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Tissue engineering 2.0: guiding self-organization during pluripotent stem cell differentiation

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Abstract

Human pluripotent stem cell (hPSC) differentiation aims to mimic development using growth factors or small molecules in a time- and dose-dependent manner. However, the cell types produced using this approach are predominantly fetal-like in phenotype and function, limiting their use in regenerative medicine. This is particularly true in current efforts to produce pancreatic beta cells, wherein robust pancreatic progenitor maturation can only be accomplished upon transplantation into mice. Recent studies have suggested that hPSC-derived cells are capable of self-organizing in vitro, revealing a new paradigm for creating mature cells and tissues. Tissue engineering strategies that provide subtle and dynamic signals to developmentally naïve cells may be applied to mimic in vitro the self-organization aspects of pancreatic development.

Introduction

Aspects of development can be simulated in vitro to generate multiple cell types from human pluripotent stem cells (hPSC). Current strategies typically involve exposing hPSC to soluble growth factors and small molecules appropriate to their developmental stage of differentiation (reviewed in [1]). In this paradigm, the sequential activation and inhibition of key signaling pathways specifies restricted cell types. This approach has been successful, however, it is becoming clear that this strategy may be limited in its ability to produce adult-like, mature cells and tissues that many potential regenerative therapies require.

It is possible that the subtle signals required by hPSC to develop into mature cell types in vitro can only be provided by self-organization. Self-organization is the ability of a cell, or cell population to assemble a tissue structure, and/or a signaling environment that is commensurate with its in vivo functional role. The challenge for tissue engineering and regenerative medicine is to find applications of existing technologies that promote and enhance the inherent self-organizing capability of hPSC while incorporating them into

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transplantable and manufacturable devices. Tissue engineering strategies that permit self-organization will complement growth factor based hPSC differentiation by incorporating co-culture with additional cell types, while improving control over three-dimensional architecture and migration. Ultimately, we propose to mobilize technologies that are responsive to dynamic feedback from cells during tissue formation and maturation. Further, characterization and modulation of feedback and communication within self-organizing tissue engineered systems will benefit from intercellular network analysis that considers both spatial and temporal measurements.

We will discuss these ideas in the context of pancreatic developmental biology and differentiation of hPSC to pancreatic beta cells for two reasons: 1) beta cell differentiation is sensitive to tissue geometry, interactions with the vasculature, and involves a cell migration step, three instances of self-organization that are not well replicated in current culture systems, and; 2) the generation of mature pancreatic beta cells has not yet been accomplished in vitro. Pancreatic beta cells derived from hPSC may be used in future cell therapies (i.e. islet transplantation) for the treatment of type 1 diabetes (reviewed in [2]).

This review will first provide a general overview of pancreatic development with reference to how this development is currently mimicked in vitro. Following that, each section will cover self-organizing cell behavior during development and illustrate how this behavior might be reproduced or investigated using tissue engineering and network biology approaches (summarized in table 1). We will then discuss current model systems for in vitro self-organized behavior in PSC-derived intestinal, optic cup, and anterior pituitary tissues.

Gastrulation and formation of the pancreatic beta cell

The mesoderm, ectoderm, and endoderm germ layers are created during the morphological and cell fate changes of gastrulation (reviewed in [3]). The lung, lining of the gut, liver, and pancreas are derived from the definitive endoderm, which is specified in vitro by controlling the concentration of bone morphogenetic protein 4 (BMP4), basic fibroblast growth factor (bFGF), activin A, and Wnt3A, or using small molecules [4], [5], [6] (figure 1d). The population differentiated using these growth factors expresses pioneer transcription factors marking definitive endoderm in 80–90% of cells (for a review of pioneer transcription factors, see [7,8]). Despite the apparent purity of this population there is likely to be significant heterogeneity arising from differences in epigenetic marks and fate-determining co-factor expression. For instance, in the mouse, cells that have not yet activated a transcriptional program of a specific definitive endoderm fate contain epigenetic chromatin patterns that reveal a propensity to differentiate to liver or pancreas [9]. In vitro simulation of gastrulation almost certainly induces epigenetic population heterogeneity that may be similar to that observed in vivo.

