Bioengineering Functional Human Jejunal Grafts for Intestinal Failure

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**Declaration**

*I, Laween Meran, confirm that the work presented in the thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.*

Signed:

Date:
**PhD Publications**


Lee S.E., Massie I., **Meran L.** & Li V.S.W. *Advances in Stem Cells and their Niches*. Volume 2, (2018), Pages 99-140. Chapter Four - Extracellular matrix remodelling in intestinal homeostasis and disease


* co-authorship

**PhD Prizes**

**Jeremy Powell-Tuck First Prize Winner for Best Abstract, June 2015**

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Abstract

Intestinal failure (IF), following extensive anatomical or functional loss of small intestine, has debilitating long-term effects on infants born with this condition. Priority of care is to increase the child’s length of functional intestine, jejunum in particular, to improve nutritional independence. Children with irreversible IF suffering complications of parenteral nutrition may be referred for intestinal transplantation. However, mortality rates are as high as 60% at 5 years. The aim of my project was to develop an innovative treatment strategy to rebuild the patients’ own jejunum for autologous transplantation. Here I report the reconstruction of transplantable intestinal mucosal grafts using primary human materials. Human jejunal intestinal organoids derived from paediatric patients with IF can be cultured and expanded efficiently in vitro with region-specific markers preserved. Decellularised human intestinal matrix with intact ultrastructure is used as biological scaffold. I show that the biochemical composition of decellularised human small intestine and colon matrix are virtually analogous, suggesting that they both can be used as scaffolds for jejunal graft reconstruction. Functional jejunal grafts with digestive enzyme-producing enterocytes can be efficiently engineered by repopulating human intestinal scaffolds with human jejunal organoids and fibroblasts in vitro, which can further survive and mature after in vivo transplantation. These primary human material based jejunal grafts provide proof-of-concept data for autologous transplantation of tissue engineered intestine in patients with IF.
To

Leylan and Dastan

You bring out the best in me

“See with your mind, hear with your heart”
Impact Statement

The small intestine is the primary site of digestion in the human body and can grow to approximately 6 metres in length. Intestinal failure occurs when disease or injury to the small intestine prevents adequate absorption of food and drink, leading to malnutrition and dehydration. A common cause of irreversible intestinal failure is short bowel syndrome, where large sections of the small intestine have been surgically removed. These patients are supported by providing essential nutrients directly into their veins. In severe cases they may be referred for intestinal transplantation, however donor organs are in shortage and survival rates are very low even after transplantation. This PhD project seeks to develop human intestinal tissue engineering techniques, to rebuild human small intestine for children with intestinal failure requiring transplantation. The research aim is to make a personalised graft using the patient’s own intestinal cells wherever possible.

Here I present my work in successfully engineering functional patient-specific jejunal grafts using autologous cells sources. This has resulted in significant impact on both clinical and the research fields. Clinically, the banking patient organoids at the point of intestinal resection in at risk cases, such as neonates with necrotising enterocolitis, may become the norm in the future. This work also introduces the new research concept of ‘recycling’ human colonic tissue to grow small intestinal grafts. The new protocols I developed of jejunal graft engineering maintained in dynamic flow bioreactors could also have a strong impact in drug screening and drug discovery fields. In addition, the physiologically relevant human intestinal grafts provide platforms for human disease modelling, as an alternative to using animals in research. Most importantly, the publication of my data represents a significant translational step forwards, pushing organoid technologies and regenerative medicine further towards clinical application. Finally, the research impact of this work was maximised by disseminating our data widely through press releases, such that its publication generated an ‘Alimetric score’ of 947 within a week, as over a hundred global news outlets reported on this work.
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Abbreviations

ALPI - Alkaline phosphatase
AB-PAS - Alcian blue and periodic acid Schiff
BMP - Bone morphogenic protein
CBC - Crypt base columnar
CHGA - Chromogranin A
COX 2 - Cyclooxygenase-2
ECM - Extracellular matrix
EGF - Epidermal growth factor
EN - Enteral nutrition
ESC - Embryonic stem cell
FEnS - Fetal enterospheres
FN - Fibronectin
GAG - Glycosaminoglycans
GI - Gastrointestinal
GFP - Green fluorescence protein
HA - Hyaluronan
hiPSC - Human induced pluripotent stem cells
HSPG - Heparan sulphate proteoglycan
HUVEC - Human umbilical vein endothelial cell
IF - Intestinal failure
ISC - Intestinal stem cell
LCT - Lactase
LRP6 - Low density lipoprotein receptor-related protein 6
LGR5 - Leucine Rich Repeat Containing G Protein-Coupled Receptor 5
MLC - Myosin light chain
MMP - Matrix Metalloproteinase
MUC2 - Mucin 2
OLFM4 - Olfactomedin 4
PDOs - Patient derived organoids
PN - Parenteral nutrition
RGD - Arginylglycylaspartic (domain)
SBS - Short bowel syndrome
SI - Small intestine
SIS - Small intestine submucosa
SOX 9 - SRY-box 9
SBS-IF - chronic intestinal failure due to short bowel syndrome
TAZ - Transcriptional coactivator with PDZ-binding motif
TESI - Tissue Engineered Small Intestine
TIMP - Tissue inhibitors of metalloproteinases
TLR4 - Toll like receptor 4
UEA1 - Ulex Europaeus Lectin 1 antibody
Wnt - Wingless-related integration
YAP - Yes association protein
## 1. Introduction

### 1.1 The Healthy Gastrointestinal Tract

A competent Gastrointestinal (GI) tract is essential for human health. This remarkable organ represents the largest mucosal surface area of the human body, estimated to be 200m$^2$, serving its chief role in nutrient digestion and absorption$^1$. In addition, its functional importance as an immunologic organ is well established as the gut is richly populated with immune cells and lymphoid tissue$^2$. As the primary interface of interaction between host and environment, the tract is densely populated with a microbial community that metabolises indigestible carbohydrates and produces essential vitamins$^3$. Whilst modulating mucosal immunity, the small intestine (SI) must also mount protective responses against invading pathogenic microorganisms in order to maintain intestinal homeostasis$^3$.$^4$. Meanwhile, the colon acts as the main reservoir of the microbial flora, the maintenance of which is essential for health and dysregulation has been implicated in multiple digestive disorders$^5$.$^6$, as well as more widespread pathological processes from neurological disorders to chronic systemic inflammatory conditions$^7$.$^9$.

Anatomically, the intestinal component of the GI tract consists of the SI (duodenum, jejunum, ileum) and large intestine (colon and rectum). Nutrient absorption primarily occurs in the SI, whereas water resorption occurs in the colon. The intestinal epithelium is constantly renewing and is organised along a crypt-villus axis. The villi, present in SI only, are covered in a range of differentiated cells that project into the lumen so that as food passes by, nutrient breakdown and absorption is maximised. Intestinal crypts are formed by invaginations of the epithelium from the mucosa into the deeper mesenchyme. Six major differentiated cell types of the intestinal epithelium perform the functions of the intestine: [1] absorptive enterocytes$^{10,11}$; [2] mucus secreting goblet cells$^{12}$; [3] hormone secreting enteroendocrine cells$^{13}$; [4] antimicrobial secreting Paneth cells$^{14,15}$; [5] taste-chemosensory tuft cells$^{16}$ and [6] immunosurveillance microfold “M” cells$^{17}$. Renewal of the epithelium is driven by
dedicated adult intestinal stem cells (ISCs) which are restricted to the base of the crypt compartment, called crypt base columnar (CBC) cells. Leucine rich repeat containing G protein-coupled Receptor 5 (LGR5), expressed exclusively in CBC cells, is a stem cell marker of the intestine. The ISCs divide and give rise to daughter cells entering the transit-amplifying (TA) zone which then proliferate and migrate upwards towards the crypt-villus junction. Here, they terminally differentiate before reaching the villus tip and are shed into the lumen, with the exception of Paneth cells that will migrate downwards back to the stem cell zone. Paneth cells are situated adjacent to the ISCs, acting as a major source of secreted growth factors such as Wnt, EGF and Notch ligands, crucial for stem cells survival. The whole ISC proliferation to differentiation journey from the base of the crypt to the villus tip lasts approximately 3-5 days in mice.

