Building gut from scratch — progress and update of intestinal tissue engineering

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Abstract

Short bowel syndrome (SBS), a condition defined by insufficient absorptive intestinal epithelium, is a rare disease, with an estimated prevalence up to 0.4 in 10,000 people. However, it has substantial morbidity and mortality for affected patients. The mainstay of treatment in SBS is supportive, in the form of intravenous parenteral nutrition, with the aim of achieving intestinal autonomy. The lack of a definitive curative therapy has led to attempts to harness innate developmental and regenerative mechanisms to engineer neo-intestine as an alternative approach to address this unmet clinical need. Exciting advances have been made in the field of intestinal tissue engineering (ITE) over the past decade, making a review in this field timely. In this Review, we discuss the latest advances in the components required to engineer intestinal grafts and summarise the progress of ITE. We also explore some key factors to consider and challenges to overcome when transitioning tissue engineered intestine towards clinical translation and provide the future outlook of ITE in therapeutic applications and beyond.

Blurb

Intestinal tissue engineering offers a potential therapeutic option for short bowel syndrome. This Review examines the progress in intestinal tissue engineering, discusses the components required for engineered intestinal grafts, preclinical progress and efforts towards clinical translation, including challenges to overcome.

Key points

- Intestinal tissue engineering has the potential to offer curative therapy for patients with short bowel syndrome

- Multiple components, including an absorptive mucosa, smooth muscle, enteric nerves and vasculature are required to generate a functional full thickness intestinal graft
- Advances in intestinal tissue engineering include endothelial cell reprogramming and vascular engineering, generation of mucosal grafts using patient-derived materials and colon mucosal repurposing using small intestinal organoids.
- Vascularisation and lymphatic engineering, generation of multi-layered personalised intestinal grafts and scaling-up of graft size present some of the future challenges in intestinal tissue engineering
- A collaborative approach, combining expertise in stem cell biology, engineering and biotechnology, is fundamental to advance engineered intestine towards clinical translation

Introduction

Regenerative medicine strives to restore cell, tissue or organ function and holds great promise for therapeutic solutions in various disease processes that lead to organ failure. Short bowel syndrome (SBS), in which patients have inadequate functional intestinal epithelium required to maintain hydration and nutrition, is one such condition. SBS affects between 0.004 and 0.4 in 10,000 people in the developed world[1], with prevalence estimated to have increased more than two-fold over the past 40 years[2]. Whilst there is no cure for SBS, current treatments include parenteral nutrition (PN) and, for those with severe disease, intestinal transplantation. PN offers a survival rate of 70% in newborn infants[3], acting as a supportive therapy whilst intestinal adaptation occurs in the early years after the insult **(Box 1).** However, in the most severe cases, when only 10% of expected intestinal length is present, 5-year survival is reduced to as low as 20%[4]. Moreover, home PN for each paediatric patient is estimated to cost between 46,000 and 230,000 Euro per year in Western Europe, $83,000 in the USA and CAD$320,000 in Canada[5]. Small intestinal transplantation is also an option, with 1 and 5-year survival of 77% and 58% respectively[6]. However, due to adverse effects of immunosuppression, shortage of organs and mismatched size (in children), this solution is still a suboptimal[7]. There is a clinical unmet need to develop an alternative approach to organ replacement therapy.

Regenerative medicine is an interdisciplinary field that combines stem cell biology, material science and tissue engineering technology with an increased complexity for the delivery of a personalised therapy designed around the specific patients' needs. A number of simple cell-based therapies such

as pigmented retinal epithelial cells derived from embryonic stem cells **[G]** (ESCs) and foetal-derived neural stem cells are undergoing phase I/II clinical trials for treatment of conditions such as macular degeneration[8,9] and motor neurone disease[10], respectively. Other therapies include epidermal skin grafts[11] and articular chondrocytes for intra-articular cartilage repair[12], whilst autologous limbal stem cells to treat corneal damage have been authorised by the European Medicines Agency as the first stem-cell based medicinal product commercially available[13-16]. On the other hand, progress for complex personalised tissue engineering is less advanced. Although preclinical work of tissue engineering in organs including oesophagus, lung and liver has been reported, organ grafts trialled clinically, such as bladder and trachea, are only demonstrated in case reports or case series[17-20].

Advances in stem cell technology have facilitated progress in tissue engineering and regenerative medicine. In particular, major advances have been made in tissue engineering of small intestine to treat SBS in the past decade. The intestine is a complex multi-layered organ that consists of functional epithelium covering the entire lumen, supporting submucosa providing a mesenchymal framework, and outer muscle layer innervated by enteric nervous system for peristalsis to facilitate intestinal transit[21]. Furthermore, native intestine is vascularised and has functional lymphatics[22]. Although the development of stem cell technology has markedly accelerated intestinal tissue engineering (ITE), it is still largely restricted to epithelium or mucosal reconstruction. Intriguingly, some important milestones have been made now in the field of intestinal tissue engineering, including engineering of functional jejunal mucosal grafts using patient-derived materials[23], generation of vascular networks via re-programming of vascular endothelial cells[24], *in vitro* 'gut-on-a-chip' techniques that offer insight into epithelial organisation in response to topography[25] and conversion of existing colon to small intestine to treat SBS in rat[26]. In this Review, we discuss various intestinal regenerative medicine strategies and the timely progress to date. In addition, we review the clinical application, the challenges and offer a future outlook of ITE strategies.

[H1] Intestinal anatomy and function

Unlike many other organs with solid consistency, intestine is a complex multi-layered hollow organ responsible for food digestion and absorption[27], barrier maintenance against gut microorganisms[28] and intestinal transit via peristalsis[29] **(Figure 1).** Understanding intestinal structure and function is fundamental for the basis of tissue reconstruction.

The self-renewing intestinal epithelium has crypt-villus architecture in small intestine and crypt only in colon, which provides an important absorptive surface and barrier function in the lumen[30]. Somatic intestinal stem cells (ISCs), expressing leucine rich repeat-containing G protein coupled receptor 5 (Lgr5), are located at the base of intestinal crypts. ISCs divide and migrate towards the lumen and differentiate into both absorptive (enterocytes) and secretory (goblet, enteroendocrine and Paneth) cell lineages[31]. Apart from epithelial cells, there is also a mesenchymal framework in the lamina propria comprised of a network of fibrous and structural extracellular matrix (ECM) proteins and cells, including fibroblasts and myofibroblasts, which constitute the stem cell niche to support ISC self-renewal and differentiation[32-34]. Multiple signalling pathways, such as the Wnt–β-catenin cascade, Notch signalling, transforming growth factor (TGF-β)–bone morphogenic protein (BMP) and hedgehog pathways, are involved in the regulation of ISCs and their adjacent niche[34-37]. The submucosa is a connective tissue layer that provides structural support to the mucosa, which also contains a lymphovascular network[38]. Blood vessels and associated lymphatics, arising in the villus as capillaries and lacteals, respectively, are fundamental for nutrient supply, fluid homeostasis, immune surveillance and transport of absorbed dietary fat[38]. The outer circular and longitudinal muscle layers give rise to peristalsis, which is regulated by the enteric nervous system (ENS) comprised of the ganglionated submucosal and myenteric nerve plexuses[29] Studies have demonstrated that the ENS also has a feedback role in regulating epithelial growth, secretion of hormones and host–microbe interactions[39,40]. The intestinal ENS, vasculature and lymphatics do not function in isolation but exist as a functional network within the body with connections to other organs. In addition, the gut microbiota also has an essential role in host metabolism and immunity, and the interaction between the microbiota, intestinal epithelium and immune cells is crucial for barrier maintenance and tissue homeostasis[41]. Recapitulating all these components and their complex cellular interactions is therefore important for engineering a functional intestine.

