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Technologies in kidney development or replacement

Pluripotent stem cell-derived kidney organoids: An *in vivo*-like *in vitro* technology

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ABSTRACT

Organoids are self-organizing, multicellular structures that contain multiple cell types, represent organ structure and function, and can be used to model organ development, maintenance and repair *ex vivo*. Organoids, derived from embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) or adult stem cells, are cultured in extracellular matrix (ECM). Organoid cultures have been developed for multiple organs and for the kidney, pluripotent stem cell (PSCs) derived organoid technology has rapidly developed in the last three years. Here, we review available PSC differentiation protocols, focusing on the pluripotent stem cells to initiate the organoid culture, as well as on growth factors and ECM used to regulate differentiation and expansion. In addition, we will discuss the read out strategies to evaluate organoid phenotype and function. Finally, we will indicate how the choice of both culture parameters and read out strategy should be tailored to specific applications of the organoid culture.

1. Introduction

Organoids are self-organizing multicellular structures that represent tissue structure and function *in vitro*, and allow assessment and modeling of development, turnover and repair *ex vivo*. Organoids are organ-like 3D structures that are cultured in a three dimensional matrix and elegantly link *in vitro* and *in vivo* models. Organoid culture technology has been in the limelight for approximately a decade. The first adult-derived epithelial intestinal organoids were described for mouse intestine in 2009 (Sato et al., 2009), quickly followed by the development of human intestinal organoids (Sato et al., 2011) and organoids derived from other organs (Bartfeld et al., 2015; Boj et al., 2015; Huch et al., 2015; Karthaus et al., 2014). Around the same time, directing differentiation of pluripotent stem cells led to the establishment of pluripotent stem cell derived organoid cultures that contain stroma in addition to the epithelial compartment, for many organs, including intestine, stomach, liver and brain (Lancaster et al., 2013; McCracken et al., 2014; Spence et al., 2011; Takebe et al., 2014).

The resemblance to the *in vivo* situation makes organoid cultures highly relevant for fundamental science as well as for clinical application. Organoid cultures have been applied to prove stemness (Stange et al., 2013), for modeling of tumorigenesis (Drost et al., 2015) and for the investigation of infectious diseases (Bartfeld et al., 2015). Clinically, organoids may be applied for drug screening, for testing the efficacy of multiple drugs on one patient as well as for high throughput screening of one compound on organoids from multiple patients (van de Wetering et al., 2015). In proof of principle studies organoids have been used as an infinite source of cells for cell therapy in preclinical models of liver (Huch et al., 2013) and colon disease (Yui et al., 2012).

The kidney is a complex organ consisting of more than 20 different cell types arranged in a configuration that is crucial for proper organ function. The distinct cell types are characterized by differential gene expression per tubular segment, segment specific morphology (*e.g.* the presence of brush border in proximal tubule cells) and function (*e.g.* transport function and hormone sensitivity). The architecture of the nephron and its vasculature enables the reabsorption of 98% of the approximately 150 l of plasma filtered in the glomerulus every day. Recently, renal organoids have been developed (Taguchi et al., 2014; Takasato et al., 2014, 2015; Xia et al., 2013) and although they cannot fully recapitulate the complex organization of the kidney, organoids enable *ex vivo* modeling of nephrogenesis, renal cell homeostasis and function. Here, we will review distinct aspects of the available kidney organoid culture systems and highlight the importance of these aspects for potential applications. We will focus on kidney organoids derived from pluripotent stem cells and we will discuss different types of pluripotent stem cells, and how type, timing and sequence of growth factor administration and matrix influence cell

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expansion, differentiation and function. In addition, we will review the techniques used to evaluate organoid phenotype, morphology and function. We will discuss how specific application of the organoid culture (research, diagnostics, or therapy) directs choices of cell source, matrix, media (growth) factors and read out strategies.

2. Pluripotent stem cells as start cells

Pluripotent stem cells (PSCs) have by definition the capacity to form all cell types of the body. Directing differentiation towards kidney cells requires careful titration of expansion and differentiation of PSCs by modifying external cues, such as growth factors and matrix components (*see below*). PSCs derived from both embryonic tissue and PSCs derived from differentiated adult cells after forced dedifferentiation (induced PSCs (iPSCs)), have been used in renal organoid culture and will be described below.

2.1. Embryonic stem cells

Embryonic Stem Cells (ESCs) are PSCs derived from the inner cell mass of the blastocyst, the part that structurally gives rise to the embryo. Mouse ESCs were isolated for the first time in 1981 (Evans and Kaufman, 1981) and nowadays, many mouse ESC lines are commercially available. The isolation and culture of human ESCs followed in 1998 (Thomson et al., 1998) and raised ethical issues including the need for destruction of a blastocyst at that time and the theoretical feasibility of human cloning (Hyun, 2010).

