Human Pluripotent Stem Cell Derived Endoderm for Modeling Development and Clinical Applications

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Abstract

The liver, lung, pancreas and digestive tract all originate from the endoderm germ layer and these vital organs are targeted by a diversity of life threatening diseases affecting millions of patients. However, primary cells from endodermal organs are often difficult to grow *in vitro*. For this reason, the development of protocols for generating endoderm cells and their derivatives has been a major a focus in the human pluripotent stem cell field. Indeed, the possibility to produce a large quantity of these cells by taking advantage of the unique properties of pluripotent stem cells and their differentiation capacity, holds great promise for the development of new therapeutics against global health care challenges such as diabetes or a diversity of infectious diseases. Here we describe recent advances in methods for generating endodermal cell types from human pluripotent stem cells and their use for disease modeling and cell based therapy.

Introduction

The endoderm is one of the three primary germ layers and along with mesoderm and ectoderm, they give rise to all adult organs. Endoderm cells are first specified through the process of gastrulation during early embryonic life and give rise to the gastrointestinal tract (gut, liver, pancreas), the respiratory system (lung and trachea) and the thyroid (Zorn and Wells, 2009). All these organs have vital functions and are susceptible to life threatening diseases. Nonetheless, most of the endodermal cells comprising these organs are challenging to grow *in vitro,* whilst the procurement of primary tissues is often difficult and ethically questionable, especially from healthy donors. Consequently, a diversity of basic studies,

disease modeling or regenerative medicine applications are currently greatly limited by the lack of high quality endodermal cells. The beta cells of the pancreas and the hepatocytes of the liver provide an exemplar of these difficulties. Beta cells regulate glucose levels in the bloodstream by producing insulin and represent the main cell type targeted by diabetes. Despite their importance, these cells are impossible to expand *in vitro,* thereby limiting the development of new therapies such as cell-based therapy. Similarly, a diversity of drugs can affect hepatocytes, leading to liver injury and ultimately liver failure. Thus, liver toxicity represents a leading cause in drug development attrition, and toxicology screen on hepatocytes is an essential part of the drug screening pipeline in the pharmaceutical industry. However, primary hepatocyte cells lose their metabolic activity after few hours in culture, display a strong functional variability and remain impossible to expand *in vitro*, thereby rendering difficult a systematic and large-scale high throughput screening approach.

For all these reasons, the production of endodermal derivatives has been a major objective in the human pluripotent stem cell (hPSC) field for the past 20 years (Thomson et al., 1998). Indeed, hPSCs can be grown almost indefinitely *in vitro* while maintaining their capacity to differentiate into the three primary germ layers, making them the perfect candidate for producing endodermal cells on a large-scale. Accordingly, a broad number of groups have developed protocols to differentiate hPSCs into endoderm derivatives. These major efforts have resulted in significant progress in our capacity to produce endoderm derivatives for translational applications. In addition, these differentiation protocols provide a unique opportunity to model and study human development, especially early organogenesis. The current review will describe the different protocols currently available, focusing on the conditions and the cocktail of growth factors which are commonly utilised to generate liver, pancreas, gut and lung cells. We will also explore the different applications of these cells, not only for disease modeling and drug screening, but also for cell-based therapy and developmental studies.

Protocols to Generate Definitive Endoderm Cells from hPSCs

A diversity of protocols is now available to differentiate hPSCs into a near homogenous population of endoderm cells, based on fundamental principles learnt from early development. Gastrulation has been broadly studied in a diversity of model organisms and the basic knowledge accumulated has firmly established that Nodal/Activin signaling, through its effector SMAD2/3, orchestrates this germ layer specification. Additional signaling pathways are involved in this process especially WNT and FGF, which are both required for the normal formation of the primitive streak (Tam and Loebel, 2007) and the induction of the key

endoderm marker SOX17 (Kanai-Azuma et al., 2002; Tam and Loebel, 2007). Following gastrulation, the endoderm layer is patterned by signaling originating from the extra-embryonic tissues and the adjacent germ layers into a primitive gut tube comprising the foregut, hindgut and midgut. From these different regions the embryonic organ buds originate, which will give rise to functional endoderm organs. In light of this knowledge, the protocols generated recapitulate these key events, resulting in efficient endoderm formation from hPSCs.

The most commonly used method relies on growing hPSCs at near confluence on mouse feeders or Matrigel, in media containing small quantity of foetal bovine serum combined with WNT3 and a high dose of Activin (Agarwal et al., 2008; Brolén et al., 2010; D'Amour et al., 2005; Kubo et al., 2004; Sullivan et al., 2010). Alternative methods rely on more defined media containing not only a high dose of Activin, but also BMP4, FGF2, the GSK3β inhibitor CHIR99021 and the PI3-Kinase inhibitor LY294002 (McLean et al., 2007; Nostro et al., 2011; Teo et al., 2012; Touboul et al., 2010). Despite their apparent divergence, these protocols respect the fundamental principles learnt from early development. They are based on elevated activation of Activin signaling, which represents the pathway necessary for inducing endoderm specification in a broad number of species (Tam and Loebel, 2007; Zorn and Wells, 2009). Of note, Nodal, which is the natural growth factor controlling endoderm differentiation during mouse gastrulation, is rarely used as a recombinant protein due to its poor stability and high cost (Schier, 2003), while GDF8 can be used as a substitute for Activin (Rezania et al., 2014). Similarly, whilst TGF-β is often used to maintain pluripotency of hPSCs and activates the SMAD2/3 signaling pathway, it is a poor inducer of endoderm differentiation. Importantly, most protocols of endoderm differentiation also modulate WNT pathway either by addition of recombinant WNT protein or the inhibition of either GSK3-β or PI3-Kinase using small molecules (McLean et al., 2007; Touboul et al., 2010). Moreover, most protocols start from colonies as opposed to single cells, since the process of EMT is inherent to the process of differentiation. Concerning extra-cellular matrix, endoderm differentiation can be efficiently performed on recombinant fibronectin or vitronectin. However, subsequent differentiation often requires additional ECM proteins such as collagen for hepatocytes. Thus, most protocols rely on relatively complex ECM.

These protocols result in a population expressing SOX17/GATA6/GATA4/CXCR4, while being negative for SOX7/SOX2/OCT4/T. The expression of FOXA2, a marker commonly used to characterise endoderm in the mouse, is less reliable in human, since it induction seems to occur after SOX17, while in the mouse, FOXA2 is a very early marker of endoderm specification. Homogeneity of differentiation is often confirmed by flow cytometry analyses for the expression of the cell surface marker CXCR4. Importantly, the resulting endoderm progenitors described by some protocols can be maintained for a prolonged period of time, while maintaining their capacity to differentiate into a diversity of endodermal derivatives

including pancreas, liver, gut and lung (Gadue et al., 2005; Hannan et al., 2013a; Pagliuca et al., 2014). Overall, methods to generate endoderm cells from hPSCs are converging and there is now a clear consensus concerning the growth factors and the culture conditions necessary to direct hPSC differentiation toward this lineage (Table 1).

