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Published version

DOSH, Rasha, JORDAN-MAHY, Nikki, SAMMON, Chris and LE MAITRE, Christine (2018). Tissue engineering laboratory models of the small intestine. Tissue engineering. Part B, Reviews, 24 (2), 98-111.

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1 **Tissue Engineering Laboratory Models of the Small Intestine**

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23 **Abstract**

24 In recent years, three-dimensional (3D) cell culture models of the small intestine
25 have gained much attention. These models support cell proliferation, migration, and
26 differentiation, and encourage tissue organization which is not possible in two-
27 dimensional (2D) culture systems. Furthermore, the use of a wide variety of cell
28 culture scaffolds and support substrates have revealed considerable differences in
29 cell behavior and tissue organization. These systems have been used in combination
30 with intestinal stem cells, organoid units or human colonic adenocarcinoma cell lines
31 such as Caco-2 and HT29-MTX to generate a number of *in vitro* and *in vivo* models
32 of the intestine. Here, we review the current 2D and 3D tissue engineering models of
33 the intestine to determine the most effective sources of intestinal cells and current
34 research on support scaffolds capable of inducing the morphological architecture
35 and function of the intestinal mucosa.

36 **Keywords**

37 Stem cells, Organoid units, Tissue engineering, Caco-2 cells, HT29-MTX cells.

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42 **Introduction**

43 Until recently, *in vitro* intestinal models have been restricted to simple two-
44 dimensional (2D) cell culture on standard cell-culture plates or transwell culture
45 inserts¹. However, three-dimensional (3D) cell culture models are currently under
46 investigation by groups worldwide to determine if these 3D cell cultures can more
47 closely mimic the *in vivo* environment and support cell differentiation and 3D tissue
48 organization which is not possible in conventional 2D cell culture systems²⁻⁷. These
49 3D cell culture models have been evaluated for their use in tissue engineering and
50 drug discovery^{8,9} and used as an alternative to *in vivo* animal models in drug toxicity
51 studies¹⁰⁻¹².

52 Tissue engineering studies have promised an improved understanding of small
53 intestinal physiology, as well as the response of the small intestine to infection,
54 toxicity and new therapies¹³. Furthermore, using these systems may be possible to
55 develop personalized intestinal tissue grafts which can be used to repair the intestine,
56 whilst avoiding the risks of immune system rejection¹⁴. The most important element
57 for successful tissue engineering of the small intestine is the use of specialized
58 biomaterial scaffolds providing cells a substrate for the deposition of extracellular
59 matrix and subsequent cell adhesion^{9,15,16}. These scaffolds are often designed to
60 biodegrade after the deposition of extracellular matrix, when the cells become
61 mechanically independent^{14,17} and could be potentially used therapeutically¹⁸,
62 however matching the degradation rates to synthesis and deposition of new matrix
63 remains a key challenge in tissue engineering¹⁹⁻²¹. **This article aims to review 2D
64 and 3D cell culture systems used to culture intestinal cells, to determine whether the
65 use of 3D scaffolds can mimic the *in vivo* environment. Furthermore, recent progress
66 in establishing intestinal stem cells and organoid units *in vitro* and *in vivo*.**

67 **The architecture of the small intestine**

68 The small intestine is essentially a tube, which can be divided into four anatomically
69 and functionally distinct layers: mucosa, submucosa, muscularis externa, and
70 serosa²² (Figure 1). The mucosa is folded into villi which increases the surface area
71 and maximizes digestion and absorption. The number of villi varies, depending on
72 the position along the length of the intestine²², with the highest frequency seen in the
73 proximal jejunum, which decreases towards the ileum. Furthermore, the villi
74 morphology differs through the small intestine, decreasing in size from the proximal
75 to the distal end of the small intestine. In the duodenum, the villi are leaf-like, whilst
76 those of the jejunum and ileum having a tongue-like, and then finger-like
77 appearance, respectively. At the base of the villi are crypts of Lieberkuhn²³. These
78 crypts are tubular glands which descend into underlying muscularis mucosa²⁴ and
79 form the intestinal stem cell niche (Figure 1). This complex villi rich mucosa layer is
80 supported by the underlying submucosa, which is composed of fibrous connective
81 tissue and a rich supply of blood and lymphatic vessels and is innervated by the
82 Meissner's nervous plexus. Beneath the submucosa is the muscularis externa,
83 composed of an inner circular and outer longitudinal smooth muscle layers
84 innervated by the Auerbach's plexus, which enables the peristaltic movement of food
85 along the intestine¹⁷. This layer is finally supported by a single layer of mesothelium
86 called the serosa²² (Figure 1).