Subcutaneous transplantation of ESCs leads to the formation of teratomas that contain differentiated tissues from all germ layers (*endoderm, ectoderm and mesoderm*) (Hentze et al., 2009). When ESCs are injected into blastocysts and transplanted into mice, they contribute to tissues from all germ layers (Bradley et al., 1984). *In vitro*, ESC lines can be differentiated into cell types of the three germ layers in 2D cell culture. 3D ESC-derived organoid cultures have been established by suspending embryonic cells – with or without pre-differentiation steps in 2D – in an ECM, often Matrigel, for organs from the three germ layers, for example intestine (Spence et al., 2011) and stomach (McCracken et al., 2014) (*endoderm*); retina (Nakano et al., 2012) and brain (Lancaster et al., 2013) (*ectoderm*); and heart (Stevens et al., 2008) and kidney (*mesoderm*).

The differentiation of ESCs into kidney organoids has been a step by step process, where differentiation into progressively more differentiated structures was achieved. First, protocols for the differentiation into kidney precursor structures, such as the nephrogenic intermediate mesoderm (Mae et al., 2013) or ureteric bud (Xia et al., 2013), were developed. The latter was created with mouse-human chimeric cultures. This was followed by protocols without chimerism that cultured specific kidney compartments that are present in adult kidney, such as the proximal tubule (Lam et al., 2014). Subsequently, it became possible to culture organoids that included multiple kidney compartments, although vasculature, nerve innervation and a convergent urinary collecting duct system remain absent (Freedman et al., 2015; Morizane et al., 2015; Taguchi et al., 2014; Takasato et al., 2014, 2015).

An advantage of the use of ESCs compared to iPSCs is that no reprogramming process which poses risks of tumor formation (*see also below*) is required. Drawbacks are the obvious ethical issues associated with the use of ESCs and that ESC-derived organoid cultures cannot be established from adult subjects.

2.2. Induced pluripotent stem cells

The discovery of the reprogramming factors by Yamanaka enabled dedifferentiation of differentiated, somatic adult cells into pluripotent cells (Takahashi and Yamanaka, 2006). This invention revolutionized the field of stem cell biology: by (viral) introduction of Oct4, Sox2, cMyc and Klf4 it became possible for the first time to obtain a pluripotent stem cell population, derived from adult cells. This technique was first developed for mouse fibroblasts (Takahashi and Yamanaka, 2006) and later also for human fibroblasts (Takahashi et al., 2007) and other somatic cell types (Aoi et al., 2008; Stadtfeld et al., 2008).

Analogous to ESC differentiation, protocols have been developed for *in vivo* and *ex vivo* differentiation of iPSC. Similar to ESCs, subcutaneous transplantation of iPSCs into mice leads to teratoma formation that contain differentiated tissues from the three germ layers (Hentze et al., 2009). After micro-injection of mouse GFP-iPSCs into blastocysts, followed by *in vivo* development, GFP+ cells contribute to tissues of all germ layers (Takahashi and Yamanaka, 2006). Differentiation protocols have been developed both for 2D and 3D cell culture, for other organs as well as for the kidney. The kidney iPSC differentiation protocols are roughly equal to the ESC protocols that are discussed above (Morizane et al., 2015). Some issues specific to iPSCs should be taken into account. Induction of pluripotency might not only require the introduction of the transcription factors mentioned above, but also epigenetic reprogramming (Kim et al., 2010). Differentiated cell types used for reprogramming into iPSC, differentiate more easily towards the organ of origin than towards other organs due to DNA methylation ("the epigenetic memory"), which ultimately affects differentiation (Polo et al., 2010). This residual DNA methylation could be overcome by differentiation and serial reprogramming; addition of methylation-modifying drugs; or use of tissue that is embryonically similar to the target tissue. iPSC lines derived from adult human kidney cells are available, both directly isolated from kidney tissue (Song et al., 2011) and isolated from urine (Zhou et al., 2012).

The use of iPSCs for the generation of renal organoids has advantages when compared to ESCs. iPSCs can be easily obtained, even from cells shed with voiding, without the ethical concerns of ESCs. The accessibility of iPSC facilitates the generation of organoids from patients as well as healthy volunteers. However, the use of iPSC for the generation of renal organoids has disadvantages. First, iPSCs are genetically instable, as most reprogramming has been done by introducing the reprogramming factors by (lenti)viral infections that integrate semi-randomly into the genome, which poses a risk for later tumor formation. Of note, recent studies show that reprogramming with episomal plasmids that do not integrate into the genome is possible (Briggs et al., 2013; Rao and Malik, 2012). Second, epigenetic characteristics may not be completely reset after reprogramming, affecting cell behavior (Kim et al., 2010) and reducing the similarity of iPSC-derived renal organoid tissue to the *in vivo* kidney, if the iPSC line has been derived from cells from organs other than the kidney. Third, although iPSC technology in theory allows the development of autologous organoids and thereby transplantation, it is a serious concern that not adequately differentiated iPSC-derived renal organoids may give rise to teratomas upon transplantation (Gutierrez-Aranda et al., 2010). Finally, iPSCs give rise to organoids with a lower efficiency than ESCs (Freedman et al., 2015).

3. Growth factors

Mimicking *in vivo* kidney development by careful composition and timing of the growth factor mix will allow the *in vitro* differentiation of pluripotent stem cells into renal lineages. Comparing protocols that are used to induce differentiation of pluripotent stem cells into renal lineages (Fig. 1), allows identification of factors that are indispensable and factors that are apparently