Components of engineered intestinal grafts

Reconstruction of a functional tissue engineered small intestine (TESI) requires both cells and the supporting scaffolds. These components include ISCs to regenerate the intestinal epithelium for digestion and absorption, stromal cells for stem cell maintenance, a scaffold for structural support, vascularisation for graft maintenance and enteric nerves for peristalsis[22,42]. Other components such as immune cells and microbiota might also need to be considered for TESI. Here, we discuss the sources of the individual components required for engineering intestinal grafts (**Figure 1**).

[H2] Epithelium. Establishing a functional mucosa represents a crucial step towards effective intestinal regeneration. Initially, intestinal 'organoid units **[G]** were derived from minced and enzymatically digested rat intestine containing a mixture of epithelium, fibroblasts and smooth muscle cells[43-45]. However, 'organoid units', generated from rat, mouse and human intestine, are limited in their expansion[45,46] and, therefore, not ideal for upscaling and regeneration. Almost in parallel, a number of exciting advances in stem cell biology were made, including derivation of pluripotent stem cells **[G]** (PSCs) from mouse and human embryos[47,48], identification of factors required to induce pluripotency in somatic cells[49-51] and discovery of the actively dividing multipotent *Lgr5*-positive ISCs **[G]** in adult intestine[31]. These major milestones are fundamental to the subsequent development of *ex vivo* intestinal organoid technology that can be generated from either PSCs or adult ISCs. These *ex vivo* organoids **[G]** can be grown three dimensionally in Matrigel in defined medium, which can differentiate into all intestinal epithelial cell types, with crypt-villus architecture, recapitulating those in the native intestine[52-55]. Importantly, these stromal-free epithelial organoids **[G]** have unlimited expansion potential, which overcome the major hurdle of the previous organoid units for ITE. The multipotent ISC-derived organoids have the potential to reconstitute all cell lineages in the intestinal epithelium and maintain regional identity. ESCs and induced pluripotent stem cell (iPSC)-derived organoids can further generate mesenchymal cells and smooth muscle cells in addition to epithelium (multi-tissue organoids) **[G]**][54-56].

[H2] Mesenchyme. Intestinal stem cells *in vivo*, are maintained by a highly structured surrounding niche, which provides essential signals for epithelial self-renewal and differentiation[57,58]. Fibroblasts and myofibroblasts are the two most abundant mesenchymal cells. Fibroblasts are essential in ECM remodelling and subepithelial myofibroblasts, expressing smooth muscle actin (SMA), are thought to be responsible for mesenchymal signals regulating epithelial homeostasis[59,60]. Studies have identified heterogenous populations of mesenchymal cells in mouse intestine contributing to the regulation of the Wnt and BMP signalling gradients, along the crypt-villus axis, responsible for proliferation and differentiation[57,61]. In particular, telocytes are a rare subset of mesenchymal cells underlying epithelial cells throughout the intestine[62,63], which express a variety of surface markers including the transcription factor FOXL-1[63], GLI1[64], CD34[65] and high levels of platelet derived growth factor receptor alpha (PDGFRα)[66]. They are sources of stromal-derived Wnt ligands, Wnt agonist R-spondin and BMP. Trophocytes, another mesenchymal cell population expressing CD81 and low levels of PDGFRα, exist below the base of crypts and secrete the BMP inhibitor GREM1 to antagonise

BMP signalling at the crypt base[67]. Mesenchymal cells can be generated from iPSCs[54,55] or isolated from primary tissue and expanded in culture *in vitro*[23]. Recapitulating the heterogeneity and complexity of the mesenchyme will be crucial for building suitable niche to promote TESI growth and differentiation. It will be important to assess if cellular heterogeneity is preserved, in the mesenchymal cells isolated from primary tissues or generated from iPSCs, prior to engineering.

Neuromusculature. The ENS comprises of submucosal and myenteric plexuses, which contain an extensive network of enteric neurons and glia cells to regulate intestinal epithelial cell functions and secretion as well as muscular wall contraction[29]. Reconstitution of the ENS is therefore crucial for functional TESI reconstruction. Major advances have been made in the past few years in the characterisation of enteric neural crest cells **[G]** (NCCs) — multilineage ENS progenitors. These cells were found in foetal (E11.5) and postnatal (D2-D14) mice and in foetal (12-15 weeks gestation), paediatric (3 weeks-7 months) and adult (26-84 years) human intestine, indicating that a population of enteric neural stem cells exist throughout life[68-71]. NCCs can be isolated and expanded from human intestinal mucosal biopsy samples[72] or derived from PSCs[42,73], leading to the generation of neurospheres with differentiated neuronal and glial cells[69,70]. Central nervous system (CNS)-derived neural progenitors, from foetal mouse tissue, also have the ability to give rise to enteric neurons following *in vitro* culture in explants of aneural embryonic wild-type (C57BL/6) mouse gut[74]. However, *in vivo* transplantation, into the muscle layer of distal colon of 2-3 week post-natal wild-type mice demonstrated less efficiency of CNS-progenitors to generate neurons as compared with transplanted ENS progenitors[74]. Studies have demonstrated that a combination of PSC-derived NCCs and human intestinal organoids in TESI can generate neuroglial structures similar to myenteric and submucosal plexuses, which demonstrate contractility both *in vitro* and *in vivo*[42,73].

Unlike ENS bioengineering, generation of smooth muscle cells for the intestinal muscle wall is less advanced. Whilst the relevance of differentiating visceral smooth muscle cells (ViSMCs) on promoting gut epithelium patterning in the developing intestine has been recognised[75,76], it remains unclear how to reliably isolate ViSMCs progenitors from the intestine. Primary ViSMCs can be isolated from mouse, rat and human intestinal tissue[77-79] with cellular phenotype and function maintained in very short-term culture up to 72 hours[77]. However, challenges lie in loss of differentiation and contractile function after prolonged ViMSC expansion *in vitro*[78,80]. Attempts to overcome these challenges include culturing ViSMCs in muscle strips, which preserves neuromuscular properties including cellular differentiation and contractile function[78]. These strips have successfully been seeded onto scaffold and transplanted *in vivo* into the omentum of wild-type

Lewis rats[78]. However, to generate sufficient smooth muscle for TESI, it would require relatively large volumes of starting material from patients in whom preservation of existing gut tissue is paramount. More promising strategies for primary ViSMC bioengineering include adapting cellular culture media[81] and co-culturing with mesenchymal cells[79]. Alternatively, it might be possible to derive ViSMCs from progenitor cells **[G]**. ViSMC regeneration might occur by recruiting multipotential vascular progenitors from bone marrow-derived stem cells as well as from less well defined sources within adult tissues[82]. Indeed, we and others have demonstrated that ViSMCs can be derived from adult somatic cells such as mesangioblasts **[G]** obtained from the blood vessels of skeletal muscle[20,83,84,85]. Similarly, ViSMCs can be obtained from other mesenchymal progenitors derived from human amniotic fluid stem cells[86] or from bone marrow mesenchymal stem cells[87]. Myogenic progenitor cells, derived from mesenchyme, can also be generated from differentiated iPSCs and ESCs[54,56] and give rise to smooth muscle following *in vivo* transplantation[55].