87 Intestinal epithelial cell types

88 The intestinal mucosa contains six main cell types, each with a specialized function.
89 The most abundant are the specialized columnar epithelial cells or enterocytes which
90 are highly polarized cells with tiny microvilli on their apical surface (Figure 1). These

91 enterocytes are responsible for producing of digestive enzymes and the absorption
92 of nutrients^{22,25,26}. The second most abundant cell types are unicellular glandular
93 cells known as mucus-secreting goblet cells. Mucins are secreted into the lumen of
94 the intestine by these goblet cells, giving rise to an adherent mucus layer which
95 surrounds and coats the intestinal villi²³. Located at the base of intestinal crypts are
96 paneth cells, which secrete antimicrobial lysozymes protecting the crypt from
97 pathological microorganisms^{26,27}. These paneth cells also play an essential role in
98 stem cell niche signals^{28,29}. There are also smaller populations of hormone-secreting
99 enteroendocrine cells and tuft cells which regulate digestion and absorption (Figure
100 1). Finally, microfold or M-cells are located within lymphoid peyer's patches and are
101 responsible for transporting antigens from the lumen to the underlying lymphoid
102 tissues^{25,26}.

103 Importantly, a small population of stem cells are located at the villus base within
104 crypts and are responsible for maintaining intestinal epithelial homeostasis (Figure 1).
105 These stem cells differentiate as they migrate along the length of the villi, replacing
106 cells which are lost at the villus tip. This process of cell renewal ensures that the
107 functions of the intestinal epithelium are maintained throughout life. The capability of
108 these stem cells to undergo self-renewal makes them particularly attractive for tissue
109 engineering and regenerative medicine applications²³.

110

111 **Why do we need to engineer a small intestine?**

112 The small intestine becomes dysfunctional in a number of diseases including
113 inflammatory driven pathologies (such as ulcerative colitis; Crohn's disease; celiac
114 disease), congenital diseases (such as lactose intolerance and short bowel

115 syndrome) and cancer. These can become extremely debilitating disorders
116 impacting on quality of life or even life threatening³⁰⁻³². Thus, the ability to replace
117 damaged and malfunctioning tissues with a tissue-engineered small intestine could
118 be of use in these conditions. Furthermore, engineered intestinal tissue could be
119 created using patient-specific explants; small samples of healthy tissue could be
120 collected from a patient and expanded within the laboratory. If these cells could then
121 be utilized in the generation of a tissue-engineered small intestine, this could then be
122 returned to the patient to enable intestinal repair or augmentation. Use of self-tissue
123 would avoid the requirement of tissue donors, and the need for lifelong
124 immunosuppression to prevent rejection of tissues³³.

125 **2D cell culture models of the small intestine**

126 *The use of cell lines in intestinal engineering*

127 Due to failed attempts to establish long-term primary cell culture of normal small
128 intestine, researchers have **successfully utilized** cell lines which are derived from
129 gastrointestinal tumours³⁴. The human colonic adenocarcinoma cell lines (**Figure 2**):
130 Caco-2 and HT29-MTX cells are probably the most frequently used cell lines due to
131 their ability to differentiate into enterocyte-like cells and mucus-producing goblet
132 cells, respectively³⁵⁻³⁷, **whilst these would not be suitable for tissue engineering**
133 **applications due to their cancerous nature they are excellent models for *in vitro***
134 **cultures.**

135 Caco-2 cells can spontaneously differentiate into cells with the ability to form tight
136 junctions and produce large amounts of digestive brush border enzymes, similar to
137 small bowel enterocytes³⁸⁻⁴⁰. Caco-2 cells express a number of digestive enzymes
138 including sucrase-isomaltase, lactase, peptidase, and alkaline phosphatase. The

139 expression of these enzymes are used as markers of intestinal differentiation and
140 digestive function⁴¹⁻⁴⁷. However, Caco-2 cells have tight junctions similar to those of
141 the colon, rather than the small intestine, this has led to criticism of their use as a
142 model for the epithelium of the small intestine^{48,49}. Furthermore, Caco-2 cell
143 behaviour can be affected by culture condition (serum supplemented and serum free
144 media), passage number, cell density and incubation times^{50,51} all of which make it
145 difficult to compare research findings between different studies^{52,53}.