The multifaceted functional dimensions of the GI tract make it one of the most fascinating organs of the human body to study, with wide reaching implications on human health. The functional differences between the SI and colon are reflected in differences in mucosal micro-architecture and epithelial cell composition. Most obviously, the colonic epithelium lacks villi structure but retains crypt compartments, where the ISCs reside. However, subtle differences in epithelial cell properties also exist. For example, Goblet cells secreting MUC2 mucin glycoproteins are more abundant in the colon than the small intestine. The secreted antimicrobial glycocalyx mucous layer not only forms a barrier against bacteria, but also serves to protect the epithelium from the physical and mechanical stress during peristalsis. The mucous layer sits directly above the epithelium acting to concentrate Paneth cell secreted antimicrobial peptides close to the epithelial cells, thereby producing an antibacterial gradient from the cells towards the lumen. Interestingly, the properties of the mucus barrier system differ between colon and SI. In the colon, a double layered mucous barrier, formed of an inner adherent layer and an outer more porous layer exists. Whilst in the SI, it is a single non-adherent mucous layer with higher porosity, allowing microbes to penetrate the mucus relatively more easily.
Paneth and goblet cells are derived from a common secretory progenitor, and as such continue to share similar characteristics\textsuperscript{23}. Antibacterial peptides secreted from crypt Paneth cells are crucial for maintaining stem cell homeostasis and modulating the microbiome\textsuperscript{15,21}. However, Paneth cells are only present in the SI. In the colon, cells termed “Paneth-like” exist, which are in fact a subset of colonic goblet cells that are distinctly cKit\textsuperscript{+} (a receptor tyrosine kinase)\textsuperscript{24}. These Paneth-like cells are predominantly situated in the proximal colon or at sites of inflammation\textsuperscript{21}. These cells mimic Paneth cells as they are interdigitated with Lgr5\textsuperscript{+} stem cells in the colon crypts. They are regulated by Notch signalling and function similarly to Paneth cells in the SI to support Lgr5\textsuperscript{+} stem cells. Since much fewer “Paneth-like” cells exist in the colon resulting in relatively less antimicrobial defence, this might explain the greater need for a more robust double-layered mucus barrier present in the colon but not the SI.

Given these functional differences, both SI and colon are required for optimal health. However, whilst many patients are able to maintain a good life following a colectomy\textsuperscript{25}, resections of the small bowel leading to intestinal failure are much more disruptive and life-threatening to patients, children in particular\textsuperscript{26}, discussed further below.

1.2 Intestinal Failure - the clinical problem

Intestinal failure (IF) is defined as a reduction of functional gut mass below the minimal amount necessary for digestion and absorption required to satisfy nutrient and fluid requirements for maintenance in adults, or growth in children\textsuperscript{27}. Short bowel syndrome (SBS) describes a malabsorptive state that often follows massive resection of the SI, and is the most common cause of chronic irreversible IF (SBS-IF)\textsuperscript{28}.

Patients with IF comprise a heterogeneous group due to the multiple underlying causes and differences in residual bowel structure and function. In the paediatric population, the causes of IF can broadly be separated into three main pathogenesis groups: (i) anatomical loss due to SBS which may be secondary to intestinal resections due to necrotising enterocolitis, Crohn’s disease or congenital malformations such as intestinal atresia and gastroschisis; (ii) dysmotility due to neuromuscular
intestinal diseases such as Hirschsprung’s disease or chronic intestinal pseudo-obstruction (CIPO); (iii) congenital epithelial defects of the intestine such as microvillus inclusion disease and tufting enteropathy. In the adult population, the causes of IF include extensive bowel resections following vascular insults to the intestine, such as in mesenteric ischaemia, thrombosis or trauma. Severe Crohn’s disease, leading to multiple resections is also a common cause for SBS-IF, as in the paediatric population. Radiation enteritis causing epithelial inflammation and scarring leading to IF is more commonly seen in adult patients with malignancy.

The role of artificial nutrition in the care of patients with IF has been evolving for over 50 years. Following significant intestinal resection, the early postoperative phase is frequently characterized by administration of parenteral nutrition (PN) with the aim to transition gradually to enteral nutrition (EN), followed by the subsequent reintroduction of oral nutrition. EN is generally favoured over PN as clear physiological advantages have been documented in both animal studies and human clinical trial data when comparing subjects receiving EN versus PN. For example, mucosal hypoplasia is observed in the SI of fasted rats receiving parenteral nutrition. This implies that food passing through the gut has an important role in preserving normal physiology, mucosal immune function and systemic inflammation.

The progression from PN to EN may be hindered by bacterial overgrowth, persistent micronutrient deficiency and metabolic complications such as electrolyte imbalances and acute kidney injury. In general, the majority of patients with less than 100cm of small bowel will become dependent on PN. Underlying intestinal conditions such as Crohn’s disease, radiation enteritis or pseudo-obstruction increase the risk of a patient being persistently dependent on PN. IF patients that become dependent on PN may suffer numerous complications including bacterial overgrowth, line sepsis, central venous access thrombosis or PN-related liver disease. Ultimately, patients with irreversible IF suffering complications of PN may be referred for small bowel transplantation. However due to shortage of suitable organs many patients die on the transplant wait list. Among children who received a
transplant, mortality at 5 years is as high as 60% due to sepsis, graft failure and complications of long-term immunosuppression\textsuperscript{39}.

At a population level, accurate international data on the epidemiology of IF in children is difficult to attain owing to the condition’s rarity and the complex heterogeneous cohort of patient backgrounds associated with the condition\textsuperscript{40}. The ‘Paediatric IF Consortium’, consisting of 14 sites across the United States of America and Canada, report SBS to be the leading cause of IF in children, as well as being the underlying cause in almost half of all patients requiring home PN\textsuperscript{40-42}. The incidence of IF in infancy was reported to be 24.5 cases per 100,000 live births, based on a single-centre study in Toronto in 2004\textsuperscript{43}. Data gathered from across 33 hospitals in the UK indicate a fourfold increase in the number of children on long-term home PN between the years 1993 to 2012, with a regional point prevalence varying from 1.76 to 41.4 patients per million\textsuperscript{44}. More recently, in 2014 the Committee for Orphan Medicinal Products (COMP) estimated that SBS affects less than 1 in 10,000 people in the European Union (equivalent to approximately 51,000 people), therefore qualifying SBS as a rare disease as defined by the European Medicines Agency (EMA). Given the growing numbers of children on home PN, the limited success of current treatment strategies and high morbidity and mortality associated with IF, there is an urgent unmet clinical need for research into novel treatments for these patients.

1.3 Intestinal adaptation and regeneration

Intestinal adaptation and regeneration are two distinct but crucially important events that take place in response to loss of or damage to the small intestine. Adaptation occurs following massive resection of the intestine, whereby the organ exhibits a morphological compensatory change in order to expand the absorptive surface area per unit length of the intestine. The main features of intestinal adaptation include an increase in the crypt depth and elongation of villus height as well as enhanced rates of epithelial proliferation and apoptosis\textsuperscript{45-47}. This is accompanied by changes in intestinal blood flow and intestinal motility, that all together increase the absorptive capacity of the remaining intestine\textsuperscript{48}. Additionally, in mouse models of SBS, where 50% of the proximal small intestine has been
resected, ileal enterocytes were able to reprogram their regional identity towards a more proximal enterocyte identity. This indicates another feature of intestinal adaptation, maximising carbohydrate, protein and lipid digestion and micronutrient absorption which occurs mostly in the duodenum and jejunum.

