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REVIEW

Generating cells of the gastrointestinal system: current approaches and applications for the differentiation of human pluripotent stem cells

Allen Wang · Maike Sander

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Abstract Human pluripotent stem cells (hPSCs), including human embryonic stem cells and induced pluripotent stem cells, are defined by their abilities to self-renew and to differentiate into any cell type of the human body. Due to these unique properties, hPSCs represent a potentially unlimited source of cells/tissues for cell replacement therapies. Use of these cells may also revolutionize the way drugs are discovered, designed, and tested. Furthermore, the study of how cells differentiate can also change our understanding of complex human biology and disease. For these reasons, scientists have dedicated significant time and effort to generate specific cell types from hPSCs with therapeutic potential, including cells derived from the definitive endoderm germ layer such as liver cells (hepatocytes) and pancreatic β cells. In this review, we will focus broadly on the most advanced differentiation strategies currently employed to differentiate hPSCs to endodermal lineages such as the liver, pancreas, and intestine as well as the principles of developmental biology around which these protocols were designed. This will be followed by a brief discussion of the vast potential of these systems as suitable in vitro models for human embryonic development and disease.

Keywords Human embryonic stem cell · Human induced pluripotent stem cell · Definitive endoderm · Liver · Pancreas · Intestine

A. Wang · M. Sander (✉)
Department of Pediatrics and Cellular & Molecular Medicine,
University of California San Diego,
La Jolla, CA 92093-0695, USA
e-mail: masander@ucsd.edu

Introduction

Within the last two decades, research involving human pluripotent stem cells (hPSCs), which encompass both human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), has progressed at an extraordinarily rapid pace. Since the first demonstration of culturing blastocyst-derived hESCs in 1998 [1], the field has seen many influential milestones such as the development of protocols to differentiate these cells towards different therapeutically relevant cell lineages [2], the ability to induce pluripotency in human somatic cells via delivery of reprogramming transcription factors [3], and the first clinical trials involving stem cell-based therapies. Driving this progress is the underlying belief that the study and use of hPSCs hold immense potential for regenerative medicine-based therapies as well as for facile human cell models for drug testing and discovery.

This review will discuss the recent progress of efforts to study and generate cell types of the definitive endoderm germ layer using hPSCs. The definitive endoderm is one of the three primary germ layers during early embryonic development and gives rise to the diverse lineages of the respiratory and gastrointestinal systems including the thymus, lung, liver, pancreas, as well as the small and large intestine. Many chronic human diseases result from the impairment or dysfunction of definitive endoderm-derived organs, several of which have been shown to be treatable by organ transplantation or cell replacement. Specific examples include cystic fibrosis, type 1 diabetes, and liver diseases, such as chronic hepatitis and alpha1 antitrypsin deficiency [4–7]. Transplant therapies, however, are limited due to the lack of donor organs and tissues making the in vitro generation of these cell types and organs from hPSCs highly desired, as they represent a possibly unlimited source of transplantable cells. Thus, numerous approaches have been

taken to derive these clinically relevant endodermal cell types from hPSCs. The most successful of these methods are inspired by decades of developmental discoveries using model organisms such as mouse, frog, and zebrafish. These approaches involve the "guiding" or "driving" of undifferentiated cells through appropriate developmental stages, mimicking those observed during fetal development. The first critical step in the generation of endoderm-derived cells and organs from hPSCs is the efficient generation of the definitive endoderm germ layer itself.

Generation of definitive endoderm

During gastrulation, the vertebrate embryo organizes itself into the three primary germ layers, specifically definitive endoderm, mesoderm, and ectoderm. During this process, the mesoderm and endoderm germ layers are specified though a common mesendoderm progenitor cell population [8–11]. Furthermore, studies from multiple animal models have demonstrated that Nodal signaling through the transforming growth factor beta (TGF-β) pathway is required for mesoderm and endoderm specification at this time [12–16]. Specifically, high levels of Nodal specify the endoderm, whereas lower levels promote mesoderm [17–20]. Hence, manipulation of the TGF-β pathway has demonstrated a high rate of success in efficiently differentiating hPSCs into definitive endoderm in vitro [21]. It has now become a standard procedure to promote the differentiation of hPSCs to definitive endoderm by treatment with high concentrations of Activin A, a potent ligand of TGF-β that mimics the effects of endogenous Nodal (Fig. 1). This treatment results in cells that express the definitive endoderm markers FOXA2, SOX17, and CXCR4, but not genes characteristic of extraembryonic endoderm such as SOX7. Furthermore, this in vitro treatment promotes definitive endoderm through an apparent mesendoderm precursor population as indicated by the transient expression of genes such as BRACHYURY (T) and MIXL1 consistent with what is thought to occur during gastrulation in vivo [21].

A variety of other factors have been applied in conjunction with Activin A to increase the efficiency of FOXA2, SOX17, CXCR4 triple positive definitive endoderm formation in vitro (Fig. 1.). Because active phosphatidylinositol 3-kinase (PI3K) signaling is thought to antagonize hPSC endodermal differentiation, PI3K inhibitors, such as LY294002 and Wortmannin, have been added to protocols to increase efficiency of endoderm formation [22, 23]. Modulation of the Wnt pathway is also common in many differentiation protocols accomplished by the addition of recombinant Wnt3a to cultures or the inhibition of the kinase GSK-3-β (an antagonist of Wnt signaling) [24–27]. Consistent with its observed role during vertebrate gastrulation [15], activation of the Wnt pathway is thought to amplify and further potentiate