146 Caco-2 cells are often used to mimic small intestinal enterocytes and have been
147 used extensively in absorption and transport studies of nutrients and drugs^{54,55}, for
148 example, insulin transport studies^{56,57}. Moreover, Caco-2 cells have been used to
149 investigate the cytotoxicity of acrylic-based copolymer protein as an oral insulin
150 delivery system⁵⁷. Caco-2 cells can also be utilized to verify the toxicology when
151 exposed to nanoparticles such as polystyrene, which resulted in increased level of
152 iron absorption⁵⁸.

153 HT29-MTX cells are also a commonly used cell line in intestinal modeling. These
154 cells are derived from human colonic adenocarcinoma cells and are resistant to
155 methotrexate (MTX). HT29-MTX cells are composed entirely of differentiated mucus-
156 secreting goblet cells. They maintain this differentiated phenotype in monolayer
157 culture and are used to mimic intestinal goblet cells, and are commonly co-cultured
158 with Caco-2 cells⁵⁹⁻⁶². HT29-MTX cells have been utilized in studies investigating the
159 diffusion of drugs across the mucus layer⁶³⁻⁶⁵, these have been used to test the
160 mucoadhesive and toxicity of nanoparticles as drug delivery systems⁶⁶, and to test
161 adhesion and invasion of Salmonella strains⁶⁷ (Table 1).

162 *2D co-culture studies*

163 In order to mimic the native small intestinal epithelium which is composed of diverse
164 absorptive and secretory cells a number of studies have co-cultured Caco-2 cells
165 alongside HT29-MTX cells^{63,64,68-71}. These studies have enabled the formation of a
166 Caco-2 derived enterocyte-like layer, which is interspersed with mucus secreting
167 HT29-MTX cells, and avoided the limitations and drawbacks previously seen in
168 mono-cultures⁶³. Walter *et al.*, (1996) co-cultured Caco-2 and HT29-MTX cells in cell
169 culture inserts in a transwell format, where they were shown to produce an adherent
170 mucus layer which covered the cell monolayer. The cells were shown to have
171 structures similar to microvilli, although they were of irregular shape and size⁶³. The
172 mucus layer formed by the HT29-MTX cells during co-culture with Caco-2 cells were
173 proposed to play an important role in digestion and bioavailability⁶⁸. Many studies
174 have exploited *in vitro* co-cultures of Caco-2 and HT29-MTX cells to provide a drug
175 absorption model^{63,68}, to study drug permeability^{56,64,70,71} and to improve alternative
176 *in vitro* systems for evaluation cytotoxicity of nanoparticles to replace animal testing⁷².
177 Furthermore, different co-culture ratios of Caco-2 and HT29-MTX cells have been
178 used to investigate the co-culture ratio most physiologically relevant to *in vivo*
179 situations^{52,70,73,74}.

180 Another significant aspect of co-culture is the facility to introduce additional cell types
181 to more closely mimic the native multicellular environment seen *in vivo*. Antunes *et al.*
182 *al.*, (2013), developed the triple co-culture model based on the use of Caco-2 and
183 HT29-MTX cells, incorporating Raji B lymphocytes. The Raji B lymphocytes were
184 selected to stimulate differentiation of Caco-2 cells to M-cells^{49,75,76}. This triple co-
185 culture system was used to investigate absorption of insulin, demonstrating insulin
186 permeability was greater in triple co-cultures compared to co-culture of Caco-2 and
187 Raji B cells alone⁷⁵. Moreover, *in vitro* triple co-culture model has been used for

188 polystyrene nanoparticle permeability studies that demonstrated the strong influence
189 of HT29-MTX cells and M-cells on the nanoparticle permeation. In this study, cellular
190 uptake of polystyrene nanoparticles was affected by the presence of mucus layers.
191 Where, nanoparticle transport was significantly increased in Caco-2/M cells due to a
192 lack of mucus secretion from M cells⁷⁷. Most recently, the Caco-2/HT29-MTX co-
193 culture and Caco-2/HT29-MTX/Raji B triple co-culture models have been
194 successfully used to investigate the intestinal permeability of different
195 biopharmaceutical characteristics of drugs. Where it was shown that higher
196 permeability of drugs were observed in more complex models compared with Caco-2
197 monoculture⁷⁶. Taken together, these studies demonstrated the importance of cell-
198 cell interactions which can impact on the physiological function in intestinal cells.
199 These models can also be combined with bacterial cells to mimic the microbiota
200 seen within the small intestine^{6,78,79}.