The glucagon-like peptides (GLP-1 and GLP-2) are synthesised and released from enteroendocrine cells in the small and large intestine, in response to nutrient ingestion. GLP-1 regulates blood glucose by stimulating insulin secretion from pancreatic islet beta cells. In addition, GLP-1 action results in the inhibition of gastric emptying and acid secretion, reduction of food ingestion and secretion of glucagon. Meanwhile, GLP-2 is recognised as a potent epithelial trophic hormone, promoting nutrient absorption by stimulating crypt cell proliferation and inhibiting enterocyte apoptosis. GLP-2 also reduces epithelial permeability and intestinal motility. The actions of GLP-2 are mediated by a distinct receptor, thought to be expressed on a subset of enteroendocrine cells in the epithelium, and on enteric nervous cells with downstream actions mediated via subepithelial fibroblasts.

Thus, GLP-2 functions as a key regulator of mucosal integrity, permeability and nutrient absorption. Both GLP-1 and GLP-2 are rapidly inactivated by the exopeptidase enzyme, dipeptidyl peptidase IV (DPP-IV), resulting in a half-life of several minutes in vivo. The process of natural intestinal adaptation having a clinically significant effect on a patient with IF (i.e. the patient becoming independent of EN or PN), is slow and can take up to 2 years. However, this process can be accelerated pharmacologically by the drug Teduglutide (Brand names Gattex or Revestive®), licenced for clinical use in PN-dependent SBS-IF patients. Teduglutide is an analog of glucagon-like-peptide-2 (GLP-2) and differs from GLP-2 by one amino acid substitution. This substitution confers a much longer half-life than endogenous GLP-2, as it is more resistant to proteolysis from dipeptidyl peptidase-4 (DPP-4). In patients with IF, Teduglutide treatment increases villus height and crypt depth of the intestinal mucosa, offering some hope for improvement in their nutritional state.
On the other hand, intestinal regeneration describes the ability of the organ to renew itself in order to maintain function. The regenerative capacity of the gut is characterised by the relatively short half-life of intestinal epithelial cells. Hence the intestinal mucosa has one of the fastest self-renewal rates of all mammalian tissues. Under normal homeostasis conditions, this rapid turnover is driven by dedicated, mitotically active Lgr5⁺ CBCs (Figure 1.1). However, upon exposure to luminal pathogens or chemical insults that cause damage to the crypts and the CBC cells, the regenerative capacity is partially preserved. This indicates that there are “back-up” mechanisms of injury-induced regeneration in place. These include (i) the presence of quiescent ‘+4’ cells; (ii) distinct populations of reserve stem cells; and (iii) cellular plasticity - whereby differentiated cells undergo “dedifferentiation” regaining their previous ISC phenotype in order to replenish the ISC pool and repair the epithelial damage quickly. Intestinal cells demonstrating plasticity in order to contribute to the regenerative process have been observed in +4/5 position progenitor cells (expressing a range of various markers that include Tert⁺, Prox1⁺, Bmi1⁺, NeuroD1⁺, Atoh1⁺, Hopx⁺, Krt19⁺, Dll⁺)64-71; +6 position Alpi⁺ enterocytes; +4 position enteroendocrine cells69,73; +3 position secretory precursors Lgr5⁺ label retaining cells; and fully differentiated Paneth cells. Most recently in 2019, a “revival stem cell” (revSC) that is able to revive the homeostatic stem cell compartment and regenerate the intestinal epithelium was described. RevSCs are marked by high clusterin expression at the +4 position and are able to give rise to all major cell types of the intestine including Lgr5⁺ CBCs, after irradiation damage or dextran sodium sulphate induced damage to the mouse intestine. Overall, these studies suggest that cellular plasticity may be a feature in distinct cell populations, in response to critical damage to intestinal crypts.

1.4 Intestinal stem cells and their niche

The fast turnover rate of intestinal epithelium is supported by the ISCs located at the intestinal crypt. The intestinal crypt is enriched by a multitude of growth factors originating intrinsically from the crypt base cells and extrinsically from the extracellular matrix surrounding the crypts (Figure 1.1).
Alongside the regulatory signalling cascades governing ISC fate, the ECM also provides important stromal cell factors and biomechanical factors that are important in maintaining the ISC niche. The proteins of the intestinal crypt basement membrane are of mixed origin, being produced by cells of the epithelial compartment as well as the stromal compartment. The basement membrane consists of two layers: (i) the basal lamina positioned directly beneath epithelial cells and (ii) the underlying reticular sheet of matrix that anchors the epithelium to the lamina propria. The basement membrane is therefore hypothesised to be a key site for epithelial-mesenchymal cross-talk. Under homeostatic conditions, the ECM enables regulated bi-directional interaction despite the physical separation between stromal cells and intestinal epithelial cells. In this section the cellular and structural components of the ISC niche are discussed.

### 1.4.1 Signalling pathways regulating intestinal stem cells

A carefully orchestrated regulatory network exists to maintain a fine balance between ISC maintenance, proliferation and differentiation, thus protecting the niche when it is confronted with environmental fluctuations (such as toxins or infections) that could potentially lead to intestinal injury and disease.

The highly conserved Wnt signalling pathway is of fundamental importance in the maintenance and proliferation of ISCs. Wnt ligands are secreted by Paneth cells (Wnt 3, 6 and 9b) and pericryptal stromal cells (Wnts 2b, 4, 5a and 5b). Stromal cells also secrete the potent Wnt agonist R-spondin. Likewise, Notch signalling is also essential for ISC maintenance whereby inhibition of the pathway results in dampened stem cell proliferation. Paneth cells express membrane bound Notch ligands (Delta-like 1 and 4) that, when presented to notch receptors of neighbouring ISCs, activate the notch signalling pathway leading to crypt proliferation and epithelial regeneration. Subsequently, inactivation of Notch signalling drives the differentiation of progenitor cells towards the secretory lineage. Conversely, Bone Morphogenetic Protein (BMP) signalling restricts ISC expansion and promotes differentiation. Higher BMP ligands are expressed in the villus compartment whilst BMP
antagonists (Noggin, Gremlins 1 and 2) are secreted by pericryptal stromal cells, thereby generating a BMP-low environment in the intestinal crypts\textsuperscript{89}. The Hippo pathway also has a central role in determining cell-fate and GI tissue homeostasis, in particular controlling organ size by regulating ISC proliferation, differentiation and apoptosis\textsuperscript{90-92}. Here, the transcriptional co-activators YAP and TAZ transduce ECM mechanical-cytoskeletal cues, leading to changes in cell shape, proliferation and differentiation, with significant crosstalk between Hippo and Wnt signalling pathways. Finally, Hedgehog signalling has a key role in stem cell homeostasis and tissue regeneration and maintenance of the crypt-villus axis\textsuperscript{93}. Inhibition of Hedgehog signalling blocks villus formation whilst boosting ISC proliferation through enhancing Wnt/\(\beta\)-catenin activity\textsuperscript{94}.

An understanding of the cross-talk between all these signalling pathways is essential in order to accurately recreate the natural ISC niche \textit{in vitro} on scaffolds for the purpose of intestinal tissue engineering.

1.4.2 Stromal - epithelial interactions

Paneth cells, as discussed above, provide essential niche signals to their neighbouring stem cells\textsuperscript{95}. Alongside Paneth cells, crosstalk between the epithelium and the subepithelial stromal cells are also of critical influence on intestinal function and regeneration in the ISC niche. The mesenchymal compartment of the intestinal lamina propria contains multiple stromal cell populations with distinct phenotypes and function. The cell populations able to secrete ECM proteins, growth factors and ligands essential in the ISC niche include fibroblasts, myofibroblasts, mesenchymal stromal cells, endothelial cells, pericytes, neural cells and smooth muscle cells\textsuperscript{96,97}.