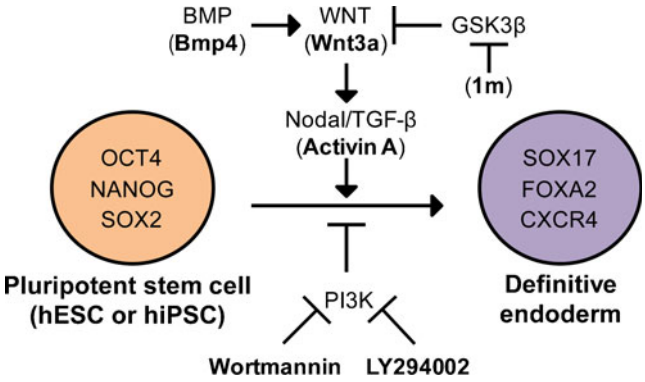

Fig. 1 Manipulations of signaling pathways during in vitro differentiation of human pluripotent stem cells to definitive endoderm. Activin A mimics endogenous Nodal signal to promote definitive endoderm formation via stimulation of the TGF-β signaling pathway. Modulation of the WNT signaling pathway through treatment with recombinant WNT3a and the GSK3β inhibitor 1 m have also been demonstrated to promote definitive endoderm formation and are thought to function upstream of Nodal. BMP4 has been suggested to modulate WNT signaling in this context. PI3K signaling inhibits definitive endoderm formation in vitro. Thus, inhibitors of this pathway, such as Wortmanin and LY294002, are frequently added in definitive endoderm differentiation protocols. *hESC* human embryonic stem cell, *hiPSC* human induced pluripotent stem cell, *TGF-β* transforming growth factor beta, *BMP* bone morphogenic protein

Nodal/TGF-β signaling during the transition of hPSCs to definitive endoderm.

In addition to optimizing differentiation efficiency, investigators have dedicated significant efforts to enrich for or isolate as well as expand the hPSC-derived definitive endoderm population. The chemokine receptor CXCR4 is commonly used to enrich for definitive endoderm using fluorescence-activated cell sorting (FACS) [21]. Recently, FACS using transcription factors has been reported with high success in hESC-derived definitive endoderm using a combination of the SOX17 and GATA4 transcription factors along with CXCR4 [28]. Furthermore, gene expression profiling of an enriched definitive endoderm population using a SOX17-GFP reporter hESC line identified CD49e, CD141, and CD238 as potential surface markers for use in endodermal purification/isolation strategies [29]. The establishment of the aforementioned protocols and technologies allowed researchers to further develop in vitro differentiation strategies to generate endoderm-derived cell types. Three of the more successful approaches that have been reported are for hepatocytes, pancreatic cells, and intestinal tissue.

Derivation of hepatocyte-like cells from hPSCs

Diseases that result in liver dysfunction are emerging as a critical issue for human health illustrated by the fact that over 40,000 deaths in the USA annually can be attributed to liver failure [30]. The most successful treatment option for

end-stage liver diseases, such as hepatitis B and C, alpha1 antitrypsin deficiency, and hepatocellular carcinoma, is orthotopic liver transplantation [31–34]. hPSCs represent a potentially unlimited source of transplantable hepatic tissue. Furthermore, since the liver plays a key role in xenobiotic metabolism, hPSC-derived hepatocytes could also be used for in vitro drug testing and toxicity screens [35].

There has been multiple reports of the efficient in vitro derivation of cells with characteristics of hepatocytes, commonly referred to as hepatocyte-like cells, from hESC and hiPSC-derived definitive endoderm. The majority of these approaches involve two stages: hepatic specification followed by maturation (Fig. 2). Multiple groups have reported protocols to accomplish in vitro hepatic specification which includes treatment of hPSC-derived definitive endoderm with chemicals thought to modify acetylation of histones such as DMSO [26, 36] and the addition of signaling factors implicated in anteroposterior patterning of the gut endoderm, including retinoic acid (RA) [37]. The most common approach is to activate the fibroblast growth factor (FGF) and bone morphogenic protein (BMP) signaling pathways. This strategy is directly derived from examination of hepatic specification in the vertebrate embryo. During in vivo embryonic development, FGF signals from cardiac mesoderm and BMP signals from the nearby septum transversum induce the hepatic lineage from the ventral foregut endoderm while suppressing the pancreatic lineage, partly through induction of sonic hedgehog (SHH) [38–40]. Elegant work utilizing mouse foregut endoderm ex vivo cultures demonstrated this process, showing that treatment of mouse foregut explants with exogenous FGF2 or FGF4 and BMP2 or BMP4 induced expression of the liver marker albumin, suggesting hepatic specification [41–45]. Inspired by these experiments, treatment of hPSC-derived definitive endoderm with intermediate concentrations of FGF2 or FGF4 in combination with high concentrations of BMP2 or BMP4 induced markers characteristic of the hepatic lineage, such as alpha-fetoprotein (AFP), alpha1 antitrypsin (A1AT), and hepatocyte nuclear factor 4-α [41–45].

To generate mature cell types of the liver, these hPSC-derived "hepatic progenitors" are subsequently subjected to an in vitro maturation step. For this stage, cells are generally grown in media previously optimized for the culture of primary hepatocytes with the addition of factors thought to promote the maturation and/or proliferation of hepatocytes during liver development. These factors include oncostatin M, dexamethasone, hepatocyte growth factor (HGF), FGF4, and epidermal growth factor (EGF) [26, 36, 37, 41–45]. Oxygen tension has also been reported to promote hepatocyte maturation [44]. During this maturation step, cells begin to acquire the polygonal morphology associated with primary hepatocytes and express hepatic markers including ALB, TAT, and CYP7A1. Furthermore, they gain hepatocyte-specific activities, such as albumin production, glycogen storage, p450 metabolic activity, and uptake and excretion of indocyanine green [41–45]. These hepatocyte-like cells have also been reported to populate the mouse liver while maintaining their hepatic characteristics [36, 43, 44, 46, 47].