201 Whilst these 2D static culture models of intestinal cells in Transwells display a
202 number of advantages, these models fail to develop villi morphology⁸⁰. Furthermore,
203 these models fail to undergo cytodifferentiation due to lack of the 3D
204 microenvironment, including luminal flow, and fluid shear stress^{80,81}.

205 **3D cell culture models of the small intestine**

206 A major shortcoming of the research utilizing intestinal cells in 2D culture is that it
207 does not mimic the complex architecture of the small intestine and fails to mimic the
208 *in vivo* phenotype. Thus, several biomaterial scaffolds have been investigated for 3D
209 cell culture and tissue engineering of the small intestine^{15,46,82-84} (Table 2). These
210 scaffolds provide a physical structure in which cells migrate and utilize topography to
211 stimulate cell development and formation of tissue networks. Scaffold porosity is a

212 critical factor in directing cell fate within the 3D scaffold architecture. Pore size is
213 essential for the diffusion of cells inside the 3D scaffolds, pores enable cells to
214 penetrate into the matrix and provide a space for cells to reside and synthesize new
215 extracellular matrix^{10,17,19,21,82}. Accordingly, many attempts have been undertaken to
216 develop porous biomaterials such as tubular constructs with mechanical and
217 physical properties well suited to the small intestine^{4,85-89}.

218 The rate of cell growth, however, varies depending on the scaffold used^{90,91}. In 3D
219 cell culture models, the interaction between cells and the scaffold is regulated by the
220 material characteristics of the scaffold. Some materials provide natural adhesion
221 sites for cells whilst others provide a substratum for the deposition of extracellular
222 matrix which subsequently provides adhesion sites for cells¹⁶. The mechanical
223 characteristics and degradation dynamics of the scaffold are important for specific
224 tissue engineering applications^{92,93}. The mechanical properties of scaffolds control
225 the shape of cells during tissue reconstruction and provide mechanical cues to cells
226 to tailor differentiation^{17,82}, whilst also providing support for load⁹⁴. Scaffolds
227 investigated to date include natural hydrogels (e.g. collagen gels and Matrigel) and
228 synthetic scaffolds (e.g. poly-lactic-glycolic acid) which have a number of key
229 advantages and disadvantages.

230 *3D cell culture using collagen gels*

231 Type I collagen gels are commonly used for 3D culture, as they are easy to prepare,
232 inexpensive, can support a range of cell types^{95,96}, and enable encapsulation of
233 cells⁹⁷. Furthermore, pore size, rigidity, and ligand density can be adjusted by
234 changing the collagen concentration or utilizing chemical cross-links⁹⁴. Li *et al.*,
235 (2013) have used collagen gels to seed fibroblasts, Caco-2 and HT29-MTX cells.

236 This 3D triple co-culture model has been used to evaluate drug permeability and has
237 been shown to have more physiologically relevant drug absorption rates⁹⁶. Pusch *et*
238 *al.*, (2011) performed 3D co-culture of Caco-2 cells and human microvascular
239 endothelial cells (hMECs), **created** multilayers of enterocyte-like cells which
240 expressed villin, E-cadherin, and the transporter p-glycoprotein at levels that were
241 similar to that of a normal human jejunum³⁴. Whilst Viney *et al.*, (2010) co-cultured
242 intestinal epithelial cell lines (IEC6: a rat small intestinal epithelial cell line; IPI-21: a
243 small boar ileum epithelial cell line, and CRL-2102: a human epithelial cell line
244 derived from colorectal adenocarcinoma) with Rat-2 (fibroblast-like cell) in collagen
245 gels alone or in combination with Matrigel. After 20 days, optimal epithelial **cell**
246 growth was seen in collagen gels supplemented with Matrigel, where multilayered
247 intestinal epithelium were seen, which included clusters of cells similar to the
248 morphology of crypts⁹⁸. This highlighted the importance of the interaction between
249 the cell lines, extracellular matrix and other cell types such as fibroblasts; and how
250 they can impact on the cell proliferation and differentiation⁹⁹. These interactions with
251 localized cells were further demonstrated when rat intestinal sub-epithelial
252 myofibroblasts (ISEMF) were co-cultured with IEC-6 cells on a collagen gel
253 scaffold¹⁰⁰, where the myofibroblasts induced differentiation of IEC-6 intestinal cells
254 to enteroendocrine cells, which was thought to be mediated by growth factors and
255 cytokines secreted by the myofibroblasts¹⁰⁰.