[H2] Vasculature and lymphatics. Vascularisation of TESI poses a substantial challenge to the survival of the grafts by maintaining sufficient nutrients and oxygen supply. Existing TESI strategies predominantly rely upon *in vivo* vascularisation of grafts[88-90]. However, incorporating vascular progenitors into grafts prior to *in vivo* transplantation[23,89] and the generation of functional blood vessels via angiogenesis is critical for meeting tissue oxygen demands. Investigations into the biology and therapeutic efficacy of endothelial stem and progenitor cells (EPCs) were largely driven by the initial observations of Asahara et al[91]. This work suggested evidence for the existence of circulating EPCs, expressing either CD34+ or Flk-1, which participate in angiogenesis[91]. Furthermore, it is now evident that in adult mammals, EPCs can be derived from bone marrow, circulation, and blood vessels[91-94]. However, endothelial cells are generally difficult to expand and senesce after a limited number of passages *in vitro*[95,96]. To overcome this problem, it has been shown that it is possible to partially reprogram vascular endothelial cells using the ETS variant transcription factor 2 (ETV2) to a more plastic and vasculogenic phenotype with increased *in vitro* and *in vivo* functional vasculogenic and angiogenic potential[24]. Alternatively, endothelial cells can also be generated from differentiation of human ESCs to form vascular-like structures[97]. Deriving endothelial cells from human PSCs has the potential advantage to capture the endothelial heterogeneity and might lead to gut-specific blood vascular endothelium. Indeed, a study published in 2020 has demonstrated that endothelial cell population can be derived from PSCs, which can be propagated and maintained in culture for up to 8 weeks[98]. Alongside vasculature, recapitulating the morphology and cellular organisation of the lymphatic network is fundamental in full thickness TESI. Specifically recreating

lacteals, the lymphatic capillary networks within intestinal villi, is necessary for absorption of dietary fat, achieving tissue fluid homeostasis and immunosurveillance. Lymphatic endothelial cells have been successfully generated from iPSCs and ESCs[99,100] whilst self-organising lymphatic networks have formed *in vitro* when co-cultured with fibroblasts in fibrin and collagen hydrogels **[G]** [101,102]. In addition, biochemical stimuli, specifically subsets of vascular endothelial growth factor (VEGF), such as VEGF-C, have been shown to stimulate lymphatic regeneration[103,104]. Whilst VEGF is most well recognised for its role in angiogenesis, both lymphatics and vasculature are endothelial cells and therefore contain VEGF-receptors, albeit their expression seems to be somewhat selective[105]. VEGF-R_3, the VEGF-receptor subset which most avidly binds VEGF-C, is predominantly expressed by lymphatic endothelial cells[105-107]. Efforts to engineer intestinal lymphatics might therefore utilise a combined strategy of generating primitive lymphatics *in vitro* via co-culture, whilst also employing biochemical induction of lymphangiogenesis and angiogenesis following TESI transplantation *in vivo*.

[H2] Immune cells and microbiome. The intestinal immune system, including both myeloid and lymphoid cells, interacts with the extensive gut microbiota to support intestinal homeostasis, whilst their dysregulation contributes to disease[108,109]. It is well recognised that the intestinal microbiota has a variety of essential functions that include regulation of host immune response via the innate immune system, mitigating against pathogen overgrowth, intestinal endocrine regulation and metabolism of bile salts[110]. Several *in vitro* intestinal co-culture models have incorporated immune cell populations including macrophages, neutrophils and intra-epithelial lymphocytes[111-113] and commensal and pathogenic microorganisms[88,114,115]. These models serve to investigate host–microbe–immune responses, specifically those governing autoimmune, inflammatory and infectious diseases. However, most of these studies present notable physiological differences to TESI, including use of immortalised epithelial cell lines. To date, most TESI constructs have not included immune cell populations or microbiota, as it is believed that the host immune cells and microbiota will infiltrate and colonise the grafts after transplantation *in vivo*. Interestingly, gut microbiota has been shown to influence epithelial cells when co-culturing organoids with various strains of commensal bacteria including *Lactobacillus*, *Escherichia Coli* (*E.Coli*) and anaerobes including *Bacteriodes Fragilis*[88,116]. Specifically, Lactobacillus and anaerobes affect epithelial cell proliferation and promote regeneration, as demonstrated by an increase in ISC gene and proliferative cell marker expression[88,117] postulated to be via activation of Wnt/β catenin[117]. *E. Coli.* and anaerobes have been shown to affect differentiation into goblet cells[88,116]. Conversely, the pathogenic bacterium *Clostridioides difficile* (formerly known as *Clostridium difficile*) has been

shown to induce colonic stem cell damage, impairing both organoid isolation from infected mice and delaying intestinal epithelial repair and regeneration *in vivo*[118]. Apart from intestinal epithelium, the intestinal microbiota also plays a part in regulating both ENS development and motility[119]. For example, bacterial metabolites; short chain fatty acids modulate serotonin release from the enterochromaffin cells of the intestinal epithelium and the neural transcription factor AHR has a role in regulating intestinal peristalsis in response to the microbiome[40,119]. In addition, the regional specific nature of both intestinal immune cell populations and microbiota would also need to be considered when generating TESI[120]. Whether immune cells and microbiota are introduced *in vitro,* or integrated within a graft following orthotopic transplantation *in vivo*, remains to be determined. More research is needed to study the role of immune cells and microbiome in intestinal epithelial cell maturation and maintenance.

[H2] Scaffolds. A bioscaffold, which provides structural support to the cellular components of TESI, needs to facilitate cellular attachment and proliferation, be robust enough to be transplanted as a graft and have similar mechanical and biochemical properties to the native tissue. Scaffolds can include: biopolymers, isolated biological polymers taken usually from the mammalian ECM such a collagen, elastin or fibrin, which have been used in exciting new areas such as hydrogels and bioinks; synthetic polymers, preferably biodegradable (such as polyglycolic acid (PGA), poly-L-lactic acid and poly(L-lactide-co-caprolactone) and natural polymer scaffolds (such as chitosan, which have been extensively utilised in TESI)[56,121,122]; or decellularized scaffolds, natural biological acellular scaffolds derived from native tissues that can be used in allogeneic or xenogeneic settings. Utilising polymers offers consistency and an ability to synthesize unlimited quantities. Furthermore, with the advent of electrospinning and 3D bioprinting, polymer scaffolds could be tailored to meet patient requirements. These fabrication techniques — electrospinning via applying voltage to produce thin polymer fibres for scaffold production and 3D bioprinting, generating scaffolds with or without cells — emulate composition and architecture of the native tissue, and have been successfully used in scaffold generation for a variety of tissue engineering applications[123,124]. Topography of the scaffold is indeed very relevant and it has been shown that microdesign can affect spatial distribution of intestinal epithelial cell-types[25]. Furthermore, ECM stiffness can also guide intestinal epithelial organisation[125] and determine ISC maintenance and differentiation[126]. However, polymer-based scaffolds generally lack the microarchitecture and biological cues responsible for cell engraftment and self-organisation, and scaling up of microdesign can be challenging. It has also been demonstrated that polymer scaffolds used so far in TESI induce an inflammatory response *in* vivo

when implanted in mice, posing a potential risk to recipient safety[88]. On the other hand, decellularized scaffolds have been derived from small and large animals and human intestine using various protocols that include enzymatic and chemicals solutions delivered by intraluminal, intravascular and/or by immersion[23,56,89,127]. Decellularized intestine is advantageous as it maintains the native microarchitecture and has the potential to offer, at least in the near future, the best physiological alternative. However, much effort is still needed towards preserving the original ECM composition, especially its minor components, assessing its functionality and scaling up for large tissues and organs. Other challenges of decellularized scaffolds include availability of donor tissue, to meet the requirements of the recipient, high variability between donor scaffolds, degradation related to long-term storage, and immunogenicity if antigen content is not completely removed during decellularization. Choosing the right scaffold is crucial for generating the appropriate TESI that suits specific patients' needs.