Differentiation of hPSCs into Endoderm Derivatives

Hepatocytes

The liver is a unique organ by the broad spectrum of its function. The liver sustains reserves of iron, vitamins and minerals. It detoxifies alcohol, drugs and other chemicals as well as removing inhaled poisons such as exhaust or smoke. The liver also produces bile for digestion of lipids, albumin (Alb) which represents the most abundant protein in the plasma, and blood clotting factors. Finally, the liver has an essential metabolic activity by storing glycogen and lipids. Most of these functions are fulfilled by hepatocytes, which represent 80-90% of the cells of the liver. Due to their broad function and interest of the pharmaceutical industry, the production of hepatocytes has been a key focus in the hPSC field, resulting in the development of a diversity of protocols to achieve this goal. These methods follow a normal path of development starting with endoderm specification, followed by patterning of the endoderm into foregut using Activin. Hepatoblast-like cells are then produced by blocking Activin and adding BMP4/FGF which are known to control liver bud formation *in vivo*. The resulting progenitors are then differentiated into hepatocytes using a diversity of media frequently, if not systematically, supplemented with Hepatic Growth Factor (HGF) and Oncostatin M (OSM), two growth factors known to be involved in liver development and functional maturation (Agarwal et al., 2008; Brolén et al., 2010; Cai et al., 2007; Chen et al., 2012; Hannan et al., 2013b; Mallanna and Duncan, 2013; Si-tayeb et al., 2010; Siller et al., 2015; Song et al., 2009; Sullivan et al., 2010; Touboul et al., 2010). The longer the cells are then grown in these conditions, the more their metabolic activity seems to increase and this phase of functional maturation can last up to 20-30 days with some protocols (Agarwal et al., 2008; Brolén et al., 2010; Hannan et al., 2013b; Mallanna and Duncan, 2013). Hepatocytes produced from hPSCs express key transcription factors and functional markers such as HNF4α, HNF6, CEBPα, PROX1, GATA4 and ALB, AAT and CYP3A4 respectively. These hepatocyte-like cells (HLCs) were also shown to have liver-specific functions such as albumin secretion, glycogen synthesis, urea production, LDL uptake and limited cytochrome P450 activity (Agarwal et al., 2008; Brolén et al., 2010; Cai et al., 2007; Hannan et al., 2013b; Mallanna and Duncan, 2013; Si-tayeb et al., 2010; Song et al., 2009; Sullivan et al., 2010). Nonetheless, HLCs are not

equivalent to primary adult hepatocytes and in fact more closely resemble foetal hepatocytes. Indeed, they express markers such as AFP and do not express adult CYP450 including CYP3A4 to the same extent as primary freshly isolated hepatocytes (Baxter et al., 2015). Thus, a broad number of approaches have been developed to further increase their metabolic function including the use of Vitamin C, 3D co-culture (Gieseck et al., 2014; Takebe et al., 2014) and small molecules (Shan et al., 2014). Nonetheless, none of these methods appear to result in the production of hepatocytes displaying the full range of adult functionality, while the transfer of these methods between laboratories has been challenging. Consequently, differentiation protocols have not fundamentally changed over the past 5 years. This lack of progress can also be explained by the scarcity of knowledge regarding hepatic functional maturation which happens very progressively after birth. Indeed, this stage is particularly difficult to study in human for obvious ethical reasons while study in animal models remains limited. Consequently, we have little information regarding the signaling pathways and factors controlling hepatocyte maturation *in vivo.* Furthermore, culture media currently available to grow primary hepatocytes only allows the maintenance of metabolic functions for few days if not hours. Consequently, even a successful process of differentiation is unlikely to generate functional cells for more than few hours. Importantly, the lack of maturity of HLCs does not imply that these cells cannot be used to model disease or transplantation (See below). On the contrary, their foetal nature could provide them with advantageous properties of proliferation and plasticity. Nonetheless, the lack of metabolic activity denotes major limitations for drug toxicity screening especially in the context of the pharmaceutical industry. In summary, the production of hepatocyte-like cells from hPSCs can be now robustly achieved using methods broadly available and despite their imperfection, the resulting cells have a direct interest for a diversity of clinical applications. Nonetheless, the production of fully functional hepatocytes continues to be a major objective and a better understanding of liver development in human will be essential to bypass the limitation of the current protocols.

Cholangiocytes

Cholangiocytes represent the second cell type of the liver which has an endodermal origin. These cells form the epithelium of the biliary three and have a key function in bile transport, inflammation and liver repair. Both hepatocytes and cholangiocytes originate from hepatoblasts, a common progenitor found in the liver bud during early hepatic development (Shan et al., 2014). Thus, protocols for cholangiocyte production follow hepatocyte differentiation up to the production of hepatoblasts which are then differentiated into cholangiocyte progenitors by modulating Notch signaling and/or TGF-β. Subsequently, these

cells are grown in a 3D environment in the presence of growth factors such as EGF/HGF/FGF10 or in co-culture with cells expressing NOTCH. The resulting cholangiocytes express biliary markers such as CK18, CK19, SOX9, HNF1B, γ-glutamyl transferase (GGT1), NOTCH2, CFTR, SCR, SSTR2 and AQP1 (Dianat et al., 2014; Ogawa et al., 2015; Sampaziotis et al., 2015a). Most importantly hPSC-derived cholangiocytes display operational MDR1 receptor, CFTR chloride transporter activity, GGT secretion as well as ALP activity (Ogawa et al., 2015; Sampaziotis et al., 2015a). Finally, they react to hormone stimuli and can transport bile acid. Transcriptomic analyses confirmed their biliary identity but also revealed that these cells continue to express foetal markers such as SOX9, suggesting that similarly to hepatocytes, hPSC-derived cholangiocytes are not equivalent to primary adult cells. Despite these limitations, these cells have been shown to model accurately genetic cholangiopathies such as Alagille's Syndrome and polycystic liver disease (Sampaziotis et al., 2015a). In addition, cholangiocytes generated from hPSCs carrying a mutation in the CFTR gene have been proven useful to validate drugs for the treatment of Cystic Fibrosis in the liver (Ogawa et al., 2015; Sampaziotis et al., 2015a). Thus, similarly to hepatocytes, hPSC-derived cholangiocytes can be useful for translational applications as well as for basic biology studies.