256 A major shortcoming of these studies is that they do not reproduce the villus-crypt
257 architecture of the small intestine. To overcome this shortcoming, **Wang *et al.*,**
258 **(2009) investigated the effect of a biomimetic crypt-like microwell on Caco-2**
259 **phenotype. A significant positive correlation between the crypt like topography and**
260 **Caco-2 metabolic activity and migration with low level of differentiation which mimics**

261 cells in crypts of native small intestine was observed¹⁰¹. In addition, a number of
262 studies have microfabricated villus-shaped collagen scaffolds into which Caco-2 cells
263 were cultured^{82,83,102,103} (Table 2). These studies demonstrated that the culture of
264 Caco-2 cells on these prefabricated villi structures led to the formation of villi which
265 were comparable to those of human jejunum after 3 weeks in culture⁸³. However, it
266 has been observed that the transepithelial electrical resistance (TEER) of cells in
267 these villus-like structures were lower than those in cells grown on 2D flat substrate.

268

269 *Synthetic Polymer Scaffolds*

270 Synthetic scaffolds have also been studied for their ability to reconstruct the small
271 intestine. Synthetic biodegradable copolymers: poly lactic acid(PLA) and poly
272 glycolic acid (PGA) forming poly lactic glycolic acid (PLGA) have been investigated
273 for scaffold fabrication in tissue engineering of the small intestine^{10,104}. The chemical
274 properties of PLGA co-polymer permitted hydrolytic degradation of the ester bond
275 into the acidic, non-toxic monomers (PLA and PGA) which are removed by natural
276 metabolic pathways. Physical properties of PLGA have been found to be related to
277 the molecular weight of the monomers, the hydrophobic PLA/hydrophilic PGA ratio,
278 the storage temperature and the exposure time to water. Demonstrating the rate of
279 degradation negatively affected cell proliferation, with the fastest degradation rates
280 displaying the poorest viability^{19,21}.

281 In addition, Costello *et al.*, (2014) used fabricated PLGA as a porous 3D tissue
282 scaffold which mimicked the shape and size of intestinal villi. They showed that co-
283 culture of Caco-2 and HT29-MTX on PLGA resulted in proliferation and
284 differentiation of co-cultured cells. However, these Caco-2 and HT29-MTX cells were

285 differentiated under the stimulation of epidermal growth factor were added to the
286 basolateral side of scaffolds⁸² (Table 2). Although the latest procedures to engineer
287 the small intestine *in vitro* have been shown to have some positive outcomes, the
288 surface area created is not adequate for human therapy and the majority of *in vitro*
289 methods created only epithelium and lacked surrounding mesenchymal structures.

290 **Recapitulating the dynamic mechanical microenvironment of the small** 291 **intestine**

292 Under *in vitro* static culture microenvironment, cells can be supplied with nutrients by
293 manual medium replacement. Thus, long term culture under static conditions
294 possesses multiple limitations such as poor delivery of nutrients, accumulation of
295 waste and risk of contamination. To overcome these limitations, and for long term
296 maintenance of intestinal cells in a healthy state, many studies have developed
297 dynamic culture microenvironments.

298 An automated perfusion system (Minucells and Minutissue) has been used to study
299 the differentiation and drug transport properties of Caco-2 cells^{105,106}. The enzymatic
300 activities and permeability coefficient of drugs in differentiated Caco-2 cells in
301 perfusion system were increased when compared to Caco-2 cells differentiated in
302 traditional culture using snapwell inserts^{105,106}.

303 Similarly, microfluidic culture methods play an important role in addressing this issue
304 and assist in the development of enhanced barrier function of Caco-2 cells¹⁰⁷.
305 Several studies have developed gut-on-a-chip microdevices to mimic the dynamic
306 motion seen in the human small intestine^{80,81,108}. Microfluidic gut-on-a-chip
307 microdevices are an alternative *in vitro* model which have the ability to recapitulate
308 the 3D structures of native human intestinal villi. In these models, Caco-2 cells

309 exposed to dynamic fluid flow and peristalsis-like motions resulted in
310 cytodifferentiation of Caco-2 cells into four main types of intestinal epithelial cells and
311 formed proliferative crypts^{80,81,108}.