In vitro generation of pancreatic endocrine cells

Diabetes mellitus is a metabolic disorder that affects over 150 million people worldwide and is characterized by absolute insulin deficiency due to autoimmune destruction of pancreatic insulin-producing β cells (type 1) or relative insulin deficiency due to defective insulin secretion or decreased insulin sensitivity (type 2). Given that type 1 diabetes results from the loss of a single cell type, the generation of a renewable source of transplantable insulin-producing β cells from hPSCs remains a major hope as a cure for this disease. For this reason, there have been numerous attempts to develop in vitro protocols to derive functional β cells from hPSCs with varying degrees of success [23, 24, 27, 48–54]. The most significant breakthrough in the development of such a protocol came from a report by D'Amour et al. in 2006 [24]. This distinct approach resulted in the generation of pancreatic insulin-producing cells and was largely influenced by principles gleaned from developmental biology. This now widely used protocol has since been revised by the same group [27] and inspired the development of additional protocols from fellow scientists in the field [51, 54]. Despite some differences, these approaches all share the general strategy published by D'Amour et al.: a stepwise in vitro differentiation protocol that mimics the stages of in vivo pancreatic development by introducing appropriate developmental cues at critical stages during differentiation (Fig. 2). During this protocol, hPSCs are guided through multiple "developmental" stages, namely definitive endoderm, primitive gut tube, posterior foregut, pancreatic progenitor, and finally pancreatic endocrine cells.

To promote the initial stage transition from definitive endoderm to primitive gut tube, hPSC-derived definitive endoderm is often cultured with FGF7 or FGF10 resulting in cells expressing *FOXA2*, *HNF4A*, and *HNF1B*. This follows developmental findings, as FGF7/10 signaling through FGFR2IIIb has been shown to be critical for formation of multiple endodermal lineages during vertebrate development [55–57]. Low levels of Wnt3a have also been added to certain protocols at this stage in order to pattern the gut tube to a more foregut-like identity [54].

Differentiation to the next stage, primitive gut tube to pancreatic foregut endoderm, is often accomplished by treatment with the pancreatic lineage-specifying factors RA, Noggin (an inhibitor of BMP signaling), and Cyclopamine

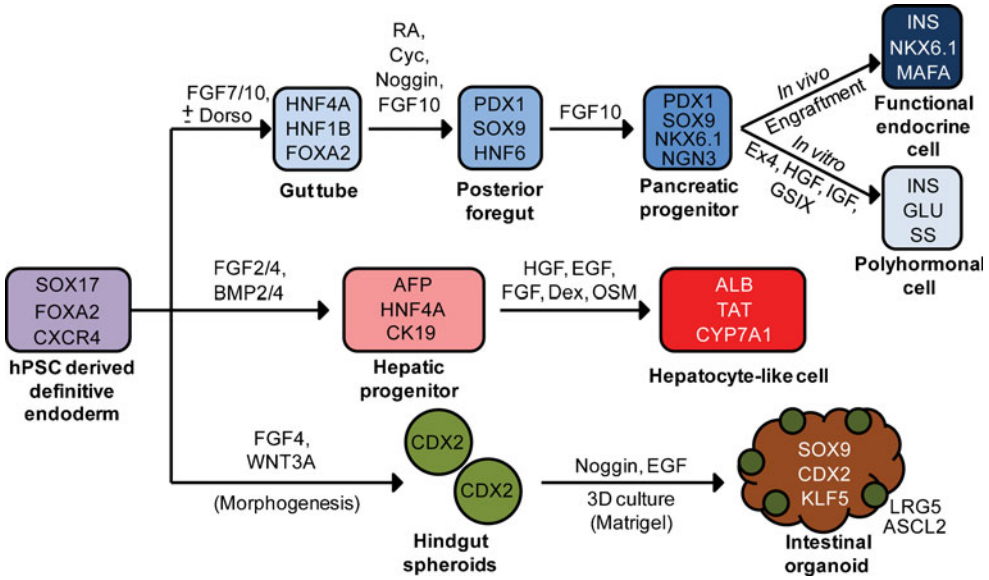

Fig. 2 Summary of approaches to derive hepatic, pancreatic, and intestinal cells from human pluripotent stem cells (hPSCs). hPSC-derived definitive endoderm can be differentiated towards the pancreatic lineage by treatment with pancreas specifying factors, such as FGF10, retinoic acid (RA), Noggin, and cyclopamine (Cyc). When implanted into mice, these hPSC-derived pancreatic cells can differentiate into glucose-responsive insulin-secreting cells. However, when cultured in vitro, they differentiate into nonfunctional polyhormonal endocrine cells. The hepatic lineage can be specified by treatment with FGF2 or 4 and BMP2 or 4, resulting in cells with characteristics of hepatic progenitors. Subsequent culture in the presence of a variety of growth factors results in the generation of cells with some hepatocyte-like characteristics. Treatment of hPSC-derived definitive endoderm with FGF4 and WNT3a generates hindgut and subsequent commitment to the intestinal lineage. Growth of these cells in 3D culturing conditions results in the formation of intestinal organoids. *hPSC* human pluripotent stem cell, *FGF* fibroblast growth factor, *Dorso* dorsomorphin, *BMP* bone morphogenic protein, *RA* retinoic acid, *Cyc* cyclopamine, *HGF* hepatocyte growth factor, *EGF* epidermal growth factor, *Dex* dexamethasone, *OSM* oncostatin M, *Ex4* exendin-4, *IGF* insulin growth factor, *GSIX* γ-secretase inhibitor, *AFP* alpha-fetoprotein, *ALB* albumin, *TAT* tyrosine amino transferase, *CYP7A1* cytochrome superfamily p450 member 7a1, *INS* insulin, *GLU* glucagon, *SS* somatostatin

(a potent inhibitor of SHH) resulting in cells expressing pancreatic genes such as PDX1, PROX1, HNF6, and SOX9. Development of this pancreatic lineage-inducing step is derived from studies of pancreas development in multiple model organisms. RA is added to the differentiating cultures to promote commitment to the pancreatic lineage, following observations that RA is required for proper pancreas specification in mice, frogs, and zebrafish [58–61]. Furthermore, it has also been suggested that RA biases pancreatic progenitors towards the endocrine lineage, which is the first step towards generating the therapeutically desired β cell [62–64].

