1 **Title**
2 Next Generation Organoids for Biomedical Research and Applications
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Abstract

Organoids are *in vitro* cultures of miniature fetal or adult organ-like structures. Their potentials for use in tissue and organ replacement, disease modeling, toxicology studies, and drug discovery are tremendous. Currently, major challenges facing human organoid technology include (i) improving the range of cellular heterogeneity for a particular organoid system, (ii) mimicking the native micro- and matrix-environment encountered by cells within organoids, and (iii) developing robust protocols for the *in vitro* maturation of organoids that remain mostly fetal-like in cultures. To tackle these challenges, we advocate the principle of reverse engineering that replicates the inner workings of *in vivo* systems with the goal of achieving functionality and maturation of the resulting organoid structures with the input of minimal intrinsic (cellular) and environmental (matrix and niche) constituents. Here, we present an overview of organoid technology development in several systems that employ cell materials derived from fetal and adult tissues and pluripotent stem cell cultures. We focus on key studies that exploit the self-organizing property of embryonic progenitors and the role of designer matrices and cell-free scaffolds in assisting organoid formation. We further explore the relationship between adult stem cells, niche factors, and other current developments that aim to enhance robust organoid maturation. From these works, we propose a standardized pipeline for the development of future protocols that would help generate more physiologically relevant human organoids for various biomedical applications.

2

1. INTRODUCTION

Strong predictability of preclinical testing is vital to success in clinical trials. Current preclinical tests for efficacy, toxicity, and pharmacokinetics are far from perfect. While animal models have been regarded as the gold standard, the use of laboratory animals continues to pose ethical questions. The considerable animal species differences in reactions to drugs (Burkina et al., 2017) and in disease phenotypes (Seok et al., 2013) also significantly lower the accuracy of preclinical predictions. For some diseases—such as infectious diseases—no relevant animal models are available. It has been reported that only 8% of the predictions of cancer drugs generated from animal models have been successfully translated into human clinical trials (Mak et al., 2014). In addition to animal models, *in vitro* two-dimensional (2D) cell cultures are also widely used for preclinical testing. The predictions from 2D cell cultures, however, are often difficult to interpret and could be misleading because cells cultured in 2D environments often lose their functionality and have altered phenotypes that are different from those in *in vivo* tissues and organs. In recent years, the United States Environmental Protection Agency (EPA), the National Institute of Health (NIH), and the Defense Advanced Research Projects Agency (DARPA) have initiated programs such as the ToxCast Programme and the Microphysiological Systems (MPS) Program to advocate the development of human cellular models to assess the safety and/or the efficacy of environmental chemicals (Kleinstreuer et al., 2014) and drugs (Fabre et al., 2014). In particular, the DARPA MPS program promotes the development of organs-on-chips and human-on-chips approaches to synthesize *in vitro* three-dimensional (3D) human tissues derived from cultured cells on bioengineered platforms to bring together native-like tissue architecture and physiology for highly-predictive and physiologically-relevant monitoring of the functions and effects of toxic substances and drugs at the organ- or body-levels. There is an urgent need to develop more physiologically relevant, efficient, and robust protocols to advance technology that synthesizes human tissues.

1.1 DEFINING ORGANOIDS. The term *organoid* was first employed in an oncology study to indicate a pathological and tumor-like tissue mass formed in a human infant (Kretzschmar and Clevers, 2016; Smith and Cochrane, 1946). The term has since been used in the medical field to describe a multisystem disorder, called *organoid nevus syndrome*, related to benign outgrowths of sebaceous glands on the skin and the eye of affected individuals (Shields et al., 1996). *Organoid* was loosely used in experiments to denote transplanted tissues or the resultant tissue masses from transplants (Waddell, 1949; Yoshida et al., 1980). Increasingly frequent in the late 1980s and early 1990s, *organoid* was employed in developmental biology experiments to denote high-density or organ-like cultures generated through aggregation and cell sorting of dissociated animal cells and tissues (Elkasaby et al., 1991; Ridgeway et al., 1986; Schroter-Kermani et al., 1991). The two decades following this were characterized by the advent of embryonic (ESC) and induced pluripotent (iPSC) stem cell cultures derived from rodent, primate, and human embryos (Martin, 1980; Thomson et al., 1998; Thomson et al., 1995) and somatic cells (Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007; Yu et al., 2007), as well as the discovery of adult stem cells in various tissues including retina (Tropepe et al., 2000), bone marrow (Wilson and Trumpp, 2006), epidermis (Clayton et al., 2007), intestinal crypts (Clevers, 2013), stomach glands (Leushacke et al., 2013), testis (Klein et al., 2010), esophagus (Doupe et al., 2012), and brain (Fuentealba et al., 2012). Following these successive breakthroughs, the definition of *organoid* evolved to describe the *in vitro* 3D structures derived from differentiating cultures of ESCs, iPSCs, or adult stem cells that bear an *in vivo* tissue-like arrangement, compartmentalization, and functionality. In this review, we adopt

89   this definition and will use the term *organoids* to describe the *in vitro* cultures of conglomerates
90   of tissue-specific cells that incorporate cell-cell and cell-matrix interactions in an orderly and 3D
91   manner. In most cases described here, *organoids* were cultured either in suspension or embedded
92   in animal-derived matrices such as Matrigel. *Organoids* can be generated from clonal derivatives
93   of adult stem cells or aggregates of stem cells and organ-specific progenitors derived from
94   primary embryonic cell types, ESCs, or iPSCs. In regular cultures, depending on the types of
95   organs, *organoids* can be spherical or irregularly shaped masses ranging in diameter from 0.1 to
96   1 mm. They demonstrate significant homology in terms of tissue architecture and gene
97   expression profiles compared to their *in vivo* counterparts. They also display growth, undergo
98   morphogenesis, mature, and in many cases, exhibit functionality similar to their tissues of origin
99   and have the ability to integrate into embryonic and adult tissues upon transplantation.
100   *1.2 MAJOR TRENDS AND CHALLENGES IN CURRENT ORGANOID RESEARCH.*
101   Studies involving organoid generation have rapidly evolved in recent years, branching into a
102   field going into multiple directions. There are studies using organoids to understand the
103   pathology of a number of genetic disorders including cystic fibrosis (Dekkers et al., 2013;
104   Hohwieler et al., 2017), polycystic kidney disease (Freedman et al., 2015), Hirschsprung's
105   disease (Workman et al., 2017), and neurodevelopmental defects like microcephaly and
106   lissencephaly (Bershteyn et al., 2017; Lancaster et al., 2013). Organoid-driven approaches have
107   been employed to investigate disease mechanisms of infectious pathogens like *Helicobacter*
108   (McCracken et al., 2014) and Zika virus (Cugola et al., 2016; Dang et al., 2016; Garcez et al.,
109   2016; Li et al., 2017; Qian et al., 2016). Not surprisingly, organoids derived from intestinal and
110   hepatic cell types have been examined as a possible source of *in vitro* tissues for regenerative
111   medicine, and these studies have been met with great success in numerous animal models
112   (Fordham et al., 2013; Fukuda et al., 2014; Huch et al., 2015; Takebe et al., 2014; Yui et al.,
113   2012). More recently, toxicology screens (Schwartz et al., 2015; Takasato et al., 2015) and
114   studies on patient-specific genetic factors and drug responses in tumorigenesis have begun
115   employing organoid technology (Cristobal et al., 2017; Fujii et al., 2016; Li et al., 2014; Matano
116   et al., 2015; van de Wetering et al., 2015; Verissimo et al., 2016; Weeber et al., 2015).
117   ESCs, iPSCs, and, to a certain extent, adult stem cells provide a virtually unlimited
118   source of raw cellular materials for organoid generation. In the case of ESCs and iPSCs,
119   organoids are derived either via the embryoid body method, in which pluripotent stem cell
120   colonies are lifted up from undifferentiated adherent cultures and fragmented into small pieces
121   for further differentiation (Freedman et al., 2015; Kadoshima et al., 2013; Lancaster and
122   Knoblich, 2014; Lancaster et al., 2013; Muguruma et al., 2015; Nakano et al., 2012; Suga et al.,
123   2011) or directly from sub-structures formed on differentiating adherent cultures (Calderon-
124   Gierszal and Prins, 2015; Dye et al., 2015; McCracken et al., 2014). Despite these advances in
125   technique, there are still tissues that are recalcitrant to organoid derivation such as the epidermis,
126   the testes, the ovaries, the bladder, the thyroid gland, and other non-epithelial tissues such as the
127   heart and the skeletal muscles (Fatehullah et al., 2016). Additional challenges demand
128   improvements to current organoid technology. Organoid derivation often results from the tedious
129   and unguided testing of empirical conditions for proper differentiation, yielding a long and costly
130   generation process. Heterogeneity in viability, size, and shape of the derived organoids plagues
131   the development of high throughput systems and algorithms that can be employed for phenotypic,
132   toxicologic, and drug screens. In many cases, techniques used for generating one type of
133   organoid cannot be easily transferred to a different organoid system, e.g. the procedures in
134   developing a brain organoid will bear little resemblance to those for a liver organoid. This lack

135 of transferability of organoid techniques across systems hinders the development of the organoid
136 field and limits its potential to incorporate other fields of study such as computational science
137 and bioengineering that require the building of mathematical/engineering models and the
138 incorporation of scalable and cross-system parameters. Importantly, organoids usually lack the
139 co-induction of the essential cell types and the associated extracellular matrices and native
140 microenvironment that will allow the recapitulation of the *in vivo* tissue sizes, structures,
141 organization, inter-cellular communication, and functionality. Lastly, very little is known about
142 the culturing conditions and specific factors driving the *in vitro* maturation of organoids, which
143 in many cases remain fetal in nature.

144   *1.3 APPLYING THE CONCEPT OF REVERSE ENGINEERING TO ORGANOID*
145 *GENERATION.* In biology, reverse engineering is the process of extracting the minimal set of
146 design parameters from existing biological systems for creating a functional mimicry (Ingber,
147 2016; Shinozawa et al., 2016). Design parameters may include cell type, soluble
148 microenvironment, insoluble microenvironment, and physical parameters such as shape, external
149 force, and fluid flow. We propose that by employing the principle of reverse engineering, we can
150 solve some of these issues, in particular: (i) recapitulating the *in vivo* cellular heterogeneity in
151 organoid systems, (ii) controlling the organoid microenvironments including extracellular matrix
152 (ECM) composition, stiffness, and architecture, and (iii) promoting the *in vitro* maturation of
153 organoids. Here, we review current developments and techniques concerning the generation of
154 human organoids and propose that by studying and employing the minimal elements required for
155 the different stages of *in vitro* organoid synthesis and maturation, we can improve and streamline
156 current organoid generation approaches as depicted in Figure 1.

157
158 **2   EXPLOITING   THE   SELF-ORGANIZING   PROPERTY   OF   EMBRYONIC**
159 **PROGENITORS**
160   Self-organization has been widely demonstrated in animal studies, whereby tissue-
161 specific progenitors, after random dispersal, aggregate, sort, and organize to reform a 3D tissue
162 mass closely resembling the organs and tissues where the cell types belong (Takeichi, 2011).
163 Recent ESC and iPSC studies have exploited this fundamental cellular principle. Self-
164 organization is a process involving the self-assembly of dissociated cells and the subsequent
165 reorganization into tissue-like structures. Using this approach, termed "dissociation-aggregation
166 approach", cell progenitors from different lineages can be recombined to form *in vitro* organoids
167 with improved representation of cellular heterogeneity and interactions. In this section, we
168 present a historical overview of the discovery of the self-organizing property of animal
169 progenitors and summarize recent works in human organoids generated based upon this principle.
170 We also review and propose studies by which the self-organizing process could be monitored
171 and regulated.

