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Tissue Engineering Laboratory Models of the Small Intestine

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Abstract

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

Keywords

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

Introduction

Until recently, *in vitro* intestinal models have been restricted to simple two-dimensional (2D) cell culture on standard cell-culture plates or transwell culture inserts[1]. However, three-dimensional (3D) cell culture models are currently under investigation by groups worldwide to determine if these 3D cell cultures can more closely mimic the *in vivo* environment and support cell differentiation and 3D tissue organization which is not possible in conventional 2D cell culture systems[2–7]. These 3D cell culture models have been evaluated for their use in tissue engineering and drug discovery[8,9] and used as an alternative to *in vivo* animal models in drug toxicity studies[10–12].

Tissue engineering studies have promised an improved understanding of small intestinal physiology, as well as the response of the small intestine to infection, toxicity and new therapies[13]. Furthermore, using these systems may be possible to develop personalized intestinal tissue grafts which can be used to repair the intestine, whilst avoiding the risks of immune system rejection[14]. The most important element for successful tissue engineering of the small intestine is the use of specialized biomaterial scaffolds providing cells a substrate for the deposition of extracellular matrix and subsequent cell adhesion[9,15,16]. These scaffolds are often designed to biodegrade after the deposition of extracellular matrix, when the cells become mechanically independent[14,17] and could be potentially used therapeutically[18], however matching the degradation rates to synthesis and deposition of new matrix remains a key challenge in tissue engineering[19–21]. This article aims to review 2D and 3D cell culture systems used to culture intestinal cells, to determine whether the use of 3D scaffolds can mimic the *in vivo* environment. Furthermore, recent progress in establishing intestinal stems cells and organoid units *in vitro* and *in vivo*.

The architecture of the small intestine

The small intestine is essentially a tube, which can be divided into four anatomically and functionally distinct layers: mucosa, submucosa, muscularis externa, and serosa[22] (Figure 1). The mucosa is folded into villi which increases the surface area and maximizes digestion and absorption. The number of villi varies, depending on the position along the length of the intestine[22], with the highest frequency seen in the proximal jejunum, which decreases towards the ileum. Furthermore, the villi morphology differs through the small intestine, decreasing in size from the proximal to the distal end of the small intestine. In the duodenum, the villi are leaf-like, whilst those of the jejunum and ileum having a tongue-like, and then finger-like appearance, respectively. At the base of the villi are crypts of Lieberkuhn[23]. These crypts are tubular glands which descend into underlying muscularis mucosa[24] and form the intestinal stem cell niche (Figure 1). This complex villi rich mucosa layer is supported by the underlying submucosa, which is composed of fibrous connective tissue and a rich supply of blood and lymphatic vessels and is innervated by the Meissner's nervous plexus. Beneath the submucosa is the muscularis externa, composed of an inner circular and outer longitudinal smooth muscle layers innervated by the Auerbach's plexus, which enables the peristaltic movement of food along the intestine[17]. This layer is finally supported by a single layer of mesothelium called the serosa[22] (Figure 1).

Intestinal epithelial cell types

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

enterocytes are responsible for producing of digestive enzymes and the absorption of nutrients[22,25,26]. The second most abundant cell types are unicellular glandular cells known as mucus-secreting goblet cells. Mucins are secreted into the lumen of the intestine by these goblet cells, giving rise to an adherent mucus layer which surrounds and coats the intestinal villi[23]. Located at the base of intestinal crypts are paneth cells, which secrete antimicrobial lysozymes protecting the crypt from pathological microorganisms[26,27]. These paneth cells also play an essential role in stem cell niche signals[28,29]. There are also smaller populations of hormone-secreting enteroendocrine cells and tuft cells which regulate digestion and absorption (Figure 1). Finally, microfold or M-cells are located within lymphoid peyer's patches and are responsible for transporting antigens from the lumen to the underlying lymphoid tissues[25,26].

Importantly, a small population of stem cells are located at the villus base within crypts and are responsible for maintaining intestinal epithelial homeostasis (Figure 1). These stem cells differentiate as they migrate along the length of the villi, replacing cells which are lost at the villus tip. This process of cell renewal ensures that the functions of the intestinal epithelium are maintained throughout life. The capability of these stem cells to undergo self-renewal makes them particularly attractive for tissue engineering and regenerative medicine applications[23].