Pancreas

Production of pancreatic β-cells from hPSCs has been explored extensively due to the great promise they hold for cell therapy against diabetes. Indeed, β-cells which secrete insulin upon glucose stimulation are the main target of the immune system in Type 1 diabetes, while there is growing evidence that their exhaustion/dedifferentiation has a major role in Type 2 diabetes. In parallel, transplantation of pancreatic islets which are the functional unit of the pancreas containing β-cells has been shown to be efficient in patients with end stage disease to restore controllable glycemia, and even in some cases to allow independence from insulin injection. Nonetheless, only a limited number of patients can benefit from this therapy since it relies on a large number of islets which can only be obtained from 2-3 cadaveric donors. Thus, major funding initiatives from industry, governmental and charity organisations have supported ambitious programs aiming to produce pancreatic cells from hPSCs. The combination of strong financial support and extensive existing knowledge on pancreatic development allowed the creation of differentiation protocols which are probably the most advanced in the endoderm field. These protocols are based on a stepwise differentiation which closely mimics embryonic development. In summary, culture conditions are changed every 2-4 days to attain a new stage of differentiation, marked by the expression of a specific combination of markers which have been functionally validated in model organisms. The first step after endoderm formation involves the induction of posterior foregut (FOXA2/HNF1B/HNF4A) using FGF7 (or KGF)

(D'Amour et al., 2006; Pagliuca et al., 2014; Rezania et al., 2014). Pancreatic progenitors (PDX1/NKX6.1/SOX9) are then obtained by adding Retinoic Acid and FGF7 while inhibiting BMP, TGF-β and SHH signaling (D'Amour et al., 2006; Pagliuca et al., 2014; Rezania et al., 2014). Importantly, Activin/TGF-β can also be used to increase the number of cells expressing NKX6.1 (Nostro et al., 2015) which is essential to obtain truly multipotent pancreatic progenitors. Endocrine progenitors (PDX1/NGN3/NeuroD1) are then induced by inhibiting the TGF-β receptor ALK5 in the presence of thyroid hormone. Further differentiation into β-like cells (INS+) is obtained by growing endocrine progenitors in the presence of a combination of Notch, SHH inhibitors and the receptor tyrosine kinase AXL inhibitor (Pagliuca et al., 2014; Rezania et al., 2014). The resulting β-cells can secrete insulin upon glucose stimulation *in vitro* while they rescue animal model for diabetes. Importantly, inhibition of ALK5 seems to be essential for avoiding the production of multi-hormonal cells which are known to represent immature cells with limited function (Rezania et al., 2014). Despite these significant advances, the resulting cells display a glucose response comparable to those foetal cells. Furthermore, these protocols are often cell line dependent, meaning that they work more efficiently on the cell lines originally used for their development. Nonetheless, hiPSC-derived pancreatic cells have been proven useful for modeling disease (see next section) and have enabled the creation of platforms for drug screening (Pagliuca et al., 2014). Finally, the biotech company Viacytes has pioneered a clinical trial to define the interest of pancreatic progenitors (PDX1/NKX6.1/SOX9) for cell based therapy (See below). Thus, pancreatic cells currently generated from hPSCs display a level of function which is likely to be sufficient for a diversity of applications including drug screening and regenerative medicine. Nonetheless, there is no doubt that the field is already working to further improve the functional profile of β-like cells currently available.

Lung

Pulmonary disease is the third leading cause of death worldwide and many of these conditions are currently without treatment. Despite this major healthcare challenge, production of lung cells from hiPSCs has only recently become a major focus with systematic studies performed to identify conditions to generate airway cells. The diversity of signaling pathways known to control lung endoderm specification, the cellular complexity of this organ as well as the close interplay with thyroid development has been a great challenge for the development of protocols for the production of lung cells. Nonetheless, several directed differentiation protocols have now managed to very efficiently produce lung and airway epithelial cells (Dye et al., 2015; Firth et al., 2014; Gotoh et al., 2014; Huang et al., 2013; Longmire et al., 2012;

McIntyre et al., 2014; Wong et al., 2012). These protocols start with patterning of endoderm cells into anterior foregut by inhibiting Activin and BMP signaling. Further stimulation of WNT, SHH, BMP4 and FGF7/10 signaling results in the acquisition of an identity of early lung epithelial progenitors expressing the master regulator NKX2.1. Alternatively, Wong and colleagues used a combination of FGF7, FGF10 and low concertation of BMP4 without direct modulation of WNT. The resulting progenitors can differentiate into cells expressing markers of proximal airway epithelium including FOXJ1, CFTR and MUC16 after cultured in air-liquid interphase condition for 5 weeks, (Wong et al., 2012). Further refinement of this protocol has improved the production of ciliated, Clara and goblet cells *in vitro* and *in vivo* with the caveat that the cultures are contaminated with mesodermal and neuronal cell types (Huang et al., 2013).

Despite these apparently divergent protocols, recent studies have clearly established that WNT signaling is essential to block thyroid cell specification expressing NKX2.1/PAX8, which are closely related to lung progenitors during early development (Serra et al., 2017). Furthermore, WNT was also shown to control the proximodistal patterning of lung endoderm (NKX2.1+/PAX8-). Of note, differentiation protocols often yield a heterogeneous mixture of lung and non-lung cell types while the lung epithelial cell types can range from basal stem cells of the proximal region to alveolar epithelial cells (AECs) from the distal region. This comes to importance when studying disease. For example, some pulmonary disorders affect specific lung epithelial cell types or regions but not others. The proximal region of the lung is most afflicted in Cystic Fibrosis and therefore the specific derivation of proximal lung epithelial from human pluripotent stem cells would be most suitable for disease modeling or drug screening in this instance. Consequently, recent protocols rely on cell sorting, taking advantage of cell surface makers CD47hi/CD26low to purify NKX2.1-expressing lung endoderm cells. Using this approach, McCauley and colleagues identified conditions of “low WNT levels” which favour the differentiation of these lung endoderm cells (NKX2.1+/SOX2+) into proximal lung cell fates while high WNT levels favour the production of progenitors (NKX2.1+/SOX9+) for distal airway cells (McCauley et al., 2017). Motile cilia were observed when these proximal lung epithelial cells were cultured in 3D with Notch inhibitors or in an air-liquid interface (ALI). The authors also demonstrated the potential of these cells in Cystic Fibrosis disease modeling and drug screening by showing that these proximal cell types can form organoids in 3D culture conditions which are able of undergoing CFTR-dependent forskolin-induced swelling.

Importantly, the difficulty to isolate and then maintain the functional characteristics of primary lung cells *in vitro*, has limited the direct comparison necessary to establish the exact level of maturation of hiPSC-derived lung cells. Furthermore, simple and quantitative standard assays for evaluating the functionality of lung cells remain to be established. Evaluation of

mucin secretion and surfactant production could be systematically applied, while electrophysiology to measure short circuit current on cells grown in air liquid interface could also provide more qualitative functional measurement. Interestingly, methods based on new imaging technologies are currently under development for measuring cilia beat frequency and such approach could provide new perspective on phenotyping lung cells. Finally, animal models for lung injury have just become available to test engraftment and function of cells generated *in vitro* (Dye et al., 2016; McIntyre et al., 2014; Miller et al., 2017; Nikolić et al., 2017). In this context, a recent study has described the generation of lung $SOX9^{+}$ bud tip-like progenitor cells which have the capacity to differentiate into multiple lineages both *in vitro* and *in vivo* providing of proof of principle for engraftment of hPSC-derived cells following lung injury (Miller et al., 2017).