The predominant cells of the intestinal stroma are fibroblasts, of which there is significant phenotypic heterogeneity. Fibroblasts drive tissue repair processes such as wound healing, but are also implicated in carcinogenesis. Intestinal subepithelial myofibroblasts (ISEMFs), a member of the fibroblast family, are located in pericryptal regions and throughout the lamina propria. Of all the stromal cells present in the ECM, ISEMFs have received the most academic attention for their importance in the ISC niche,
given their ability to secrete Wnt ligands and BMP antagonists\textsuperscript{81,89,97,98}. TGFβ is thought to be a key inducer of myofibroblast differentiation, which exhibit qualities of smooth muscle cells as they are able to exert a contractile force\textsuperscript{98}. Upon normal wound healing, ECM remodelling is mediated by an inflammatory trigger of matrix catabolism by matrix metalloprotease enzymes (MMPs) secreted by ISEMFs\textsuperscript{98}. Once the healing process resolves, ISEMFs undergo apoptosis, mediated in part by IL-1β\textsuperscript{98}. Meanwhile, excessive ECM deposition is associated with a pathological persistence of activated ISEMFs, such as in inflammatory bowel diseases (IBD)\textsuperscript{99,100}.

Multipotent mesenchymal stromal cells (MSCs) are non-haematopoietic progenitor cells that were originally isolated from bone marrow but have been found to reside in all post-natal organs and tissues\textsuperscript{101,102}. They have functional roles in immunomodulation and regeneration\textsuperscript{102-104}, however their specific function in the gut has not been extensively explored. In mouse models of radiation injury, the systemic administration of MSCs was shown to improve intestinal epithelial repair\textsuperscript{105-107}. Indeed, several clinical trials are underway to investigate the therapeutic utility of MSCs in several diseases, including IBD, with contradictory long term results\textsuperscript{108-112}. It will be crucial to understand the exact role of MSCs in the maintenance of the ISC niche \textit{in vivo}, in order to improve their clinical applications for intestinal diseases.

Smooth muscle cells, present in close association with ISEMFs, form a thin layer of muscle (muscularis mucosa) to separate the lamina propria from the submucosa. The smooth muscle cells contract and relax to keep the muscularis mucosal layer under constant agitation\textsuperscript{97}. This function serves to expel potentially toxic luminal contents out of the crypts and away from the ISC niche. Similar to ISEMFs, smooth muscle cells also secrete BMP antagonists to repress the differentiative BMP signal and maintain the Wnt activity in the crypt base\textsuperscript{89}.

Endothelial cells present in the lamina propria are important in maintaining epithelial homeostasis. Previously published mouse model studies showed that radiation-induced injury triggered rapid endothelial apoptosis prior to epithelial death \textit{in vivo} \textsuperscript{111}. Importantly, loss of epithelial stem cells did
not occur when endothelial apoptosis was blocked by administration of a basic fibroblast growth factor (bFGF) treatment or by genetic deletion of the acid sphingomyelinase (ASMase) - a gene that is required for radiation-induced endothelial apoptosis. Endothelial cells are also implicated in the induction of intra-mucosal immune responses\textsuperscript{114,115}. However, their full role in homeostasis of the ISC niche remains to be discovered.

Pericytes are peri-endothelial myofibroblast-like contractile cells wrapping around the capillaries, which regulate angiogenesis and capillary wall permeability via paracrine signalling\textsuperscript{116}. However, the identity of the pericytes remains controversial, specifically regarding their ontogeny and progeny. Distinction between populations of pericytes and myofibroblasts is challenging since they express similar molecular markers\textsuperscript{117}. Subsets of pericytes have been reported to be multipotent progenitors that may participate in tissue regeneration\textsuperscript{118}. The comprehensive role of pericytes in the ISC niche remains unclear. It is thought that pericytes may function similarly as ISEMFs based on their close developmental origin and identity\textsuperscript{116,117}.

Neural cells are also present in the ISC niche and important for intestinal epithelial growth. The enteric nervous system consists of a large number of neurons and enteric glia cells (EGCs) that are interconnected to form the two ganglionated plexuses – the myenteric and the submucosal plexuses. EGCs are located both within the ganglia and in the extraganglionic regions, such as the lamina propria with close proximity to the intestinal crypts\textsuperscript{119,120}. Bjerknes and colleagues showed that enteric neurons participate in the feedback loop that regulates epithelial growth and repair by expressing the glucagon-like peptide 2 (GLP-2) receptor\textsuperscript{121}. In addition to their neuroprotective function, these mucosal EGCs are thought to play crucial roles in maintaining the intestinal epithelial barrier. Recent data show that EGCs homeostasis postnatally is dependent on functional host-microbe interactions, indicating their role in regulating immune responses in the gut\textsuperscript{122}. The EGCs also exert protective functions on the intestine by secreting factors such as epidermal growth factor (EGF) and TGFβ isoforms following inflammation or injury\textsuperscript{123,124}.
Finally, it is important to acknowledge the lamina propria is also rich in immune cells such as dendritic cells, macrophages and T-regulatory cells\textsuperscript{2,125}. A full discussion of the intestine’s mucosal immune system is not within the remit of this thesis, but has been reviewed extensively in the literature\textsuperscript{125,126}. Most recently, an important study used single-cell RNA sequencing to identify MHC class II machinery enrichment in two subsets of Lgr5\textsuperscript{+} ISCs, followed by functional studies co-culturing intestinal organoids with CD4\textsuperscript{+} T helper cells\textsuperscript{127}. It was demonstrated that pro-inflammatory signals (IFN\textgamma, IL-17a IL-13) promote intestinal epithelial cell differentiation whilst regulatory signals (IL-10) promoted ISC self-renewal. Furthermore, both in T cell depleted mice and in an MHCII knock out mouse model, the pool of ISCs expands. This indicates the important role of the Th1/2/17 immune axis in the ISC niche.
Figure 1.1 - The extracellular matrix and cellular components of the intestinal stem cell niche: The crypt surrounding microenvironment is made up of both physical/structural and cellular niche to regulate ISC homeostasis. The physical niche includes collagen fibres, integrins, fibronectin filaments, laminins, and glycosaminoglycan, which form a highly structured network named as the extracellular matrix (ECM). The cellular niche includes pericryptal myofibroblasts, fibroblasts, mesenchymal stromal cells, endothelial cells, pericytes, neural cells, smooth muscle cells and immune cells. The ECM and cellular niche interact and communicate with each other via different signalling pathways to maintain homeostasis of the intestinal epithelium, which includes: enterocytes, goblet cells, enteroendocrine cells, Paneth cells, tuft cells, M cells and intestinal stem cells.
1.4.3 The extracellular matrix of the intestinal stem cell niche

In addition to the cellular residents of the intestine’s ECM, the biomechanical parameters of the ECM are also important influences on the ISC niche (Figure 1.1). Several structural proteins make up the ECM, including fibronectins, laminins, collagens, glycosaminoglycans (e.g. heparan sulphate proteoglycans - also known as percalan) and integrins are reported to be enriched at the intestinal crypt base, suggesting their potential role in ISC regulation\(^{78,128-138}\). All these components influence intestinal epithelial proliferation and differentiation during migration from crypts to villus tips, healing following mucosal insult and injury, inflammatory bowel disease, and malignant transformation.

Laminin is a major glycoprotein constituent of the intestinal crypt basement membrane (together with fibronectin, type IV collagen and heparan sulphate proteoglycan) and is recognised to be particularly important in the establishment of epithelial cell polarity\(^{132,139}\). Laminin subtypes are key for the constitution of SI and colon basement membranes. Laminin \(\alpha 1\) and \(\alpha 2\) were shown to be enriched at the crypt regions, while laminin \(\alpha 5\) was expressed strongly at the villus basement membrane\(^{139-141}\). Laminin \(\alpha 5\) is believed to play a crucial role in establishing the mucosal pattern of the SI by maintaining the villus architecture\(^{140,142}\). The recent study on the designer matrices for intestinal organoid culture has further demonstrated that laminin-111 (\(\alpha 1\beta 1\gamma 1\)) is important to enhance ISC survival and proliferation\(^{143}\).