172   *2.1 REGENERATIVE POTENTIAL OF DISSOCIATED CELLS FROM ANIMAL*
173 *EMBRYONIC TISSUES AND ORGANS.*

174   *2.1.1 Pioneering Experiments in Sponges and Hydroids.* In the first series of
175 experiments conducted over a century ago, Henry van Peters Wilson showed that sponges broken
176 down into randomly dispersed single cells were able to reorganize into live sponges (Wilson,
177 1907). Similar results, detailing the generation of live hydranths, were found in a study
178 performed by Charles Wesley Hargitt (Hargitt, 1915). A similar conclusion was drawn with
179 some essential differences by a later study performed by De Morgan and Drew using different
180 species. In their experiments, the resulting tissue aggregates, or restitution mass, developed the

181 characterized tissue and cellular architecture of hydranths, e.g. well defined endodermal tubules,
182 the outer perisarc, and the interstitial ectodermal cells, but soon underwent partial disintegration
183 and were never able to give rise to live hydranths (De Morgan and Drew, 1914). Despite these
184 differences, these early experiments sparked enormous interest in the regenerative ability of
185 animal tissues and clearly demonstrated that dissociated cells carried the potential to reform
186 tissues and structures. Tissue cells from different cellular origins, e.g. ectoderm and endoderm,
187 also possessed the ability to attach to one another and undergo cellular rearrangements to achieve
188 an advanced tissue morphology and architecture resembling those found endogenously.
189 Interestingly, Wilson also found that cells from different species of sponges sorted out from each
190 other and only recombined with cells of their own species (Wilson, 1907). This principle is not
191 strictly adhered to in vertebrate cells where chimeric tissues and organs of different species
192 origin could quite frequently be generated.
193   *2.1.2 Regenerative Potential of Embryonic Tissues in Other Animal Species.* Following
194 this initial discovery, there remained an important question of whether animal cells generally
195 possess a similar self-organizing ability. Testing of this hypothesis was not feasible until the
196 early 1940s when Johannes Holtfreter discovered a way to conveniently dissociate and re-
197 associate *Xenopus* frog embryonic cells by exploiting the changes in cell adhesion of frog cells to
198 changes in environmental pH (Steinberg and Gilbert, 2004). Thereafter, Aaron Moscona and
199 colleagues employed trypsin, at the time a novel enzymatic tool, for more efficient cell
200 dissociation in avian and mammalian organs and tissues (Moscona, 1957b; Moscona and
201 Moscona, 1952). Culturing conditions for the resultant tissue aggregates however posed
202 additional requirements for more finely regulated nutrient and thermal conditions. This led to the
203 development of more sophisticated culture media and increased precision on the controls for pH
204 and heating to promote prolonged maintenance of tissue masses *in vitro*.
205   Other advances in culturing techniques such as the advent of rotation cultures (Moscona,
206 1961) and the use of chorioallantoic membrane as an incubator for organoid tissues (Garber and
207 Moscona, 1964) had facilitated widespread employment of the dissociation-aggregation method
208 and had advanced cellular differentiation of the resulting organoids. Rotation cultures, for
209 instance, involved incubation of trypsin-dissociated cell suspension in a swirling flask, assisting
210 single cells to collide, adhere, and form cell aggregates. It had been used to form mouse
211 embryonic brain cell aggregates displaying a high degree of cellular organization (DeLong,
212 1970), undergoing the regular biochemical differentiation and expressing enzymes responsible
213 for neural transmission (Seeds, 1971), forming myelination of axons and displaying signs of
214 proliferation and synapse formation (Seeds and Vatter, 1971). Due to concerns that the
215 aggregative features observed in dissociated embryonic cells was an artificial phenomenon
216 created by *in vitro* cultures, a series of studies were carried out aiming to test if dissociated cells
217 could aggregate in an embryonic environment such as the chorioallantoic membrane. In those
218 cases, dissociated embryonic kidney, liver, and skin cells were tested and successfully
219 differentiated as aggregates (Garber et al., 1968; Garber and Moscona, 1964; Weiss and Taylor,
220 1960).
221   In another classic embryological experiment, epidermis and neural tube from early
222 amphibian embryos were dissociated and allowed to re-aggregate (Townes and Holtfreter, 1955).
223 The two cell populations sorted out from each other and self-organized into epidermal cells
224 covering the outside of the tissue aggregate containing a neural tube-like structure. Interestingly,
225 there were other studies producing chimeric organoid structures with different animal species or
226 even organs with little relation to each other under normal developmental contexts. For instance,

227 chimeric experiments were carried out for chick nephrogenic and mouse chondrogenic cells;
228 ultimately concluding that the nephrogenic and chondrogenic cells aggregate according to their
229 tissue types, but disregard their species-specificity (Moscona, 1957a). In another study, mouse
230 skin cells at an advanced stage were found to suppress feather formation in chick-mouse
231 chimeric aggregates, while mouse skin cells from a younger stage appeared to incorporate into
232 the feather structures (Garber et al., 1968). Further, chimeric aggregates of human and mouse
233 cells derived from the same organs (lung, liver, and brain) integrated well with each other—
234 similar to those derived from the same species—suggesting that, between human and mouse,
235 tissue specificity dominates over species specificity (Cassiman and Bernfield, 1974). A similar
236 phenomenon was observed for embryonic chick and rat heart cells, in which chimeric aggregates
237 were formed (Nag et al., 1980). These experiments highlighted the versatility and flexibility of
238 the embryonic and fetal progenitors in chimeric species organ synthesis and inspired more recent
239 experiments that employed the successful integration of embryonic progenitors derived from
240 ESC and iPSC cultures in animal transplantation as a readout for functionality (e.g. Cohen et al.,
241 2016).

242   ***2.1.3 Recent Development Using Dissociated Embryonic Progenitors for Organoid***
243 ***Generation.*** Over the past four to five decades, numerous experiments have continuously
244 demonstrated the tremendous propensity of embryonic and fetal progenitors to self-renew,
245 differentiate, self-organize, and regenerate. In particular, organoid structures have been generated
246 from aggregating embryonic and fetal progenitors derived from virtually all types of embryonic
247 organs and tissues from higher vertebrates including those that are ectoderm (brain, spinal cord,
248 inner ear, submandibular gland, retina, lens), mesoderm (heart, limb bud, kidney), and endoderm
249 (lung, pancreas, liver) derived (Table 1). In one particular example, fragments and aggregates of
250 dissociated cells of embryonic day 13 mouse submandibular gland epithelium were able to self-
251 organize and undergo branching morphogenesis, forming tissues with structural features and
252 differentiation markers characteristic of the intact gland. The study also reported that the self-
253 organization process was sensitive to perturbation by integrin and E-cadherin signaling (Wei et
254 al., 2007). In another well-studied example, dissociated embryonic chick retina cells in rotation
255 cultures were able to reconstitute all the essential retinal layers including differentiating cone and
256 rod cells aligned in rosette structures within the tissue aggregates. These tissue aggregates, so-
257 called rosette spheres, could reach a size of 0.3-0.5 mm and included up to half a million cells.
258 The tissue organization of these rosette spheres was further improved by the inclusion of the
259 retinal pigmented epithelium, which promoted laminar reconstitution and the proper alignment of
260 the different retinal layers (Layer et al., 2001). Many current studies have shifted their focus
261 from simple aggregation experiments to a number of different directions including investigating
262 (1) the identity of the soluble intercellular factors and the intracellular molecular mechanisms
263 that promote the aggregation and the subsequent cell-sorting processes, (2) the role of
264 developmental ages of embryonic and fetal progenitors in organoid formation, (3) the effects of
265 specific dissociation techniques, and (4) the functionality of the resulting tissue aggregates or
266 organoids with more sophisticated biochemical and morphological studies as readouts for
267 function. Documenting these studies is out of the scope of this review, but some of them have
268 been summarized and discussed (Takeichi, 2011).
269

2.2 AGGREGATION EXPERIMENTS EMPLOYING CELL PROGENITORS DERIVED FROM PLURIPOTENT STEM CELLS.

Given these strong data in animal works and the rich resources of efficient adherent differentiation protocols, recent studies have started to employ dissociated embryonic cell types derived from human ESCs and iPSCs as raw materials for organoid generation. In these studies, ESC and iPSC colonies were differentiated either directly as colonies or after a re-plating step as a monolayer of evenly spread-out, single cells. These differentiating adherent cultures, after reaching the progenitor stages of their respective lineages, were dissociated, dispersed, and re-aggregated to form suspension 3D organoids. In some cases, organoids were derived from progenitors of single germ layers, like in the case of pancreatic (Hohwieler et al., 2017; Kim et al., 2016) and kidney organoids (Takasato et al., 2016; Takasato et al., 2015). In other cases, composite organoids were made from progenitors of multiple germ layer lineages such as liver, cerebral cortical, and intestinal organoids (Schwartz et al., 2015; Shinozawa et al., 2016; Takebe et al., 2013; Takebe et al., 2014; Workman et al., 2017). Many of these organoids have been successfully employed in toxicology studies, disease modeling, and animal transplantation studies. Timed addition of progenitor cell types to composite organoids has allowed increased control over the timing and incorporation of specific cell-cell interactions during organoid generation and differentiation to mimic *in vivo* situations. Cryopreservation of progenitor cell types for these organoids has been successfully performed, thus permitting a consistent and convenient supply of progenitors for organoid formation.

2.2.1 Organoids Derived from Single Germ Layers. KIDNEY. Mammalian kidneys are derived from the intermediate mesoderm that gives rise to the major kidney progenitors including the ureteric epithelium, metanephric mesenchyme, and the renal stroma. Ureteric epithelium forms the collecting tubes whereas the metanephric mesenchyme forms the nephrons, which include substructures like the proximal and distal tubules and the glomeruli. The mature kidney epithelial structures are surrounded by a renal interstitium containing a vascular network derived from the renal stroma. To generate kidney progenitors, human ESCs were differentiated from the primitive streak stage to form $PAX2^+LHX2^+$ intermediate mesoderm by manipulation of WNT and FGF signaling (Takasato et al., 2014). Differentiating embryonic kidney cells, resulting from the further differentiation of $PAX2^+LHX2^+$ intermediate mesoderm progenitors, were dispersed and aggregated to form organoid structures that displayed evidence of development of the ureteric epithelium, the proximal tubules, and the renal vesicles. However, no definitive nephron structures could be identified in these organoids. In a subsequent study, by manipulating the strength and the duration of retinoic acid and WNT signaling, the same group identified a way to preferentially induce ureteric epithelium development over metanephric mesenchyme (Takasato et al., 2016; Takasato et al., 2015). By dissociating progenitors at an earlier time point (day 6 instead of day 18) and optimizing WNT and FGF signaling post-organoid formation, kidney organoids matured to form nephron-like structures including the essential components of collecting ducts, distal and proximal tubules, and glomeruli. Endothelial vascular networks, together with pericyte-like and mesangial-like cells, were also identified and appeared to invade some of the glomeruli. Advanced morphological patterning in terms of the arrangement of the collecting ducts to nephron-like structures and the presence of cortical versus medullary stroma patterning indicated the formation of a complex kidney-like structure. Furthermore, these organoids were tested positive for endocytic function to dextran and for response to nephrotoxic agents like Cisplatin. Comparisons to a range of human fetal tissues

315 suggested that these kidney organoids resembled first trimester kidney tissues suggestive of their
316 fetal nature and the need for further maturation.

