific regional identities. For example, Matrigel® promotes the formation of a rigid, continuous neuroepithelium, which upon addition of the growth factor Nodal self-organizes into optic cups composed of a properly patterned retinal pigment epithelium and a neural retinal epithelium.[43] Similar strategies have been successfully employed to generate organoids representing diverse regions of the neuroepithelium, including the retina,[44] adenohypophysis,[45] midbrain,[46] cerebellum,[47] and hippocampus[48] (Supplementary Appendix Table 1).

The growth of brain cortical organoids is limited by free diffusion of oxygen, nutrients, and growth factors. Consequently, cells in deep areas of organoids undergo apoptosis.[9,49] A protocol in which the organoid culture is kept spinning in a bioreactor was developed to enhance nutrient exchange, thus substantially improving growth and development of neuroepithelia. These neuroepithelia spontaneously form regions reminiscent of the cerebral cortex in the absence of inductive signals.[49] Morphological, histological and transcriptional analyses proved that these so-called cerebral organoids contained interdependent domains recapitulating various brain regions.[49] Importantly, human specific features including the outer subventricular zone were observed in cerebral organoids [49,50] (Supplementary Appendix Table 1).

Single-cell RNA sequencing (scRNAseq), a powerful tool for studying cellular identity, revealed that cerebral organoids contain neural and mesenchymal cells with progenitor or differentiated phenotypes. A remarkable level of transcriptional similarity was found between cells in organoids and fetal tissues, lending credence to cerebral organoid as a model for human cortical development.[51] Furthermore, scRNAseq showed that cortical radial glial-like cells in organoids are similar to outer radial glial cells, which are unique to humans.[52]

Organoids of the mesodermal kidney have been reported. The ureteric epithelium renal progenitor can be generated from human pluripotent stem cells using defined media conditions for intermediate mesoderm induction.[53,54] These progenitors can self-organize into ureteric bud structures once aggregated with dissociated mouse embryonic kidney cells.[53] Another renal progenitor, the metanephric mesenchyme, can be differentiated in embryoid bodies by patterning the posterior intermediate mesoderm through phasic

activation of Wnt and FGF signaling. The resulting metanephric mesenchyme can form nephron-like structures with tubules and glomeruli in 3D culture.[30] It is also possible to simultaneously induce ureteric epithelium and metanephric mesenchyme through directed differentiation of human pluripotent stem cells.[55] The correctly patterned renal progenitors then self-organize in 3D culture to generate nephrons with defined glomeruli and segmented tubular structures, which are associated with a collecting duct network and surrounding interstitial and endothelial cells.[56] While this progress is exciting, further work is needed to ascertain the functional maturity of the organoid nephrons, as it is difficult to distinguish immature pro- and mesonephric nephrons from mature metanephric nephrons using only lineage markers.[57] It is also desirable to develop defined conditions for ureteric epithelial progenitors, which have not been propagated as a pure population in culture. Their presence is inferred by gene expression and immunofluorescence of known markers.[53,55,56] Once isolated, ureteric epithelial progenitors could then be tested for their ability to undergo reciprocal induction with nephron progenitor cells, or to reconstitute collecting ducts in embryonic kidney re-aggregation assay.[58]