Progress to date

Historical perspective

The lack of a definitive curative therapy for SBS has led researchers' attempts to harness innate developmental and regenerative mechanisms to engineer intestine. In the early 1990s, Vacanti and colleagues can be credited with the first attempts at modern intestinal engineering (**Figure 2**)[43,128,129]. The enzymatically digested intestinal "organoid units", isolated from rats, were seeded onto a tube of biodegradable PGA[43]. With further development and refinement of this technique, Grikscheit and colleagues were able to show that these engineered intestinal constructs could form crypt-villus structures, rescue the weight loss caused by massive small bowel resection in a rat model of SBS[44] and subsequently went on to adapt the technique to human organoid units[90]. Although these efforts provided a promising start, the technique has a major limitation of requiring a large amount of source tissue relative to the amount of engineered intestine produced. Furthermore, duration of *in vitro* culture of organoid units is relatively limited and initial studies failed to demonstrate *in vitro* survival beyond 1 month[45], although a study published in 2018 showed that mouse organoid units can now be cultured up to 3 months[46].

Engineering intestinal mucosa

The establishment of stromal-free ISC-derived intestinal organoids in 2009 was a major advancement for ITE[52,53]. These long-lived organoids maintain their multipotency and genetic

stability in culture with unlimited expansion potential, which is the ideal cell source of TESI. In 2016, the first TESI with Lgr5-ISC organoids was achieved by seeding mouse cells onto PGA scaffolds, which were implanted in the peritoneal cavity of recipient mice[121]. These transplanted grafts showed preservation of the ISC compartment as well as all mature intestinal epithelial lineages on somewhat immature crypt-villus structures, with myofibroblasts and smooth muscle cells recruited into the graft from the host animal[121,130]. However, despite well-defined protocols for derivation and expansion, including good manufacturing practice (GMP)-compliant protocols[131], use of human ISC organoids for TESI has been somewhat limited. In 2020, the first patient-derived TESI has been generated using cells and scaffolds obtained from patients with intestinal failure[23]. Patient-derived organoids (PDOs) and fibroblasts were established from duodenum, jejunum, and ileum of children with intestinal failure. These organoids could be expanded exponentially, whilst retaining their regional identity, as assessed by characteristic brush border enzymes. Importantly, seeding of jejunal organoids on decellularized human small intestinal and colonic scaffolds can both functionally differentiate into jejunum that shows protease and disaccharidase activity, and barrier function. The TESI, transplanted into immunodeficient mice for up to 2 weeks, retained jejunal epithelial identity and recruited host vessels to the graft. Although the work has brought TESI a step closer to the clinic, the epithelium of the TESI after *in vivo* transplantation did not fully recapitulate a mature crypt-villus morphology and was enterocyte dominant[23]. Future study will be needed to further differentiate the patient-derived TESI into fully functional jejunum *in vivo*.

In parallel, several studies reported that intestinal organoids can also be derived from ESCs and iPSCs in 2011[54,132]. Organoids derived from different stem cell sources, adult ISCs or PSCs, have all been shown to have the capacity to regenerate functional intestinal epithelium *in vivo*[55,132,133] (**Figure 3**). Human intestinal organoids (HIOs) derived from PSCs contain all mature epithelial cell subtypes, mesenchymal cells, $SMA^{+}desmin^{+}$ smooth muscle cells, and evidence of epithelial function, but demonstrate a level of maturity more comparable to foetal intestine[54,55]. In 2015, Finkbeiner et al first successfully transplanted a PGA scaffold seeded with ESC-derived HIOs into an immunodeficient mouse model[56]. The TESI survived for 12 weeks with an organised crypt-villus morphology, expression of all epithelial cell subsets and subepithelial myofibroblasts, but an absence of a substantive ENS or demonstration of function in the TESI. However, transplantation of these HIOs seeded on porcine decellularized matrix did not show CDX2 expression, indicating that intestinal cell fate of ESC-derived HIOs was not preserved *in vivo* under those experimental conditions[56]. It will be

important to further investigate how terminal differentiation of ESC-derived HIOs can be improved when seeded on decellularized scaffolds.

Engineering muscle, enteric nerves, and vasculature

Most of the ITE work discussed so far has focused predominantly upon engineering the mucosal layer, whilst reconstruction of full thickness TESI requires additional neuromuscular elements. Several groups have reported the incorporation of a neural element to HIOs to generate TESI, most of which have involved seeding HIOs onto hydrolysable synthetic scaffolds[42,56,134]. In 2017, Workman et al. showed that iPSC-derived NCCs and ENS progenitors can be incorporated into HIOs with evidence neuronal function (by calcium transients) and nerve-mediated contractile activity *in vitro* and *in vivo*[73]. In parallel, Schlieve et al demonstrated that combining iPSC-derived NCCs with HIOs on PGA scaffolds in immunodeficient mice can establish submucosal and myenteric ganglia that showed neuroepithelial connections and neuron-dependent contractility and relaxation[42]. A study published in 2021 further showed that non-enteric pre-migratory NCCs can be functionally combined with HIOs to regulate peristalsis in TESI[134], suggesting that cell candidates for ENS reconstruction can be expanded to non-enteric origin. Apart from ENS, an organised and contractile smooth muscle coat is also vital to the propulsion of the food bolus. Zakhem et al combined duodenal smooth muscle cells with intestinal neural progenitors into a functional wavy sheet wrapped around a chitosan scaffold and transplanted the construct into the omentum of athymic rats for 4 weeks, followed by anastomosis to a bypass loop of native bowel[122]. Despite being incompletely epithelialized from the host, histological analysis showed the presence of digested food in the lumen of the construct, suggesting functional propulsion of luminal content. Furthermore, studies on muscle cell culture, including the use of mesoangioblasts to generate skeletal muscle in engineered oesophagus[20] and the first report of isolation and characterisation of mesoangioblasts from small intestinal tissue[135], offer insights into the continued improvements of smooth muscle cells for ITE.

As alluded to already, vascularisation in TESI has thus far mostly depended on *in vivo* vascularisation from the host animal, limiting the scale of the pre-transplant engineered construct[23,42,56,136]. Two studies provided major advancements in pre-vascularisation of TESI. In the first study, the Ott group produced a pre-vascularised TESI graft by repopulating decellularized rat intestine with iPSC-derived HIOs in the lumen, and human umbilical vein endothelial cells (HUVECs) through the superior mesenteric artery and vein[89]. $CD31^+$ cells were visible at the subepithelial level and the vessels of

the TESI were perfusable, albeit at 24% of the perfusability of freshly isolated cadaveric rat mesentery. Following heterotopic transplantation and anastomosis to the carotid artery and jugular vein under systemic heparinisation, glucose could be absorbed from the TESI and utilised by host tissues, as measured by ^{18}F-fluorodeoxyglucose-positron emission tomography[89]. In a subsequent study, Palikuqi et al showed that transient activation of ETV2 could reset vascular endothelial cells (R-VEC) to embryonic-like malleable vasculogenic endothelial cells[24]. R-VECs could repopulate decellularized rat mesentery down to the capillary level at the intestinal wall and formed stable networks *in vivo* that were perfusable with human blood. Upon co-culture, R-VECs could further vascularise ISC-derived organoids with adaptation of vascular-niche transcriptome, suggesting that co-culture of organoids with R-VECs prior to transplantation could improve the subepithelial vascular network of TESI[24].