Fibronectin (FN) is a versatile high molecular weight adhesive glycoprotein found in a wide range of tissues and plays important roles in cell adhesion, migration, growth, and differentiation. In the intestinal ECM, it is secreted by myofibroblasts as well as being expressed by epithelial cells, and is located predominantly in the basement membrane, but also throughout the lamina propria\(^{131,136,144}\). Changes in the deposition pattern of FN correlates with several intestinal disease states. For example, an upregulation of fibronectin throughout epithelial cells is seen in conditions associated with intestinal fibrosis such as IBD\(^{129}\). Strain forces exerted in the ECM in vitro have been shown to induce FN mediated epithelial cell migration by activating the extracellular signal-regulated kinase (ERK) and
myosin light chain (MLC) signalling pathways, indicating the importance of FN in wound closure and epithelial migration\textsuperscript{145}. Furthermore, FN is postulated to be an activator of the nuclear factor-κB signalling pathway in the context of intestinal inflammation\textsuperscript{129}. FN is likely to have multifunctional roles since it contains binding sites for other ECM proteins such as collagens and glycosaminoglycans, as well as RGD binding sites for cell surface receptors of the integrin superfamily\textsuperscript{129}.

The integrin superfamily is a group of structurally and functionally related heterodimeric receptors, consisting of α and β subunits that link the ECM with the intracellular cytoskeleton as part of the RGD-adhesion system, and are particularly relevant in discussion of the ISC niche\textsuperscript{128,146}. Drosophila studies demonstrated integrin signalling to be obligatory for the maintenance and proliferation of ISCs, reputedly anchoring them in the crypts during the physical compression and stretching forces associated with peristaltic muscle contraction as food is propelled across the length of the gut\textsuperscript{147}. In the same study, several integrin subunits and signalling components were found to be expressed at high levels in ISCs but low levels in differentiated cells of the Drosophila midgut\textsuperscript{147}. β1 integrins are essential in regulating the proliferation of ISCs of a genetic mouse model, by mediating Hedgehog signalling\textsuperscript{148}. In human tissue, many biological effects of fibronectin are mediated through the transmembrane α1β5 integrin receptor. In addition, integrin α8β1 is thought to be a crucial mediator of intestinal crypt cell-matrix interaction, through the focal adhesion kinase (FAK) signalling pathway\textsuperscript{149-151}. Human intestinal epithelial cells have also been shown to be regulated by Integrin-linked kinase (ILK) through a fibronectin dependent mechanism\textsuperscript{152}. Overall, these studies provide evidence of an essential role for integrins in promoting ISC homeostasis and potentially intestinal tumorigenesis when integrin mediated mechanisms are disturbed.

Collagen is the most abundant structural ECM protein in the human body. The collagen superfamily contains at least 19 different subtypes, with types I, III, IV and VIII being secreted by fibroblasts being uniformly distributed in the health intestinal ECM\textsuperscript{135,153,154}. However, increasing evidence points to type VI collagen (which interacts closely with type IV collagen of the basement membrane) as a chief...
regulator of the mechanical microenvironment of the intestinal crypt cells, via fibronectin and RGD-dependent crypt cell interactions\textsuperscript{128,133}. Indeed, intestinal crypt epithelial cells have been demonstrated to secrete type VI collagen into the basal lamina of the intestinal basement membrane\textsuperscript{133}. Increases in ECM collagen deposition leads to increased tissue stiffness which alters integrin focal adhesions, growth factor receptor signalling and acto-myosin and cytoskeletal dependent cell contractility\textsuperscript{155}. However, in a dysregulated environment, as is seen in IBD, histological findings of excessive collagen cross-linking is found, associated with intestinal fibrosis and stricturing disease\textsuperscript{156,157}.

Glycosaminoglycans (GAGs) are present abundantly in the ECM and provide lubrication and structural integrity to cells owing to their high viscosity and low compressibility. These properties enable them to form a passageway between cells, facilitating cell migration\textsuperscript{158,159}. GAGs can operate as organisers of collagen fibre deposition, stimulating angiogenesis and inhibiting coagulation\textsuperscript{160}. The specific GAGs of physiological interest in the intestine are heparan sulphate proteoglycan (HSPG), hyaluronic acid, heparin, and chondroitin sulphate.

One of the most intensely studied GAGs in the intestine is HSPG, which are present in the ECM as linear polysaccharides of repeating disaccharide units of glucuronic acid and N-acetyl glucosamine, able to bind to Wnt, Hedgehog, TGF-β, and FGF in Drosophila and Xenopus studies\textsuperscript{134,161-163}. Intestine-specific HSPG is found on the basolateral surface of intestinal epithelial cells. Their role in the ISC niche has been implicated to be through their ability to modulate the Wnt/β-catenin signalling pathway during regeneration of small intestinal crypts\textsuperscript{134,164}. This study demonstrated that γ-radiation induced intestinal epithelial injury in mice deficient in intestine specific-HSPG led to a loss of Wnt target gene expression and reduced nuclear staining of β-catenin. This coupled with a reduced phosphorylation of low-density lipoprotein receptor-related protein-6 (LRP6) by stimulation with Wnt3a in the epithelial cells of HSPG-deficient mice, strongly proposes that HSPG present on the cell surface enhances binding of Wnt ligands to intestinal cells, thereby promoting intestinal regeneration\textsuperscript{134}. 

\textsuperscript{16}
In the healthy gut, another GAG named hyaluronic acid (HA) exists abundantly throughout the matrix as a chemically simple, high molecular weight, non-branching polymer of repeating units of N-acetyl-glucosamine. However, in disease processes such as in excessive inflammation, these polymers are cleaved to fragments of lower molecular weight that take on signalling roles. Though the mechanisms for HA degradation are incompletely understood, it has been recently demonstrated that fibroblasts in the guts of active Crohn’s disease secrete the protein KIAA1199, which leads to excessive degradation of HA and the generation of pro-inflammatory HA fragments. HA binds to CD44, which is expressed on the plasma membrane of many cell types, and also binds to the Toll-like receptors TLR2 and TLR4, which are widely distributed in the gastrointestinal tract and are important in mediating the host response to both commensal and pathogenic bacteria. Indeed, it has been shown that HA injected intraperitoneally to mouse models of radiation enteritis demonstrated a TLR4 and COX-2 mediated increase in intestinal crypt survival.

Application of knowledge on the intestinal ECM was demonstrated by Gjorevski et al. when they devised a modular synthetic hydrogel to study the effect of various ECM parameters on ISC behaviour in vitro. They showed that enrichment of hydrogels individually with ECM components including FN, laminin-111, collagen IV and HSPG enhanced ISC survival, when compared with inert matrix gels providing physical support alone. The combination of Laminin-111 together with FN’s RGD (Arg-Gly-Asp) peptide in mechanically dynamic gels that demonstrated the greatest influence on intestinal organoid culture efficiencies in a dose dependent fashion. Central to the study of biomechanics of the ECM is the Hippo signalling pathway, which incorporates mechanosensing and interacts with most of the major signalling pathways including Notch and Wnt. Cells interact with physical stimuli via cell adhesion molecules at their periphery, which allow the cytoskeleton to connect with the adjacent ECM structures. This enables microenvironmental forces to be sensed and translated into intracellular messages, in a process termed mechanotransduction. ECM signals act on the two transcriptional co-activators YAP (yes-associated protein) and TAZ (transcriptional co-activator with PDZ-binding motif). Indeed, YAP displays nuclear translocation and activation in
response to mechanical tension, hence is important in cellular mechanosensing and mechanotransduction\textsuperscript{170,171}. Gjorevski et al. demonstrated that ISCs embedded in hydrogel-based matrices of low stiffness correlated with poor proliferation and cytoplasmic localisation of YAP. Whereas increasing stiffness leads to the nuclear translocation and activation of YAP. Initial higher matrix stiffness enhanced YAP activation and ISC expansion, while the formation of organoids was vitally dependent on a dynamic softening profile of the matrix\textsuperscript{143}.