For endodermal lineages, pluripotent stem cells are first differentiated into definitive endoderm through exposure to Activin/Nodal and low serum, and the resulting gut tube can be patterned along the anterior-posterior axis by temporal and spatial manipulation of the Wnt, FGF, retinoic acid, TGFβ/BMP, and Notch pathways.[59,60] Organoids representing tissues that originate from the foregut, including the lung,[61] thyroid,[62] stomach,[37] pancreas,[63-65] and liver,[66] as well as those from the mid/hindgut, such as the small and large intestines,[67,68] have been reported and recently reviewed[13] (Supplementary Appendix Table 1). One of the unique features of pluripotent stem cell-derived organoids is that tissue-specific epithelia are differentiated *de novo* through a series of progenitors, a process that entails interaction between different germ layers. In pluripotent stem cell organoids, the residual mesodermal cells present after endodermal induction become fibroblasts and smooth muscle cells around the epithelium.[37,61,67] This phenomenon is reminiscent of the mesenchymal tissues found around endodermal organ primordia during organogenesis.[69] In support of the role of mesodermal cells in epithelial morphogenesis, purposely mixing mesenchymal stem cells and endothelial cells with human pluripotent cell-derived hepatic progenitors results in 3D liver buds that become vascularized and functional upon transplantation (Fig. 2). ScRNAseq revealed that communications between different lineages in the liver bud help to make them become transcriptionally similar to their counterparts in human fetal liver.[38] Recently, this heterotypic culture approach was shown to support vascularized organ bud formation from the pancreas, kidney, intestine, heart, lung and brain.[63]

Applications of Organoid Technologies

Disease Modeling

Organoid technology is useful in a wide range of applications, bridging a gap between 2D cultures and animal models. Compared to 2D cultures, organoids may provide better fundamental insights into development, homeostasis, and pathogenesis, and offer new translational approaches to diagnose and treat diseases (Fig. 2). For instance, cerebral

organoids recapitulate human-specific neurogenic processes, thereby offering an unprecedented opportunity for studying human brain development.[49] Human cerebral organoids have recently been grown in a microfabricated compartment that allows long-term *in situ* imaging. This system was used to model the physics of cortical folding and study the mechanism underlying lissencephaly when a disease-causing mutation was introduced using CRISPR/CAS9 genome editing.[6] Another study found that cerebral organoids generated from lissencephaly patient induced pluripotent stem cells have a mitotic defect in outer radial glial cells, which are poorly represented in mouse models.[52] Brain organoids were also used to show that Zika virus preferentially infects neural progenitors, resulting in reduced proliferation and apoptosis, which suggests a mechanism for Zika virus-associated microcephaly.[50,70-72]

Anticancer Drug Screening

Organoids have been successfully grown from primary tumors of the colon, prostate, breast and pancreas.[13] These patient tumor-derived organoids (tumoroids) have emerged as robust preclinical models that have potential to predict an individual patient's response to treatment (Supplementary Appendix Table 2). For example, a living biobank of tumoroids from metastatic gastrointestinal cancer patients recapitulated the response of contributing patients to anticancer agents in clinical trials.[7] Tumoroids can also be used to study the tumor niche. An organoid library representing different colorectal tumor grades revealed a decreasing dependency on niche factors along the normal-adenoma-carcinoma transition. Such niche dependency is found to be primarily determined by the genetic makeup of the tumors.[73] This and other examples (Supplementary Appendix Table 2) suggest that tumoroids may constitute a versatile platform for linking cancer genomic data to tumor biology to develop personalized drug screen and treatment.

Identification of Drug Toxicity

Organ toxicity is the primary reason for failures in drug development and post-approval withdrawals.[74] Current toxicology screens using cell lines and animal models are often inadequate for predicting adverse effects in humans. Three-dimensional organoids offer a near-physiological representation of human tissues that may offer more accurate toxicity prediction. Renal and hepatic toxicities are among the most common adverse effects of drugs. Encouragingly, kidney organoids have been shown to recapitulate nephrotoxic effects of cisplatin[56] and gentamicin.[4] Other advantages of organoids include their genetic stability[1-4] and scalability for high-throughput screens. For instance, human nephron progenitor cells have a nearly unlimited ability to self-renew,[4] which could be a boon for standardization of nephrotoxicity screens. Recently the US Food and Drug Administration has started testing if 3D liver-on-a-chip constructs can be used to screen for hepatic toxicity of compounds used as food additives, supplements, and cosmetics. The organoid-on-a-chip technology may lend itself to the development of many drug toxicity screen programs.