Organ domains in the definitive endoderm are marked by the expression of organ-specific transcription factor combinations, and are specified by tightly controlled spatial and temporal gradients of cell signaling molecules (reviewed in [10]). For instance, in the mouse, BMP signaling from the septum transversum mesenchyme in the ventral foregut first inhibits pancreatic differentiation and promotes liver fate. Three hours later, the converse occurs; BMP signaling inhibits liver differentiation and promotes pancreatic fate [11]. Despite the detailed knowledge of the transcriptional program activated during specification of the endodermal organ domains in the mouse [12,13], the specification of endodermal fate in hPSC culture is poorly understood. This is potentially due to unknown differences between the mouse and human transcriptional programs, and because the addition of growth factors in vitro only approximates the control exerted during in vivo cell fate specification.

During patterning of the foregut endoderm, two ventral and one dorsal pancreatic regions are marked by the transcription factors Pdx1 and Ptf1a, which are in part induced by the presence of endothelial cells [14] (figure 1a, b). This pancreatic progenitor population proliferates and creates a branched epithelial plexus in which cell fate is concurrently specified (figure 1c). Cells found in the tips of the epithelial cords are fated to become exocrine acinar cells, while those of the trunk become either duct cells or endocrine cells. Endocrine progenitors expressing the transcription factor neurogenin 3 (Ngn3) delaminate from the trunk region of the epithelial cords, migrate and coalesce into islets (reviewed in [15], figure 1c). In vitro protocols recreate the process of pancreatic specification without explicitly inducing branching morphogenesis, migration, or cell-cell interactions, which are all self-organizing cell behaviors (figure 1). The following paragraphs will review the importance of these self-organizing phenomena to the development of the pancreas and how they may be mimicked in vitro to improve the creation of mature pancreatic beta cells.

Branching morphogenesis affects epithelial-mesenchymal interactions

Branching morphogenesis accompanies the specification of all pancreatic cell types, and creates the ductal system that transports digestive enzymes to the duodenum after being produced by exocrine cells. In addition to having a transport function, it appears that the tissue architecture created by branching morphogenesis in the pancreas indirectly affects cell fate specification through the Rho-GTPase Cdc42. Cdc42 knockout results in two times fewer endocrine cells because branching morphogenesis puts endocrine progenitors in contact with laminin-1 produced by mesenchymal cells, impairing their differentiation [16]. The chemical ablation of mesenchymal cells rescues endocrine cell differentiation.

While direct contact with the mesenchyme-produced laminin-1 appears to impair endocrine progenitor differentiation, pancreatic mesenchyme plays an important role in expansion of all cell populations in the developing pancreas. Ablation of the mesenchyme resulted in uniformly reduced exocrine and endocrine populations, an effect that appears to be mediated by β -catenin signaling [17]. Interestingly, mesenchymal ablation affected branching morphogenesis, but appears to be a distinct phenotype from the Cdc42 knockout discussed above because it did not result in a propensity towards endocrine differentiation. Thus, the mesenchyme acts on all pancreatic cell types to promote expansion, but its effects on cell fate specification appear to be dependent on the architecture of epithelial morphogenesis.

While developmental biology posits a role for tissue architecture in the derivation of endocrine cells from hPSC, an appropriate system for examining branching morphology in hPSC-derived cells has not yet been developed. In vitro epithelial branching morphogenesis typically requires cells embedded in an extracellular matrix (ECM), most commonly collagen or Matrigel. For instance, self-organizing branching morphogenesis occurs in Matrigel-embedded mouse pancreatic explants and mouse embryonic stem cell (mESC)-derived pancreatic progenitors [18]. The ECM may provide a basement membrane to promote apicobasal polarity, or it may help create mechanical forces that promote branching tubulogenesis [19]. In addition to ECM, other cell populations may promote branching morphogenesis in vitro. For example, combining the developing kidney with other tissues that it normally encounters in development allows for the exchange of developmental cues that result in the successful induction of epithelial budding, branching, and capillary structures [20], [21].

Tissue engineering approaches can be used to promote and investigate branching morphogenesis. Microfabrication techniques can be used to create branched epithelium of particular geometries, revealing that areas of high mechanical stress are a key determinant of branch points [22]. Computational modeling of mammary epithelial tubule branching within

microfabricated devices suggested that the location of branch points was determined by a lower concentration of an inhibitory molecule(s), one of which was confirmed experimentally to be TGF β -1 [23].