Why do we need to engineer a small intestine?

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

syndrome) and cancer. These can become extremely debilitating disorders impacting on quality of life or even life threatening[30–32]. Thus, the ability to replace damaged and malfunctioning tissues with a tissue-engineered small intestine could be of use in these conditions. Furthermore, engineered intestinal tissue could be created using patient-specific explants; small samples of healthy tissue could be collected from a patient and expanded within the laboratory. If these cells could then be utilized in the generation of a tissue-engineered small intestine, this could then be returned to the patient to enable intestinal repair or augmentation. Use of self-tissue would avoid the requirement of tissue donors, and the need for lifelong immunosuppression to prevent rejection of tissues[33].

2D cell culture models of the small intestine

The use of cell lines in intestinal engineering

Due to failed attempts to establish long-term primary cell culture of normal small intestine, researchers have successfully utilized cell lines which are derived from gastrointestinal tumours[34]. The human colonic adenocarcinoma cell lines (Figure 2): Caco-2 and HT29-MTX cells are probably the most frequently used cell lines due to their ability to differentiate into enterocyte-like cells and mucus-producing goblet cells, respectively[35–37], whilst these would not be suitable for tissue enginering applications due to their cancerous nature they are excellent models for *in vitro* cultures.

Caco-2 cells can spontaneously differentiate into cells with the ability to form tight junctions and produce large amounts of digestive brush border enzymes, similar to small bowel enterocytes[38–40]. Caco-2 cells express a number of digestive enzymes including sucrase-isomaltase, lactase, peptidase, and alkaline phosphatase. The

expression of these enzymes are used as markers of intestinal differentiation and digestive function[41–47]. However, Caco-2 cells have tight junctions similar to those of the colon, rather than the small intestine, this has led to criticism of their use as a model for the epithelium of the small intestine[48,49]. Furthermore, Caco-2 cell behaviour can be affected by culture condition (serum supplemented and serum free media), passage number, cell density and incubation times[50,51] all of which make it difficult to compare research findings between different studies[52,53].

Caco-2 cells are often used to mimic small intestinal enterocytes and have been used extensively in absorption and transport studies of nutrients and drugs[54,55], for example, insulin transport studies[56,57]. Moreover, Caco-2 cells have been used to investigate the cytotoxicity of acrylic-based copolymer protein as an oral insulin delivery system[57]. Caco-2 cells can also be utilized to verify the toxicology when exposed to nanoparticles such as polystyrene, which resulted in increased level of iron absorption[58].

HT29-MTX cells are also a commonly used cell line in intestinal modeling. These cells are derived from human colonic adenocarcinoma cells and are resistant to methotrexate (MTX). HT29-MTX cells are composed entirely of differentiated mucus-secreting goblet cells. They maintain this differentiated phenotype in monolayer culture and are used to mimic intestinal goblet cells, and are commonly co-cultured with Caco-2 cells[59–62]. HT29-MTX cells have been utilized in studies investigating the diffusion of drugs across the mucus layer[63–65], these have been used to test the mucoadhesive and toxicity of nanoparticles as drug delivery systems[66], and to test adhesion and invasion of Salmonella strains[67] (Table 1).

2D co-culture studies

In order to mimic the native small intestinal epithelium which is composed of diverse absorptive and secretory cells a number of studies have co-cultured Caco-2 cells alongside HT29-MTX cells[63,64,68–71]. These studies have enabled the formation of a Caco-2 derived enterocyte-like layer, which is interspersed with mucus secreting HT29-MTX cells, and avoided the limitations and drawbacks previously seen in mono-cultures[63]. Walter *et al.,* (1996) co-cultured Caco-2 and HT29-MTX cells in cell culture inserts in a transwell format, where they were shown to produce an adherent mucus layer which covered the cell monolayer. The cells were shown to have structures similar to microvilli, although they were of irregular shape and size[63]. The mucus layer formed by the HT29-MTX cells during co-culture with Caco-2 cells were proposed to play an important role in digestion and bioavailability[68]. Many studies have exploited *in vitro* co-cultures of Caco-2 and HT29-MTX cells to provide a drug absorption model[63,68], to study drug permeability[56,64,70,71] and to improve alternative *in vitro* systems for evaluation cytotoxicity of nanoparticles to replace animal testing[72]. Furthermore, different co-culture ratios of Caco-2 and HT29-MTX cells have been used to investigate the co-culture ratio most physiologically relevant to *in vivo* situations[52,70,73,74].