312 **Intestinal stem cell isolation and its importance in engineering the small** 313 **intestine**

314 In recent years, there has been an increasing interest in Intestinal stem cells which
315 are found at the base of the crypts within the proliferative compartment (Figure 1).
316 These stem cells give rise to the four **main** cell lineages: enterocytes, goblet,
317 enteroendocrine and paneth cells and are classified as crypt base columnar cells^{109–}
318 ¹¹¹. Adult stem cells residing within the crypts have the ability to undergo cell
319 proliferation into transit-amplifying progenitor (TA), which terminally differentiate and
320 give rise to all six intestinal cell types of the mammalian intestine^{31,112–114}. The
321 proliferative capacity of these stem cells ensures there are sufficient cells to
322 regenerate any damaged tissue¹¹⁵ and continually maintain digestion and absorption
323 process. These stem cells are ideal candidates for use in regenerative medicine¹¹⁶.
324 The use of stem cell markers is essential for isolation of pure stem cell populations
325 for use in tissue engineering. In the small intestine, there are two stem cell
326 populations within the crypt, classified by location and cycling dynamics^{24,25,117}. The
327 first of these stem cells are cycling, slender cells found at the bottom of the crypt
328 between paneth cells, these are known as crypt base columnar cells. These cells
329 express several stem cell markers including *Lgr5*; CD133 (Prom1); *Ascl2*; *Olfm4*;
330 *Smoc2* and *Sox9*^{low} ^{118–122}. The second stem cell population are quiescent stem cells,
331 which are located in the crypt directly above the terminally differentiated paneth
332 cells¹²³. These quiescent stem cells express *Bmi-1*, *Hopx*, *mTert*, and *Lrig1*, and
333 *Sox*^{high} ^{124,125}. The locations where stem cells are located in the small intestine are

334 known as the stem cell niche, which is maintained by a range of cells (pericryptal
335 myofibroblast, adjacent epithelial cells, immune cells (lymphocytes) endothelial cells,
336 enteric neurons), which together with basement membrane derived extracellular
337 matrix regulate stem cell differentiation and fate¹²⁶⁻¹²⁸. Several regulatory pathways
338 play a role in the maintenance, and proliferation of stem cells²⁴; these include: Wnt;
339 Notch; Hedgehog and bone morphogenetic protein (BMP) pathways¹²⁹⁻¹³¹.
340 Canonical Wnt signaling is well recognized as the main regulator of epithelial
341 renewal in the small intestine¹¹⁸, with epidermal growth factor (EGF) signaling
342 maintains stemness and prompts proliferation^{110,132,133}. Whilst Notch signaling
343 controls differentiation to enterocytes, inhibition of Notch signaling leads to the
344 differentiation **towards** secretory lineages (including: goblet, paneth, enteroendocrine
345 and tuft cells)¹³⁴. Bone morphogenetic protein (BMP) signaling negatively regulates
346 stem-cell **characteristics and promotes differentiation of progenitor cells in the villus**
347 **compartment, but has no effect on stem cells located in** the crypts¹³⁵. Thus the
348 manipulation of these signaling pathways *in vitro* culture can be used to maintain
349 stem-cell characteristics or drive differentiation of cells to appropriate lineages.

350 For the successful extraction of stem cells from intestinal crypts a clear stem cell
351 marker is essential to enable purification of intestinal stem cells, and whilst there are
352 a variety of stem cells markers, Lgr5 (also known as GPR67) has been suggested
353 the most appropriate marker for purification of stem cells^{23,24,26,28,116,120}. Lgr5 is
354 expressed in cycling columnar cells in the base of the crypts, but not in the villi^{116,136}.
355 Lgr5 is a target of Wnt signaling and these cells are capable of generating all
356 epithelial lineages in *in vitro* culture^{18,116,137}. **Furthermore**, intestinal stem cells are
357 capable of self-organizing into organoid units that recapitulate the intestinal villi and

358 crypt domains and reflect main structural and functional properties of the small
359 intestine^{138,139}.