In pancreas differentiation protocols, RA treatment is combined with the addition of Noggin and Cyclopamine during posterior foregut patterning to suppress alternative foregut fates, such as the hepatic lineage. As mentioned, ventral pancreas and liver emerge from a common cell population during embryonic development. Liver is specified by BMP and FGF signaling [40], while the pancreas emerges from a SHH negative domain. Thus, in contrast to hepatic specification, induction of the pancreatic lineage requires suppression of BMP and SHH signaling by Noggin and Cyclopamine, respectively. The importance of this hepatic lineage suppression step for the development of protocols aimed at generating pancreatic β cells was demonstrated by multiple groups using different hESC lines [51, 54]. These studies showed variant propensities of different hESC lines in favoring hepatic over pancreatic lineage induction and that efficient pancreatic differentiation of some hESC lines requires the addition of Noggin or other inhibitors of BMP signaling, such as dorsomorphin, even earlier during the transition of definitive endoderm to primitive gut tube [51, 54]. The exact mechanisms of how these two closely related lineages emerge from a common multipotent embryonic progenitor during human development remain a fascinating biological question that is particularly relevant to the refinement of these pancreatic differentiation protocols.

Even less is known about how to efficiently induce further development of posterior foregut endoderm towards the pancreatic endocrine lineage. In some protocols, this transition is carried out without adding specific signaling factors to the differentiating cultures [27]. However, other studies have shown that the addition of FGF10 has beneficial effects [54], mimicking in vivo development during which mesenchymal FGF10 signaling stimulates proliferation of pancreatic progenitors [56]. Also still unresolved is how to efficiently instruct multipotent pancreatic progenitors in vitro

to adopt endocrine cell characteristics while suppressing alternative exocrine pancreatic fates. Though the signals mediating this fate decision are still unknown, studies in developing mouse embryos have shown that endocrine specification requires induction of the transcription factors Nkx6.1 and Ngn3 and concomitant repression of Ptf1a [65, 66]. Thus, NKX6.1 and NGN3 serve as markers to identify correctly specified endocrine precursors during in vitro differentiation.

The final step of pancreas differentiation protocols is the transition of pancreatic progenitors into mature pancreatic endocrine cells, including glucose-responsive insulin-secreting β cells. This final step, however, remains to be achieved in vitro. Current protocols have only resulted in the generation of non-glucose-responsive cells that simultaneously express multiple hormones and lack expression of key β cell genes, such as NKX6.1 and MAFA [24]. Given the relevance of this step for therapeutic applications, many attempts are underway to generate proper endocrine cells in vitro. Inspired by studies of murine endocrine differentiation [67], γ-secretase inhibitors (GSIX) have been added to the cultures to promote endocrine cell formation by suppression of Notch signaling activity [24]. Recently, beneficial effects have been reported by adding the inhibitors of TGF-β signaling Noggin and SB431542 [54]. In addition, investigators have tried to promote β cell formation by treatment with a wide variety of factors thought to be involved in β cell maturation, such as exendin-4 (Ex4), HGF, Nicotinamine, and IGF1 [23, 24, 27, 48–54]. While these treatments may influence the efficiency of differentiation towards immature multihormonal cells, they do not result in the generation of glucose-responsive β cells.

The only reported method to successfully promote proper differentiation and maturation of in vitro-derived pancreatic progenitors into glucose-responsive insulin-producing cells involves in vivo engraftment of these cells into mice [27]. Several weeks posttransplantation, implanted progenitors begin to display insulin secretory properties and glucose responsiveness and can restore glucose homeostasis in diabetic mouse models similar to engrafted adult human islets. In contrast to the endocrine cells generated in vitro, these cells express the β cell markers PDX1, NKX6.1, and MAFA. Little is known, however, about the mechanisms that underlie proper differentiation and maturation of these cells in vivo. Further studies are warranted to better understand how pancreatic endocrine cells differentiate and mature in vivo and how to mimic this process in vitro. This is an important goal as the generation of glucose-responsive β cells in vitro promises therapeutic applications beyond cell replacement therapies. These cells would also be extremely useful for high throughput screens to identify drugs that stimulate insulin secretion or promote β cell expansion for novel treatments of both type 1 and type 2 diabetes.

Intestinal tissue from hPSCs

Human diseases associated with intestinal dysfunction or diseases requiring surgical removal of the small bowel, such as short bowel syndrome, are initially treated by total parenteral nutrition (TPN) therapy (intravenous feeding). However, if these patients fail to respond to TPN, they become candidates for intestinal transplantation [68–70]. The paucity of donor organs remains a critical issue that could be alleviated by the in vitro generation of intestinal tissue from hPSCs.

Recently, principles of developmental biology have been instructive in the development of a novel protocol for the generation of intestinal tissue from hPSCs [71] (Fig. 2). This study applied observations in mouse and chick that FGF4 and Wnt/β-catenin signaling actively promotes hindgut patterning and subsequent formation of the intestinal lineage. Accordingly, culture of hPSC-derived definitive endoderm in high concentrations of FGF4 and WNT3A preferentially induced the hindgut/intestinal marker CDX2, while at the same time repressing the markers for the pancreatic and hepatic lineages PDX1 and ALB, respectively. Remarkably, these cultures began to undergo morphogenetic changes similar to those observed during hindgut formation in mouse embryos, resulting in the formation of CDX2-expressing "spheroids." These spheroids were then placed in three-dimensional culture conditions permissive to intestinal development, where they formed intestinal "organoids" that expressed the intestinal markers CDX2, SOX9, and KLF5 and displayed columnar organization with villus-like structures similar to embryonic intestine. After prolonged culture, crypt-like structures arose expressing genes characteristic of adult intestinal stem cells, such as *LGR5* and *ASCL2*, which subsequently generated intestinal tissue containing crypts, villi, and all differentiated cell types of the intestinal epithelium. To date, this protocol comes closest to completely recapitulating the development of an organ by directed differentiation of hPSCs in vitro.

Modeling human endodermal development and disease

In addition to the frequently discussed therapeutic applications for cell/organ replacement, hPSC differentiation platforms have emerged as exceptionally powerful for modeling human embryonic development and disease in vitro. These platforms allow for detailed investigation of human biology previously not feasible due to the lack of appropriate cells or tissues that could be cultured and studied in vitro. Importantly, there exists a genetic component for multiple diseases associated with endoderm-derived organs, such as alpha1 antitrypsin deficiency, inflammatory bowel disease, and aforementioned type 1 and type 2 diabetes [72–76].