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

polystyrene nanoparticle permeability studies that demonstrated the strong influence of HT29-MTX cells and M-cells on the nanoparticle permeation. In this study, cellular uptake of polystyrene nanoparticles was affected by the presence of mucus layers. Where, nanoparticle transport was significantly increased in Caco-2/M cells due to a lack of mucus secretion from M cells[77]. Most recently, the Caco-2/HT29-MTX co-culture and Caco-2/HT29-MTX/Raji B triple co-culture models have been successfully used to investigate the intestinal permeability of different biopharmaceutical characteristics of drugs. Where it was shown that higher permeability of drugs were observed in more complex models compared with Caco-2 monoculture[76]. Taken together, these studies demonstrated the importance of cell-cell interactions which can impact on the physiological function in intestinal cells. These models can also be combined with bacterial cells to mimic the microbiotia seen within the small intenstine[6,78,79].

Whilst these 2D static culture models of intestinal cells in Transwells display a number of advantages, these models fail to develop villi morphology[80]. Furthermore, these models fail to undergo cytodifferentiation due to lack of the 3D microenvironment, including luminal flow, and fluid shear stress[80,81].

3D cell culture models of the small intestine

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

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

The rate of cell growth, however, varies depending on the scaffold used[90,91]. In 3D cell culture models, the interaction between cells and the scaffold is regulated by the material characteristics of the scaffold. Some materials provide natural adhesion sites for cells whilst others provide a substratum for the deposition of extracellular matrix which subsequently provides adhesion sites for cells[16]. The mechanical characteristics and degradation dynamics of the scaffold are important for specific tissue engineering applications[92,93]. The mechanical properties of scaffolds control the shape of cells during tissue reconstruction and provide mechanical cues to cells to tailor differentiation[17,82], whilst also providing support for load[94]. Scaffolds investigated to date include natural hydrogels (e.g. collagen gels and Matrigel) and synthetic scaffolds (e.g poly-lactic-glycolic acid) which have a number of key advantages and disadvantages.

3D cell culture using collagen gels

Type I collagen gels are commonly used for 3D culture, as they are easy to prepare, inexpensive, can support a range of cell types[95,96], and enable encapsulation of cells[97]. Furthermore, pore size, rigidity, and ligand density can be adjusted by changing the collagen concentration or utilizing chemical cross-links[94]. Li *et al.*, (2013) have used collagen gels to seed fibroblasts, Caco-2 and HT29-MTX cells.

This 3D triple co-culture model has been used to evaluate drug permeability and has been shown to have more physiologically relevant drug absorption rates[96]. Pusch *et al.,* (2011) performed 3D co-culture of Caco-2 cells and human microvascular endothelial cells (hMECs), created multilayers of enterocyte-like cells which expressed villin, E-cadherin, and the transporter p-glycoprotein at levels that were similar to that of a normal human jejunum[34]. Whilst Viney *et al.,* (2010) co-cultured intestinal epithelial cell lines (IEC6: a rat small intestinal epithelial cell line; IPI-21: a small boar ileum epithelial cell line, and CRL-2102: a human epithelial cell line derived from colorectal adenocarcinoma) with Rat-2 (fibroblast-like cell) in collagen gels alone or in combination with Matrigel. After 20 days, optimal epithelial cell growth was seen in collagen gels supplemented with Matrigel, where multilayered intestinal epithelium were seen, which included clusters of cells similar to the morphology of crypts[98]. This highlighted the importance of the interaction between the cell lines, extracellular matrix and other cell types such as fibroblasts; and how they can impact on the cell proliferation and differentiation[99]. These interactions with localized cells were further demonstrated when rat intestinal sub-epithelial myofibroblasts (ISEMF) were co-cultured with IEC-6 cells on a collagen gel scaffold[100], where the myofibroblasts induced differentiation of IEC-6 intestinal cells to enteroendocrine cells, which was thought to be mediated by growth factors and cytokines secreted by the myofibroblasts[100].