Endothelial-pancreatic interactions

Endothelial cells promote expansion of and induce insulin expression in the mouse dorsal pancreas when the pancreatic endoderm is beginning to form at embryonic day 8.5 (E8.5). The endothelium of the dorsal aorta initiates expression of Ptf1a in the dorsal pancreatic endoderm [24]. In addition, increased vascular endothelial growth factor (VEGF) expression in Pdx1-expressing dorsal pancreatic progenitors causes hypervascularization of the pancreas, and islet hyperplasia [25]. It has been suggested that the pancreatic transcription factor program induced by dorsal aorta endothelium is attributable more specifically to the presence of angioblasts, which are attracted by chemokine (CXCL12) secretion by the dorsal pancreatic endoderm in the chick. Angioblasts appear to act through an unknown interaction to expand expression of Pdx1 and insulin in the pancreatic endoderm [26]. Interestingly, the ventral pancreatic bud does not require endothelial cell influences to develop normally. This difference between dorsal and ventral pancreatic regions is partly due to different transcriptional programs in their specification. Some transcription factors are required for ventral but not dorsal pancreas specification and vice versa [27]. The molecular basis for the effects of endothelial cells on the dorsal pancreatic field could be mediated by connective tissue growth factor [28], BMP [29], or Notch signaling [30,31], but detailed work has not been done at this stage in vivo. Alternatively, they may act through an ECM-dependent pathway, given their role in producing a basement membrane for beta cells in the islet ([32] and reviewed in [33]), and their expression of netrin-4, which is implicated in pancreatic progenitor differentiation [34]. The feasibility of replicating the effects of endothelial cells via addition of growth factors or ECM to hPSC culture will become clearer as the mechanism for the effects of endothelial cells is elucidated.

In contrast to the pro-pancreatic events induced by endothelial cells at E8.5, endothelial cells restrict the size of the expanding pancreatic endoderm during later development (E12.5) [35]. Pdx1-dependent over-expression of VEGF resulted in decreased pancreatic mass in response to hypervascularization and chemical ablation of endothelial cells at this point caused in hyperbranching and expansion of pancreatic epithelium [36]. Thus, endothelial-pancreatic interactions appear to have opposing effects at different time points, promoting pancreatic endoderm expansion early in development (E8.5) and restricting it later (E12.5).

Recently developed technologies could be used to promote the self-organization of a co-culture containing endothelial cells and hPSC-derived pancreatic endoderm. At least two cell types can be encapsulated in a hydrogel in defined ratios, enabling control over the strength of the signal created by co-culture [37]. Other ways to accomplish co-culture of cells with controllable initial geometries include microfluidics [38], and with polyethylene glycol (PEG) hydrogels containing cells assembled into multiple shapes including a lock and key type interaction [39]. Triculture of endothelial cells, fibroblasts, and skeletal myoblasts was used to create a fibrin-gel based, polymer supported vasculature that was perfusable in vivo [40]. Star-PEG-heparin or hyaluronic acid hydrogels can simultaneously modulate migration, growth factor presentation, and degradation to control the morphogenesis of human endothelial cells [41,42]. These temporally variable and cell-responsive hydrogels may be useful for studying time-sensitive endothelial-pancreatic progenitor interactions.

Cell migration creates islets

The delamination of endocrine progenitors from the trunk of the epithelial cord is a distinctive step in the formation of islets. In vivo, insulin gene expression precedes

delamination, and an epithelial to mesenchymal (EMT) transition triggered by the transcription factors Ngn3 and Snail results in migration and organization into islet structures [43], [44]. The self-organization of endocrine progenitors into islet structure appears to be mediated by G-protein coupled receptors, non-canonical Wnt, and receptor tyrosine kinase (RTK) signaling [45]. While the importance of delamination and migration to in vitro derivation of endocrine cells from hPSC is not known, endocrine differentiation is promoted in the presence of focal adhesion kinase inhibitor, suggesting that modulation of EMT-related pathways may control endocrine specification in hPSC culture [46].

Human Gata4 and Gata6 are also implicated in an endodermal EMT [47], but have been relatively unstudied in hPSC pancreatic differentiation protocols. In the mouse, Gata4 is required for liver and ventral pancreas formation, but is dispensable for dorsal pancreas formation [48]. Gata6 is essential for both dorsal and ventral pancreatic domains, and appears to be segregated to the endocrine lineage as development continues. The early Gata6 knockdown phenotype consists of a lack of pancreatic budding, while the late and milder phenotype consists of enlarged pancreatic ducts, with a higher proportion of cells expressing endocrine progenitor markers [49]. Many of these endocrine progenitors are in the incorrect location (i.e. in or near ductal epithelium), suggesting impaired migration. Gata6 mutants also exhibit an unusual branching structure [49]. It appears that Gata6 also plays an important role in human development, as its heterozygous mutation accounts for 56% of cases of pancreatic agenesis in humans [50]. These observations point to an unexplored role related to EMT for Gata6 in endocrine cell specification during hPSC differentiation, and suggest that Gata6 deficiency impairs endocrine differentiation with a phenotype similar to Cdc42-knockout disruption of branching morphogenesis.