317 ***PANCREAS.*** The pancreas is a glandular organ, which serves dual functions in
318 regulating blood sugar level, by its exocrine glands that secrete insulin and glucagon and in
319 digesting proteins, lipids, carbohydrate, and nucleic acids by excreting enzymes from its
320 endocrine glands. Exocrine (Hohwieler et al., 2017) and endocrine (Kim et al., 2016) pancreatic
321 progenitors have been respectively derived from human ESC and iPSC 2D cultures. These
322 cultures were dissociated and re-aggregated in suspension to form functional and transplantable
323 endocrine and exocrine organoids. Both endocrine and exocrine pancreatic progenitors were
324 differentiated from ESCs and iPSCs via activation of WNT and Activin signaling to prompt a
325 definitive endoderm fate, followed by treatment with retinoid acid, FGF ligand, and BMP
326 inhibitor to generate $PDX1^+$ pancreatic endoderm, a common progenitor for endocrine and
327 exocrine cell types. $PDX1^+$ cells were then skewed towards either exocrine or endocrine
328 progenitors using distinct growth factor and small molecule cocktails.

329 Pancreatic endocrine cells expressed proprotein convertase 1/3, glucose transporter 1, and
330 the majority of pancreatic hormones such as insulin, somatostatin, and pancreatic peptide, with
331 the exception of glucagon (Kim et al., 2016). Detailed marker profiling, however, suggested that
332 these endocrine cells were still immature. For instance, the authors observed a low expression
333 level of mature β-cell marker NKX6-1 and the coexpression of endocrine progenitor marker
334 MAFB with insulin, indicating that the endocrine cells derived were immature. Upon organoid
335 formation by aggregation of endocrine cells, these cells continued to express pancreatic
336 hormones and the above mentioned functional markers, but, in addition, lost expression of
337 MAFB in $insulin^+$ cells, gained expression of the mature β-cell marker MAFA (Nishimura et al.,
338 2006), and increased expression of glucose sensor genes (*SLC2A1* and *GCK*). All these were
339 indicative of improved β-cell function and maturation. Further, these pancreatic endocrine
340 organoids displayed sensitivity to high glucose concentration and responded by inducing insulin
341 and c-peptide secretion. Functionally, transplantation of pancreatic endocrine organoids
342 prolonged the life span of streptozotocin treated mice, which had their pancreatic islet destroyed
343 by streptozotocin treatment. The mice with organoid transplants displayed a response to high
344 blood glucose and expressed at least one endocrine hormone, c-peptide, in the blood stream.
345 Function of the transplants however gradually became unstable after 2 weeks. Mice survived
346 more than 40 days, but a more long-term survival study was not conducted and ultimate cause of
347 transplant failure was not concluded.

348 In contrast to endocrine organoids, human pancreatic exocrine organoids formed by
349 aggregation of dissociated exocrine cells contained acinar-like and ductal-like cell types and
350 structures (Hohwieler et al., 2017). Activities for key exocrine enzymes such as carbonic
351 anhydrase, amylase, trypsin, and elastase were detected. Cystic fibrosis patients display
352 increased probability of pancreatitis, pancreatic exocrine insufficiency, and pancreatic cancer.
353 Patient iPSCs carrying *CFTR*[a] mutations were used to derive pancreatic exocrine organoids for
354 modeling cystic fibrosis disease progression. Forskolin is known to induce rapid swelling of
355 control intestinal organoids but not in organoids derived from a cystic fibrosis mouse model

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[a]Cystic fibrosis transmembrane conductance regulator (*CFTR*), the gene mutated in cystic fibrosis,
encodes a chloride channel protein, a member of ATP-binding cassette transporter superfamily. CFTR
mediates fluid homeostasis in the epithelia of organs such as pancreas, liver, intestine, and lung.

356 (Dekkers et al., 2013). Expectedly, forskolin/IBMX treatment[b] did not induce much swelling in
357 the lumens of human cystic fibrosis patient-derived organoids. Encouragingly, the swelling
358 defect could be ameliorated in a pre-selected set of CFTR correctors and potentiators with known
359 functions in improving CFTR activity, or with chemically modified *CFTR* mRNA that confers
360 high stability and low immunogenicity, demonstrating a proof of principle for these organoids in
361 disease modeling and compound screening. However, upon orthotopic transplantation to a mouse
362 host, the human organoids remained fetal in gene expression, and, because of a lack of functional
363 assays, it was difficult to assess the maturity and purity of the resulting human grafts.
364   ***2.2.2 Germ Layer Composite Organoids. INTESTINAL SYSTEM.*** Attention has often
365 been paid to parenchymal cells in organoid generation. The role of non-parenchymal cells, such
366 as neural cells, mesenchymal cells, and vascular cells, has been well documented in development
367 and tissue functionality (Cleaver and Melton, 2003; Furness, 2012). A recent study, which
368 appreciates the inclusion of these non-parenchymal cell types, generated human intestinal
369 organoids with an intact enteric nervous system (Workman et al., 2017). In this study,
370 dissociated caudal neural crest cells were recombined with human intestinal organoids by low
371 speed centrifugation. Neuronal and glial differentiation was observed in intestinal organoids
372 seeded with neural crest cells. The overall spatial relationship of the resulting composite
373 organoid resembled human fetal or mouse embryonic day 11.5 intestine. These organoids were
374 cultured *in vitro* for 28 days before transplantation. Importantly, the graft displayed neuronal
375 calcium oscillations, contractile activity, and neuromuscular coupling suggestive of a functional
376 enteric nervous system. These grafts were also used to model the phenotype of *PHOX2B*
377 mutations in the etiology of Hirschsprung's disease, a genetic disorder resulting in
378 agangliogenesis of the human bowel.
379   ***LIVER.*** Hepatocytes are the major cell type in the liver, which make up around 80% of
380 the parenchymal mass in the adult organ and display both endocrine and exocrine properties (Si-
381 Tayeb et al., 2010; Zhao and Duncan, 2005). Non-hepatocyte cell types such as cholangiocytes,
382 sinusoidal endothelial cells, Ito cells (hepatic stellate cells), Kupffer cells (tissue macrophages),
383 and pit cells (natural killer cells) constitute the rest of the liver. The liver buds, which are derived
384 from the foregut endoderm and containing the adult liver progenitors, are formed around
385 embryonic day 8.5 in mouse. At embryonic day 9.5, hepatoblasts delaminate from the liver buds
386 to invade the surrounding septum transversum (mesoderm derived), which contains progenitors
387 for non-hepatocyte cell types such as Ito cells and endothelial cells. To recapitulate the *in vivo*
388 cellular heterogeneity of adult liver and to promote the vascularization that is essential for
389 normal liver function and transplantation, Takebe et al. attempted to generate *in vitro* human
390 liver buds by aggregating cell types from two germ layer lineages: hepatic endoderm derived
391 from ESC/iPSC cultures, and from the mesoderm, umbilical vein endothelial cells and
392 mesenchymal stem cells (Takebe et al., 2013; Takebe et al., 2014). A 10: 7: 2 ratio of human
393 HNF4A^{+} hepatic endoderm, umbilical vein endothelial cells, and mesenchymal stem cells were
394 mixed and resuspended in hepatocyte culture medium. The cells in the suspension aggregated
395 and self-organized to form liver-bud organoids containing an endothelial network and maturing
396 hepatocytes with increased expression of early liver specific genes like *alpha-fetoprotein*, *retinol*
397 *binding protein 4*, *transthyretin*, and *albumin*. Microarray analyses suggest that the resulting

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[b] Forskolin increases the amount of intracellular cAMP, which in turn activates CFTR that mediates fluid
secretion into the lumen of small intestinal organoids. IBMX (3-isobutyl-1-methylxanthine) is a
phosphodiesterase inhibitor. Because of its effect in inhibiting phosphodiesterase, IBMX may potentiate
the accumulation of intracellular cAMP by the action of forskolin.

liver-bud organoids resembled early embryonic liver buds in mouse (embryonic day 10.5 to 11.5). Upon transplantation into mouse brain, connectivity with host vasculatures was quickly observed. Prolonged cultures of transplants up to 60 days led to development of hepatic cord-like structures, which contained cells expressing tight junction proteins and collagens that are normally found along the entire length of the liver sinusoids. Albumin production by day 45 of transplantation was comparable to or surpassed *in vitro* cultures of adult human hepatocytes. Ketoprofen[c] and debrisoquine[d] were used to distinguish human hepatic metabolic functions from mouse hepatic functions since these drugs are metabolized differently in human and mouse livers. Human specific metabolites, upon challenge with ketoprofen or debrisoquine, were found in the transplanted mouse blood serum and urine, suggestive of the presence of functional human liver tissues in the organoids. An additional minimally-invasive mesenteric transplantation model was carried out and the transplanted liver buds promoted survival of mice with ganciclovir-induced liver failure by ~50% at day 30 after transplantation. No specific markers or assays however were used to detect the presence of other non-hepatocyte cell types either under *in vitro* cultures or upon transplantation. These non-hepatocyte cell types play important roles in liver function and pathology. Cholangiocytes, for example, are important for bile metabolism and hepatocyte survival and have been successfully derived from human iPSC cultures (Dianat et al., 2014). For other non-hepatocyte cell types, such as pit cells and Kupffer cells, there are no existing differentiation protocols.

   CENTRAL NERVOUS SYSTEM. To mimic *in vivo* brain development, neural constructs (organoids) were generated by aggregating dissociated neural progenitors, mesenchymal stem cells, endothelial cells, and microglial/macrophage precursors (Schwartz et al., 2015). Neural constructs were formed with neural progenitors embedded in polyethylene glycol hydrogels, followed by seeding of endothelial cells and mesenchymal stem cells at day 9 and lastly with microglial/macrophage precursors at day 13 to mimic the *in vivo* recruiting sequence of blood vessels and macrophage after the formation of the neural tube. Timed addition of cell precursors to the initial neural constructs allowed temporal control of the incorporation of different cell types. The resultant neural organoids bore gene signatures for forebrain and hindbrain development and displayed markers for GABAnergic neurons, glutamatergic neurons, and glia. Extensive vascular networks and phagocytic activities were observed in the neural organoids suggestive of functional vascular and macrophage cell types. Machine learning refers to studying and developing algorithms that can iteratively learn from data without being explicitly programmed and make predictions. The neural organoids were employed for machine learning using RNA sequencing datasets with known neurotoxins and control compounds. Subsequent blind trials found that toxicity of 9 out of 10 known compounds could be correctly predicted suggesting that such neural organoids could be used for *in vitro* drug toxicity screens, although the authors admitted that a functional perfused blood-brain barrier could further improve predictability of drug toxicity. The blood-brain barrier is lined by specialized endothelial cells, called brain microvascular endothelial cells that regulate the transport of substances into and out of the brain. To reconstruct a functional adult-like blood-brain barrier, additional cell types are required, such as pericytes and brain microvascular endothelial cells

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[c] Ketoprofen is primarily metabolized to ketoprofen-glucuronide by human hepatocytes and metabolized to hydroxyl-ketoprofen by mouse hepatocytes.

[d] Debrisoquine is metabolized to 4-hydroxy debrisoquine by CYP2D6 in human hepatocytes. Mouse liver lacks 4-hydroxylase activity and thus cannot convert debrisoquine into its 4-hydroxy metabolite.