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

cells in crypts of native small intestine was observed[101]. In addition, a number of studies have microfabricated villus-shaped collagen scaffolds into which Caco-2 cells were culutred[82,83,102,103] (Table 2). These studies demonstrated that the culture of Caco-2 cells on these prefabricated villi structures led to the formation of villi which were comparable to those of human jejunum after 3 weeks in culture[83]. However, it has been observed that the transepithelial electrical resistance (TEER) of cells in these villus-like structures were lower than those in cells grown on 2D flat substrate.

Synthetic Polymer Scaffolds

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

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

differentiated under the stimulation of epidermal growth factor were added to the basolateral side of scaffolds[82] (Table 2). Although the latest procedures to engineer the small intestine *in vitro* have been shown to have some positive outcomes, the surface area created is not adequate for human therapy and the majority of *in vitro* methods created only epithelium and lacked surrounding mesenchymal structures.

Recapitulating the dynamic mechanical microenvironment of the small intestine

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

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

Similarly, microfluidic culture methods play an important role in addressing this issue and assist in the development of enhanced barrier function of Caco-2 cells[107]. Several studies have developed gut-on-a-chip microdevices to mimic the dynamic motion seen in the human small intestine[80,81,108]. Microfluidic gut-on-a-chip microdevices are an alternative *in vitro* model which have the ability to recapitulate the 3D structures of native human intestinal villi. In these models, Caco-2 cells

exposed to dynamic fluid flow and peristalsis-like motions resulted in cytodifferentiation of Caco-2 cells into four main types of intestinal epithelial cells and formed proliferative crypts[80,81,108].

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

In recent years, there has been an increasing interest in Intestinal stem cells which are found at the base of the crypts within the proliferative compartment (Figure 1). These stem cells give rise to the four main cell lineages: enterocytes, goblet, enteroendocrine and paneth cells and are classified as crypt base columnar cells[109–111]. Adult stem cells residing within the crypts have the ability to undergo cell proliferation into transit-amplifying progenitor (TA), which terminally differentiate and give rise to all six intestinal cell types of the mammalian intestine[31,112–114]. The proliferative capacity of these stem cells ensures there are sufficient cells to regenerate any damaged tissue[115] and continually maintain digestion and absorption process. These stem cells are ideal candidates for use in regenerative medicine[116]. The use of stem cell markers is essential for isolation of pure stem cell populations for use in tissue engineering. In the small intestine, there are two stem cell populations within the crypt, classified by location and cycling dynamics[24,25,117]. The first of these stem cells are cycling, slender cells found at the bottom of the crypt between paneth cells, these are known as crypt base columnar cells. These cells express several stem cell markers including *Lgr5;* CD133 (Prom1); *Ascl2;* Olfm4; *Smoc2 and Sox9*^{low} [118–122]. The second stem cell population are quiescent stem cells, which are located in the crypt directly above the terminally differentiated paneth cells[123]. These quiescent stem cells express *Bmi-1*, *Hopx*, *mTert,* and *Lrig1*, and Sox^{high} [124,125]. The locations where stem cells are located in the small intestine are

known as the stem cell niche, which is maintained by a range of cells (pericryptal myofibroblast, adjacent epithelial cells, immune cells (lymphocytes) endothelial cells, enteric neurons), which together with basement membrane derived extracellular matrix regulate stem cell differentiation and fate[126–128]. Several regulatory pathways play a role in the maintenance, and proliferation of stem cells[24]; these include: Wnt; Notch; Hedgehog and bone morphogenetic protein (BMP) pathways[129–131]. Canonical Wnt signaling is well recognized as the main regulator of epithelial renewal in the small intestine[118], with epidermal growth factor (EGF) signaling maintains stemness and prompts proliferation[110,132,133]. Whilst Notch signaling controls differentiation to enterocytes, inhibition of Notch signaling leads to the differentiation towards secretory lineages (including: goblet, paneth, enteroendocrine and tuft cells)[134]. Bone morphogenetic protein (BMP) signaling negatively regulates stem-cell characteristics and promotes differentiation of progenitor cells in the villus compartment, but has no effect on stem cells located in the crypts[135]. Thus the manipulation of these signaling pathways *in vitro* culture can be used to maintain stem-cell characterisitcs or drive differentiation of cells to appropriate lineages.