\textit{In vivo} ECM morphology, and therefore its mechanical properties, is under constant dynamic remodelling, whereby components are deposited, degraded or modified by cues conveyed to the matrix by cells. The process of intestinal ECM remodelling is strongly associated with angiogenesis, cell migration and differentiation, deposition and destruction via the matrix metalloproteinases (MMPs) family\textsuperscript{172}. MMPs comprise a large family of at least 25 zinc-dependent endopeptidases capable of degrading all components of the ECM. They are classified according to substrate specificity, and are themselves inhibited by tissue inhibitors of metalloproteinases (TIMPs). The role of MMPs in maintaining homeostasis of the ISC niche was confirmed through \textit{in vitro} studies showing that intestinal organoids cultured in RGD based hydrogels that are susceptible to rapid degradation by MMPs, promoted a pro-inflammatory phenotype in the organoids\textsuperscript{143}. These findings provide direct evidence that the physical properties of the ECM influence ISCs regulation.

\textbf{1.5 Intestinal tissue engineering}

Tissue engineering is an interdisciplinary field that comprises the combination of biomaterials to form scaffolds and stem cells for cellular regeneration, in order to create a construct to replace lost human tissues. Previous studies have reported pre-clinical developments of tissue engineering in many soft tissue organs including oesophagus, skeletal muscle, liver and lung reconstruction\textsuperscript{173-179}. Whilst engineering of simpler tissues such as skin and cornea are well established in clinical practice\textsuperscript{180,181}, examples of successful clinical applications of more complex organs have only been demonstrated in a few clinical case reports of trachea and bladder reconstruction\textsuperscript{182,183}. Intestinal
tissue engineering, through combination of biomaterials and patient-derived cells, offers a novel treatment approach for IF patients. This approach could render immunosuppression needless, thereby circumventing complications of graft-host rejection and malignancy. To date there have been no clinical studies of bioengineered SI grafts in humans. Furthermore, tissue engineering strategies are adaptable to enable personalised grafts whereby length, diameter and composition may be modified based on the individual patient’s underlying disease aetiology. For example, in SBS inadequate enteral absorption follows massive full thickness anatomical resection of the small intestine. The most predominant pathologies resulting in SBS include necrotizing enterocolitis (NEC), intestinal atresia, gastroschisis, malrotation with volvulus and Crohn’s disease. Hence in these circumstances, constructing a full thickness intestinal wall graft (including mucosa, submucosa and muscularis layers) in neonatal dimensions is essential. In purely neuromuscular intestinal diseases, such as extensive Hirschsprung’s disease or chronic intestinal pseudo-obstruction (CIPO), patients have a healthy intestinal epithelium but dysfunctional neuromuscular wall. Hence constructing a neuromuscular graft capable of peristalsis is paramount. Finally, in congenital epithelial defects such as in Microvillus Inclusion Disease or Intestinal Epithelial Dysplasia, the patient retains good neuromuscular function. Hence reconstruction of a purely mucosal graft would be of utmost significance in these patient groups.

Overall, tissue-engineered grafts are most suited to patients who have lost nutritional autonomy following an acute insult to the gut, as is the case in congenital malformations or necrotising enterocolitis. In these situations, the graft is unlikely to be vulnerable to ongoing disease processes. In contrast, chronic relapsing and remitting conditions such as Crohn’s disease will render the tissue-engineered graft susceptible to systemic inflammatory responses and end organ damage. In these clinical cases, optimisation of medical therapies prior to surgery will be crucial for the success of transplanted gut (whether cadaveric or tissue engineered). Similarly, in conditions such as Hirschsprung’s disease or congenital epithelial defects, *ex-vivo* correction of the cellular and genetic defects prior to recellularisation will be an essential step to longevity of the engineered intestinal graft.
In all cases, in order to maintain long-term viability after transplantation the engineered grafts require a vascular network to facilitate graft survival. Inducing neo-angiogenesis is also a key priority for intestinal tissue engineering. Similarly, the lymphatic system of the intestine must not be overlooked during tissue engineering studies. Intestinal lymphatics are essential for dietary lipid and fat-soluble vitamin absorption, as well as transporting antigens and antigen presenting cells\(^{185}\). Lymphatic vasculature is a one-way drainage system for interstitial fluid and immune cells. Fluid drains in to blind ending permeable lymphatic capillaries to form “lymph”. Lymph is then transported to lymph nodes by collecting vessels, before re-entering the blood circulation via the thoracic duct\(^{185}\). Indeed, loss or damage to the intestinal lymphatics results in severe gut inflammation, sepsis and death in mouse studies using diphtheria toxin administered selectively to ablate intestinal lymphatics\(^{186}\).

Finally, since this thesis focuses on infants and children with IF, it is important to consider the need for a tissue-engineered construct to grow in dimension at an appropriate rate for the child’s age and size. Previous studies of transplanted tissue-engineered constructs in children offer some insight into this question. The 4-year follow up study of one child who successfully received a tissue-engineered trachea at the age of 10 as a treatment of last resort, reported that engineered tracheal graft was vulnerable to malacia and stenosis\(^{187,188}\). This observation may be related to the growth dynamics and properties of cartilaginous tissue. Interestingly, long-term follow up publications on patients receiving engineered smooth muscle bladder constructs have not reported similar findings\(^{189,190}\). In long-term follow up canine studies of bladder augmentation with porcine SI matrix scaffolds indicated that the grafts can be remodelled and replaced by host tissue\(^{191}\). More longitudinal studies tracking the growth of the matrix as well as cells on engineered graft will be an essential step prior to adopting tissue-engineered therapies in situations other than ‘the last resort’. There have been important pre-clinical studies on tissue engineering of the intestine\(^{192-198}\). The ongoing debate regarding the best source of cells and scaffolds for intestinal tissue engineering is discussed further below.
1.5.1 Sources of intestinal cells

There are several sources of intestinal epithelial cells for ex-vivo expansion and transplantation purposes (Figure 1.2). These include human adult somatic intestinal stem cells (hISCs); human induced pluripotent stem cells (hiPSCs); human embryonic stem cells (hESCs) and human fetal enterospheres (FEnS). This body of work focuses on the use of adult somatic hISCs for tissue engineering and transplantation purposes. All these cellular sources are discussed in this section.

Previous attempts to establish the in vitro expansion of ISCs were extremely difficult without inducing genetic transformations. In 1992, Evans et al., first demonstrated primary adult intestinal crypt cultures that consisted of both epithelial and mesenchymal cells on collagen-type 1 coated plates\textsuperscript{199}. However, only short-term cultures lasting up to two weeks was possible using this method\textsuperscript{199-201}. In 2007, Barker et al., made the landmark discovery that ISCs were marked by the Wnt target gene Lgr5 in CBC cells of both the SI and colon\textsuperscript{18}. The increasing knowledge of the dynamics of ISC biology paved the way for the establishment of novel organoid “mini-gut” culture systems, first established by Sato et al., in 2009\textsuperscript{202}. The study showed that a single Lgr5-expressing ISC was able to grow three-dimensionally into crypt-villus budding structures in a Matrigel-based culture, and in the absence of a mesenchymal niche. This has proven to be another key milestone in the field of ISC biology, with significant research applications\textsuperscript{203,204}. Prior to this publication it was not possible to reliably culture ISCs that retained multipotency of all differentiated intestinal cell types in the lab. One month after the Sato et al. publication, another group led by Calvin Kuo in Stanford published an alternative collagen matrix-based air-liquid interface culture system, which also enabled long-term in vitro intestinal epithelial cultures. A key advantage of this method is preservation of epithelial-mesenchymal interactions the immune microenvironment. Once established, these organoids could be maintained for at least 1 year exhibiting proliferation and multi-lineage intestinal epithelial differentiation. However, this method has only been demonstrated to work for embryonic or neonatal murine cultures and the organoids cannot be expanded by passaging. Therefore Sato et al. methods
for both mouse and human intestinal organoid cultures have been more widely adopted in the field, since this method demonstrates unconstrained capacity for expansion with karyotype stability\textsuperscript{202,205}.