Emerging technologies towards translation

Apart from the standard combination of cells and either polymer or decellularized scaffolds, several alternative approaches have been reported in the past five years that are of relevance to the production of multi-layered ITE. Work from the Lutolf lab has successfully engineered rationally designed functional mini-intestines at organoids-on-a-chip scale through microfabrication of crypt-villus-like channels, whereby ISC-derived organoids self-organised to form tube-shaped epithelia with an accessible lumen[25]. Although this process is not directly translatable for TESI reconstruction, such organoids-on-a-chip technology could benefit TESI by offering valuable physiological *ex vivo* models to study spatial and mechanical cues on epithelial cell heterogeneity and organisation in response to topography. Similarly, the same group has also generated centimetre-scale intestinal tube through 3D bioprinting of mouse intestinal organoids, recapitulating the tissue organisation in native intestine[137]. These micro- and macro-fabricated intestines have generated invaluable models for study of intestinal disease, drug discovery and regenerative medicine. Other emerging technologies might provide alternative scaffold or biofabrication strategies, and include intravital 3D printing, electrospinning, and the use of complex ECM-derived hydrogels[126,131,138] **(Box 2).** Furthermore, numerous studies have proven the beneficial effects of dynamic culture conditions, especially in perfusion bioreactors, on maturation of epithelial, muscular, and vascular components in TESI *in vitro*[20,23,89,139].

In 2021, Sugimoto and colleagues have taken ITE a step further via organ repurposing for SBS treatment[26]. This study involved generation of functional small intestinalised colon (SIC) in rat by

replacing a segment of colonic epithelium with ileal ISC organoids whilst retaining the native colonic muscular coat and neurovascular supply as an endogenous scaffold (**Figure 3**). The epithelium of the SIC retained its ileal phenotype, expressing sucrase-isomaltase and NPC1L1 that mediated cholesterol absorption, and formed mature crypts and villi with lacteals. Transplantation of SIC to rat SBS model reduced body weight loss and substantially increased the survival rate at 10 days from 0/4 in controls to 5/7, with 2/7 rats with SIC surviving over a month[26]. Together, these myriad works highlight the progress made, but also the complexities we must consider, when moving ITE towards the clinic.

Bench to bedside

Transitioning tissue engineering from the bench to the bedside necessitates an approach that incorporates the reliable delivery of an adequate functioning graft that is safe for patients[140]. Whilst major conceptual advances have been made towards ITE strategies for SBS, there remain several challenges to overcome before these might provide a viable treatment option for clinical application. Here, we highlight several of these key aspects that require consideration when transitioning TESI from the bench to the bedside.

Personalised TESI grafts

For treatment of *SBS*, the ultimate goal is to generate a full thickness functional intestinal graft for transplantation. Whilst this step is a considerable way off being achieved, the progress towards generating individual intestinal components offers the opportunity to employ targeted engineering strategies for specific diseases. It is therefore likely that the first clinically translatable therapies will be cell-based or partial reconstruction, rather than full thickness TESI, and perhaps in some cases prevent causative pathologies progressing to irreversible intestinal failure. Examples that illustrate the feasibility of this approach include generation of intestinal PDOs utilising CRISPR-Cas9 based gene editing in patients with cystic fibrosis[141] and colonic mucosal defects corrected using organoid therapies[133,142-144]. Such strategies have the potential to offer therapeutic benefit in mucosal disorders such as microvillus inclusion disease, inflammatory bowel disease or radiation-induced mucosal injury. The treatment could be either organoid-only transplantation or mucosal graft reconstruction for more severe disease with larger damaged surface areas. Similarly, neural crest cell therapies or neuromuscular layer ITE could be of value in the treatment of intestinal enteropathies such as Hirschsprung disease. Transplantation of enteric neural stem cells has been shown to rescue nitric oxide synthase deficient mouse colon[145]. Similarly, *in vivo* engraftment and

migration of human PSC-derived ENS precursors led to rescue disease-related mortality in Hirschsprung disease (*Ednrb*$^{s\text{-}l/s\text{-}l}$) mice[146]. For such therapies, demonstrating success and feasibility in larger animal models is essential before considering translation into human clinical trials.

Moving beyond cell-only therapies, the composition of TESI constructs will need to be personalised to both the patient and the underlying pathology. For instance, in patients with SBS, full thickness TESI will need to be region-specific reflecting the native bowel resected. In addition, as the majority of patients with SBS have preserved colon, it might be possible to adopt the previously discussed SIC approach by substituting colonic mucosa with engineered small intestinal mucosa, which can overcome the hurdle of engineering a fully functional neo-intestine[26]. This approach could be useful also in the setting of mucosal diseases in which engineered mucosal sheets containing functional intestinal epithelium and supportive subepithelial mesenchyme would be sufficient. Choosing the right engineering approach for TESI reconstruction could offer personalised strategies to treat specific intestinal disease.

Cell selection

Engineered intestine is comprised of cells derived either from the intestine, from PSC differentiation *in vitro* or from a mixture of the two. When approaching clinical translation, consideration needs to be given to selection of cell source, whether to pursue allogenic or autologous therapies and GMP requirements (**Box 3**).

Whilst ESCs are advantageous due to their pluripotency, they do not offer the possibility of autologous therapy and also come with substantial difficulties regarding ethical and political controversy[147], both of which might limit their clinical application in ITE. Directed differentiation of iPSCs has given rise to multi-layered primitive intestine[55] with ENS *in vivo*[73]. Whilst limited by scale, these are the closest cellular constructs to full thickness human TESI achieved to date. iPSC-based therapies are advantageous for those who lack sufficient intestine, such as patients who have complete jejunal and/or ileal loss, and provide an 'off the shelf' solution in TESI. However, the expansion capacity of iPSC-derived organoids is much less efficient than the mesenchymal-free ISC-derived organoids, somewhat limiting their upscaling. Furthermore, concerns regarding utilising iPSCs include variability across iPSC lines, epigenetic status and tumorigenic potential[148-150]. For instance, intestine generated from iPSCs, despite differentiation, retains a foetal signature[54,55,151]. Application of mechanical forces has achieved some maturation with a transcriptome shifted towards that of paediatric tissue[152]. With the risk of undesirable cell types developing *in vitro* and *in vivo*, efficient and reliable iPSC generation and differentiation protocols are needed prior to clinical

translation. Such protocols will also need to be robust for generating composite cells in a clinically relevant number for TESI reconstruction[153]. In addition, stringent quality controls including screening for genome instability, markers of pluripotency and tumorgenicity will be crucial to meet requirements for GMP compliance and be safe for transplantation[154]. Considering the approach to clinical translation, the immunogenic potential of autologous iPSCs remains unclear. Autologous engineered constructs offer clear clinical advantages, specifically avoiding immunosuppression and its associated morbidity. However, reprogramming, expansion, differentiation and fabrication protocols to generate GMP compliant, patient-specific autologous iPSCs will be both complex to establish and prohibitively expensive, with estimated costs of US$800,000 for an iPSC-derived cellular product[154]. Initial iPSC therapies are, therefore, likely to be allogeneic, so-called 'off the shelf' and would necessitate immunosuppression. Generating biobanks of iPSCs from screened and HLA-matched donors or multiple iPSC lines to cover all MHC classes, might overcome some challenges of immunogenicity. Ethical issues regarding donor selection and screening will also need to be considered.

By contrast, autologous intestinal epithelial cells can be established easily either as stromal-free ISC-derived organoids or multicellular organoid units for use in TESI[23,90,155]. Absence of mesenchyme enables robust expansion and maintenance of ISC-derived organoids, whilst multicellular organoid units have limited expansion potential. However, addition of mesenchymal cells are advantageous as they avoid the need for extrinsic growth factors and molecules in generation of TESI, some of which are animal-derived and hence not GMP-compliant[90,155]. Progress has also been made towards organoid culture protocols to achieve GMP compliance, including replacement of Matrigel with defined matrices such as ECM or synthetic hydrogel[126,131,156]. Whilst the use of ISCs has made strides towards clinical application[23], full thickness TESI will require ISCs in combination with mesenchyme, muscle, vasculature and ENS. This step has not yet been achieved *in vitro* and likely represents the next step towards generating autologous TESI.