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

crypt domains and reflect main structural and functional properties of the small intestine[138,139].

Intestinal organoids and tissue engineering of the small intestine

3D cell culture of organoids

Studies over the past two decades have provided promising results in tissue engineering of small intestine due to the successful isolation of intestinal crypts which could form organoid units (Figure 3). The ability to extract complete crypts, which contain progenitor cells, from intestinal tissues has excellent potential to expand *in vitro* to form organoid units and differentiate following transplantation. Kim *et al.,* (2007) harvested neonatal rats intestinal epithelial organoid units and seeded them on biodegradable polyglycolic acid scaffolds and maintained them within a perfusion bioreactor for 2 days. The cells were shown to distribute and adhere to the polymer scaffold[140]. Sato and colleagues (2009) developed a 3D culture system of mouse intestinal crypt known as 'mini-gut' culture or organoid culture. These 3D cultures in Matrigel supplemented with growth factors (R-spondin-1, epidermal growth factor, and the BMP inhibitor: Noggin)[18]. In this system, the crypt-villus organoids developed not only from whole crypts but also from single $Lgr5^{+}$ stem cells. These single intestinal stem cells were shown to form crypt-like structures by day 1-4, and then crypt-buds by day 5[18]. In a similar studies conducted by Jabaji *et al.,* (2013),(2014), compared type 1 collagen with Matrigel as an alternative scaffold for growing of isolated crypt units. They showed that the intestinal crypts enlarged and formed enteroids *in vitro* when cultured in both scaffolds as monoculture and when cultured with myofibroblast for 1 week[141,142]. Intestinal crypts not only isolated from human and mice but also isolated from juvenile and adult porcine. Khalil *et al.,*

(2016) developed long-term culture model of juvenile and adult porcine intestinal crypts to generate budding enteroids[143]. More recently, Pastula *et al.,* (2016), modified Sato's 3D culture methods using a combination of Matrigel and collagen and co-cultured the epithelial organoid with myofibroblast, and neuronal cells. Where myofibroblast and neuronal cells supported the growth of epithelial organoids. However, the presence of collagen led to a reduction in the budding of epithelial organoids[7].

A major disadvantage of these systems is they form closed organoid units, this has recently been overcome by Sachs *et al.,* (2017), where tube formation was induced by culturing the organiods in a contracting floating collagen gel. They concluded that these systems enabled the organoids to align and fuse forming the macroscopic hollow structures. However this model although cellular differentiation was observed villi structures were still missing[144], whilst Wang *et al.,* (2017) has successfully generated crypt-villus architecture from intestinal stem cells cultured on a fabricated collagen scaffold[145]. Furthermore, application of chemical gradients which were applied to the scaffold promoted and supported cell migration along the crypt-villus axis[145]. Demonstrating a combined approach of microengineered scaffolds together with biophysical cues and chemical gradients could hold the potential for tissue engineering a small intestinal model *in vitro*[145].

In vivo implantation of organoid seeded scaffolds

A number of studies have directly seeded these organoid units onto biodegradable scaffolds to test their ability to regenerate the intestine post-implantation in rodents[85,146,147] and large animals[148]. In 1988, Vacanti *et al.,* isolated organoid units 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 structures[149]. 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 structures[146]. 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 identified[150].
415 Levin *et al.,* (2013) seeded multicellular organoid units derived from postnatal human
416 small intestine resections onto a biodegradable PGA / PLA polymer[151]. 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 markers[151]. *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 weeks[141]. 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 villi[152].
430 The main limitations of these attempts to generate small intestinal tissue are the high

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

3D cell culture of pluripotent stem cells

Multipotent stem cells can generate numerous tissues in the body and have the high proliferative capacity, making them attractive for use in regenerative medicine[153]. Mesenchymal stem cells have been investigated as a promising source of smooth muscle layer for small intestinal tissue engineering. Hori *et al.,* (2002) investigated mesenchymal stem cells to study the feasibility of muscle regeneration, the limitations considered the lack of ability to regenerate smooth muscle layer[154].

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

into immunocompromised mice for 12 weeks. While these promising findings, tissue engineered intestine were supplemented with further neuronal cell types to generate physiologically functional tissue engineered intestine[147].

Conclusion

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

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