To conclude this part, the lung field has developed robust methods for directing the differentiation of hPSCs into a diversity of lung cells which will be very useful to uncover mechanisms controlling embryonic development and disease. The production of these cells could also open the possibility to cell based therapy applications which have been relatively unexplored until now.

Gastrointestinal Tract

The gastrointestinal tract has a complex and diverse set of functions such as digesting food, absorbing nutrients and energy as well as providing immune protection. There is a rising global incidence of diseases affecting the gastrointestinal tract a well as an increase of patients lacking gastrointestinal function altogether; there is therefore a high demand for effective models of human intestinal development, disease and regeneration to improve our understanding of their aetiology, and screening of potential therapeutics for inflammatory bowel disease and intestinal transplant for short gut syndrome. To address this objective, human intestinal organoids (HIOs) have been successfully generated from hPSCs using different approaches that mainly rely on driving differentiation of endoderm cells into mid/hindgut using WNT and FGF (Hannan et al., 2013a; Spence et al., 2011). The resulting cells are then grown in 3D culture conditions, as had been previously established for primary gut organoids (Sato et al., 2009). These organoids rely on EGF, R-Spondin and WNT signaling modulation, as well as extracellular matrix support from collagen and laminin rich gels such as Matrigel. Whilst Matrigel provides an excellent basement matrix for organoid growth, there is variation in the characteristics of the matrix, as well as contamination with various cytokines. Recent efforts have been made to switch to more reproducible and defined synthetic gels, improving the potential of these organoids to be used in regenerative medicine (Cruz-Acuña

et al., 2017). Importantly, the method used to reach the first step of the protocol has a major impact on the subsequent organoids generated. Indeed, methods allowing a near homogeneous population of endoderm cells results in gut organoids that closely resemble organoids derived from foetal tissue (Fordham et al., 2013) which can be grown almost indefinitely *in vitro* and which can acquire expression of functional markers over time (Forbester et al., 2016). On the other hand, the most established and characterised system by Wells and colleagues allows the production of endoderm cells contaminated by a small fraction of mesoderm cells. These cells, when grown in 3D conditions, form a mesenchyme layer supporting differentiation, organisation and functional maturation of gut cells. Of note, this co-culture system allows only a limited number of passaging and is thus not compatible with large scale amplification the cells. Nonetheless, hiPSC-derived intestinal-like structures are highly proliferative, express the characteristic markers of the intestinal epithelium including functional markers such as lysozyme and mucins, the intestinal transcription factors KLF5, CDX2 and SOX9 as well as the stem cell markers LGR5 and ASCL2 (Spence et al., 2011). The organoids contain the main cell types comprising the adult intestinal epithelium including enterocytes, goblet cells, Paneth cells and enteroendocrine cells. Functional analysis of the gene NGN3 in these organoids, showed that its knockdown results in loss of enteroendocrine cells whereas its overexpression leads to 5-fold higher levels of endocrine cells (Spence et al., 2011). This study provides a proof of principle that the intestinal organoids provide a powerful tool to study gene function and their role in disease development. Nonetheless, it is important to underline that HIOs still exhibit a gene expression profile similar to foetal cells, while the functionality of these organoids can be further increased by transplantation under the kidney capsule of immune deficient mice (Watson et al., 2014).

The initial organoid systems appeared to represent the proximal small intestine, with subsequent attempts to optimise protocols to derive other regions of the gastrointestinal tract. Gastric organoids were generated using WNT3A, CHIR99021, and Noggin to generate foregut from definitive endoderm, followed by FGF4, Noggin, and finally retinoic acid to generate gastric organoids that express key markers such as SOX2, PDX1, GATA4 and KLF5 (McCracken et al., 2014). More recently, attempts were made to direct intestinal organoids to a more colonic nature (Munera et al., 2017), using BMP to generate organoids that express colonic epithelial markers and can produce colon-specific cell types after engraftment in the kidney capsule. However, transcriptome wide analysis has shown limited similarity to *in vivo* tissue, and there is still much scope for optimisation of all systems to greater physiological maturity. This lack of maturity of the model can also be turned to advantage, allowing further differentiation of the systems to specific intestinal regions, and exploring development (Finkbeiner et al., 2015; Tsai et al., 2017).

Overall, the development of protocols of hPSCs-derived gut organoids has been remarkable by its rapid progress and its diverse applications in disease modeling and developmental studies, and represents a highly promising tool in regenerative medicine. However, further optimisation of the model is needed, to more closely resemble mature region-specific areas of the gastrointestinal tract, particularly on a transcriptome and epigenome wide level (Table 2 and Figure 1).

Endoderm Differentiation of hPSCs as an *in vitro* Model of Development

While translational applications have been the main driver to develop protocols for differentiating hPSCs into endoderm, these methods have also been useful to uncover molecular mechanisms directing this early cell fate decision during development. Indeed, *in vitro* hPSC differentiation recapitulates developmental processes and waves of expression of key transcription factors, thereby providing a unique opportunity for studying mechanisms impossible to study otherwise, especially in human. This aspect is particularly important in view of the recent observations that despite significant conservation, the mechanisms controlling cell fate decisions could also show divergence even between mammalian species such as human and mouse. As an example, recent reports have shown that GATA6 is necessary for endoderm differentiation and pancreatic development in hPSCs (Fisher et al., 2017; Shi et al., 2017; Tiyaboonchai et al., 2017) while heterozygous mutation in humans results in pancreatic agenesis. On the other hand, this mutation has no effect on pancreas development in the mouse. Only the combined absence of GATA6 and GATA4 seems to affect mouse pancreatic organogenesis (Carrasco et al., 2012; Xuan et al., 2012). Interestingly, GATA6 could be controlled by the WNT pathway through its interaction with LEF1 in human endoderm cells (Sun et al., 2017) suggesting additional divergence at the signaling pathway level. Thus, transcriptional networks controlling early endoderm differentiation could vary between species. hPSC endoderm differentiation has also been used to perform studies impossible or challenging with model organisms. As an example, ChIP-seq analyses have revealed the extend of the network controlled by SMAD2/3 during endoderm differentiation and also their cooperation with the transcription factor EOMES and epigenetic modifiers JMJD3 (Brown et al., 2011; Kim et al., 2011). Recent studies also confirm that pluripotency factors such as OCT4 cooperate with signaling pathways to enable the expression of endoderm markers (Ying et al., 2015). Interestingly, comparative analyses between *in vitro* data and studies in the mouse, suggest that EOMES function in endoderm could be conserved between species (Teo et al., 2011). Further analyses have uncovered the cross talk between PI3-Kinase, WNT and TGF-β (Singh et al., 2012), while other studies have uncovered the epigenetic regulations directing endoderm differentiation including the mechanisms by which

SMAD2/3 could direct the activity of MLL complexes through its interaction with DPY30. Interestingly, genetic studies in the mouse suggest that similar interplays between DPY30 and SMAD2/3 could also take place *in vivo* (Bertero et al., 2015) thereby demonstrating that hPSCs can be useful to inform evolutionary conserved mechanisms controlling gastrulation.