Multi-layers, upscaling and challenges

Multi-layered intestinal tissue has been demonstrated with transplantation of iPSC-derived HIOs in mouse kidney capsule with differentiation into mucosa, submucosal and smooth muscle layers[55]. Subsequent addition of NCCs and formation of primitive neuronal plexuses illustrates promise regarding feasibility of generating a functional multi-layered intestinal graft[42,73], albeit the full diversity of enteric neuron cell types has yet to be determined[157]. However, such approach lacks scaffolding with limited upscaling potential to generate robust constructs which might subsequently

be amenable to surgical transplantation. TESI mucosal grafts using PDOs seeded onto scaffold are larger in size and sufficiently robust[23], yet the neuromuscular layers have yet to be combined to generate a fully functional multi-layer TESI graft in a clinically translatable fashion.

Intestinal grafts generated to date have predominantly relied upon *in vivo* vascularisation following implantation[55,73,123] which is not feasible for larger constructs. Further progress, therefore, needs to address the issue of vascularisation, either via a pedicled flap or engineered vasculature *in vitro*. Recent progress has been made generating vascular networks *in vitro* from re-programmed endothelial cells that anastomose with native vasculature when transplanted *in vivo*[24]. This step is promising toward engineered vasculature. To date, however, this has been generated in isolation, and the next step would be to combine such engineered vasculature with other intestinal components. For instance, TESI could be pre-vascularised by repopulating decellularized vascular networks with endothelial cells in perfusion bioreactors followed by seeding organoids into the decellularized intestinal lumen[24,89]. Alternatively, *in situ* vascularisation of constructs with omental flaps could be applied, as demonstrated *in vivo* in other multi-layered engineered organs, including trachea and oesophagus[19,20]. Introducing vasculature will be important for upscaling multi-layered TESI in the future to meet clinical needs.

Future perspectives

For treatment of SBS, multiple parameters need to be considered for generating clinically relevant TESI, including, size, absorptive, peristaltic, endocrine, barrier and immune functions, as well as genetic stability of the cells in the construct[22]. Major progress has been made in the ITE field in the past decade, ranging from advancements in stem cell technology and biomimetic scaffolding, to neuromuscular and lymphovascular engineering. These advancements have brought us a step closer to the reconstruction of full thickness multi-layered TESI. It has been previously established that presence of minimal 10% of neonatal small bowel length (~200cm) would help patients weaning off PN[4,158]. The goal is, therefore, to engineer 20cm functional multi-layered TESI to treat children with SBS. In adults, the capacity for adaptation of existing small bowel following resection seems to be much more variable than that of children[159]. Thus, it might be more difficult to quantify the length of TESI graft required. In a study of 268 adults with SBS, multivariate analysis demonstrated that small bowel length of <75cm was significantly associated with permanent dependence on PN ($p=0.001$) [160]. Length of TESI in adults will need to be customised to the individual for SBS treatment. In both children and adults, multi-layered TESI will also need to be size matched to the patient at

time of implantation, in terms of intestine lumen diameter. This step is most likely to be achieved via size-matching of the scaffold. As a consequence, transplantation of full thickness TESI grafts should have a similar technical surgical feasibility to current intestinal transplants. Engineered constructs will require appropriate populations of progenitor cells for each tissue type, so that the graft might be self-sustaining following implantation and will grow with the individual. Although there is limited clinical data available, a study reporting transplanted bio-engineered trachea in a child[19] lends support to this approach. Despite substantial growth and weight gain of the child during follow-up, 11cm in height and 5kg in weight, no upsizing of the graft was required[19] At 2-year follow-up endoscopy demonstrated complete epithelialisation, with respiratory epithelium, of the graft, no clinical or serological evidence of rejection and the patient had returned to school[19]. Vascular integration might occur in a similar way to transplantation; via microvascular anastomosis, or alternatively via use of a 2-stage vascularised flap as previously described[44]. The regenerative capacity of ENS has been demonstrated following both mouse and rat bowel anastomosis, including, in rats, a migratory response of neurons towards the anastomotic site[161,162]. This approach should facilitate amalgamation of engineered and native ENS following transplantation.

Whilst researchers are working towards upscaling of TESI grafts, it might be worth considering other alternative strategies to expedite translation of TESI to clinic (**Table 1**). Work on the SIC by transforming the host's existing colon to small intestine could offer innovative alternative treatment solution[26]. It is arguably more achievable by engineering mucosa only instead of full thickness multi-layered TESI. This technique is of particular translational interest as surgical mucosectomy and endoscopic mucosal resection are already established procedures in current clinical practice[163-165]. Furthermore, there is substantial functional redundancy in the colon, as demonstrated by adaptation following colectomy for malignancy[166]. Importantly, the ability of small intestinal organoids to retain their regional identity in culture and following *in vivo* transplantation in the colon has been demonstrated[23,26,142], highlighting the feasibility of this technology. Future studies will be needed to further optimise such organ repurposing strategies.

Other practical considerations for clinical translation of TESI include generation and storage of cells and scaffolds. Hydrolysable synthetic scaffolds, such as PGA, are available as GMP-compliant off-the-shelf products, whilst methods have been developed to cryopreserve decellularized scaffolds for future seeding[167]. *In vitro* expansion, vitrification and storage of cell lines used in production of experimental TESI need to be standardised. Any processes used to generate TESI for human

transplantation would all need to meet regulatory requirements[22]. Should these requirements be met, most cell and biological scaffold technologies currently used in ITE could be amenable to biobanking, including at the time of initial treatment for a condition that might lead to intestinal failure.

Whilst the current review has focussed on scientific advancements in ITE, the ethical and governance issues surrounding clinical translation of TESI are equally important. Given the global collaborative scientific effort required for success in ITE, there is a need for multilateral regulatory consensus with respect to stem cell products and engineered therapies. For example, agreed criteria for reporting degree of maturity and function will be required before clinical trial, as well as 'release criteria' for biological components of TESI constructs, such as an absence of pluripotency markers in iPSC-derived tissues[22,140]. Ideally, such regulatory consensus also includes consideration of equitable access to engineered therapies, given that initially such therapies will be extremely expensive and only available in geographically restricted areas, while substantial burden of disease exists in low and middle-income countries due to poor access to treatment[168]. It is also vital to consider the need for robust informed consent procedures when enrolling patients in clinical trials, including any relationship or associated commercial interests with the donors.

Apart from the continued development in ITE technology, advancing our mechanistic understanding of intestinal regeneration might also help improve TESI generation. For instance, it has been shown that stem and progenitor populations expand dramatically following intestinal resection[169] and that GLP-2 agonists have beneficial effects not only in patients with intestinal failure, but also in TESI constructs[23]. It is also well-reported that intestinal epithelium is highly plastic and can de-differentiate and replenish ISCs upon damage[57,170-173]. Studies have further demonstrated the similarity between foetal intestinal development and the regeneration programme following injury, including the importance of mechanical cues[174,175]. Understanding these regenerative processes might offer insights into advancing TESI development. In addition, research on mechanical impact on ISC maintenance and differentiation is also crucial for ITE. For example, high matrix stiffness promotes ISC expansion[126], whilst incorporation of uniaxial strain into HIO culture improves the epithelial morphology, barrier and muscle function of HIOs[152]. Current clinical practice could offer clues as well, particularly if we can advance our mechanistic understanding of intestinal adaptation following bowel lengthening surgery[176,177]. It is exciting to speculate how these discoveries could be harnessed in future TESI constructs.