The Use of Organoids for Gene and Cell Therapy

Functional integration of transplanted organoids (or cells from organoids) has been demonstrated for organoids of the colon,[75] liver,[18,66] pancreas, retina,[76] and thyroid.[77] In such demonstrations different levels of evidence were used to support functionality, including morphological similarity[4,63,75,76] to native tissues, connection to the host (via the vasculature[4,63] or synapses[76]), epithelial permeability,[75] and rescue of a disease or injury.[18,77] Additionally, CRISPR/Cas9 genome editing has been used to correct mutations in the CFTR gene in colon organoids of cystic fibrosis patients, which restored CFTR function.[78] Such proof-of-concept studies suggest that organoids might be a source of functional cells that could complement stem cells in future cell therapy. However, more studies are needed to evaluate the efficacy and safety of organoid transplantation. Recent efforts to bring induced pluripotent stem cells to the clinic could offer valuable lessons and guidance in this area.[79]

Challenges and Future Directions

Organoids presently have many shortcomings. They are limited in physical dimension and exhibit considerable variability. For instance, pluripotent stem cell-derived organoids still have a large degree of randomness in their cellular composition, tissue architecture, and cell-matrix and cell-cell interaction. Standardization of methodologies is still far in the future. Additionally, organoids are devoid of important physiological input, such as innervation, vascularization, and immune regulation, thus making them a reductionist system. A recent example of discrepancy in drug resistance[2] between a mouse tumor and its organoids highlight the importance of validating results obtained from organoids. These limitations may present major challenges for basic research and clinical applications. Fortunately, biological and bioengineering solutions are being invented at a rapid pace to address these challenges.

Extracellular Matrix and Organoids

The most common extracellular matrix support for organoids is Matrigel®, which is derived from mouse sarcoma cells.[80] Variability of such chemically undefined material could confound high-throughput screens.[81] Risks from such xenobiotics may also be problematic for clinical translation. Additionally, the isotropic Matrigel® is unable to mimic the morphogen gradients and dynamic changes in local biomechanical forces that exist *in vivo*. To overcome these issues, designer synthetic matrices that can change their biophysical and biochemical properties on demand are being developed. One such mechanically dynamic matrix has been developed to provide tunable stiffness and degradability for stem cell expansion and differentiation in intestinal organoids.[82] In the future, extracellular matrix engineering may be expected not only to replace xenobiotics but also provide information concerning how tissues are organized, thus improving the consistency and quality of organoids.

Cellular Composition of Organoids

Although the absence of certain tissue-resident cell types in organoids could sometimes be advantageous,[9] that can also be a hindrance. For example, lack of vasculature is not

physiological. Without vasculature the central portions of organoids may become hypoxic and apoptotic.[9,42] Bioavailability of growth factors and drugs is likely different between a homogenous culture medium and tissues, in which signaling molecules are delivered via a vasculature or through local diffusion. While several types of organoids become vascularized if transplanted into an animal,[4,63,66,83] an *in vitro* organoid culture with a perfusable vasculature has not been reported. Stroma-tumor interaction is important for tumorigenesis and metastasis.[84] Various stromal components, such as immune cells[85] and endothelial cells,[86] have been added to tumoroids to study how they regulate tumorigenesis. Organoids are lacking in modeling interaction with the immune system. The peripheral nervous system plays an integral role in tissue homeostasis and repair.[87] However, it is not typically represented in organoids. To this end, a recent study generated human intestinal organoids with a functional enteric nervous system by combining pluripotent stem cell-derived neural crest cells and intestinal organoids.[88]

The cells from which organoids are developed, especially cells from patients,[5,49] could introduce variability to the production and growth of organoids. Several studies reported small but detectable variation between organoids derived from patient induced pluripotent stem cells,[49,89,90] as might be expected due to differences in patient age, genetic background and culture conditions. Innovations in culture method (e.g., the use of bioreactors[50]) may minimize variation in culture conditions. CRISPR/Cas9 technology can be used to engineer isogenic organoid models to control for genetic background, thus reducing variability (Supplementary Appendix Table 2). Tumoroids generated from patient tumors vary considerably, likely due to the heterogeneous nature of tumors.[5] There are also questions about how medium composition affects growth of tumor and non-tumor cells.[73,91,92] Systematic studies of a large number of tumoroid models (e.g., the TUMOROID trial, NL49002.031.14, and the SENSOR study, NL50400.031.14) may answer how variability of organoids will impact their clinical application.