On the other hand, hPSC differentiation into endoderm can be exploited to uncover novel mechanisms which are either masked by functional redundancy *in vivo* and/or are only relevant for human development. As an example, detailed molecular analyses has revealed that endoderm specification is controlled by interplays between SMAD2/3 and the cell cycle regulators Cyclin D/CDK4/6 (Pauklin and Vallier, 2013). Furthermore, the same cell cycle regulators seem to orchestrate the recruitment of transcriptional regulators and epigenetic modifiers on endoderm genes (Pauklin et al., 2016). These mechanisms suggest that the cell cycle machinery directly orchestrates cell fate decisions in hPSCs. However, knockout of these cell cycle regulators in the mouse embryo has no effect on gastrulation, thereby suggesting these mechanisms could be either more important *in vitro* or human specific.

Importantly, hPSCs are also frequently used to uncovered mechanisms controlling development of endoderm organs. *In vitro* differentiation has been applied to study the function of a diversity of transcription factors and signaling pathways controlling pancreatic (Cebola et al., 2015; Kee et al., 2015; Zhu et al., 2016) or hepatic development (DeLaForest et al., 2011). Similarly, hPSCs have been used to uncover the signaling pathways controlling lung or intestinal specification from endoderm in human (Chen et al., 2017; Huang et al., 2013; McCauley et al., 2017; Serra et al., 2017; Snoeck, 2015). Thus, hPSCs offer unprecedented opportunities to study human organogenesis.

Taken together, these studies have uncovered new molecular mechanisms regulating human endoderm formation and its derivatives, and thus demonstrate the importance of hPSC as a model system to not only validate known molecular mechanisms, but also to uncover new aspects of embryonic development.

Disease Modeling Using Endoderm Derivatives

The remarkable advances in the development of protocols for generating a repertoire of endoderm derivatives *in vitro* has been associated with translational applications which can be divided in 2 groups: Disease modeling/drug screening and cell based therapy (Figure 2).

Genetic Disorders

A broad number of inherited diseases has been modelled using endodermal derivatives. Of particular interest, the liver received great attention in recent years due to various genetic

disorders, alcohol induced and non-alcoholic fatty liver disease, hepatotropic infections and drug-mediated toxicity. Accordingly, a diversity of reports have shown that hepatocytes derived from hiPSCs can be used to model a variety of disorders including inherited metabolic disorders (IMDs) such as α1-antitrypsin deficiency (A1ATD), familial hypercholesterolemia (FH), glycogen storage disease type 1a (GSD1a) and Wilson's disease (WD) (Cayo et al., 2012; Sampaziotis et al., 2015b; Yusa et al., 2011; Zhang et al., 2011). Successful modeling of monogenetic disorders has also been achieved with hiPSC-derived cholangiocytes (Ogawa et al., 2015; Sampaziotis et al., 2015a) and lung cells for Cystic Fibrosis (Wong et al., 2012). Importantly, those culture systems have also been used to screen drugs for hepatic maturation, hepatoxicity (Hannoun et al., 2016), control of cholesterol levels (Cayo et al., 2017), Cystic Fibrosis (Sampaziotis et al., 2015a; Wong et al., 2012), α1-antitrypsin deficiency (Choi et al., 2013) or even neonatal diabetes (Zeng et al., 2016).

HPSC-derived intestinal organoids also present a great opportunity for exploring intestinal pathophysiology using genetically modified PSC lines. For example, deriving iPSCs from patients with dyskeratosis congenita (DC) and gene correction by CRISPR/Cas9, enabled disease modeling and pathway interrogation using the organoid model (Woo et al., 2016). Similarly, hiPSC-derived intestinal organoids have been used to model Hirschsprung's disease caused by a mutation in the gene PHOX2B. In this last study, hiPSCs lines with mutations in PHOX2B were differentiated into neural crest cells and combined with HIOs. Further transcriptional analysis revealed downregulation of genes relevant to muscle development in organoids carrying the genetic anomaly (Workman et al., 2016). These results provide of proof of concept that HIOs could be useful to further shed light on the molecular mechanisms driving disease.

Finally, pancreatic cells have been used by a broad number of studies to model a genetic form of diabetes (MODY) or pancreatic agenesis (Teo et al., 2013). Overall these reports show that hPSC-derived pancreatic cells can be used to model genetic disorders affecting pancreatic development including mutations in HNF1b (Teo et al., 2016), GATA6 (Fisher et al., 2017; Shi et al., 2017; Tiyaboonchai et al., 2017), PDX1 (Kee et al., 2015; Zhu et al., 2016), NGN3 (Zhu et al., 2016) and β-cell function (Zeng et al., 2016). Importantly, such a model could be used advantageously to uncover the mechanisms by which haploinsufficiency induces diabetes in humans and will ultimately help to functionally validate the diversity of genetic variants associated with diabetes by GWAS studies.

Accordingly, several consortia have generated large scale banks of hiPSC lines (Soares et al., 2014) to study human genetic diversity in a dish. Of particular interest, the NextGen consortium (Warren et al., 2017a) has shown that a large cohort of hiPSC lines differentiated into hepatocytes could allow the identification of new expression Quantitative Trait Loci (eQTL) involved in lipid metabolism (Pashos et al., 2017) and also to validate the function of genetic

variants identified by Genome Wide Association Studies (GWAS) (Warren et al., 2017b). Of note, hiPSC lines have also been generated from patients with Type 1 (Chen et al., 2009) and Type 2 (Ohmine et al., 2012) diabetes without revealing a clear phenotype after differentiation. Nonetheless, these studies were based on a limited number of cell lines while large scale experiments could be informative in uncovering novel genetic mechanisms driving diabetes. Taken together, these studies demonstrate the feasibility of using endoderm derivatives to not only model monogenetic disorders affecting endodermal organs, but also to study genetic mechanisms controlling the onset and penetrance of complex disorders.

Infectious Disease

Endodermal organs are also the target of major infectious diseases such as malaria and hepatitis. Thus, modeling these diseases using their cellular target such as liver cells has attracted a lot of attention in recent years. Accordingly, several groups have shown that hiPSC-derived hepatocytes can support the Hepatitis C virus (HCV) life cycle including replication, release of infectious virions and an inflammatory response to the infection (Carpentier et al., 2014; Schwartz et al., 2012). Interestingly, microarray analyses of non-permissive cells and permissive cells for infection, has revealed upregulation of known cofactors supporting virus infection and downregulation of antiviral genes, providing a platform for further identification of key genes involved in survival of the virus, and potential therapeutic applications (Wu et al., 2012). hPSC-derived hepatocytes were also successfully infected with HBV (Shlomai et al., 2014) or *Plasmodium* parasites which cause malaria (Ng et al., 2015). The common caveat with these studies is the low infection and replication rate of the pathogens. This limitation could be attributed to immaturity of the host hepatocytes, highlighting the importance of further improvement of current differentiation protocols. HIOs have also been used to model the interface of the intestine with infectious agents such as interaction of *Salmonella enterica* serovar Typhimurium with intestinal epithelium (Forbester et al., 2015). This study shows that the bacteria could infect hiPSC-derived intestinal cells while provoking a change in expression profile indicating the initiation of a pro-inflammatory response. Additionally, HIOs have been used to study more physiological colonisation of the gut, and how this might affect development, taking advantage of the relative immaturity of the model (Woo et al., 2016). Similarly, hPSC-derived gastric organoid infection by *H. pylori* results in the rapid association of the virulence factor CagA with the c-Met receptor inducting epithelial cell proliferation (McCracken et al., 2014). These data clearly establish the interest of hPSC-derived gut cells to model host-pathogen interaction. Finally, there is no doubt that hPSC-derived lung cells will soon be used as a model for infections of the lung tissue.