360 **Intestinal organoids and tissue engineering of the small intestine**

361 ***3D cell culture of organoids***

362 Studies over the past two decades have provided promising results in tissue
363 engineering of small intestine due to the successful isolation of intestinal crypts
364 which could form organoid units (Figure 3). The ability to extract complete crypts,
365 which contain progenitor cells, from intestinal tissues has excellent potential to
366 expand *in vitro* to form organoid units and differentiate following transplantation. Kim
367 *et al.*, (2007) harvested neonatal rats intestinal epithelial organoid units and seeded
368 them on biodegradable polyglycolic acid scaffolds and maintained them within a
369 perfusion bioreactor for 2 days. The cells were shown to distribute and adhere to the
370 polymer scaffold¹⁴⁰. Sato and colleagues (2009) developed a 3D culture system of
371 mouse intestinal crypt known as ‘mini-gut’ culture or organoid culture. These 3D
372 cultures in Matrigel supplemented with growth factors (R-spondin-1, epidermal
373 growth factor, and the BMP inhibitor: Noggin)¹⁸. In this system, the crypt-villus
374 organoids developed not only from whole crypts but also from single Lgr5⁺ stem
375 cells. These single intestinal stem cells were shown to form crypt-like structures by
376 day 1-4, and then crypt-buds by day 5¹⁸. In a similar studies conducted by Jabaji *et*
377 *al.*, (2013),(2014), compared type 1 collagen with Matrigel as an alternative scaffold
378 for growing of isolated crypt units. They showed that the intestinal crypts enlarged
379 and formed enteroids *in vitro* when cultured in both scaffolds as monoculture and
380 when cultured with myofibroblast for 1 week^{141,142}. Intestinal crypts not only isolated
381 from human and mice but also isolated from juvenile and adult porcine. Khalil *et al.*,

382 (2016) developed long-term culture model of juvenile and adult porcine intestinal
383 crypts to generate budding enteroids¹⁴³. More recently, Pastula *et al.*, (2016),
384 modified Sato's 3D culture methods using a combination of Matrigel and collagen
385 and co-cultured the epithelial organoid with myofibroblast, and neuronal cells. Where
386 myofibroblast and neuronal cells supported the growth of epithelial organoids.
387 However, the presence of collagen led to a reduction in the budding of epithelial
388 organoids⁷.

389 A major disadvantage of these systems is they form closed organoid units, this has
390 recently been overcome by Sachs *et al.*, (2017), where tube formation was induced
391 by culturing the organoids in a contracting floating collagen gel. They concluded that
392 these systems enabled the organoids to align and fuse forming the macroscopic
393 hollow structures. However this model although cellular differentiation was observed
394 villi structures were still missing¹⁴⁴, whilst Wang *et al.*, (2017) has successfully
395 generated crypt-villus architecture from intestinal stem cells cultured on a fabricated
396 collagen scaffold¹⁴⁵. Furthermore, application of chemical gradients which were
397 applied to the scaffold promoted and supported cell migration along the crypt-villus
398 axis¹⁴⁵. Demonstrating a combined approach of microengineered scaffolds together
399 with biophysical cues and chemical gradients could hold the potential for tissue
400 engineering a small intestinal model *in vitro*¹⁴⁵.

401 *In vivo* implantation of organoid seeded scaffolds

402 A number of studies have directly seeded these organoid units onto biodegradable
403 scaffolds to test their ability to regenerate the intestine post-implantation in
404 rodents^{85,146,147} and large animals¹⁴⁸. In 1988, Vacanti *et al.*, isolated organoid units
405 from neonatal rat intestine and seeded these onto a tubular scaffold of polyglycolic