Cell migration relies on directional cues in the form of soluble chemokines or immobilized gradients of growth factors or ECM. Traditional methods for creating soluble gradients include the under-agarose assay, the Boyden chamber, and the Dunn chamber (reviewed in [51]). More recently, soluble gradients have been created using convection microfluidics, or through a source-sink system that creates a non-convective concentration gradient [52] (reviewed in [53]). Alternatively, convective microfluidic systems can be used to control the concentration of endogenously produced autocrine or paracrine signals, which is important for controlling for cell pluripotency and differentiation [54,55]. Three-dimensional (3D) encapsulation technology is capable of providing fate-determining cues to mESC [56], and 3D patterning of multiple growth factors has been shown to guide migration of neural precursor cells [57]. The temporal and spatial control of 3D presented factors, such as RGD peptide can be controlled using a photodegradable hydrogel, which has been shown to modulate mesenchymal stromal cell activity [58]. These new 2D and 3D technologies offer novel ways of inducing and manipulating migratory cell activities, and could be applied to facilitate in vitro self-organizing delamination and migration of pancreatic endocrine progenitors derived from hPSC.

Producing mature beta cells in vivo

Current protocols for differentiation of hPSC result in cell populations that are incapable of glucose-stimulated insulin secretion, a hallmark of beta cell function. Human PSC-derived cells express insulin in granules that appear ready for secretion, but only secrete insulin in response to direct depolarization, not increased glucose concentration [59], [60]. Competency for glucose-stimulated insulin secretion is developed by hPSC-derived pancreatic progenitors that mature in vivo over approximately 3 months [61]. It has been suggested that a population within transplanted pancreatic progenitors marked by the cell surface marker CD142 is capable of giving rise to all pancreatic cell types in vivo [62]. Transplanted CD142 positive cells give rise to a sub-population that adopts a beta-cell-like

phenotype that controls blood glucose levels in mice but it is possible that the CD142 negative populations play a role in transplant maturation [62]. Insight into the degree of proliferation, death, and heterogeneity of endocrine precursors within the CD142 positive populations would be useful for unraveling dynamics of the maturation process.

Maturation of pancreatic progenitors in vivo appears to occur in both the kidney capsule and the epididymal fat pad, suggesting that a blood-borne systemic factor(s) or intercellular signals within the transplant are responsible for the adoption of beta cell-like properties. Beta cells interact extensively with each other through ephrins, cell adhesion molecules, and gap junctions (reviewed in [63]). The islet contains dense parasympathetic innervation, but interestingly, alpha cells also secrete acetylcholine that primes beta cells to respond to glucose challenge [64]. Other recently discovered paracrine interactions include serotonin-mediated beta cell proliferation [65], and an IL-6 dependent activation of GLP-1 secretion by alpha cells, which has the effect of increased insulin secretion [66]. Hormonal influence on beta cells include secretion of incretins by intestinal L and K cells, pituitary secretion of prolactin and growth hormone, and bone secretion of osteocalcin, among others [63]. It is probable that metabolic factors such as amino acid and glucose concentration also play a role in the proper development of functional beta cells.

The importance of the systemic factors listed above could be determined using an encapsulation approach that modulates the signals received by maturing pancreatic progenitors. Encapsulation will play a key role in a cell therapy approach for treating type 1 diabetes because it provides a means of isolating the transplanted cells from the antibodies and cells of the host immune system [67]. Hydrogels used for encapsulation can present ECM that may enhance the maturation of pancreatic progenitors such as collagen IV, laminin, or peptide motifs that simulate their presence [68–71]. They can also mimic cell-cell contact [72], or present molecules that promote beta-cell function such as GLP-1 [73]. Controlling the pore size of the capsule could also modulate the environment experienced by the maturing pancreatic progenitors by excluding molecules larger than a particular size [74,75]. The pore size of capsules can be controlled to a minimum of 7 nm [76]. The effect of eliminating signals on the maturation of transplanted pancreatic progenitors may be determined by encapsulating in hydrogels that sequester particular molecules [77–79], or eliminate metabolites with immobilized enzymes [80]. The signals inducing pancreatic progenitor maturation into beta-like cells in vivo may be investigated using these tissue engineering technologies. The formulation of intercellular networks that describe the local and systemic environment of the maturing pancreatic progenitor may also contribute to understanding the maturation process.