Overall, hPSC-derived endodermal cells have demonstrated their importance and capacity to model a diverse set of diseases and certainly represent a complementary if not an advantageous alternative to previous systems. Nonetheless, these platforms are just starting to be exploited to uncover new mechanisms of disease and the transfer of this technology to more disease-oriented laboratories will certainly accelerate their exploitation for clinical application.

Cell-based Therapy

The second major translational application of endoderm derivatives is cell-based therapy. Indeed, multiple diseases affecting organs originating from the endoderm layer could be treated if not cured using cell replacement therapy. Accordingly, primary hepatocytes have already been transplanted into nearly 100 patients to treat a variety of inherited liver diseases including A1ATD, glycogen storage disorders (GSD), Crigler-Najjar syndrome and factor VII deficiency. Unfortunately, cell-based therapy of the liver is limited by the scarce number and quality of available donor cells. Therefore, a broad number of studies have analysed the capacity of hPSC-derived hepatocytes to colonise the liver and restore vital functions in animal models for liver failure. Interestingly, these studies have shown variable results depending on the type of liver injury. For instance, liver colonisation and rescue of the fumarylacetoacetate hydrolase (Fah)-mice which provide a model for inherited metabolic disorders, remains extremely challenging with hPSC-derived hepatocytes, while they represent the gold standard for primary adult hepatocyte transplantation. Other models such as the uroplasminogen activator (uPA) mice which mimic acute liver failure have been successfully engrafted with hPSC-derived hepatocytes but the persistence of the transplanted cells was limited to few weeks (Touboul et al., 2010). Finally, the best results have been obtained with liver injuries induced by chemical modeling of liver cirrhosis such as tetrachloride or paracetamol treatment. In these experiments, hPSC-derived hepatocytes were able to colonise more than 5-10% of the liver while improving mice survival (Cai et al., 2007; Chen et al., 2015; Liu et al., 2011; Tolosa et al., 2015).

Considered together, these results demonstrate that hPSC-derived hepatocytes could be used in cell based therapies targeting specific liver pathology. Indeed, these cells could be a useful therapy in the context of acute liver failure induced by drug induced liver injury for example. Nonetheless, such application will require a large number of cells which could be challenging to produce in GMP conditions. In addition, the injection of such number of cells will have to be done in the portal vein and thus could result in cells engrafting in other organs. Thus, risks associated with uncontrolled growth and teratoma formation will have to be

carefully monitored. Finally, such approach might not work for a majority of end stage liver diseases which involve important remodelling of the liver architecture due to fibrosis/cirrhosis.

For these reasons, alternative approaches to intra-hepatic transplantation are currently explored including the transplantation of bags or patches containing liver cells (Stevens et al., 2017). As an example, hPSC-derived hepatoblasts combined with mesenchymal stem cells and endothelial cells in a 3D patch of Matrigel have been shown to rescue TK-NOG mice (Takebe et al., 2014). Thus, extra-hepatic transplantation could also provide an interesting alternative to treat liver failure. However, such approach has also its own limitations since the 3D patch requires extensive spontaneous vascularisation to be plugged to the blood flow, while the absence of connection with the biliary system will be problematic to evacuate toxic bile. Thus, while promising, cell-based therapy using hiPSC-derived liver cells still requires new innovations which could involve the *in vitro* generation of mini-livers using decellularized organs (Pla-Palacín et al., 2017). In addition, a step by step clinical strategy with systematic testing in animals will help to address the challenges associated with safety and intra-hepatic injection.

Concerning HIOs, these endodermal derivatives have also been shown to grow successfully on tube shaped PGA/PLLA scaffolds and integrate *in vivo* (Finkbeiner et al., 2015) thereby advancing the use of these organoids as potential therapies for intestinal regeneration. Recently, HIOs with a functional enteric nervous system were generated by combining the HIOs with hPSC-derived neural crest cells. These organoids after *in vivo* transplantation formed neuroglial structures and had electrochemical coupling that regulated waves of propagating contraction. Despite the successful engraftment of the cells, their phenotype appears to be immature and more similar to the foetal gut, indicating that further optimisation is required (Workman et al., 2016).

The other endodermal organ which has been shown to benefit from cell based therapy is the pancreas. Indeed, islet transplantation (see introduction) has already established the interest of such approach for treatment of diabetes. In parallel, several groups have shown that pancreatic cells generated from hPSCs can alleviate diabetes in a mouse model (Jiang et al., 2007; Pagliuca et al., 2014; Rezania et al., 2014). Based on these promising results, the biotechnology company Viacyte has developed a major program aiming to transplant hESC-derived pancreatic progenitors encapsulated in a bag in patients with advanced Type 1 diabetes. The use of pancreatic progenitors allows the production of functional cells *in vivo* thereby avoiding the lack of functional maturation associated with *in vitro* differentiation, while the encapsulation device protects cells against immune rejection and precludes risks associated with abnormal growth. Extensive animal model experiments have demonstrated the efficacy of this approach and the first human clinical trial is currently ongoing. This trial which is one of the most advanced using hPSC derivatives has enrolled 20 patients to confirm

the safety of this approach and to evaluate the quantity of cells necessary for maximum efficacy. Interestingly, recent information seems to suggest that the encapsulation device could present some challenges. Indeed, the encapsulation device requires extensive vascularisation without inducing fibrosis of surrounding tissues to enable long term functioning of the pancreatic cells. Nonetheless, this trial represents the first of its kind and there is no doubt this program and other initiatives such as Semma therapeutics will drive the field towards the development of novel therapies against diabetes.

To conclude this part, endodermal derivatives have generated promising results in preclinical models for cell based therapy applications while pancreatic cells have already reached the clinic. Nonetheless, additional studies are still needed to improve encapsulation method and also to produce a large quantity of cells in conditions compatible with the clinic.