There have since been several intestinal organoid transplantation studies demonstrating regenerative medical applications of the ‘mini-gut’ organoids. For example, mouse intestinal organoids were injected into the colon lumen where they demonstrated a capacity for mucosal healing and regeneration, by integrating into the damaged mucosa of the mouse colon\textsuperscript{204}. Particularly noteworthy, is the ability of the engrafted SI organoids to maintain their original tissue identity to form villi when transplanted ectopically in the mouse colon\textsuperscript{206}. However, this finding was not replicated when GFP labelled murine fetal enterospheres (derived from the proximal fetal intestine) were implanted in a colonic injury model. These fetal-derived cells in this model engrafted at sites of denuded colon epithelium and expressed the marker carbonic anhydrase, specific to colon\textsuperscript{207}. This introduced new debate as to whether the regional and functional identities of the ISCs are determined by extracellular signals or the intrinsic cellular programme of the transplanted organoids were more important in determining the final functional identity of the cells. More recently, human colon organoids have been xenotransplanted orthotopically, using a Lgr5-CreER knockin to enable genetic lineage tracing\textsuperscript{208}. Here, they demonstrated that the human colon cells maintained multipotency and self-renewal, with a slower cycling status than the mouse colonic epithelium.

HiPSCs are another attractive source for cell therapy. They are generated by forced expression of OCT3/4, SOX2, KLF4 and c-MYC in differentiated adult cells such as fibroblasts\textsuperscript{209}. Once in their pluripotent state, they have been used to generate intestinal epithelium of all lineages \textit{in vitro}\textsuperscript{210,211}. These cells were ectopically transplanted under the kidney capsule of NSG NOD SCID mice, in the absence of a scaffold, where crypt-villus structures formed\textsuperscript{211}. Although not clinically relevant for human transplantation at these small scales without scaffolds, this \textit{in vivo} model using human pluripotent stem cells (either embryonic or induced) has proven to be an important system for modelling intestinal diseases. When combined with human vagal neural crest cells (NCCs) that were
differentiated into neurons and glial cells, and implanted in the kidney capsule of mice for 6-10 weeks, a functional enteric nervous system developed. This was further used to investigate the cell and molecular basis for Hirschsprung’s disease\textsuperscript{212}.

However, translation of this technology as cellular therapy in human clinical trials faces major hurdles. For example, viral vectors are commonly used for efficient reprogramming\textsuperscript{213}. Furthermore, transplantation of hiPSCs poses a risk of teratoma formation \textit{in vivo}\textsuperscript{214}. In contrast, since adult somatic hISCs are multipotent and fate restricted, their potential to induce teratoma formation is abolished. Additional concerns have also been raised over genetic mutations and chromosomal aberrations evolving during the reprogramming process, which may lead to malignancy\textsuperscript{215-218}. On the other hand, a key advantage of hiPSCs is the ability to non-invasively obtain donor cells from autologous sources, which is particularly important for IF patients who have very little or no small intestine left \textit{in situ}. Recently, a pre-clinical study on intestinal reconstruction using hiPSCs on rat scaffolds was reported\textsuperscript{193} (discussed in further detail in section 1.5.2). hiPSCs are currently in clinical trials for adult diseases such as age-related macular degeneration\textsuperscript{219}, Parkinson’s disease\textsuperscript{220} and severe heart failure\textsuperscript{221}.

However, for conditions of infancy and childhood, substantial concerns persist over the use of hiPSCs and hESCs not only due to the risk of teratoma formation, but also because of chromosomal aberrations during the reprogramming process which increase the risk of malignancy. In addition, it is accepted that iPSCs retain a degree of epigenetic memory of their original tissue, which may have influence on the behaviour of cells \textit{in vivo}\textsuperscript{222}. Finally, hiPSCs have been shown to be immunogenic in mice\textsuperscript{223}. This may mean that their uses in regenerative medicine may be limited as patients receiving such transplants will require immunosuppression.

Embryonic stem cells (ESCs) are derived from totipotent cells of the blastocyst stage of the early mammalian embryo (i.e. less than 8 weeks after fertilisation) and are capable of unlimited, undifferentiated proliferation \textit{in vitro}\textsuperscript{224}. Mouse ESCs have been induced to differentiate into functional intestinal tissue\textsuperscript{225,226}. Using similar protocols, human ES cell lines (commercially available)
have also been used to form intestinal tissue when engrafted in the kidney capsule of NSG mice. After 6 weeks in vivo, the cellular grafts contained most of the intestinal epithelial, mesenchymal and smooth muscle cell types found in the developing gut\textsuperscript{211}. Interestingly, when seeded into synthetic scaffolds (discussed further in section 1.5.2), smooth muscle cells were not present implying some loss of pluripotency in these conditions\textsuperscript{227}. Since the derivation of ESCs from human embryos is associated with significant ethical obstacles, this is likely to hinder their widespread use in translational clinical research.

Somewhat related is the development of protocols to generate human FEnS\textsuperscript{207}, derived from immature gut epithelium of first-trimester human fetuses (i.e. after 8 weeks from fertilisation) following elective abortions. However, the functional characterisation of human FEnS and fetal organoids in vitro remains relatively limited. Most of the data in this paper focuses on mouse FEnS, where Fordham et al. show that they are refractory to differentiation in vitro but demonstrate some potential for differentiation in a mouse model of colonic injury. Whilst mouse FEnS have been passaged for up to 180 days showing a normal karyotype\textsuperscript{207} and can be passaged for up to 2 years, human FEnS have thus far been reported to be maintained for over 2 months. In addition, it is still unclear whether fetal cells transplanted for regenerative medicine purposes will display immune privilege, echoing the maternal acceptance of the fetus during pregnancy. There is some evidence that allogeneic transplantation of fetal cells may evoke an immune response in the recipient, as demonstrated in some in vitro studies with human fetal retinal cells\textsuperscript{228}. This may represent challenges regarding their use in clinical translation.
Figure 1.2 - Sources of human intestinal stem cells for tissue engineering: Schematic diagram summarising that intestinal organoids have been generated from (i) Embryonic Stem Cells (ESCs) or Fetal Enterospheres (FEnS); (ii) induced pluripotent stem cells derived from skin fibroblasts and (iii) adult somatic intestinal stem cells (ISCs).
Scaffolds are essential for tissue engineering as they act as a physical platform and support network, guiding three dimensional cell growth (Figure 1.3)\(^2\). Scaffolds should ideally mimic the natural ECM that exists \textit{in vivo} as closely as possible. Biodegradability of the scaffold is also an important consideration, since this is important to limit ongoing foreign body reactions and inflammation. In the context of the intestine, retaining the characteristic crypt-villus axis is of utmost importance. Biomaterials used to generate scaffolds may be either synthetic or biological or a combination of the two. Furthermore, scaffold composition and design significantly impact cell behavior and identity\(^{173,176,196-198,227,229-231}\).