"Disease in a dish" models using patient-derived hPSCs could provide novel insight into disease mechanisms as well as drive the development of novel treatments.

The application of efficient endodermal lineage differentiation protocols, such as the ones described in this review, already provides remarkable mechanistic insight into human development. A recent study by Teo et al. [77] employed protocols to differentiate hPSCs into definitive endoderm and combined this approach with in vivo mouse experiments to dissect the transcription factors that regulate endoderm specification. It was shown that NANOG acts upstream of EOMES, which subsequently associates with SMAD2 and 3 to initiate definitive endoderm formation. Other examples of the use of endodermal differentiation protocols to understand human biology include the recent report showing that *HNF4A* is required for specification of the hepatic lineage in humans [78] and the demonstration that *NGN3* is necessary for enteroendocrine formation during human intestinal development [71]. The latter finding is of particular significance because the function of *NGN3* might not be entirely conserved between mice and humans. While *Ngn3* deletion in mice causes both pancreatic endocrine and intestinal dysfunction [65, 79], *NGN3* mutations in humans are associated with congenital malabsorptive diarrhea, but rarely diabetes [80–82].

hPSC differentiation systems are also emerging as platforms to study the role of chromatin modifications in human development. For example, a recent report demonstrated that the chromatin of critical definitive endoderm gene loci is poised for future activation by depositing both the repressive H3K27me3 and the activating H3K4me3 modification in undifferentiated hESCs [83]. Upon definitive endoderm formation, the repressive H3K27me3 mark is removed, resulting in activation of endodermal genes. Future studies of this kind will prove informative for dissecting the role of epigenetic modifications for the formation of endodermal organs and tissues as well as the diseases associated with these organs. Genetic variants associated with both type 1 and type 2 diabetes identified by genomewide association studies often occur in noncoding parts of the human genome [73–75]. Thus, epigenetic profiling could provide insight into the functional significance of many of these variants.

Patient-derived hiPSC technology has recently allowed scientists to generate in vitro models for studying a "disease in a dish." To gain a better understanding of diseases associated with endoderm-derived tissues, a recent landmark study isolated hiPSCs from patients diagnosed with A1AT deficiency [84]. This study first demonstrated that these hiPSCs were deficient in the synthesis and secretion of A1AT when differentiated towards hepatocytes, and subsequently employed Zinc Finger Nucleases as a genome editing technique to correct the mutation causing the disease. The corrected cells properly synthesized A1AT when differentiated into hepatocyte-like cells, and populated the liver in mice while maintaining their hepatocyte-like characteristics. This study demonstrates how disease mechanisms can be dissected in vitro and provides proof-of-principle that genetically corrected patient-derived hiPSCs maintain functional properties in vivo.

Perspectives and conclusions

The approaches for differentiating hPSCs towards the hepatic, pancreatic, and intestinal lineage, as discussed in this review, reveal both the remarkable progress that has been made as well as the many challenges that persist. One conclusion to be drawn from current studies is that properly differentiated mature human cells can only be derived by closely mimicking normal development in the culture dish. Indeed, all methods discussed in this review involve the addition of soluble agonists or antagonists for signaling pathways known to control corresponding developmental steps in vivo. A key difference in the approaches for generating hepatic and pancreatic cells compared to intestinal cells is that hepatic and pancreatic protocols aim to differentiate a specific cell type in two-dimensional cultures, while intestinal protocols generate an entire intestinal crypt comprising multiple cell types by differentiating cells in 3-D space. Because in vivo development of the pancreas, liver, and other endodermal organs does occur within a 3-D epithelium, 3-D culture conditions could help generate functional hepatocytes and β cells in vitro. Additionally, the in vitro culturing conditions described in this review also lack other cell types or tissues present in developing organs in vivo, such as mesenchymal and endothelial cells. Given their importance during hepatic and pancreatic development [85–88], co-culture with endothelial and mesenchymal cells should be considered for maturing cells in vitro.

Methods for endodermal differentiation from hPCSs are rapidly evolving with frequent publications reporting novel methodologies. For example, although not detailed in this review, the generation of lung and airway progenitors from hiPSCs derived from patients with cystic fibrosis has recently been reported [89]. The rapid progress has fueled great enthusiasm for this emerging field with the hope of utilizing in vitro-derived human cell types for cell therapies as well as disease modeling and drug testing. Although at present, evidence for clinical efficacy of any of the in vitro-derived cell types is still missing, the immense therapeutic potential of this research warrants even more intense efforts in years to come.

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Conflict of interest There are no conflicts of interest to disclose.