Current Challenges for Basic Studies and Clinical Applications

Variation Between hPSCs Lines

Variability in capacity of differentiation between hPSC lines is a major challenge and while the origin of this limitation remains unclear, genetic mechanisms appear to be a leading cause (Ortmann and Vallier, 2017). Nonetheless, endoderm differentiation seems to be particularly affected by this phenomenon. Indeed, while current protocols have been proven to work in multiple labs, endoderm differentiation remains strenuous, mainly due to the inductive nature of this process. Contrary to neuroectoderm specification which results from the inhibition of signaling pathways opposing differentiation, endoderm specification is driven by the inductive activity of Activin/Nodal signaling, which plays a central role in establishing the entire transcriptional network characterising endoderm cells. This role is obscured by the essential and apparently contradicting role of Activin signaling in maintaining pluripotency (Pauklin and Vallier, 2015). Thus, any factors affecting the level of Activin signaling or its efficacy can impact on the differentiation efficiency and increase contamination by undifferentiated cells. Furthermore, multiple factors can influence endoderm specification some of which are difficult to control. Firstly, cell density and size of plated colonies can interfere with the necessary EMT process characterising endoderm differentiation. Of note, this EMT also precludes differentiation from single cells and thus easy quantification of cell population. Secondly, the stability and quality of recombinant growth factors can interfere with the inductive nature of Activin. Thirdly, the composition of the extracellular matrix can interfere with differentiation. For example, Matrigel contains high levels of TGF-β, which can interfere with Activin/Nodal signaling. Lastly, the endogenous levels of key growth factors including Nodal which level of expression could vary between different hiPSC lines (Ortmann and Vallier, 2017).

Consequently, all these parameters need to be optimised in each lab and for each individual hPSC line to obtain a robust and homogeneous differentiation. Of note, the development and more importantly the standardisation of chemically defined culture such as the E8 culture system is also helping to decrease these variability issues, while allowing a better control of differentiation conditions. In addition, protocols for deriving self-renewing endodermal progenitors (Cheng et al., 2012) and foregut stem cells (Hannan et al., 2013a) could help to bypass the limited differentiation capacity of some hPSCs lines. Indeed, these multipotent endodermal cells can be isolated from heterogeneous differentiation and then expended almost indefinitely thereby bypassing the need of constantly differentiating hPSCs.

Functional Maturation

Despite the advances in generating these cell types *in vitro*, the hPSC-derived cells display a foetal signature as opposed to their mature adult counterparts. This lack of functionality represents a limitation especially for specific *in vitro* applications such as toxicology screens or modeling of chronic disorders. Importantly, the lack of basic knowledge concerning the mechanisms controlling organ maturation especially in human, have hindered the further optimisation of current protocols. Consequently, new developments are often based on a trial and error approach. In addition, an increasing number of studies now utilises the development of 3D culture systems where multiple cell types can be cultured together. Indeed, most endoderm cell types work in a cooperative way, receiving signals from neighbouring cells in a 3D environment. Of particular interest, endothelial and mesenchymal cells often provide important support for cellular function of endoderm derivatives. Accordingly, several groups have established 3D co-culture system to differentiate liver cells (Dye et al., 2015; Gieseck et al., 2014; Ogawa et al., 2015; Sampaziotis et al., 2015a; Takebe et al., 2014) or pancreatic β-cells (Chmielowiec and Borowiak, 2014) and lung organoids (Franzdóttir et al., 2010). Nonetheless, such systems seem to improve function of the endodermal cells without conferring full maturation. Furthermore, 3D culture systems are more complex to set up while cells of interest are more difficult to access for phenotyping analyses, thereby precluding high throughput applications. GMP conditions can also be difficult to achieve despite recent progress in the development of artificial Matrigel based on PGA/PLLA scaffolds (Finkbeiner, 2015). Using simpler systems that generate a pure population of endoderm derivatives enables a greater focus on specific biological aspects and direct comparison to primary tissue. These challenges highlight the need to perform further basic research to unravel the factors and biology involved in maturing functional cell types in endodermal organs.

A solution to these challenges could be provided by transdifferentiation or forward programming approaches. These methodologies have been applied successfully to bypass the requirements for functional maturation *in vitro* for pancreas, intestine and liver cells (Benthuysen et al., 2016; Huang et al., 2014; Miura and Suzuki, 2017). Of particular interest, Du and colleagues used up to 7 hepatic factors including HNF1A, HNF4A and HNF6 along with the maturation factors ATF5, PROX1 and CEBPA to generate hiHeps from fibroblasts. The resulting cells display the hallmarks of primary hepatocytes including expression of phase I and phase II drug-metabolising enzymes as well as phase III drug transporters. Furthermore, these cells can repopulate the liver *in vivo*, expressed albumin and showed CYP enzyme activity (Du et al., 2014). These encouraging results and provide a proof of principle that such approach could bypass the need of laborious and lengthy culture systems of differentiation. Nonetheless, these methods are generally very complex, rely on proprietary media difficult to obtain and work specifically on embryonic fibroblasts. In addition, there are specific aspects associated with endodermal cells which make the transdifferentiation approach particularly challenging when compared to other cell types such as neurons. Indeed, endoderm cells are often quiescent while proliferative intermediates equivalent to neuronal stem cells are difficult to identify or to expand. Furthermore, endoderm cells are often epithelial cells that depend on strong cell to cell interactions, precluding single cell isolation or purification. Thus, transdifferentiation methods need to be extremely efficient or include oncogenes promoting proliferation with the risk of affecting basic cellular biology and normal function of the cells. Finally, there is currently no culture system to grow primary endodermal derivatives such as hepatocytes or β-cells. Thus, even extremely efficient methods of reprogramming could only produce cells losing rapidly their function.

To conclude, generating fully functional cells from hPSCs remains a challenge which can be alleviated only partially by 3D culture systems and transdifferentiation approaches. Only a better understanding of the mechanisms orchestrating functional maturation of endodermal organ will enable to improve current protocols of differentiation.

Conclusions and Perspectives

The field of stem cell biology and regenerative medicine has made considerable progress over the last decade in developing protocols for the production of a diversity of endoderm originating cell types from hPSCs. We can now produce liver, pancreas, gut (intestinal, stomach, colon), lung and thyroid cells (Agarwal et al., 2008; Cai et al., 2007; Dye et al., 2015; Jacob et al., 2017; McCauley et al., 2017; Ogawa et al., 2015; Sampaziotis et al., 2015a; Si-tayeb et al., 2010; Snoeck, 2015; Song et al., 2009; Sullivan et al., 2010). Our knowledge of

basic developmental biology has been essential for the generation of these protocols. Importantly, methods of differentiation are now converging based on common standards for cell characterisation and culture systems. Consequently, hPSC endodermal derivatives are starting to be used by a growing number of groups to uncover novel mechanisms either controlling development or disease.

The main obstacle in the field of disease modeling and regenerative medicine remains the lack of functionality of the different cell types generated. This is partly due to our limited understanding of the mechanisms driving organ maturation in humans, especially as there is growing evidence that the molecular events that drive these processes could diverge between species (Snoeck, 2015). Thus, further basic studies are crucial for the development of novel approaches for the generation of fully functional cells from hPSCs. More specifically, it will be essential to identify signaling pathways and transcription factors that drive functional maturation of endoderm cells such as hepatocytes and β-cells.