439 (Lippmann et al., 2013). These cell types have been derived from human ESC and iPSC cultures
440 (Lippmann et al., 2012; Orlova et al., 2014).
441   *2.2.3 Monitoring and Regulating the Self-organization Process to Facilitate Organoid*
442 *Formation.* We envisage more widespread usage of the dissociation-aggregation method in
443 future studies, especially for organ systems whose full functionality depends on the incorporation
444 of multiple germ layer lineages. To improve this method, we propose timed and sequential
445 incorporation of tissue- and organ-specific cell progenitors to better mimic the events occurring
446 during embryonic development (Figure 2). Self-organization of the aggregating cells during
447 organoid formation can be broken down into steps of temporally-overlapping and interrelated
448 processes that could be monitored and regulated. These include the chemotactic movement of the
449 aggregating cells (Chen et al., 2007), the cell surface interactions to matrices and to neighboring
450 cells (Takebe et al., 2015), the cell-sorting process, the formation of the apical-basal orientation
451 of individual cells, and the process of lumenogenesis (Odenwald et al., 2017; Taniguchi et al.,
452 2015) (Figure 3). Time-lapse imaging of aggregating cell populations labeled transiently with
453 fluorescent dyes or permanently with genetically-engineered reporters could allow tracking of
454 the kinetics of organoid formation, whether they form under certain conditions or not, and, if
455 they do form, how fast they form, and the dynamic changes of their shape along the time course
456 of their formation. The speed of condensation and the changes in the shape of the forming
457 organoids could be monitored by quantitative analyses (Takebe et al., 2015). Additionally,
458 pharmacological interventions can be applied to regulate the speed of aggregation. Takebe et al.
459 found that manipulation of myosin II activity controlled the collective movement of cells during
460 the aggregation process (Takebe et al., 2015). Mesenchymal stem cells within the initial
461 aggregating mix were identified as a key cell population that was essential to drive the
462 aggregating process (Takebe et al., 2015). By closely monitoring the structural parameters of the
463 forming organoids, such as their diameter and morphology, Arora and colleagues were able to
464 promote the yield of successful intestinal organoids using an automated micro-peptide aspiration
465 and release system (Arora et al., 2017). More studies in these areas are urgently needed to
466 translate findings from organoid monitoring into promoting the successful formation of
467 organoids.
468
469 **3 UTILIZING DESIGNER MATRICES AND SCAFFOLDS FOR ORGANOID**
470 **FORMATION**
471   *3.1 DESIGNER MATRICES AND MATRIX PARAMETERS.* When considering the
472 minimal elements for forming a functional organ or tissue, one must consider not only
473 parenchymal cells and the less-represented and essential cell types like immune, vascular, and
474 neural cells, but also the secreted ECM framework and its associated growth factors, cell
475 interacting molecules, enzymes, and extracellular vesicles that produce the necessary physical
476 and biochemical signals to support organ and tissue maintenance, growth, and morphogenesis.
477 To mimic the physical and biochemical properties of *in vivo* cellular microenvironment, designer
478 matrices (Gjorevski et al., 2014), either derived from natural sources or artificially synthesized
479 with physical and biochemical properties specially designed for a certain cell type can be
480 employed. Controls exercising on organoid formation can be greatly facilitated by incorporating
481 designer matrices. For example, micropores present in hydrogels and scaffolds would allow
482 organoid formation at a defined shape and size. The physical and biochemical properties of
483 designer matrices can be spatially and/or temporally controlled to enable stem cells to self-
484 organize, and this topic has been elegantly reviewed (Gjorevski et al., 2014). Together with

12

485 advanced 3D printing technology, studies can be conducted to fabricate designer matrices with
486 controlled nano- and micro-structures to standardize and improve organoid formation. We
487 propose that by studying the endogenous composition and dynamics of ECM expression during
488 the development of animal and human organs and tissues and empirically testing the
489 requirements of these ECM and other tailor-made matrices in *in vitro* cultures, one would be able
490 to determine the minimal and essential elements and signals from the ECM that are required for
491 the cellular activity, organization, and differentiation of organoid cultures.
492   A number of parameters dictate the ability of matrices to modulate organoid formation.
493 These parameters include stiffness, composition, topology (or geometry), degradability, the
494 ability to bind growth factors, and the capacity to modulate growth factor activities. Regulating
495 these parameters may prove advantageous to promoting organoid formation, standardizing
496 organoid production, and moving organoid technology to clinics. Organoids are currently
497 cultured either matrix-free or by embedding them in matrices like Matrigel. Matrix-free
498 suspension organoid cultures display superb nutrient diffusion, but lack certain physicochemical
499 supports, resulting in organoids that may clump and causing lower yields, and may develop
500 necrosis within the core. Matrix provides an initial guidance to the aggregating cells and serves
501 as a subsequent physical support and constraint for organoid formation. Matrigel and collagen
502 type I are the most commonly used matrices in organoid cultures derived from ESCs, iPSCs, and
503 adult stem cells. However, there are few studies investigating the precise roles of these matrices
504 in supporting organoid formation. On top of this, the animal origin and lot-to-lot variation of
505 Matrigel hinders the use of the derived organoids in clinical applications. Fully synthetic or
506 recombinant matrices are chemically defined and can be standardized for organoid production
507 and better facilitate downstream clinical applications. Well-defined matrices, such as those
508 incorporating synthetic ECM peptides with artificial hydrogels, allow precise control of the
509 cellular microenvironments.
510   Matrix stiffness is an important parameter regulating cell behavior. Matrix stiffness
511 reflects the resistance that a cell experiences when it deforms the matrix, and it can be measured
512 by the elastic constant (Young's modulus, presented as Pascal). Cells sense matrix stiffness
513 through mechanoreceptors, such as integrins (Humphrey et al., 2014). It was found that a stiffer
514 hydrogel (1.3 kPa) supported intestinal stem cell expansion, whereas softer hydrogel
515 (approximately 190 Pa) promoted differentiation and intestinal organoid formation, suggestive of
516 a role played by matrix stiffness in regulating cell proliferation versus differentiation in 3D
517 environments (Gjorevski et al., 2016). Earlier studies also revealed an important function of
518 matrix stiffness in lineage specification of mesenchymal stem cells in 2D (Engler et al., 2006)
519 and 3D environments (Huebsch et al., 2010) and ESCs in 2D environments (Chowdhury et al.,
520 2010). As a prime example, precise temporal control of matrix stiffness can be achieved by
521 synthesizing a composite with specific proportions of degradable and non-degradable synthetic
522 hydrogels, thus providing additional guidance to stem cell renewal and differentiation as shown
523 in intestinal organoid cultures (Gjorevski et al., 2016).
524   *3.2 CHARACTERIZATION OF ECM IN IN VITRO CULTURES AND DURING*
525 *DEVELOPMENT.* ECM composition and architecture is under constant remodeling during
526 normal development (Daley et al., 2008) and ESC differentiation. Data from human embryonic
527 liver development demonstrated dynamic expression pattern of integrins and ECM components
528 (Couvelard et al., 1998). During embryoid body formation from human ESCs, the expression of
529 fibronectin was spatiotemporally correlated with the expression of a definitive endoderm marker
530 GATA4 (Taylor-Weiner et al., 2013). A reverse correlation was found with the expression of a

531 pluripotent marker NANOG. Directed differentiation of ESCs to definitive endoderm produced
532 fibrillar fibronectin whereas other lineage differentiation or ESC cultures produced punctate
533 fibronectin. Kanninen et al. have recently discovered that integrin expression was dynamic
534 during hepatic differentiation of human ESCs and iPSCs (Kanninen et al., 2016). The expression
535 of laminin-511/521-specific integrins increased during definitive endoderm induction and
536 hepatic specification. They showed that laminin-511 and laminin-521 promoted hepatic
537 specification from human definitive endoderm cells (Kanninen et al., 2016). Recombinant
538 laminin-511 and laminin-521 would presumably provide an optimal biochemical cue as a
539 designer matrix to assist generation of liver organoids.
540   To understand the dynamic changes in ECM, temporal expression studies need to be
541 carried out to examine the changes in ECM molecule expression during consecutive
542 developmental stages. Studying the secretome of a cell population that includes growth factors,
543 extracellular matrices, extracellular proteinases, and enzymes may prove to be extremely useful.
544 For example, transcriptomic analyses have been performed to determine the changes in
545 secretome across several early kidney developmental stages (Martinez et al., 2006). More
546 advanced technology involving the use of automated and high-throughput tandem mass
547 spectrometry (MS) can be employed to identify the secretome and the proteome of the ECM of
548 cultured cells under various experimental conditions and of *in vivo* tissues across developmental
549 stages (Byron et al., 2013; Ngounou Wetie et al., 2013). Steps such as sample or peptide
550 fractionation and liquid chromatography (LC) prior to MS can help reduce sample complexity
551 and ease the identification of ECM components. Such approaches had been successfully used to
552 identify tens to hundreds of ECM components from tissues ranging from eyes to mammary
553 glands to cartilages (Byron et al., 2013). Solid-state NMR spectroscopy and matrix-assisted laser
554 desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) have been suggested
555 for the compositional analysis of natural ECM and bioengineered tissues (Schiller and Huster,
556 2012). Low abundant proteins can be detected directly with high-resolution MS or a coupled LC
557 and LC-MS/MS system (i.e. ESI-Q-TOF MS, FT-ICR-MS) (Personal communication with Dr.
558 Andrzej Ciechanowicz). To investigate a more dynamic biological system, as occurs in
559 development, quantification of the relative or absolute amount of particular ECM and secretome
560 components can be achieved by MS using isotope labeling or other label-free strategies. Key
561 ECM and secreted biologically-active protein components identified from secretome assay could
562 be tested for functionality in promoting organoid formation and development via loss-of-function
563 experiments or ectopic administration. Recently, an ECM array platform has been developed to
564 empirically examine the composition of ECM molecules permissive for definitive endoderm
565 differentiation towards the liver and pancreatic lineages (Braga Malta et al., 2016). Coupling
566 array studies with the protein expression data from MS assays will provide valuable information
567 for formulating designer matrices in organoid formation.
568   *3.3 APPLICATIONS OF DESIGNER MATRICES.* To incorporate the dynamic
569 changes in ECM composition into organoid differentiation protocols, one may passage
570 dissociated organoid cells at defined time points onto predesigned matrices and scaffolds or may
571 incorporate relevant ECM components into a bio-inert scaffold or hydrogel with degradable
572 matrices, as described previously (Gjorevski et al., 2016). The same study found that a synthetic
573 hydrogel, polyethylene glycol, did not support organoid formation (Gjorevski et al., 2016).
574 Polyethylene glycol cannot interact with cells; it only provides physical support. This finding
575 indicates that additional biochemical signals generated by the biologically-active matrices were
576 critical for organoid formation. Biologically-active matrices can generate signals either by