Tissue Architecture of Organoids

Organoid cultures rely on self-organization of stem cells in the absence of precise modulation of their physical environment (e.g., shear force, pressure, and matrix stiffness). This results in some degree of randomness and sometimes non-physiological arrangements in tissue architecture. For instance, gastrointestinal organoids contain a lumen that is isolated from the external environment.[9] Whether organoids are suitable for studying an epithelium that have an open-system native environment in which cells are constantly being shed and interact with their surroundings is unknown. Recently, microfluidics-based co-culture of human gut epithelial cells with gastrointestinal bacteria was used to study interactions between the gut and its microbiota.[93] Although such a system was designed for monoculture, it may be adapted for organoids. Tissue architecture of organoids could be guided by scaffolds made of biomaterials or decellularized tissues, or 3D printed with bioinks.[94,95] The latter technique has been used to print 3D proximal tubules in a perfusable tissue chip to mimic *in vivo* kidney environment.[96] One might speculate that 3D kidney constructs with native tissue architecture could be achieved via self-organization of kidney progenitors aided by bioprinted scaffold and vasculature.[4,53,56]

Organ-on-a-chip systems that are based on microfluidics and organoid technologies afford precise control over biomechanical variables and the delivery of bioactive molecules. Such developments may facilitate real-time studies, as well as high-resolution monitoring of the behavior of single cells, cell-cell interaction, and metabolic processes at the tissue level. However, such modalities are generally missing in current organoid studies. Developing such techniques currently will necessitate close collaboration between bioengineers and developmental biologists. In the future, mature commercial solutions of biomaterial and bioprinting may accelerate the pace of discovery. Organoid technology has been effectively integrated with other cutting-edge technologies, such as CRISPR/CAS9 genome editing, single cell genomics, microfluidics and live imaging (Fig. 2 and Supplementary Appendix Table 2). Current challenges may ultimately be resolved and head toward increased precision in recapitulating human physiology with organoids.

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FIGURE LEGENDS

Figure 1. Comparison of organoid cultures with 2D cell culture and animal models. Organoids can be generated using adult tissue-resident stem cells or pluripotent stem cells as the cellular source. As a bridge between the conventional 2D culture and animal models, organoids provide multiple unique advantages (summarized in the table in the bottom half) that afford experimental manipulability and at the same time capture biological complexity.

Figure 2. Schematics of the mainstream human organoid models and their applications. Human organoids can be generated from normal or malignant primary tissues. R-spondin method: the essential design principle for generating small intestine organoids, namely extracellular matrix support (Matrigel), activation of the Wnt signaling pathway (R-spondin1, Wnt3a), growth factors for organ-specific epithelial proliferation (EGF), and stem cell self-renewal factors (inhibitors of BMP and TGF-beta). Alternatively, somatic cells can be reprogrammed into induced pluripotent stem cells, which are sources of organoids of all three germ layers via directed differentiation. SFEBq: serum-free floating culture of EB-like aggregates with quick reaggregation. SFEBq entails dissociation of pluripotent stem cells into a homogenous single-cell suspension to minimize any endogenous inductive signals, followed by quick aggregation in serum- and growth factor-free medium. Organoid technologies have integrated well with other technologies

including genome editing, single cell genomics, live imaging, and microfluidics, thus providing more accurate insights into developmental processes, disease pathogenesis, as well as enabling translational approaches to diagnose and treat diseases.

Abbreviations: MM: metanephric mesenchyme. NPC: nephron progenitor cell. UE: ureteric epithelium. A/P: anterior/posterior. HUVEC: human umbilical vein endothelial cell. MSC: mesenchymal stem cell. When provided with appropriated mechanical support, MSCs facilitate condensation of other input cells.

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