Network modeling of beta cell maturation

A lack of ability to mimic the highly integrated in vivo environment of the beta cell may explain why attempts to mature these cells in vitro have not succeeded. An improved understanding of the hormonal and paracrine interactions of the maturing pancreatic progenitors with other organs and cell types will be necessary to isolate components of the environment that are necessary for in vitro beta cell maturation. These signals will in part be determined from modulating the transplant scaffold as discussed in “Producing mature beta cells in vivo”, providing knowledge that will contribute to the formation of a network model of pancreatic beta cell maturation.

We expect cell-cell interaction network modeling to be useful in understanding the maturation process of pancreatic progenitors in vivo. Intercellular networks are constructed from data about the expression of ligands and their receptors in distinct cell populations. Construction of intercellular networks has been useful in the umbilical cord blood field,

where it has revealed multiple signaling pathways that promote or inhibit the expansion of haematopoietic stem cells (HSCs). Characterization of the transcriptome and secretome resulted in the mapping of ligand-receptor interactions among four cell populations [81]. Perturbation of the intercellular network revealed that a negative feedback loop inhibited expansion of HSCs which could be overcome by a fed-batch bioreactor culture, promoting rapid expansion of HSCs [82]. In a similar fashion, the transcriptome of all the major cell types in the pancreas has revealed numerous receptor-ligand pairs that activate signaling pathways [83]. This transcriptome is one time-point in a mature cell population. A dynamic time course of the transcriptome of maturing pancreatic progenitors may reveal intercellular interactions that reveal key drivers of maturation. The transcriptomes of organs known to interact with the beta cell should also be analyzed to reveal additional regulatory pathways. Network analysis of beta cell maturation may identify nodes or regulatory loops that can enhance beta cell expansion, accelerate maturation, or suggest drivers of beta cell maturation that can be mimicked in vitro.

In vitro self-organization of pluripotent cells

Most in vitro differentiation protocols do not react to behaviors that cells initiate according to their developmental programs, such as those outlined above. Evidence is growing that hPSC-derived cells may be capable of self-organization in vitro. Enabling self-organizing behavior requires a dynamic environment, in contrast with the traditional tissue engineering paradigm which focuses on providing cells with instructive signals without a mechanism for feedback regulation of the signal.

Self-organization of cell types in the endoderm was discovered in the intestine and colon after a screen for Wnt-responsive genes necessary for self-renewal of the intestine revealed that Lgr5 was exclusively expressed in the stem cell population of the intestinal crypt [84]. Isolated Lgr5 expressing stem cells are capable of forming self-organizing crypt-villus organoids containing many of the intestinal cell types, including enterocytes, goblet cells, Paneth cells, and enteroendocrine cells [85]. These stem cells also create their own niche in vitro, giving rise to Paneth cells that secrete factors essential for Lgr5 cell survival such as Wnt3A [86]. A similar behavior is observed in hPSC differentiation when organoids of intestinal progenitors bud from posterior endoderm [87]. The organoids mature over 14–28 days to contain functional versions of most cells found in the intestine in a morphology that is reminiscent of the intestinal lumen (figure 2b).

Self-organizing systems have also been demonstrated with mESC. Induction of the retinal anlage in serum free aggregate culture depends on the presence of either Matrigel or entactin, laminin and Nodal signaling (figure 2a). The provision of these signals was not indicated by a specific developmental mechanism, but was inspired by the general importance of basement membrane proteins to tissue morphogenesis and cell fate. Under these conditions, mESC aggregates form a neural retina and retinal pigment epithelium. These two layers spontaneously create an optic cup-like morphology in a process that is initially dependent on actin-myosin contractility, and later dependent on cell proliferation [88]. The optic cup matures after 20 days in culture into an invaginated structure composed of all major retinal cell types (figure 2a).