577 directly interacting with cell membrane receptors such as integrins or by binding to growth
578 factors. Many ECM components have integrin-binding domains and/or growth-factor-binding
579 domains, which can be incorporated into designer matrices. It is well-known that the ECM binds
580 and influences the activities of growth factors (Rozario and DeSimone, 2010). More
581 sophisticated design of matrices can be used to create concentration gradients of growth factors,
582 thereby guiding morphogenetic events. Growth factors can also be conjugated on matrices to
583 sustain their releases to cells during prolonged organoid cultures (Lutolf and Hubbell, 2005).
584   To achieve endogenous matrix architecture, a recent study used multi-photon excited 3D
585 printing technology to produce an ECM scaffold that mimics the developing mouse heart tissues
586 with submicron resolution (Gao et al., 2017). The scaffold was seeded with component cell types
587 of developing heart derived from human ESC and iPSC, such as cardiomyocytes, endothelial
588 cells, and smooth muscle cells. The resultant so-called cardiac muscle patches, although much
589 smaller in size than regular organoids, tested positive for cell engraftment and increased cardiac
590 function in a mouse model for cardiac infarction. In this study, however, only fibronectin was
591 used. Incorporation of multiple relevant ECM molecules mimicking the endogenous composition
592 of ECM in the heart and other organ systems will enhance the applicability of this 3D scaffold
593 printing technology.
594   Collectively we propose that designer matrices fabricated based on the understanding of
595 the dynamic changes in ECM parameters during *in vivo* organ and tissue development and robust
596 empirical testing could maximize the self-organizing capacity of cells and provide an optimal
597 environment for human organoid formation.
598
599 **4 PROMOTING THE MATURATION OF ORGANOIDS**
600   *In vitro* organoid cultures have thus far failed to produce fully-mature cell types.
601 Intestinal, gastric, lung, kidney, and cerebral organoids derived from human ESCs and iPSCs
602 exhibit immature phenotypes, resembling human fetal organs (Camp et al., 2015; Dye et al.,
603 2015; Finkbeiner et al., 2015; Hohwieler et al., 2017; McCracken et al., 2014; Takasato et al.,
604 2015). To promote the maturation of *in vitro*-derived organoids, maturation factors need to be
605 introduced and culturing conditions must be adjusted at subsequent stages of differentiation to
606 drive the transition of fetal organoids into more adult-like phenotype. Narrowly, "maturation
607 factors" could refer to proteins or chemical factors secreted from cells that promote the
608 maturation of fetal tissues towards more adult-like tissues. In a broader sense, they could refer
609 broadly to factors ranging from cell-cell interacting factors, circulating hormones, microbial-
610 derived factors (Avior et al., 2015), electrical signals to other unknown factors that could
611 promote the same process for specific cell types. There is ongoing debate on how maturation of
612 embryonic tissues is achieved. One hypothesis suggests that tissue maturation is driven by the
613 induction of adult stem cells in fetal tissues. An alternative hypothesis suggests that authentic
614 tissue-specific fetal stem cells, distinct from adult stem cells, exist during development and
615 contribute to tissue maturation. In this section, we review studies and propose a number of
616 approaches with which maturation of organoids could potentially be achieved. Furthermore, we
617 review current studies that attempt to promote the *in vitro* maturation of organoids with variable
618 successes.
619   *4.1 ORGANOID MATURATION UPON TRANSPLANTATION.* There are concerns
620 that organoids derived from human ESC and iPSC, even after prolonged cultures, might be
621 unable to mature *in vitro* to become adult-like tissues. However, that transplantation into animal
622 tissues in many cases induces maturation of human organoids (Cho et al., 2017; Finkbeiner et al.,

623 2015; Takebe et al., 2013; Watson et al., 2014; Workman et al., 2017) suggests that functional
624 maturation of *in vitro* organoids could be achieved. For instance, transplantation into the kidney
625 capsule of mice promoted the maturation of both the intestinal and the enteric nervous system of
626 human intestinal organoids (with neural crest-derived enteric neurons) after engraftment for 6 to
627 10 weeks (Workman et al., 2017). Highly mature intestinal tissues, with villi and crypts
628 containing functional intestinal stem cells, were formed *in vivo*. Enteric neurons and glia
629 were organized into ganglionic structures in close proximity to the submucosal and myenteric
630 layers of smooth muscle fibers. Nitric oxide synthase expression was detected upon
631 transplantation, another sign of maturation of the enteric nervous system. One possible
632 explanation is that vascularization of organoid structures brings in by the blood stream active
633 components like hormones and serum proteins that could assist a general tissue maturation
634 process. This notion is supported by the enhanced maturation of human intestinal and liver
635 organoids upon ectopic transplantation into adult tissue sites away from their organs of origin,
636 such as to kidney capsule and brain respectively when compared with *in vitro* cultured organoids
637 (Finkbeiner et al., 2015; Takebe et al., 2013). Another possibility is that this transplantation-
638 induced maturation could be due to the direct contact of immature organoid cell types with an
639 adult environment. For instance, adult stem cell niche factors, present in the *in vivo* tissues, may
640 play a role during the maturation process (section 4.2).

641   *4.2 ADULT STEM CELL NICHE FACTORS AS POTENTIAL MATURATION*
642 *FACTORS.* Adult stem cells (also called tissue stem cells) reside in a tissue-specific
643 environment, called the stem cell niche. A stem cell niche maintains the self-renewal of adult
644 stem cells and is comprised of ECM, soluble factors, and in some cases niche support cells
645 (Rojas-Rios and Gonzalez-Reyes, 2014). Organoids have been derived from adult stem cell-
646 containing tissues, such as the intestinal crypts (Sato et al., 2009), the colonic crypts (Sato et al.,
647 2011), the gastric glands (Barker et al., 2010; Bartfeld et al., 2015), the biliary ducts (Huch et al.,
648 2013b), and the pancreatic ducts (Boj et al., 2015; Huch et al., 2013a). To demonstrate the self-
649 renewal and multipotency of adult stem cells, organoids have also been clonally derived from
650 single proliferative adult stem cells isolated from a number of mouse and human organs and
651 tissues including the small intestines (Sato et al., 2009), the colons (Sato et al., 2011), the gastric
652 pyloric glands (Barker et al., 2010), the gastric corpus tissues (Bartfeld et al., 2015), the
653 pancreatic ducts (Huch et al., 2013a), the biliary ducts (Huch et al., 2013b), the liver (Huch et al.,
654 2015), the lungs (Kumar et al., 2011; McQualter et al., 2010), the prostate gland (Chua et al.,
655 2014; Karthaus et al., 2014), the mammary glands (Jamieson et al., 2016; Shackleton et al.,
656 2006), the salivary glands (Nanduri et al., 2014), the retina (Tropepe et al., 2000), and the
657 fallopian tubes (Kessler et al., 2015). Adult stem cell-derived organoid structures were formed
658 either via the curling up of adult stem cell-containing tissue fragments or via cell division of
659 single stem cells into closed cyst-like structures. Most of the clonally derived adult stem cell
660 organoids were embedded within laminin-rich Matrigel and comprised only of epithelial cells
661 without the presence of stromal and mesenchymal cell types. In rare cases when intestinal tissue
662 fragments that contained stroma were cultured, the derived organoids would consist of polarized
663 epithelial cells surrounded by myofibroblasts and the epithelial compartment developed both
664 crypt-like structures and villus-like protrusions into the lumens (Ootani et al., 2009). Adult stem
665 cell-derived gastric organoids showed higher expression of differentiated markers for glandular
666 cell types such as mucous neck, chief or endocrine cells (*Muc6*, *Pgc*, *Gif*, *Chga*, *Chgb*, *Sst*, and
667 *Gast*) (Fernandez Vallone et al., 2016). Transcriptomes of organoids derived from adult stem
668 cells clustered with their corresponding adult cells based on gene expression profiling studies.

669 While global gene expression profiling by RNA sequencing revealed that human gastric
670 organoids derived from human ESCs/iPSCs resembled human fetal instead of adult stomach
671 tissues (McCracken et al., 2014). There is no single study to our knowledge that has directly
672 compared the transcriptomes of organoids derived from human ESCs/iPSCs and adult stem cells.
673   Adult stem cell-derived organoids are cultured in conditions that attempt to mimic the
674 paracrine signals and the ECM components provided by their corresponding stem cell niches.
675 Identification of these culturing parameters and conditions may prove beneficial in identifying
676 cues to control the expansion and maturation of organoids derived from human ESCs and iPSCs.
677 The commonly used niche factors in mouse intestinal organoid cultures are EGF, Noggin, and R-
678 spondin 1 (abbreviated as ENR condition). ENR condition was sufficient to maintain mouse
679 intestinal stem cell self-renewal and promoted differentiation to all intestinal epithelial lineages.
680 To further promote self-renewal and suppress differentiation of stem cells, two small molecules,
681 a glycogen synthase kinase 3β inhibitor CHIR 99021 and a histone deacetylase inhibitor valproic
682 acid, have been employed (Yin et al., 2014). Under this condition, morphology of organoids
683 became homogenous and formed elongated crypt (budding) structures. Intestinal stem cells
684 (Lgr5-GFP) were also found throughout the organoids. In contrast, manipulation of Wnt and
685 Notch signaling directed differentiation of organoids into specific lineages such as enterocytes,
686 goblet cells or Paneth cells (Yin et al., 2014).
687   Other types of adult stem cell organoids required not only the ENR condition but also
688 additional factors for self-renewal and growth. Some of these additional factors may suppress
689 differentiation. For example, on top of ENR, mouse colon organoid cultures required exogenous
690 Wnt3A (Sato et al., 2011); human intestinal and colon organoid cultures Wnt3A, gastrin,
691 nicotinamide, a TGFβ inhibitor A-83-01, and a p38 inhibitor SB202190 (Sato et al., 2011);
692 mouse gastric organoid cultures Wnt3A and FGF10 (Barker et al., 2010); and human gastric
693 organoids Wnt, gastrin, FGF10, and A-83-01 (Bartfeld et al., 2015). To induce or control the rate
694 of differentiation, some of the niche factors were removed or their concentrations adjusted.
695 Mouse colon organoids and human intestinal and colon organoids under growth conditions did
696 not produce differentiated cells. To induce differentiation, Wnt3A, A-83-01, and SB202190
697 needed to be withdrawn (Sato et al., 2011). Also, mouse gastric organoids under growth
698 condition expressed gastric epithelial markers but lacked markers of pit and enteroendocrine
699 lineages (Barker et al., 2010). Reduction in Wnt3A concentration resulted in the formation of pit
700 cells, mucus neck cells, and enteroendocrine cells. Differentiation of human gastric organoids
701 could be controlled by addition of nicotinamide for gland-type organoids and withdrawal of Wnt
702 for pit-type organoids (Bartfeld et al., 2015).
703   In the normal adult liver, stem cells have been identified in the pericentral (Wang et al.,
704 2015) and periportal regions (Miyajima et al., 2014). These stem cells can give rise to both
705 hepatocytes and biliary epithelial cells (cholangiocytes). EPCAM is a marker for these liver stem
706 cells (Schmelzer et al., 2007). EPCAM^{+} cells from bile ducts of adult human liver produced
707 organoids in the presence of EGF, R-spondin 1, FGF10, nicotinamide, HGF, a TGFβ inhibitor A-
708 83-01, and a cAMP pathway agonist Forskolin (Huch et al., 2015). *In vitro* hepatocyte
709 differentiation of the organoids required BMP7 treatment followed by the withdrawal of R-
710 spondin 1, FGF10, nicotinamide, and Forskolin and the addition of FGF19, DAPT, and
711 dexamethasone. The resulting cells exhibited hepatocyte morphology, expressed high levels of
712 hepatocyte markers and performed liver functions, such as albumin secretion, CYP3A4 activity,
713 LDL uptake, glycogen storage, bile acid salt secretion, and ammonia detoxification. Upon
714 transplantation, human liver organoid cells also became hepatocyte-like cells and produced

715 human albumin, though the level was lower than that produced by transplanted primary human
716 hepatocytes.