References

1. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147
2. Murry CE, Keller G (2008) Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. Cell 132:661–680
3. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872
4. Rosenblatt RL (2009) Lung transplantation in cystic fibrosis. Respir Care 54:777–786, discussion 786-777
5. Shapiro AMJ, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP, Secchi A, Brendel MD, Berney T, Brennan DC et al (2006) International trial of the Edmonton protocol for islet transplantation. N Engl J Med 355:1318–1330
6. Gane EJ, Portmann BC, Naoumov NV, Smith HM, Underhill JA, Donaldson PT, Maertens G, Williams R (1996) Long-term outcome of hepatitis C infection after liver transplantation. N Engl J Med 334:815–821
7. Samuel D, Muller R, Alexander G, Fassati L, Ducot B, Benhamou J-P, Bismuth H (1993) Liver transplantation in European patients with the hepatitis B surface antigen. N Engl J Med 329:1842–1847
8. Lawson KA, Pedersen RA (1987) Cell fate, morphogenetic movement and population kinetics of embryonic endoderm at the time of germ layer formation in the mouse. Development 101:627–652
9. Lawson KA, Meneses JJ, Pedersen RA (1991) Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. Development 113:891–911
10. Rodaway A, Takeda H, Koshida S, Broadbent J, Price B, Smith JC, Patient R, Holder N (1999) Induction of the mesendoderm in the zebrafish germ ring by yolk cell-derived TGF-beta family signals and discrimination of mesoderm and endoderm by FGF. Development 126:3067–3078
11. Zorn AM, Wells JM (2009) Vertebrate endoderm development and organ formation. Annu Rev Cell Dev Biol 25:221–251
12. Green JB, Smith JC (1990) Graded changes in dose of a Xenopus activin A homologue elicit stepwise transitions in embryonic cell fate. Nature 347:391–394
13. Clements D, Friday RV, Woodland HR (1999) Mode of action of VegT in mesoderm and endoderm formation. Development 126:4903–4911
14. Aoki TO, David NB, Minchiotti G, Saint-Etienne L, Dickmeis T, Persico GM, Strahle U, Mourrain P, Rosa FM (2002) Molecular integration of casanova in the Nodal signalling pathway controlling endoderm formation. Development 129:275–286
15. Ben-Haim N, Lu C, Guzman-Ayala M, Pescatore L, Mesnard D, Bischofberger M, Naef F, Robertson EJ, Constam DB (2006) The Nodal precursor acting via activin receptors induces mesoderm by maintaining a source of its convertases and BMP4. Dev Cell 11:313–323
16. Hagos EG, Dougan ST (2007) Time-dependent patterning of the mesoderm and endoderm by Nodal signals in zebrafish. BMC Dev Biol 7:22
17. Tremblay KD, Hoodless PA, Bikoff EK, Robertson EJ (2000) Formation of the definitive endoderm in mouse is a Smad2-dependent process. Development 127:3079–3090
18. Lowe LA, Yamada S, Kuehn MR (2001) Genetic dissection of nodal function in patterning the mouse embryo. Development 128:1831–1843
19. Vincent SD, Dunn NR, Hayashi S, Norris DP, Robertson EJ (2003) Cell fate decisions within the mouse organizer are governed by graded Nodal signals. Genes Dev 17:1646–1662
20. Tada S, Era T, Furusawa C, Sakurai H, Nishikawa S, Kinoshita M, Nakao K, Chiba T (2005) Characterization of mesendoderm: a diverging point of the definitive endoderm and mesoderm in embryonic stem cell differentiation culture. Development 132:4363–4374
21. D'Amour KA, Agulnick AD, Eliazer S, Kelly OG, Kroon E, Baetge EE (2005) Efficient differentiation of human embryonic stem cells to definitive endoderm. Nat Biotechnol 23:1534–1541
22. McLean AB, D'Amour KA, Jones KL, Krishnamoorthy M, Kulik MJ, Reynolds DM, Sheppard AM, Liu H, Xu Y, Baetge EE et al (2007) Activin a efficiently specifies definitive endoderm from human embryonic stem cells only when phosphatidylinositol 3-kinase signaling is suppressed. Stem Cells 25:29–38
23. Zhang D, Jiang W, Liu M, Sui X, Yin X, Chen S, Shi Y, Deng H (2009) Highly efficient differentiation of human ES cells and iPS cells into mature pancreatic insulin-producing cells. Cell Res 19:429–438
24. D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, Moorman MA, Kroon E, Carpenter MK, Baetge EE (2006) Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol 24:1392–1401
25. Bone HK, Nelson AS, Goldring CE, Tosh D, Welham MJ (2011) A novel chemically directed route for the generation of definitive endoderm from human embryonic stem cells based on inhibition of GSK-3. J Cell Sci 124:1992–2000
26. Hay DC, Fletcher J, Payne C, Terrace JD, Gallagher RCJ, Snoeys J, Black JR, Wojtacha D, Samuel K, Hannoun Z et al (2008) Highly efficient differentiation of hESCs to functional hepatic endoderm requires Activin A and Wnt3a signaling. Proc Natl Acad Sci 105:12301–12306
27. Kroon E, Martinson LA, Kadoya K, Bang AG, Kelly OG, Eliazer S, Young H, Richardson M, Smart NG, Cunningham J et al (2008) Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. Nat Biotechnol 26:443–452
28. Pan Y, Ouyang Z, Wong WH, Baker JC (2011) A new FACS approach isolates hESC derived endoderm using transcription factors. PLoS One 6:e17536
29. Wang P, Rodriguez RT, Wang J, Ghodasara A, Kim SK (2011) Targeting SOX17 in human embryonic stem cells creates unique strategies for isolating and analyzing developing endoderm. Cell Stem Cell 8:335–346
30. Cao S, Esquivel M, Carlos O, Keeffe M, Emmet B (1998) New approaches to supporting the failing liver. Annu Rev Med 49:85–94
31. Fox IJ, Roy-Chowdhury J (2004) Hepatocyte transplantation. J Hepatol 40:878–886
32. Puppi J, Dhawan A (2009) Human hepatocyte transplantation overview. Methods Mol Biol 481:1–16
33. Penn I (1991) Hepatic transplantation for primary and metastatic cancers of the liver. Surgery 110:726–734, discussion 734-725
34. Mazzaferro V, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, Montalto F, Ammatuna M, Morabito A, Gennari L (1996) Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. N Engl J Med 334:693–699
35. Stummann TC, Bremer S (2008) The possible impact of human embryonic stem cells on safety pharmacological and toxicological assessments in drug discovery and drug development. Curr Stem Cell Res Ther 3:118–131
36. Hay DC, Zhao D, Ross A, Mandalam R, Lebkowski J, Cui W (2007) Direct differentiation of human embryonic stem cells to