Tissue engineering is a complex regenerative technology that requires collaborative effort across disciplines. To address the remaining biological, translational and governance issues, we advocate a multi-disciplinary consortium approach, as exemplified by the INTENS team (https://www.intens.info). INTENS brings together biologists, clinicians, biotechnologists and engineers across academia and industry, from five continents, to advance ITE through transparent collaboration. The consortium is supported by funding from the European Union's Horizon2020 research and innovation programme, which has resulted in advancements in matrix-epithelium interaction[126,131], foetal stem cell development and regeneration-induced reprogramming[174,175], organoids-on-a-chip and 3D bioprinting technology[25,137], as well as TESI mucosal graft using primary materials derived from intestinal failure patients[23]. More cross-disciplinary consortia should be encouraged to further advance ITE technology and bring TESI to clinical trials.

Finally, it is worth mentioning other alternative use of TESI beyond direct therapeutic applications. One of the key applications of organoids is disease modelling[178]. For instance, ISC organoids have been used to model malignancies by isolation directly from tumours[52,179], by introduction of oncogenic mutations into healthy organoids[180], by combination with endothelial cells[24], and by using air-liquid interface cultures to retain fibroblasts and immune cells in patient-derived cancer organoids[181]. In addition, patient-derived ISC organoids have been used to investigate hereditary multiple intestinal atresia[182], whereas iPSC-derived HIOs have been used to create an *in vitro* model of Hirschsprung disease[73]. Whilst disease modelling using organoids alone has provided some mechanistic insights, the lack of surrounding microenvironment might not fully capture cellular processes in response to stimuli such as drug screening and infection. Development of multi-layered diseased TESI *in vitro* will offer a more physiological model to faithfully recapitulate the pathology and treatment responses. In fact, several studies have demonstrated the use of ITE for disease modelling, including engineered human colon cancer model for invasion-driver gene screening[183], microfabrication of mini-intestine for injury and infection model[25] and 3D bioprinting of intestinal tube for drug treatment[137]. Further research is needed to perfect TESI for disease modelling, drug discovery, personalised and regenerative medicine.

Conclusions

Advances in stem cell and organoid technology, in particular, have fuelled progress in ITE over the past decade. The latest work, including TESI mucosal grafts generated with patient-derived

materials and intestinal repurposing strategies, have brought ITE a step closer to clinical translation and offer an innovative solution to overcome the challenge of engineering multi-layered TESI. Future research is likely to focus upon optimising these repurposing techniques, expediting TESI translation to clinic, alongside pursuing strategies to generate and upscale multi-layered TESI grafts. Adopting a collaborative approach, via combining expertise in stem cell biology, engineering and biotechnology, will be fundamental to the successful application of TESI in clinic for treatment of SBS.

Table 1 | Summary of intestinal engineering strategies: progress, limitations and future directions towards clinical translation

Engineering strategy	Progress to date	Limitations of strategy	Steps towards translation
Cell-based therapy	Generation of intestinal organoids from adult ISCs or PSCs *in vitro* Heterotopic and orthotopic transplantation of intestinal organoids[55,133,144] *In vivo* differentiation and generation of multi-layered intestinal graft from PSCs[55] Demonstrated absorptive and barrier function and adaptive response to intestinal resection (increased villus height and crypt fission)[55] Generation of primitive ENS with some neuronal function[42,73]	Small size of graft Absence of lymphovascular system and reliance upon in vivo vascularisation[55] Foetal signature of PSCs-derived grafts[55,151] Autologous grafts prohibitively expensive, whereas allogenic grafts would require immunosuppression	Upscaling graft size Orthotopic transplantation – large animal models GMP compliance Targeted therapy to meet specific patients' needs High likelihood of clinical translation of component grafts (e.g. organoids, ENS)
Engineering intestinal grafts using stem cells and scaffolds	Generation of mucosal grafts *in vitro* using ISCs and PSCs[23,56,121] Heterotopic transplantation of mucosal grafts *in vivo*[23,89] Partial revascularisation of grafts *in vivo*[89]	Mucosal layer only – lack ENS, musculature and lymphatics[23,56,89,121] Challenges in full thickness graft reconstruction Largely reliant upon *in vivo* vascularisation, limiting size of constructs[23,56] Foetal signature of PSCs used for grafts[151]	Progress to full thickness construct Personalised grafts for targeted therapy; e.g. mucosal engineering for mucosal disorders Strategies for *in vitro*/*in vivo* vascularisation[24] Orthotopic transplantation GMP compliance Collaborative approach harnessing multidisciplinary expertise

	Demonstrated digestive and absorptive properties and barrier function[23,89] Feasibility of engineering autologous grafts using patient derived materials[23]	Variability and/or availability of decellularized native tissue as scaffold Polymer-based scaffolds lack the microarchitecture and biological cues for cell engraftment	High likelihood of clinical translation of component grafts (e.g. mucosa, ENS); full thickness graft engineering will take longer time to achieve in clinical trials
Intestinal repurposing	Successful colonic mucosal removal and transplantation of small intestinal stem cells *in vivo*[26,142] Demonstrated engrafted cells maintain small intestine phenotype[26,142] Adaptation of existing vasculature and lymphatics and retention of ENS function[26] Demonstration of efficacy *in vivo* short gut model[26]	Not feasible if no/limited colon Removal of colonic epithelia may not be efficient Limited size of organoid delivery and mucosal replacement	Alternative epithelial removal/mucosal delivery techniques and upscaling Trial in larger animal models GMP compliance High likelihood of clinical translation of organ repurposing with preserved endogenous neuromusculature

ENS, enteric nervous system; GMP, good manufacturing practice; ISC, intestinal stem cell; PSC, pluripotent stem cell.

Figure 1 | Intestinal structure and components of engineered intestinal grafts
Schematic depicting intestinal structure and organisation. The self-renewing epithelium, derived from a stem cell population at the base of the crypts is supported by mesenchymal cells in the mucosal and submucosal layers. Neural crest cells recapitulate the submucosal and myenteric nerve plexi of the enteric nervous system and smooth muscle cells generate the circular and longitudinal muscle layers, both required for peristaltic graft function. Vasculature and lymphatics, required for nutrient absorption, may be derived from endothelial cells.

Figure 2 | Timeline highlighting significant advances in the field of intestinal tissue engineering.
Progress of intestinal tissue engineering (ITE) from the discovery of pluripotent stem cells (PSCs) and early grafts engineered from "organoid units" to more recent tissue engineered small intestine (TESI) strategies utilising human intestinal organoids (HIOs) and intestinal stem cell (ISC)-derived organoids. ESC, embryonic stem cell; iPSC, induced pluripotent stem cell.

Figure 3 | Cellular and scaffold sources used to generate TESI and summary of engineering strategies to date
Tissue engineered small intestine (TESI) grafts could be generated from a variety of cellular components including organoids, mesenchyme and neural crest cells derived from embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and primary cells. Sources of scaffold include decellularized intestinal tissue or synthetic or natural polymers. Intestinal engineering strategies employed to date include cell-based therapies, cell combinations seeded onto scaffold to produce mucosal grafts and mucosal repurposing to generate a small intestinalised colon.