717   Pancreatic ductal organoids have been generated from duct fragments or ductal cells of
718 normal mouse pancreas using ENR, FGF10, nicotinamide, and gastrin; human pancreatic
719 organoids additionally required Wnt3A, A-83-01, and prostaglandin E2 (Boj et al., 2015; Huch
720 et al., 2013a). These organoids exhibited budding structures with an enriched ductal cell
721 population, but they were devoid of acinar and endocrine lineages (Boj et al., 2015). Following
722 orthotopic transplantation into the tail region of the mouse pancreas, both mouse and human
723 organoids developed ductal structures evidenced by the expression of CK19, but the presence of
724 other lineages was not reported (Boj et al., 2015).

725   Regeneration of ectoderm-derived tissues has enormous value in treatments of
726 degenerative diseases such as age-related macular degeneration and Alzheimer's disease. By
727 colony-forming assay, retinal stem cells were identified from the ciliary margin in the adult
728 mouse eye (Tropepe et al., 2000). These cells were pigmented cells that could clonally
729 proliferate and give rise to spheres *in vitro*. FGF2 signaling promoted their proliferation and
730 colony forming ability. A single pigmented cell from the ciliary margin generated in 7 days a
731 large sphere consisting of 13,000 pigmented and non-pigmented cells. They proliferated as
732 spheres and acquired the expression of CHX10 and nestin. Under differentiation conditions, the
733 cells in spheres differentiated into MAP2-expressing neuronal cells and GFAP-expressing glial
734 cells. Some undifferentiated nestin-positive cells remained in the center of spheres. When the
735 cells from spheres were cultured in 2D under the same differentiation condition, they became
736 flattened pigmented cells without neural markers.

737   Collectively, the use of adult stem cells in organoid generation has been mostly from
738 endoderm-derived epithelial tissues (intestine, colon, stomach, liver, pancreas, lung, and
739 prostate), a few ectoderm-derived epithelial tissues (mammary gland, retina, and salivary gland),
740 and mesoderm-derived epithelial tissue (fallopian tubes) (summarized in Table 2). There is no
741 current attempt to bridge our understanding on the culturing conditions for adult stem cell-
742 derived and ESC- and iPSC-derived organoid cultures. Owing to the functions of stem cell
743 niches in controlling proliferation and differentiation of adult stem cells, conditions mimicking
744 the paracrine signals and the ECM components provided by their corresponding stem cell niches
745 would be ideal for adult stem cell-derived organoids. However, only the design of intestinal
746 organoid cultures was based on its niche. Other types of organoid cultures were developed by
747 modifying the existing intestinal organoid conditions. Essential niche factors for other organ and
748 tissue systems are not well characterized.

749   *4.3 EXPLORING DEVELOPMENTAL CUES IDENTIFIED DURING NORMAL*
750 *FETAL TO ADULT TRANSITION.* Recent studies on a number of human fetal tissues suggest
751 the existence of fetal stem cells, which display distinct features and differentiation potentials
752 from adult stem cells. For instance, stem cell populations have been identified and isolated from
753 a number of human fetal tissues like neuroretina (Zhou et al., 2015), kidney (Da Sacco et al.,
754 2017), heart muscle (Leung et al., 2015), skeletal muscle (Alexander et al., 2016), pancreas
755 (Bonfanti et al., 2015), intestine (Fernandez Vallone et al., 2016), and brain (Kallur et al., 2006;
756 Kim et al., 2006), which are proliferative, self-renewing, and maintain tissue specific gene
757 signatures when cultured *in vitro*. For instance, fetal enterospheres can be established from
758 proliferative progenitors derived from human fetal intestinal tissues at gestational week 10 and
759 the correspondingly-aged mouse fetal intestinal tissues at embryonic day 16.5 (Fordham et al.,
760 2013). These fetal enterospheres could be passaged for an extended period of time (up to 2

months for human and 2 years for mouse), were cultured in specific medium conditions distinct from adult organoids (ENR condition with the addition of PGE2 for human and ENR medium alone for mouse), and exhibited a smooth spheroid morphology in contrast to those organoids derived from Lgr5+ adult stem cells, which had budding protrusions on their surfaces. Apart from morphology, there are also distinct gene signatures that define adult stem cells and fetal progenitors. For instance, *LGR5* specifically labeled adult stem cells in the intestine and the stomach (Barker et al., 2010; Huch et al., 2013b; Sato et al., 2009) but was expressed at relatively low levels in fetal tissues (Fernandez Vallone et al., 2016). Instead, fetal tissues were characterized by high expression of genes such as *TROP2* and *TNFRSF19* (Fernandez Vallone et al., 2016). So far, the differences found in organoid morphologies, culturing requirements, and gene expression profiles have been mainly characterized in adult and fetal intestines. Further investigation needs to be performed on other developing and adult organ systems to validate the generality of this phenomenon. These results so far support the notion that immature proliferative progenitors exist in mammalian fetal tissues and are developmentally distinct from adult stem cells in terms of the organoid structures they derive, their gene expression profiles, and their signaling requirements.

Studying the natural fetal to adult transition of embryonic tissues bears enormous implications on how to promote terminal differentiation of ESC- and iPSC-derived tissue-specific cell types, which very often suffer from an insufficient degree of cellular maturation. It is therefore imperative to promote studies in mammalian species to understand the signaling events and gene expression dynamics during the normal developmental transition of fetal tissues towards adulthood. Studies focusing on analyzing the stage-wise morphological and transcriptomic changes of fetal and perinatal tissues are particularly useful in identifying tissue-specific and functionally-relevant gene markers to label and isolate stem cell and progenitor populations from differentiating human ESCs and iPSCs. By performing a three-way comparison between the transcriptomes of human intestinal organoids derived from pluripotent stem cells and human fetal and adult intestinal tissues, Finkbeiner et al. identified *OLFM4* as a marker for intestinal tissue maturation, with enhanced specificity compared to the traditional adult stem cell marker *LGR5* (Finkbeiner et al., 2015). In another study, microarray datasets of developing mouse heart tissues were collected and their transcriptomes were analyzed to reconstruct a gene regulatory network involved in heart maturation (Uosaki et al., 2015). Pathway analyses were employed to identify key pathways and upstream regulators activated at subsequent embryonic and adult stages. For example, the authors identified that the peroxisome proliferator-activated receptor (PPAR) pathway became increasingly active during subsequent developmental stages in the heart and therefore correlated to cardiac tissue maturation. In a similar study, matched human fetal atrial and ventricular heart tissues at first and second trimester stages corresponding to 7, 15, and 20 weeks of gestation were analyzed using microarrays (van den Berg et al., 2015). Ventricular- and atrial-specific gene signatures were identified. Interestingly, genes encoding histone H1 variants such as *HIST1H3I*, *HIST1H2BM*, and *HIST1H2A1*, and those for the ECM collagens such as *COL1A2*, *COL2A1*, and *COL15A1* were downregulated and upregulated respectively in second trimester heart tissues as compared to those from the first trimester, suggestive of a developmental change in gene expression pattern during human heart tissue maturation. In order to translate these transcriptomic studies into useful information for *in vitro* organoid cultures, gain- and loss-of-function experiments can be carried out in animals to identify key molecules and pathways regulating the maturation of specific tissues.

806 ***4.4 CURRENT ATTEMPTS ON PROMOTING ORGANOID MATURATION.*** A
807 number of studies have attempted to promote *in vitro* maturation in human organoids. One
808 approach aims to generate reporter lines that allow isolation of the rare adult stem cell
809 populations from differentiated human ESC and iPSC cultures. Using zinc finger genome editing
810 technology, Forster et al. engineered a GFP reporter insertion into the endogenous locus of the
811 intestinal adult stem cell marker *LGR5* (Forster et al., 2014). By flow cytometry-assisted sorting
812 of teratoma tissues derived from this knock-in human iPSC line, the authors were able to isolate
813 LGR5$^+$ progenitors that could form organoids similar in culturing conditions and differentiation
814 potentials to intestinal organoids derived from LGR5$^+$ adult stem cells. They additionally found
815 that maintenance of these adult stem cell-like derived organoids required both WNT and Notch
816 signaling. A second approach aims to identify conditions for maturation either empirically or by
817 inference from culturing conditions of adult stem cells. For instance, it was found that maturation
818 medium containing T3 hormone significantly promoted the maturation of *in vitro* human ESC-
819 and iPSC-derived cardiomyocytes, which corresponded to second trimester human heart tissues
820 (van den Berg et al., 2015). The maturation status of those cardiomyocytes not treated with the
821 maturation medium remained at first trimester. One key feature of primate cerebral cortex is the
822 presence of gyrification. Human cerebral organoids generated so far lack cortical folding. A
823 recent study suggests that enhanced AKT signaling caused by PTEN mutation promotes
824 formation of folds in human cerebral organoids (Li et al., 2017). It is unknown whether *in vitro*
825 activation of AKT pathway may promote maturation of cerebral cortical organoids. Many adult
826 stem cell organoid markers are WNT targets, suggesting that adult organoids likely have high
827 WNT signaling and activation of WNT signaling could promote fetal-to-adult transition of fetal
828 organoids. Indeed, it has been shown that inclusion of Wnt3A ligand in cultures for fetal
829 progenitor derived enterospheres could promote their maturation into adult stem cell-like derived
830 organoids (Fordham et al., 2013). Some other studies have started to exploit whether ECM and
831 bioengineered scaffolds may provide cues for maturation. For instance, salivary gland spheres
832 were expanded in Matrigel and then differentiated into organoids in a mixture of Matrigel and
833 collagen (Nanduri et al., 2014). Another recent study reports improved maturation and
834 engraftment of lung organoids by using a microporous poly(lactide-co-glycolide) scaffold (Dye
835 et al., 2016). These experiments strongly suggest that, to a certain extent, maturation of
836 organoids is achievable *in vitro*.
837
838 **5 BIOMEDICAL APPLICATIONS AND PERSPECTIVES OF NEXT GENERATION**
839 **HUMAN ORGANOIDS**
840   A number of excellent reviews have recently been published on the usage of organoids in
841 pharmaceutical and clinical research. Here, we aim to highlight the potential improvements on
842 organoid synthesis that our review discusses and propose studies that will benefit a range of
843 biomedical applications including disease modeling, drug discovery, and tissue transplantation.
844   ***5.1 MIMICKING THE COMPLEX CELLULAR HETEROGENEITY OF***
845 ***UNDERSTUDIED ORGANS AND TISSUES.*** So far, most of our discussion has been limited
846 to internal organs and brain tissues. Organoids or 3D cultures for many other tissues, especially
847 those that involved the incorporation of ectoderm-derived cell types, such as inner ears, cranial
848 ganglia, skin, limbs, and eyes, have been less commonly attempted. The major difficulty
849 associated with synthesizing organoids for these tissues is the lack of robust differentiation
850 approaches for the co-induction of specific progenitors in the right proportions from the same or
851 different germ layers. For instance, the mammalian ear, which includes the outer, middle, and

inner ear, is derived from cell types generated from all three germ layers. Defects in any of the cell components could lead to conductive or sensory hearing loss. Another example is the ganglionic structures that develop in the head region, such as the trigeminal and vestibulocochlear ganglia, the latter of which contributes to the inner ear. Disorders like sensory or age-related hearing loss and migraine arise from malfunctions of these ganglia. These ganglia are composed of sensory neurons, which are derived from cranial placodes, and glial cell types, from neural crest. Both cranial placodes and neural crest are ectoderm derived but their induction regimes in ESCs and iPSCs differ dramatically (Dincer et al., 2013; Leung et al., 2013; Leung et al., 2016; Menendez et al., 2011; Mica et al., 2013). Neurovascular networks for internal organs are composed of autonomic neurons, endothelial cells, and vascular smooth muscle cells. The introduction of an intact neurovascular network into organoids for internal organs will allow more precise disease modeling, for instance, for Hirschsprung disease (Workman et al., 2017) or other genetic disorders with a disrupted neurovascular development. A recent study found that proper differentiation of hESC-derived autonomic neurons was dependent on contacts with endothelial cells and vascular smooth muscle cells and that the co-cultures of all three cell types led to the development of an organized neurovascular network (Acevedo et al., 2015). This again demonstrates that physiologically relevant progenitors can self-organize to reform endogenous tissue-like structures *in vitro*.