hepatocyte-like cells exhibiting functional activities. Cloning Stem Cells 9:51–62
37. Touboul T, Hannan NR, Corbineau S, Martinez A, Martinet C, Branchereau S, Mainot S, Strick-Marchand H, Pedersen R, Di Santo J et al (2010) Generation of functional hepatocytes from human embryonic stem cells under chemically defined conditions that recapitulate liver development. Hepatology 51:1754–1765
38. Jung J, Zheng M, Goldfarb M, Zaret KS (1999) Initiation of mammalian liver development from endoderm by fibroblast growth factors. Science 284:1998–2003
39. Rossi JM, Dunn NR, Hogan BL, Zaret KS (2001) Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. Genes Dev 15:1998–2009
40. Deutsch G, Jung J, Zheng M, Lora J, Zaret KS (2001) A bipotential precursor population for pancreas and liver within the embryonic endoderm. Development 128:871–881
41. Zhao D, Chen S, Cai J, Guo Y, Song Z, Che J, Liu C, Wu C, Ding M, Deng H (2009) Derivation and characterization of hepatic progenitor cells from human embryonic stem cells. PLoS One 4: e6468
42. Song Z, Cai J, Liu Y, Zhao D, Yong J, Duo S, Song X, Guo Y, Zhao Y, Qin H et al (2009) Efficient generation of hepatocyte-like cells from human induced pluripotent stem cells. Cell Res 19:1233–1242
43. Cai J, Zhao Y, Liu Y, Ye F, Song Z, Qin H, Meng S, Chen Y, Zhou R, Song X et al (2007) Directed differentiation of human embryonic stem cells into functional hepatic cells. Hepatology 45:1229–1239
44. Si-Tayeb K, Noto FK, Nagaoka M, Li J, Battle MA, Duris C, North PE, Dalton S, Duncan SA (2010) Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. Hepatology 51:297–305
45. Brolen G, Sivertsson L, Bjorquist P, Eriksson G, Ek M, Semb H, Johansson I, Andersson TB, Ingelman-Sundberg M, Heins N (2010) Hepatocyte-like cells derived from human embryonic stem cells specifically via definitive endoderm and a progenitor stage. J Biotechnol 145:284–294
46. Duan Y, Catana A, Meng Y, Yamamoto N, He S, Gupta S, Gambhir SS, Zern MA (2007) Differentiation and enrichment of hepatocyte-like cells from human embryonic stem cells in vitro and in vivo. Stem Cells 25:3058–3068
47. Asgari S, Moslem M, Bagheri-Lankarani K, Pournasr B, Miryounesi M, Baharvand H (2011) Differentiation and transplantation of human induced pluripotent stem cell-derived hepatocyte-like cells. Stem Cell Rev Rep: 1–12. doi:10.1007/s12015-011-9330-y
48. Xu X, Browning VL, Odorico JS (2011) Activin, BMP and FGF pathways cooperate to promote endoderm and pancreatic lineage cell differentiation from human embryonic stem cells. Mech Dev 128:412–427
49. Jiang J, Au M, Lu K, Eshpeter A, Korbutt G, Fisk G, Majumdar AS (2007) Generation of insulin-producing islet-like clusters from human embryonic stem cells. Stem Cells 25:1940–1953
50. Johannesson M, Stahlberg A, Ameri J, Sand FW, Norrman K, Semb H (2009) FGF4 and retinoic acid direct differentiation of hESCs into PDX1-expressing foregut endoderm in a time- and concentration-dependent manner. PLoS One 4:e4794
51. Mfopou JK, Chen B, Mateizel I, Sermon K, Bouwens L (2010) Noggin, retinoids, and fibroblast growth factor regulate hepatic or pancreatic fate of human embryonic stem cells. Gastroenterology 138:2233–2245
52. Cai J, Yu C, Liu Y, Chen S, Guo Y, Yong J, Lu W, Ding M, Deng H (2010) Generation of homogeneous PDX1(+) pancreatic progenitors from human ES cell-derived endoderm cells. J Mol Cell Biol 2:50–60
53. Jiang W, Shi Y, Zhao D, Chen S, Yong J, Zhang J, Qing T, Sun X, Zhang P, Ding M et al (2007) In vitro derivation of functional insulin-producing cells from human embryonic stem cells. Cell Res 17:333–344
54. Nostro MC, Sarangi F, Ogawa S, Holtzinger A, Corneo B, Li X, Micallef SJ, Park I-H, Basford C, Wheeler MB et al (2011) Stage-specific signaling through TGFβ family members and WNT regulates patterning and pancreatic specification of human pluripotent stem cells. Development 138:861–871
55. De Moerlooze L, Spencer-Dene B, Revest JM, Hajihosseini M, Rosewell I, Dickson C (2000) An important role for the IIIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis. Development 127:483–492
56. Bhushan A, Itoh N, Kato S, Thiery JP, Czernichow P, Bellusci S, Scharfmann R (2001) Fgf10 is essential for maintaining the proliferative capacity of epithelial progenitor cells during early pancreatic organogenesis. Development 128:5109–5117
57. Elghazi L, Cras-Meneur C, Czernichow P, Scharfmann R (2002) Role for FGFR2IIIb-mediated signals in controlling pancreatic endocrine progenitor cell proliferation. Proc Natl Acad Sci U S A 99:3884–3889
58. Stafford D, Prince VE (2002) Retinoic acid signaling is required for a critical early step in zebrafish pancreatic development. Curr Biol 12:1215–1220
59. Chen Y, Pan FC, Brandes N, Afelik S, Solter M, Pieler T (2004) Retinoic acid signaling is essential for pancreas development and promotes endocrine at the expense of exocrine cell differentiation in Xenopus. Dev Biol 271:144–160
60. Martin M, Gallego-Llamas J, Ribes V, Kedinger M, Niederreither K, Chambon P, Dolle P, Gradwohl G (2005) Dorsal pancreas agenesis in retinoic acid-deficient Raldh2 mutant mice. Dev Biol 284:399–411
61. Molotkov A, Molotkova N, Duester G (2005) Retinoic acid generated by Raldh2 in mesoderm is required for mouse dorsal endodermal pancreas development. Dev Dyn 232:950–957
62. Kobayashi H, Spilde TL, Bhatia AM, Buckingham RB, Hembree MJ, Prasadan K, Preuett BL, Imamura M, Gittes GK (2002) Retinoid signaling controls mouse pancreatic exocrine lineage selection through epithelial–mesenchymal interactions. Gastroenterology 123:1331–1340
63. Tulachan SS, Doi R, Kawaguchi Y, Tsuji S, Nakajima S, Masui T, Koizumi M, Toyoda E, Mori T, Ito D et al (2003) All-trans retinoic acid induces differentiation of ducts and endocrine cells by mesenchymal/epithelial interactions in embryonic pancreas. Diabetes 52:76–84
64. Shen CN, Marguerie A, Chien CY, Dickson C, Slack JM, Tosh D (2007) All-trans retinoic acid suppresses exocrine differentiation and branching morphogenesis in the embryonic pancreas. Differentiation 75:62–74
65. Gradwohl G, Dierich A, LeMeur M, Guillemot F (2000) neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. Proc Natl Acad Sci U S A 97:1607–1611
66. Schaffer AE, Freude KK, Nelson SB, Sander M (2010) Nkx6 transcription factors and Ptf1a function as antagonistic lineage determinants in multipotent pancreatic progenitors. Dev Cell 18:1022–1029
67. Duvillie B, Attali M, Bounacer A, Ravassard P, Basmaciogullari A, Scharfmann R (2006) The mesenchyme controls the timing of pancreatic beta-cell differentiation. Diabetes 55:582–589
68. Reyes JD (2006) Intestinal transplantation. Semin Pediatr Surg 15:228–234
69. Buchman AL, Scolapio J, Fryer J (2003) AGA technical review on short bowel syndrome and intestinal transplantation. Gastroenterology 124:1111–1134