Box 1 | Summary of short bowel syndrome

Patients with short bowel syndrome (SBS) have a substantial reduction in functioning small bowel length. In adults this is defined as <200cm of small intestine[184] whereas in children this varies depending upon their stage of growth, with estimates suggesting a residual small bowel length <25% expected for gestational age[184]. This reduction occurs as consequence of either substantial surgical resection in response to disease, infection or necrosis (for example, Crohn's disease, necrotising enterocolitis or small bowel volvulus) or due to congenital absence (for example, intestinal atresia or gastroschisis)[3,185]. With a substantially reduced absorptive intestinal epithelium, patients with SBS fail to maintain hydration, electrolyte homeostasis and nutrition. Without adequate nutritional support, symptoms of diarrhoea, dehydration, malnutrition and weight loss ensue.

The mainstay of treatment in SBS is nutritional support in the form of parenteral nutrition (PN); intravenous feeding delivered via a central line into a large vein[186]. In SBS, there is some adaptation of remaining intestine with increased villus height and elongation of crypts serving to increase the surface area for absorption, which enables some patients to subsequently achieve enteral autonomy[187]. The remainder, however, are maintained upon PN long-term. Whilst PN sustains growth and nutrition, it has notable complications including PN-related liver disease, leading to cirrhosis and liver failure and central-line-associated morbidity including line sepsis and central venous thrombosis in the vessels with intravenous access required to deliver PN[1,188,189].

Other treatments include pharmacological therapies such as glucagon-like-peptide-2 (GLP2) analogues and surgical interventions in SBS, encompassing various intestinal lengthening procedures, to increase both intestinal length, and epithelial surface area for absorption and slow intestinal transit[190,191]. However, these interventions rarely achieve substantial increases, the majority less than a twofold increase in intestinal length. Furthermore, whilst remaining small bowel length is important, factors including the site of the resected bowel (jejunum versus ileum), presence of ileocaecal valve and colon and the quality of the remaining bowel are also key determinants of outcomes in patients with SBS[185]. Intestinal transplantation is an alternative treatment, particularly in those with sequelae of PN. However, organ shortage, high rates of graft rejection (60% at 5 years), and morbidity and mortality due to long-term immunosuppression, give rise to poor survival[7,192]. Alternative approaches in the treatment of SBS are, therefore, needed.

Box 2 | Engineering techniques use to generate multi-layered grafts for clinical application

3D printing and/or bioprinting, using polymers (printing) or biomaterials and cells (bioprinting), has been used to engineer simpler tissues such as skin, cartilage and bone[124,193-195]. It is advantageous as, via precise positioning of biomaterials and cells, it is possible to mimic the structural complexity of native tissue and can occur at relatively small scales. Intravital bioprinting, with direct fabrication of constructs within defects and/or existing tissue, has been described, demonstrating the potential application of these techniques *in vivo*[138].

However, generation of more complex multi-layered organ grafts using 3D bioprinting, such as tissue engineered small intestine (TESI), remains in early development. This approach is due to difficulties in reproducing both the functional and biomechanical properties of tissue whilst also capturing the heterogeneous structural and cellular microenvironments within grafts. For example, hydrogels based on extracellular matrix are compatible with bioprinter technologies and facilitate intestinal stem cell growth[131]. However, they do not recapitulate the mechanical properties of native tissue. Similarly, generating vascular networks within 3D-printed tissue is also a challenge. Current strategies for promoting vascularisation, including incorporation of angiogenic growth factors and optimisation of pore size and/or channels, rely on the growth of vasculature from native tissue. The trajectory of bioprinting technology is towards increasingly fine resolution, but printing the hierarchical vascular network down to capillaries is not currently feasible[124].

To date, 3D printing and bioprinting has predominantly been used to generate scaffolds for both tracheal grafts[196] and TESI[197]. Generation of multi-layered grafts has harnessed other techniques to enable precise seeding of cells and/or growth factors into the relevant scaffold regions, for example via microinjection, which has been used in trachea, oesophagus and TESI[19,20,23]. Bioreactors can also enable culture of different cell types within defined compartments, such as concurrent culture of endothelial cells and intestinal epithelium in TESI[89]. Furthermore, both bioengineered oesophagus and TESI have illustrated the importance of dynamic culture to facilitate *in vitro* cellular maturation and engraftment[20,23,89,198]. *In vivo* heterotopic transplantation, for example into omentum of mice and pigs, has been used to stimulate oesophageal graft maturation[20,199] and, into kidney capsule of mice, has similarly been used generate primitive intestinal grafts via iPSC organoid maturation[55]

Box 3 | Good manufacturing practice

Good manufacturing practice (GMP) comprises guidelines, regulations and standards issued by international organisations and national regulatory bodies. In the UK, this body is the Medicine and Healthcare products Regulatory Authority (MHRA), the European Medicines Agency (EMA) in the EU and the Food and Drug Administration (FDA) in the USA[200-202]. GMP requirements aim to deliver a consistent level of efficacy, quality and safety of products generated from a range of industries including medical, food and drug manufacturing. In the UK and EU, cell-based therapies, gene therapy and tissue engineering constructs covered as Advanced Therapy Medicinal Products[203,204].

The general aims of GMP guidance are to ensure products: are of consistent high quality; are appropriate to their intended use; meet the requirements of the marketing authorisation or product specification

Products need to be manufactured in specific GMP-approved facilities (specific clean rooms classified A-D) run by personnel who have undergone training and competence requirements. Within these facilities there are stringent quality assurance systems whereby products are tested for purity, sterility (bacterial, fungal, mycoplasma and endotoxin contamination) functionality and/or efficacy and stability. For allogeneic products, there is additional donor testing for transmissible diseases. Thorough documentation ensures traceability of generated products. Specifically related to cell therapies and engineered grafts, the source of the donor cells needs to be known, reagents and products used for the production of cells or grafts need to be animal product-free. Regenerative medicine and cellular-based therapies have specific GMP challenges including biological variability giving rise to variable quality and yield and, as products with live cells, are of variable stability and have short shelf lives that might pose hurdles or difficulties for product storage and distribution.

Glossary

Terms	Definition
Stem cells	Cells with the ability to divide and produce further stem cells (self-renewal) and cells that can differentiate into specialised cell types (potency).
Pluripotent stem cells	Cells with the ability to be cultured indefinitely in an undifferentiated state, whilst retaining the ability to be differentiated into endoderm, mesoderm, and ectoderm.
Multipotent stem cells	Cells capable of self-renewal and the ability to develop into multiple specialised cell types but restricted to a certain organ or tissue type. Intestinal stem cells are an example.
Progenitor cells	A transitional cell type between stem and fully differentiated cell types, that has lost the ability for self-renewal but retained capacity for differentiation.
Mesangioblasts	Blood vessel associated multipotent progenitor cells with the capacity to differentiate into a variety of mesodermal cell types.
Neural crest cells	Neural progenitor cells derived from the cranial and sacral neural crest which migrate to the gut and give rise to the submucosal and myenteric plexuses of the ENS.
Organoids	Cluster of cells growing in 3D containing stem, progenitor, and differentiated cells that self-organise to resemble aspects of native tissue.
Epithelial organoids	Organoid containing stem, progenitor, and differentiated cells from epithelium only (single germ layer).
Multi-tissue organoids	Organoids containing cells of multiple germ layers, established through the co-culture of different cell types or differentiation of pluripotent stem cells. iPSC-derived intestinal organoids are an example.
Organoid units	Aggregates of intestinal epithelial cells with a core of mesenchyme obtained by mechanical and enzymatic digestion of small intestinal mucosa.
Hydrogel	A 3D structural network composed of natural (e.g. Matrigel®) or synthetic (e.g. polyglycolic acid) polymer units that can absorb large amounts of water relative to the dry weight of the component polymers.

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