406 acid and poly-L-lactic acid prior to implantation into the omentum of the syngeneic
407 adult rat. These organoid units survived, proliferated and had a characteristic villus-
408 crypt structures¹⁴⁹. Choi and Vacanti, (1997) demonstrated that the organoid units
409 isolated from 6-day-old neonatal rat intestines, seeded on PGA and then implanted
410 into adult rats survived, proliferated, and regenerated small intestine-like
411 structures¹⁴⁶. Similarly, organoid units isolated from 7-week old Yorkshire swine and
412 cultured on biodegradable scaffolds tubes and then implanted intraperitoneally in the
413 autologous host. In these implants, differentiated intestinal cells innervated
414 muscularis mucosa and intestinal sub-epithelial myofibroblasts were identified¹⁵⁰.
415 Levin *et al.*, (2013) seeded multicellular organoid units derived from postnatal human
416 small intestine resections onto a biodegradable PGA / PLA polymer¹⁵¹. Following
417 transplantation into NOD/SCID gamma chain-deficient mice, the human tissue
418 formed a villus-crypt architecture similar to that of the mature human small intestine,
419 which contained all differentiated epithelial cell types and mesenchyme cells which
420 expressing muscular and neural markers¹⁵¹. *In vivo* subcutaneous implantation using
421 PGA scaffolds of one week old collagen based enteroids derived from 3D co-cultures
422 of small intestinal crypts and myofibroblast resulted in sustainable re-formed
423 intestinal organoids with differentiated lineages after 5 weeks¹⁴¹. Cromeens *et al.*,
424 (2016) produced neomucosa by seeding enteroids derived from LGR5-EGF
425 transgenic mice on Matrigel for 10-14 day and then these enteroids released from
426 Matrigel and seeded onto PGA scaffolds and implanted into the peritoneal cavity of
427 immunocompromised NOD/SCID mice. After 4 weeks, neomucosa was produced
428 with a clear crypt domains and blunted villi. The shortcoming of this study was the
429 villi were blunted and did not extend to the length of native small intestinal villi¹⁵².
430 The main limitations of these attempts to generate small intestinal tissue are the high

431 number of cells required for engineering functional tissue and the absence of
432 scaffolds which mimic the native intestine and capable of generating intestinal stem
433 cell niche.

434 **3D cell culture of pluripotent stem cells**

435 Multipotent stem cells can generate numerous tissues in the body and have the high
436 proliferative capacity, making them attractive for use in regenerative medicine¹⁵³.

437 **Mesenchymal stem cells have been investigated as a promising source of smooth**
438 **muscle layer for small intestinal tissue engineering.** Hori *et al.*, (2002) investigated
439 mesenchymal stem cells to study the feasibility of muscle regeneration, the
440 limitations considered the lack of ability to regenerate smooth muscle layer¹⁵⁴.

441 Over recent years, several studies have provided evidence that human induced
442 pluripotent stem cells (iPSCs) can be used to generate intestinal tissue^{147,155,156}. A
443 study published in 2011 aimed to direct the differentiation of human pluripotent stem
444 cells to generate fetal intestinal-like **immature properites** using manipulation of
445 growth factors¹⁵⁵. Similarly, Yoshida *et al.*, (2012) demonstrated that mice pluripotent
446 stem cells were successfully differentiated into smooth muscle *in vitro*¹⁵⁷. Whilst,
447 Watson *et al.*, (2014) **generated** human intestinal organoids from human iPSCs. In
448 this model, these organoids were embedded in collagen type I and then transplanted
449 into immunocompromised mice for **a period of 6 weeks**. Following transplantation,
450 iPSCs fully differentiated into all types of small intestinal **cells and smooth muscle**
451 **layers when compared with *in vitro* human intestinal organoids¹⁵⁶.** A recent study
452 **conducted by Finkbeiner *et al.*, (2015) also has been shown that the human**
453 **intestinal organoids derived from iPSCs generated a tissue that looks resemble the**
454 **native human intestinal tissue when seeded onto PGA/PLA scaffolds and implanted**

455 into immunocompromised mice for 12 weeks. While these promising findings, tissue
456 engineered intestine were supplemented with further neuronal cell types to generate
457 physiologically functional tissue engineered intestine¹⁴⁷.

458 **Conclusion**

459 In spite of the current limitations, attempts at tissue engineering intestinal tissues *in*
460 *vitro* have provided initial knowledge on the behaviour of intestinal cells in 2D and 3D
461 culture (Figure 4&5) and the performance of stem cells and organoid units following
462 implantation into animals. These models are extremely useful for the study of
463 intestinal physiology, drug absorption studies and toxicity studies. However, to date,
464 they fall short of successfully modeling the *in vivo* environment. Advanced studies
465 and new approaches are required to provide intestinal tissue composed of mucosa
466 and neuromuscular tissue before treatment of patients with intestinal failure can be
467 achieved. The potential ability of stem cells to differentiate into many intestinal cell
468 types provides the intestinal mucosa an amazing reconstruction capacity, and
469 exploitation of this role might make it possible to treat a variety of intestinal diseases.

470 **Acknowledgments**

471 This work was supported by Ministry of Higher Education and Scientific Research/
472 Iraq for the PhD scholarship.

473 **Author Disclosure Statement**

474 No competing financial interests exist.

475

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