70. Kaufman SS, Atkinson JB, Bianchi A, Goulet OJ, Grant D, Langnas AN, McDiarmid SV, Mittal N, Reyes J, Tzakis AG (2001) Indications for pediatric intestinal transplantation: a position paper of the American Society of Transplantation. Pediatr Transplant 5:80–87
71. Spence JR, Mayhew CN, Rankin SA, Kuhar MF, Vallance JE, Tolle K, Hoskins EE, Kalinichenko VV, Wells SI, Zorn AM et al (2011) Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. Nature 470:105–109
72. Khor B, Gardet A, Xavier RJ (2011) Genetics and pathogenesis of inflammatory bowel disease. Nature 474:307–317
73. Wellcome Trust Case Control Consortium (2007) Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 447:661–678
74. Scott LJ, Mohlke KL, Bonnycastle LL, Willer CJ, Li Y, Duren WL, Erdos MR, Stringham HM, Chines PS, Jackson AU et al (2007) A genome-wide association study of type 2 diabetes in Finns detects multiple susceptibility variants. Science 316:1341–1345
75. Saxena R, Voight BF, Lyssenko V, Burtt NP, de Bakker PI, Chen H, Roix JJ, Kathiresan S, Hirschhorn JN, Daly MJ et al (2007) Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels. Science 316:1331–1336
76. Crystal RG (1990) Alpha 1-antitrypsin deficiency, emphysema, and liver disease. Genetic basis and strategies for therapy. J Clin Invest 85:1343–1352
77. Teo AK, Arnold SJ, Trotter MW, Brown S, Ang LT, Chng Z, Robertson EJ, Dunn NR, Vallier L (2011) Pluripotency factors regulate definitive endoderm specification through eomesodermin. Genes Dev 25:238–250
78. DeLaForest A, Nagaoka M, Si-Tayeb K, Noto FK, Konopka G, Battle MA, Duncan SA (2011) HNF4A is essential for specification of hepatic progenitors from human pluripotent stem cells. Development 138:4143–4153
79. Jenny M, Uhl C, Roche C, Duluc I, Guillermin V, Guillemot F, Jensen J, Kedinger M, Gradwohl G (2002) Neurogenin3 is differentially required for endocrine cell fate specification in the intestinal and gastric epithelium. EMBO J 21:6338–6347
80. Pinney SE, Oliver-Krasinski J, Ernst L, Hughes N, Patel P, Stoffers DA, Russo P, De Leon DD (2011) Neonatal diabetes and congenital malabsorptive diarrhea attributable to a novel mutation in the human neurogenin-3 gene coding sequence. J Clin Endocrinol Metab 96:1960–1965
81. Rubio-Cabezas O, Jensen JN, Hodgson MI, Codner E, Ellard S, Serup P, Hattersley AT (2011) Permanent neonatal diabetes and enteric anendocrinosis associated with biallelic mutations in NEUROG3. Diabetes 60:1349–1353
82. Wang J, Cortina G, Wu SV, Tran R, Cho JH, Tsai MJ, Bailey TJ, Jamrich M, Ament ME, Treem WR et al (2006) Mutant neurogenin-3 in congenital malabsorptive diarrhea. N Engl J Med 355:270–280
83. Kim SW, Yoon SJ, Chuong E, Oyolu C, Wills AE, Gupta R, Baker J (2011) Chromatin and transcriptional signatures for Nodal signaling during endoderm formation in hESCs. Dev Biol 357:492–504
84. Yusa K, Rashid ST, Strick-Marchand H, Varela I, Liu PQ, Paschon DE, Miranda E, Ordonez A, Hannan NR, Rouhani FJ et al (2011) Targeted gene correction of alpha1-antitrypsin deficiency in induced pluripotent stem cells. Nature 478:391–394
85. Matsumoto K, Yoshitomi H, Rossant J, Zaret KS (2001) Liver organogenesis promoted by endothelial cells prior to vascular function. Science 294:559–563
86. Landsman L, Nijagal A, Whitchurch TJ, Vanderlaan RL, Zimmer WE, Mackenzie TC, Hebrok M (2011) Pancreatic mesenchyme regulates epithelial organogenesis throughout development. PLoS Biol 9:e1001143
87. Yoshitomi H, Zaret KS (2004) Endothelial cell interactions initiate dorsal pancreas development by selectively inducing the transcription factor Ptf1a. Development 131:807–817
88. Nikolova G, Jabs N, Konstantinova I, Domogatskaya A, Tryggvason K, Sorokin L, Fassler R, Gu G, Gerber HP, Ferrara N et al (2006) The vascular basement membrane: a niche for insulin gene expression and beta cell proliferation. Dev Cell 10:397–405
89. Mou H, Zhao R, Sherwood R, Ahfeldt T, Lapey A, Wain J, Sicilian L, Izvolsky K, Musunuru K, Cowan C et al (2012) Generation of multipotent lung and airway progenitors from mouse ESCs and patient-specific cystic fibrosis iPSCs. Cell Stem Cell 10:385–397