  5.2 ENHANCING SAFETY, EFFICACY, AND COST-EFFECTIVENESS OF DRUG TESTING. Drug development is an expensive (1.78 billion US dollars for a new molecular entity) and lengthy (average 13.5 years) process (Paul et al., 2010). The biggest challenge facing the pharmaceutical industry is the high attrition rate in drug development. The product failing rates in the United States, Europe, and Japan between 1990 and 2004 had drastically increased, approximately 75% at preclinical phase, 70% at phase I, 55% at both phase II and phase III (Ledford, 2011). A recent study analyzing drug attrition from four major pharmaceutical companies shows that drug toxicity was the primary cause of attrition at preclinical phase and clinical phase I (Waring et al., 2015). The likelihood of final approval counting from the start of clinical phase I was only about 10% (Hay et al., 2014; Kola and Landis, 2004). The main causes of attrition were lack of efficacy and safety, each contributing to approximately 30% of failures (Kola and Landis, 2004). During 2007-2010, 66% of phase III submission failures were attributable to lack of efficacy, particularly in the therapeutic areas of oncology and neurodegeneration, and 21% of failures were caused by safety issues (Arrowsmith, 2011). Moreover, drug withdrawal from the market due to toxicology has a remarkable impact on the pharmaceutical industry and also on patients. Adverse drug reactions are serious problems and considerably increase morbidity, mortality, and health care costs (Pirmohamed et al., 2004). Drug-induced liver injury was the most common reason for the withdrawal of clinical drugs from the worldwide market during 1953-2013 (18% of all withdrawals) (Onakpoya et al., 2016). Another study reported the top three reasons for the post-marketing withdrawal in the EU during 2002-2011 were cardiovascular toxicity, hepatotoxicity, and neurotoxicity (McNaughton et al., 2014). To improve R&D productivity and quality of health care, obtaining human proof-of-concept data early in drug development and identifying risks at the preclinical phase are crucial.

  An ideal *in vitro* model for preclinical drug testing should mimic human pathophysiology to promote accurate prediction of drug efficacy and toxicity. Next generation human cell-derived organoids generated by the incorporation of the correct cellular heterogeneity, maturation factors, and designer matrices would bear a closer resemblance to the *in vivo* tissues and organs than conventional 2D cultures and 3D organoids. They would have close to native cellular

898 composition and could better predict drug efficacy and toxicity at a tissue or organ level. Also
899 many drug-induced injuries are not caused by direct effects on parenchymal cells but rather
900 caused by immune responses or non-specific systemic effects from non-parenchymal cell types.
901 For instance, some compounds show liver toxicity *in vivo* but are not toxic to hepatocytes or only
902 at extremely high concentrations, indicating that *in vitro* testing using only hepatocytes is not
903 adequate to predict hepatotoxicity (Godoy et al., 2013). Non-parenchymal cells in the liver, such
904 as Kupffer cells and hepatic stellate cells, may contribute to inflammation during drug-induced
905 liver injury (Godoy et al., 2013). Liver organoids with Kupffer cells or hepatic stellate cells
906 would predict such hepatotoxicity more accurately than those with primary human hepatocytes,
907 which is the current gold standard for *in vitro* drug testing. Hepatic stellate cells also contribute
908 to the development of liver fibrosis. Recently human hepatic organoids derived from HepaRG
909 and primary human hepatic stellate cells have been used to evaluate drug-induced liver fibrosis
910 (Leite et al., 2016). Liver organoids derived from human ESCs/iPSCs with normal genotypes
911 would be superior to HepaRG, which is a human hepatocellular carcinoma-derived hepatic
912 progenitor cell line. Organoids derived from human iPSCs of different genetic backgrounds
913 would help develop personalized toxicology, which will pave the way to precision medicine. A
914 study successfully reproduced individual differences in drug metabolism capacity and
915 responsiveness by using a panel of human iPSC-derived hepatocyte-like cells (Takayama et al.,
916 2014). Liver organoids with bile duct-like structures formed by including cholangiocytes would
917 predict drug-induced cholestasis. 3D hepatocyte spheroids derived from human ESCs and iPSCs
918 exhibited increased expression of drug metabolizing enzymes and transporters and increased
919 sensitivity to tested drug compounds, compared to traditional hepatocellular carcinoma-derived
920 cell lines like HepG2 cells (Takayama et al., 2013). Similarly, neurotoxicity has contributed to
921 attrition of drug candidates. A recent study by (Schwartz et al., 2015) has constructed neural
922 organoids using multi-lineage progenitors to mimic the normal composition of cell types during
923 brain development. The system has successfully predicted known toxins in blind screens. Due to
924 the technical difficulty in establishing and maintaining organoid cultures, 2D cell models still
925 dominate the early phases of drug development, such as the lead generation phase. However, due
926 to their enhanced ability to mimic human physiology, organoid cultures are especially valuable
927 in lead optimization and preclinical development.
928   Increasing demands from patient advocacy groups and the anticipation of changing
929 government policies to introduce new competition among pharmaceutical companies have
930 continuously added tremendous pressure to reduce drug prices and a demand to enhance cost
931 effectiveness in the drug development pipeline. Stem cell differentiation relies heavily on growth
932 factors, which are expensive and short-lived. 2D differentiation allows easy access of growth
933 factors to cultured cells and, in many cases, allowed close to homogeneous induction of
934 progenitor cell types from human ESCs and iPSCs. Coupling an initial 2D differentiation
935 protocol followed by organoid formation would enhance cost-effectiveness. On the other hand,
936 small molecules have been developed to replace growth factors. They are smaller in molecular
937 mass, cheaper and more stable than growth factors. A study testing small molecules for replacing
938 growth factors used in hepatic differentiation of hESCs has shown that the cost could be reduced
939 by 67% (Tasnim et al., 2015). Small molecules will have better ability to penetrate organoid
940 structures compared to growth factors. Therefore, they are better suited to be employed at later
941 stages of organoid cultures.
942   *5.3 FACILITATING THE SETUP OF VARIOUS BIOENGINEERING*
943 *APPROACHES.* In static cultures, cells in organoids do not experience mechanical signals as

they do in the body. Dynamic (perfusion) cultures can provide the necessary fluid shear stress and other forces. They also can facilitate the efficient delivery of nutrients and oxygen. Perfusion bioreactors, which allow cultures in much larger scales than microfluidic devices, have been used in tissue engineering for bones (Gaspar et al., 2012) and intestinal tissues (Kim et al., 2007). A recent study reports human liver organoids formed with diameters up to a few millimeters from the self-organization of a conglomerate of genetically engineered adult hepatocytes, liver sinusoidal endothelial cells, and mesenchymal stem cells in a perfusion bioreactor (Ramachandran et al., 2015), suggesting that the principle of self-organization might be applicable to adult cell populations. On the other hand, microfluidic devices have also been used for perfusion cultures. Organ-on-a-chip is a microfluidic cell culture device with hollow microchannels onto which cells are cultured and through which medium is flowed (Bhatia and Ingber, 2014). Organ-on-a-chip devices can be used to culture one or more types of cells simultaneously and to mimic pathophysiological conditions at different levels, such as at the tissue/organ level, where cells from the same tissues and organs are cultured on a chip, or at organism level, where cells from different tissues and organs are used (Huh et al., 2011). Organ-on-a-chip and human-on-a-chip approaches enable high throughput assays. By using an organ-on-a-chip device, the organoid-based *in vitro* model can predict drug response and toxicity at an organismal level. A pioneering human-on-a-chip study has combined multiple cell types in a multi-channel 3D microfluidic cell culture system (Zhang et al., 2009). This system included cell lines representing liver, kidney, lung, and adipose tissues grown in separate channels and cultured with a common medium mimicking blood flow. Similarly, different types of organoids could be cultured in such a multi-channel microfluidic device to build a human-on-a-chip. Organoids could be formed in other culture systems and then transferred to the microfluidic device or could be formed directly in the microfluidic device. Two recent reviews have described the potential of organoid-on-a-chip in biomedical research and applications (Konar et al., 2016; Skardal et al., 2016). Microfabrication can produce topological patterns that are particularly useful for the study of neuronal behavior. Jeong, et al. have created a deep hemicylindrical, microchannel-networked, concave array system for the formation of nerve-like networks (Jeong et al., 2015). Rat embryonic neural progenitor cells self-aggregated into host neurospheroids in concave microwells and satellite neurospheroids in deep hemicylindrical channels. Neurites grew along channels and were bridged by satellite neurospheroids to connect host neurospheroids together. The neural network was shown to transmit signals from one neurospheroid to another. Taken together, other components of our approach, such as the use of empirically tested design matrices to control the microenvironments and the administration of maturation factors specific for different organs and tissues, should also be considered in these systems.

5.4 MEETING THE DEMANDS FOR HUMAN TISSUES FOR TRANSPLANTATION. Demand for raw tissue materials for transplantation calls for a supply of *in vitro* derived, xeno-free, and high-quality human tissues. For instance, for end-stage organ failure, such as liver and heart failure, organ transplantation is the only available treatment. Renal replacement therapy can treat end-stage renal diseases, but kidney transplantation represents the best treatment both for patients' quality of life and cost-effectiveness. Over 4,500 people in Canada (2015 data, https://www.cihi.ca/en/types-of-care/specialized-services/organ-replacements/e-statistics-on-organ-transplants-waiting), 120,000 people in the U.S. (2015 data, www.organdonor.gov), 86,000 in the European Unions plus Iceland, Norway, and Turkey (December 2013 data, http://ec.europa.eu/health//sites/health/files/blood_tissues_organs/docs/ev_20141126_factsfigure

s_en.pdf), and 1.5 million in China (2007 data, http://www.ghgj.org/Living%20Organ%20Transpl.pdf) are on the waiting list for organ transplant. Most of these people need kidney transplantation (>80%), followed by liver (~10%), heart (<5%), lung (~1%), and pancreas (~1%). In the U.S., from early 1990s onwards, the gap between the number of people waiting for a transplant and the number of organ donors has continued to widen to over 7 times. Added to the demand, organ donation is still less common in many countries such as China and Japan. Apart from internal organs, there is also increasing demand for human cell and tissue materials to regrow missing bones, muscles, connective tissues, neural plexi, and skin in the face, neck, and extremities resulting from injuries in wars, motor vehicle-related accidents, burns, and natural disasters (Jalali et al., 2014; van Zuijlen et al., 2015).