Using similar methods, mESC aggregates have been shown to self-organize in the presence of sonic hedgehog and BMP4 signaling into Rathke's pouch-like tissue (an anterior pituitary precursor). The endogenous production of BMP4 in large aggregates exceeded the threshold required for Rathke's pouch specification [89] (figure 2a). Corticotrophs were observed when Notch signaling was inhibited, and co-culture with a mesenchymal cell line derived from the fetal head induced luteinizing hormone (LH) and follicle stimulating hormone

(FSH) expression, while growth hormone (GH) and prolactin expressing cells were also created by culture with steroid hormone-containing medium signaling activation [89].

In vitro derivation of mature pancreatic beta cells may be dependent on self-organizing cell behaviors, given that these behaviors lead to mature beta cells in vivo (figure 2c). This proposition is supported by the observation that self-organizing populations in vitro are more capable of creating mature cell types. Intestinal organoids derived from hPSC mature to produce structures that resemble mature intestinal tissue, and contain all the major cell types of the intestinal epithelium [87]. Self-formation of the anterior pituitary produced cells that were capable of releasing adrenocorticotrophic hormone (ACTH) in response to corticosteroid-releasing hormone (CRH) stimulation, just as mature corticotrophs do in vivo, suggesting that these cells have developed a mature stimulus-response for hormone release. Further, these cells could regulate blood ACTH levels in vivo after only 1 week [89]. Compare this to in vitro differentiation of insulin expressing cells that are not capable of insulin secretion in response to glucose stimulation, and take on the order of months to mature in vivo.

A deeper understanding of self-organizing behaviors in PSC populations can be derived from construction of intercellular communication networks such as those developed in the umbilical cord blood field. The controllability of self organization will depend on detailed knowledge of how populations interact with one another, and this understanding will need to have a dynamic, temporally dependent component [90]. A geometric localization of cells within microtissues will also be necessary to study how the function and interactions between each population is affected by the architecture of the tissue. Thus far, the controllability of hPSC differentiation is variable, most likely owing to a superficial understanding of these components.

Conclusions

We have identified key self-organizing behaviors that occur in vivo, along with tissue engineering strategies to induce these behaviors. Those strategies include regulation of cell-cell interactions, promoting the formation of distinct tissue architecture, and permitting cell migration. We anticipate that analyzing differentiation in vivo and in vitro using a network structure that incorporates both temporal and spatial aspects will permit heightened levels of control in hPSC differentiation and self-organization. Tissue engineered systems will incorporate materials that are capable of integrating feedback from cells in order to provide the responsive environment needed for self-organization.

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Highlight

- Self-organization is a property of developmental systems and pluripotent stem cells
- Tissue engineering approaches can be applied to control self-organization in vitro
- Maturation of pluripotent stem cells in vitro may be promoted by self-organization
- Subtle, dynamic signals are provided to pluripotent cells through self-organization

Figure 1.

In vivo and in vitro beta cell development and additional cues for incorporation. a) In vivo development is characterized by pancreatic endoderm formation during interaction with the dorsal aorta, followed by branching morphogenesis and delamination/migration of endocrine progenitors. b) In vitro beta cell development currently follows a soluble-factor protocol in 2D. c) Potential additions to the in vitro differentiation of beta cells. Adapted from [91,92].

Figure 2. Self-organization in vitro. a) Pluripotent stem cell aggregates self-organize into optic cup-like (top) or anterior pituitary-like tissues (bottom). b) hPSC derived intestinal tissue buds off a monolayer of posterior endoderm-like cells and matures into a lumen-containing organoid containing all major intestinal cell types in the proper architecture. c) Pdx1-expressing pancreatic progenitors branch and differentiate when embedded in Matrigel. LEN = laminin, entactin, Nodal signaling. Adapted from [88], [89].

Table 1

Summary of critical in vivo signals to be replicated in vitro, and how to accomplish with new technologies

Critical signals to replicate in vitro	Strategies to replicate these signals
EMT/Migration [43–50]	Convective microfluidics [54], [55]
	Gradient formation via source/sink of chemokine [52]
	Immobilized growth factors in hydrogels [56], [57]
Endothelial cell interactions [25–27]	Co-encapsulation in hydrogel [37]
	Self-assembly of geometrically controlled hydrogel [39]
	Microfluidic 3-D assembly [38]
	Creation of vasculature-like structure [40]
	Bioresponsive hydrogels [41], [42]
Branching tubulogenesis [16]	Microfabrication [22], [23]
	Embedding cells in ECM [18], [20]
	Branch-inducing cell populations [20], [21]