Importantly, many organs in the body such as the intestine, muscle and hematopoietic system have resident adult stem cell populations necessary for tissue homeostasis and repair. Thus, organoids generated from primary tissues have been proposed as an alternative to hPSCs for producing liver, pancreatic, gut and lung cells *in vitro*. Many labs have described the generation of organoids from primary tissue including the intestine, liver and pancreas (Broutier et al., 2016; Rookmaaker et al., 2015). These systems can also aid in disease modeling (Schwank et al., 2013) and drug screening (Dekkers et al., 2013). However, the capacity/origin of other primary systems still requires further characterisation. For example, hepatocyte like cells produced from liver organoids can be used for modeling disease but their functionality remains limited (Broutier et al., 2017; Huch et al., 2015). The same applies to pancreatic organoids which originate from the pancreatic duct and which have a limited natural capacity of differentiation toward β-like cells. Beyond these technical aspects, primary organoids and hiPSC-derived endodermal organs provide a complementary system. Indeed, hPSC-derived cells can be used to model embryonic development and early organogenesis, while primary organoids could provide a platform to study functional maturation of specific cell types by comparison of foetal vs adult organoids derived from the same organ. In addition, hPSCs allow the study of disease progression while organoids provide information on end stage disease. Finally, hPSCs from the same donor can be differentiated into a diversity of cell types which is extremely challenging from organoids. Thus, the two systems could advantageously complement each other to study disease and development.

To conclude, the advances made in producing endodermal cell types is offering new opportunities to investigate developmental regulations and to uncover disease mechanisms impossible to study before. Moreover, endoderm derived cell types hold unique promises for regenerative medicine of diseases currently incurable. Nonetheless, key challenges remain to

be addressed to fulfil this potential. Therefore, the constant effort from the stem cell field must continue not only on clinical applications but also on basic studies aiming to understand basic characteristics of endodermal organ development.

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Figure Legends

Figure 1. Endoderm Derivatives Generated from hPSCs

Schematic representation of differentiation of hPSCs into endoderm cell types and signaling pathways required for their generation. Key transcription factors expressed by each lineage is also outlined.

Figure 2. Endoderm Derivatives for Clinical Application and Disease Modeling

Schematic overview of translational application of hPSCs-derived endoderm cell types. hPSC-derived endoderm cells can be used to model genetic disorders, infectious disease, validate genes associated with disease and drug screening. Additionally, they can be used for cell therapy.

Table 1. Protocols to Generate Endoderm Cells from hPSCs

Factors used	Markers Expressed	Cells Generated	Reference
FBS, Activin A	SOX17, FOXA2, GATA4, CXCR4	Hepatocytes, Pancreatic cells	(Agarwal et al., 2008; D'Amour et al., 2005, 2006)
FCS, Activin A, FGF2 or WNT3A	SOX17, HNF3β, CXCR4, HNF4α, α1-antitrypsin and αFP	Hepatocytes	(Brolén et al., 2010)
SR, Activin A	SOX17, FOXA2,	Hepatocytes	(Kubo et al., 2004)
Activin A, LY294002	SOX17, FOXA2, GSC, GATA4, GATA6	In vivo transplantation showed expression of hepatic, lung and intestinal markers	(McLean et al., 2007)
Activin A, BMP4	SOX17, FOXA2, CXCR4	Pancreatic progenitors	(Teo et al., 2012)
Activin A, LY294002, FGF2, BMP4	SOX17, FOXA2, GSC, GATA4, N-Cad	Hepatocytes	(Touboul et al., 2010)
Activin A, bFGF, BMP4, VEGF	SOX17, FOXA2	Pancreatic cells	(Nostro et al., 2011)

Table 2. Markers/Function Displayed in Endoderm Derivatives Generated from hPSCs

Cell Type	Markers Expressed	Function Displayed	Reference
Hepatocytes	**Fetal hepatocytes:** HNF4α, HNF6, CEBPα, PROX1, ASGPR1, AFP **Adult hepatocytes:** HNF4α, ALB, AAT, CYP3A4, CK8, CK18	• Albumin secretion • Glycogen storage • Urea metabolism • LDL uptake • Cytochrome P450 activity	(Agarwal et al., 2008; Brolén et al., 2010; Cai et al., 2007; Hannan et al., 2013b; Mallanna and Duncan, 2013; Si-tayeb et al., 2010; Siller et al., 2015; Song et al., 2009; Sullivan et al., 2010; Touboul et al., 2010)
Cholangiocytes	**Cholangiocyte progenitors:** SOX9, HNF1b, CK19 **Adult Cholangiocytes:** CK7, CFTR, SCR, SSTR2, AQP1, AE2	• Rhodamine 123 transport by MDR1 receptor • CFTR activity • GGT secretion • ALP activity • Transport of bile acid • Hormonal response	(Dianat et al., 2014; Ogawa et al., 2015; Sampaziotis et al., 2015a)
Pancreatic β-cells	**Pancreatic progenitors:** PDX1, NKX6.1, SOX9, FOXA2, HNF1B, HNF4A **Beta cells:** PDX1, NKX6.1, NGN3, INS, GCG, SST	• Insulin secretion upon glucose stimulation	(Cheng et al., 2012; Chmielowiec and Borowiak, 2014; D'Amour et al., 2006; Jiang et al., 2007; Pagliuca et al., 2014; Rezania et al., 2014)
Lung cells	**Proximal/Airway progenitors:** NKX2.1, FOXA2, SOX2, P63 **Proximal Airway cells:** SCGB1A1, SCGB3A2, FOXJ1, P63, KRT5, MUC5AC **Distal/Alveolar progenitors:** NKX2.1, FOXA2, SOX9, FOXP2, ETV5, SFTPC **Distal/Alveolar cells:** SFTPC, PDPN, AQP5, HOPX	• Apical localization and functionality of CFTR • Surfactant protein B (SP-B) uptake and release • Presence of lamellar bodies • Surfactant protein C (SP-C) secretion • Ciliogenesis and motile cilia	(Dye et al., 2015; Firth et al., 2014; Ghaedi et al., 2013; Gotoh et al., 2014; Huang et al., 2013; Jacob et al., 2017; Longmire et al., 2012; McCauley et al., 2017; McIntyre et al., 2014; Mou et al., 2012; Wong et al., 2012)
Gut	**Intestinal stem cells:** LGR5, ASCL2 **Differentiated intestinal cell types:** VIL, MUC2, LYZ, CHGA **Stomach progenitors:** SOX2, PDX1, LGR5, SOX9, GATA4 **Differentiated stomach cell types:** MUC5AC, MUC6, TFF1/3, GKN1, TFF2	• Presence of apical microvilli • Functional peptide transport of enterocytes • Mucin secretion by goblet cells • Pathophysiological response to *H. Pylori* infection	(Finkbeiner et al., 2015; Fordham et al., 2013; Munera et al., 2017; Spence et al., 2011; Watson et al., 2014; Workman et al., 2016) (McCracken et al., 2014)