There are ongoing clinical trials sponsored by the U.S. NIH to conduct transplantation trials using human ESC- and iPSC-derived cardiac progenitors and retinal pigmented epithelial cells (www.clinicaltrials.gov). Clinical data for other more highly demanded tissues for transplantation such as kidney, liver, and pancreas are urgently needed. In animal studies, xenographic transplantation into adult mouse tissues of human liver organoids generated from a composite aggregate of liver progenitors, endothelial cells, and mesenchyme stem cells has been conducted with success (Takebe et al., 2014). To extend its application, such a multi-lineage recombination method using organ-specific progenitors and vascular and mesenchymal stem cell types has been used to successfully reconstruct composite organoids for intestine, lung, kidney, heart, and brain (Takebe et al., 2015). Liver and other organoids derived from these and other studies suffer from a limitation in size, which usually ranged in the millimeter scale, thereby restricting their direct usage in transplantation. To resolve this issue, de-cellularized liver scaffolds from animals and human cadavers or cell-free scaffolds generated from 3D printing technology could be made and seeded with dissociated organ-specific precursors or directly with organoids containing parenchymal, vascular, and other supporting cell types (Collin de l'Hortet et al., 2016). Together with physiologically relevant culturing methods and bioreactor cultures, these scaffolds could physically support the growth of larger organ structures *in vitro*. Of interest, whole organ de-cellularization has been achieved for liver and a number of other organs including heart, lung, kidney, and pancreas (Scarritt et al., 2015). One of the main challenges in this type of study is to maintain the architecture and composition of the ECM during the de-cellularization process, such as collagen, laminin, elastin, and fibronectin, and biologically-active molecules, such as growth factors. Synthesizing cell-free scaffolds with xeno-free designer matrices and 3D printing technology is an alternative to using de-cellularized organ scaffolds. A few studies have attempted to re-cellularize lung and kidney scaffolds derived from rhesus monkey's organs with undifferentiated human ESCs (Scarritt et al., 2015). The resultant chimeric organs contain ESCs expressing non-specific markers. Differentiated ESC- and iPSC-derived organ progenitors can instead be used for re-cellularization to promote tissue-specific differentiation. For advanced clinical studies, patient-specific iPSCs derived with xeno-free methods should be used to respond to ethical concerns regarding preservation of human embryos and to reduce chances of immune rejection upon transplantation.

6 CONCLUSION

In this review, we have highlighted new perspectives and directions that have been initiated in recent organoid studies. The new development will act as a catalyst for the organoid field and provide new directions to other fields, particularly for developmental biology, embryonic stem cell studies, and adult stem cell biology.

1036　　　　By applying the principle of reverse engineering, we propose a new pipeline for human
1037　organoid generation that potentially can greatly enhance the efficiency of generation, the
1038　physiological relevance, and the functionality of *in vitro* derived human organoids. By exploiting
1039　the self-organizing property of embryonic progenitors, the dissociation-aggregation approach
1040　allows the generation of organoids with enhanced native cellular composition as well as more
1041　flexible experimental designs and multi-stage quality controls as progenitor raw materials can be
1042　generated in batches, cryopreserved, thawed, and expanded before organoid formation. Designer
1043　matrix, whether it is tailor-made, native or a composite of both, helps construct the native micro-
1044　and matrix environment organoid cell types encountered during aggregation, self-organization,
1045　and differentiation. Lastly, a rigorous maturation schematic for different organoid systems
1046　permits the generation of fully-functional and terminally-differentiated cell types from human
1047　organoids that can be used in a wider range of applications and more closely mimic normal
1048　human physiology.

1049　　　　To significantly advance human organoid synthesis technology for various applications,
1050　there is an urgency to synthesize knowledge and technologies developed in different fields
1051　including stem cell biology, developmental biology, matrix biology, systems biology,
1052　bioengineering, material science, biostatistics, and bioinformatics. Such endeavors have been
1053　made in various fronts and have to be continuously encouraged by government institutions,
1054　sponsoring institutions, and other private funding agencies. Continuous education of the public
1055　and communications among scientists, the public media, and the public are also paramount in
1056　reducing unwarranted skepticism for using stem cell-derived cell types and tissues for basic
1057　research and biomedical applications. Various scientific fields, when proposing research, on the
1058　other hand, should also avoid over-optimism and fairly evaluate the beneficial effects of stem
1059　cell products.

1070 **CONFLICT OF INTEREST**
1071 The authors declare no conflict of interest.

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1702 **Figure Captions**
1703 **Figure 1.** A novel pipeline for developing future protocols to generate organoids.
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1705 **Figure 2.** Increased control on the timing of progenitor incorporation into organoid structures.
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1707 **Figure 3.** Dissection of the self-organization process.

1708 **Table 1 – Summary of pioneered animal studies for organ progenitor aggregation**

Germ layer	Tissues	Species	Embryonic stage	Length of culture (days)	Reference
Ectoderm	Inner ear	Gallus gallus			(Orr, 1968)
	Inner ear	Mus musculus	E13-E14	6	(Bianchi et al., 2002)
	Cerebral cortex, brainstem spinal cord	Mus musculus			(Crain and Bornstein, 1972)
	Retina	Gallus gallus			(Layer et al., 2001)
	Submandibular gland	Mus musculus	E13-E17	4	(Wei et al., 2007)
Mesoderm	Kidney	Mus musculus	E11.5	21	(Benedetti et al., 2016; Xinaris et al., 2012)
	Heart	Gallus gallus	2.5, 4 or 7d		(McDonald and Sachs, 1975)
		Gallus gallus, Rat	50h, 12d		(Nag et al., 1980)
Endoderm	Pancreas	Mus musculus	E10.5	14	(Greggio et al., 2013; Greggio et al., 2014)

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1710 **Table 2. Adult stem cell-derived organoids**

Germ layer	Tissue	Species	Niche factors for expansion	Matrix	Conditions for *in vitro* differentiation	Reference
Endoderm	Intestine	Mouse	EGF, Noggin, R-spondin 1, Y-27632 (for single cell culture)	Matrigel	Same as expansion condition	(Sato et al., 2009)
Endoderm	Intestine	Human	EGF, Noggin, R-spondin 1, Wnt3A, gastrin, nicotinamide, A-83-01, SB202190	Matrigel	Withdrawal of Wnt3A, nicotinamide and SB202190	(Sato et al., 2011)
Endoderm	Colon	Mouse	EGF, Noggin, R-spondin 1, Wnt3A, Y-27632 (for single cell culture in the first 2 days)	Matrigel	Withdrawal of Wnt3A	(Sato et al., 2011)
Endoderm	Colon	Human	EGF, Noggin, R-spondin 1, Wnt3A, gastrin, nicotinamide, A-83-01, SB202190	Matrigel	Withdrawal of Wnt3A, nicotinamide and SB202190	(Sato et al., 2011)
Endoderm	Stomach	Mouse	EGF, Noggin, R-spondin 1, Wnt3A, gastrin, FGF10, Y-27632 (for single cell culture in the first 2 days),	Matrigel	Wnt3A reduction	(Barker et al., 2010)
Endoderm	Stomach	Human	EGF, Noggin conditioned medium, R-spondin 1 conditioned medium, Wnt conditioned medium, gastrin, FGF10, A-83-01, nicotinamide (for single cell culture), Y-27632 (for single cell culture)	Matrigel	Nicotinamide for gland-type organoids; Withdrawal of Wnt for pit-type organoids	(Bartfeld et al., 2015)
Endoderm	Liver	Mouse	Gastrin, EGF, R-spondin 1, FGF10, nicotinamide, HGF, Y-27632 (for single cell culture in the first 4 days), Noggin and Wnt3a (for the first 4 days)	Matrigel	Withdrawal of R-spondin 1, HGF and nicotinamide and addition of EGF, FGF10, A-83-01, DATP and dexamethasone	(Huch et al., 2013b)
Endoderm	Liver	Human	Gastrin, EGF, R-spondin 1 conditioned medium, FGF10, nicotinamide, HGF, A-83-01, Forskolin (a cAMP pathway agonist); For the first 3 days also including Y-27632, Noggin and Wnt conditioned medium	Matrigel	First BMP7 for 2-4 days and then the withdrawal of N-acetylcysteine, R-spondin 1, FGF10, nicotinamide and Forskolin and addition of FGF19, DAPT and dexamethasone	(Huch et al., 2015)
Endoderm	Pancreas	Mouse	Gastrin, EGF, R-spondin 1, Noggin, FGF10, nicotinamide, Y-27632 (for single cell culture in the first 4 days)	Matrigel	In suspension 0.2% FBS, Activin A for 3 days, then with indolactam-V, FGF10, 2% FBS for 4-5 days, and then with B27, Noggin, retinoic	(Huch et al., 2013a)

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					acid, KAAD-cyclopamine for 6 days, finally with B27 and DBZ for 2-4 days before *in vivo* transplantation in kidney capsule	
Endoderm	Pancreas	Mouse	Gastrin, EGF, R-spondin 1, Noggin, FGF10, nicotinamide, Y-27632 (for single cell culture in the first 4 days)	Matrigel		(Boj et al., 2015)
Endoderm	Pancreas	Human	EGF, R-spondin 1 conditioned medium, Wnt3a conditioned medium, Noggin conditioned medium, gastrin, FGF10, nicotinamide, A-83-01, prostaglandin E2	Matrigel		(Boj et al., 2015)
Endoderm	Lung	Human	10% FCS, hydrocortisone, insulin, transferrin, triiodothyronine, choleragen, adenine, EGF	3T3-J2 feeder	Self-assembly or Matrigel with retinoic acid, cholera toxin, knockout serum replacement	(Kumar et al., 2011)
Endoderm	Lung	Mouse	Insulin, transferrin, cholera toxin, EGF, bovine pituitary extract, 5% FBS, retinoic acid	Matrigel at air-liquid interface	Coculture with primary PDGFRα$^+$ lung stromal cells	(Barkauskas et al., 2013)
Endoderm	Prostate	Mouse/human	EGF, Y-27632, 5% Matrigel, 5% charcoal-stripped FBS, DHT	Flotation	Same as expansion condition	(Chua et al., 2014)
Endoderm	Prostate	Mouse	EGF, Noggin, R-spondin, A-83-01, DHT	Matrigel	Same as expansion condition	(Karthaus et al., 2014)
Endoderm	Prostate	Human	EGF, Noggin, R-spondin, A-83-01, DHT, FGF10, FGF2, prostaglandin E2, nicotinamide, SB202190	Matrigel	Same as expansion condition	(Karthaus et al., 2014)
Ectoderm	Mammary gland	Mouse	Insulin, hydrocortisone, prolactin, 1% FCS	Matrigel	Same as expansion condition	(Shackleton et al., 2006)
Ectoderm	Mammary gland	Mouse	Hydrocortisone, insulin, EGF, FGF2, FGF10, Wnt3A, heparin, R-spondin 2, Y-27632	Basement membrane extract	Same as expansion condition	(Jamieson et al., 2016)
Ectoderm	Retina	Mouse	With or without FGF2	Suspension	On poly-L-ornithine-coated glass in EGF containing serum-free medium	(Tropepe et al., 2000)
Ectoderm	Salivary gland	Mouse	EGF, FGF2, insulin, dexamethasone, Y-27632	Matrigel	Intact spheres in collagen-Matrigel mixture (4:6) and	(Nanduri et al., 2014)

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| Mesoderm | Fallopian tube | Human | Wnt3A conditioned medium, R-spondin 1 conditioned medium, EGF, noggin, FGF10, nicotinamide, Y-27632, SB431542 | Matrigel | 10% FCS containing medium Same as expansion condition | (Kessler et al., 2015) |

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