The choice of scaffold source depends on the desired mechanostructural properties required for the engineered organ, as well as cellular behaviour on the scaffold. Synthetic scaffolds are generally derived from biodegradable polymers and are moulded into the desired organ shape, or designed using 3D printers. The great advantage of synthetic scaffolds is the potential to develop an “off the shelf” product that is widely available and can be manufactured in large scale production lines. However, these polymers do not retain any of the natural nanotopography of the native intestine, nor any bioactivity. In contrast, biological scaffolds are those that are derived directly from the ECM of native organ through a process of decellularization, which describes the process whereby an organ is perfused with various chemical or biological solutions in order to remove all cellular and immunogenic material\(^2\). Professor Badylak’s research group have led extensive preclinical studies in the use of several biological scaffolds, including porcine small intestine submucosa (SIS), for use in a wide range of tissue environments such as oesophageal, skeletal muscle, tendon, vascular, abdominal wall and bladder repair\(^{229,233-236}\). Notably, the bioactivity of important growth factors is retained in studies of porcine SIS scaffolds, including transforming growth factor-\(\beta\) (TGF-\(\beta\)), basic fibroblast growth factor (b-FGF), and vascular endothelial growth factor (VEGF) following decellularization\(^{237-239}\). Therefore, an understanding of the mechanisms by which biological scaffolds promote site appropriate tissue
reconstruction, as well as their remodelling in vivo are instrumental to anticipating the host response and the long-term graft survival post-transplantation. Retaining bioactivity of the ECM is important after decellularization since excessive processing can reduce the amount of ECM molecules present. Badylak’s group also showed that the use of ionic detergents can reduce the amount of glycosaminoglycans present. It is therefore important to achieve the fine balance between complete decellularization and over-processing or using excessively harsh biochemical solutions that may also damage the microarchitecture and remove important organic signals of biological scaffolds. In addition, several clinical studies have demonstrated the use of porcine SIS in promoting wound healing and restoration of a wide range of tissue function, from chronic skin wounds such as burns to chest wall reconstruction. As such, porcine SIS is commercially available and approved by the Food and Drug Administration for a variety of clinical applications. However, porcine SIS is not currently routinely used for intestinal reconstruction. There is growing evidence for the use of SIS in SI tissue repair in rat, rabbit and canine models. In canine models, SIS grafts measuring approximately 7cm by 3cm in dimension were shown to encourage tissue repair in a model of partial small bowel wall resection. Encouragingly, this study demonstrated in vivo remodelling of the SIS to include histologically discreet villi on the mucosa as well as submucosa, smooth muscle and serosal layers by 6 months. Similar results were also seen in the rat models when 2cm-length tubular rat-SIS grafts were implanted. Meanwhile, porcine-SIS tubular grafts, 3cm in length, assessed in rat models were reported to enable rapid regeneration of mucosa and well as smooth muscle layers of the gut. However, full characterisation data of the neo-intestinal morphology was limited and it is unclear whether the central portion of the tubular graft was fully recellularised. All together, these studies indicate the intestinal matrix to be rich in biological characteristics, favourable for cell growth and regeneration in vivo and support the use of human biological intestinal decellularized scaffolds for intestinal tissue engineering in this thesis.
1.5.3 Recent advances in intestinal tissue engineering

To date, intestinal biological scaffolds have been derived from native rat\textsuperscript{193,230}, pig\textsuperscript{193,227} and human intestines\textsuperscript{227,249,250}. Despite this, published studies on engineering transplantable human intestinal grafts using biological scaffolds are surprisingly limited. The most sophisticated and pertinent of these studies is from 2017 by Kitano \textit{et al.}, where they described the seeding of hiPSC-derived intestinal organoids onto rat decellularised scaffolds with intact vasculature\textsuperscript{199}. Characterisation of a polarised columnar epithelium \textit{in vitro} showed differentiation to enterocytes, enteroendocrine cells, stromal cells and proliferative cells. However, goblet and Paneth cell differentiation \textit{in vitro} and crypt-villus morphology was missing. In parallel, the rat scaffold vascular tree was re-endothelialised by infusing human umbilical vein endothelial cells (HUVECs). The functional capacity of the regenerated intestinal constructs to absorb basic nutrients (glucose and medium-chain fatty acids) from the lumen was demonstrated \textit{in vitro}. The engineered grafts were then transplanted heterotopically in the cervical region of immunodeficient rats, whereby the superior mesenteric artery and vein of each graft was anastomosed to the right carotid artery and
right internal jugular vein respectively. The epithelial morphology following 4 weeks of in vivo transplantation showed presence of all differentiated cells (Paneth cells, goblet cells, enteroendocrine cells and enterocytes), indicating more optimal conditions for maturation of the grafts in vivo. Functional tests were repeated after 2 weeks in vivo, and showed glucose and fatty acid absorption. Positron emission tomography and computer tomography (PET-CT) was further used to trace the incorporation of glucose analog 18F-fluorodeoxyglucose delivered into the lumen of the graft, which showed prominent accumulation in the brain of the rats.

Another important study in 2016 utilised biological intestinal scaffolds to model colorectal cancer. Chen et al., described the engineering of an organotypic ex vivo colon cancer model using a biological human colon scaffold that was recellularised with primary human colon organoids and colon myofibroblasts derived from healthy individuals250. Commercially acquired microvascular endothelial cells were also used to re-populate the scaffolds. However, the co-culture of human colon organoid and myofibroblast cells were immortalised with human telomerase reverse transcriptase (hTERT) to prevent premature senescence and ensure their long-term growth potential. This may have artificially conferred an advantage to repopulating the scaffolds, a method which would not be translatable for clinical transplantation studies. Overall, their ex vivo system captures the pathological progression from APC-mutant neoplasia to a submucosal invasive tumour. Their data also included characterisation of the engineered colon epithelium with good crypt structure and demonstrated Lgr5+ proliferating cells at the base of crypts, alongside differentiated goblet cells and enteroendocrine cells higher up. However, absorptive enterocyte differentiation was not demonstrated in this paper and scaffold integrity could have been compromised since their seeding methods involved detachment of the mucosa from the submucosa. Details on the methods of culture were somewhat limited but implied that the engineered grafts were less than 1cm in size and were maintained in static conditions for up to 7 weeks in mutagenesis experiments. Though not directly related to tissue engineering for transplantation purposes, this study highlighted human biological intestinal scaffolds to be superb physiological platforms for cellular regeneration.
The use of synthetic scaffolds for intestinal tissue engineering has also been demonstrated in several notable studies\textsuperscript{227,251-254}. The scaffolds were derived from polyglycolic/poly L lactic acid (PGA/PLLA) polymers that were moulded into a tubular shape\textsuperscript{251}. Initial studies with these scaffolds used rat intestinal cells called “organoid units” are multicellular units derived from neonatal rat intestine containing a mesenchymal core surrounded by a polarised intestinal epithelium\textsuperscript{201}. These units contain mucosa, submucosa and muscle layers of the intestine (first described by Evans et al in 1992)\textsuperscript{199}. The intestinal cultures were seeded onto synthetic scaffolds to form “Tissue Engineered Small Intestine” (TESI) constructs. There was no in vitro culture period as the TESI was immediately transplanted into rat models of SBS. Results showed that rats receiving TESI had reduced mortality compared to controls\textsuperscript{254}. In subsequent studies, the same technique was used to construct and test TESI in vivo, in a Yorkshire swine model\textsuperscript{255}. Here, the seeded scaffolds were implanted by wrapping the scaffolds into the omentum or mesentery. Seven weeks later, the animals were sacrificed and the TESI constructs were harvested for analysis. The results demonstrated that all constructs retained engineered portions of intestine with crypt-villus axis preservation and stromal cell presence. Proliferation, as indicated by PCNA, was constrained to the crypt compartments. In addition, goblet and enteroendocrine cells were identified as well as the ganglion cells in Auerbach’s and Meissner’s plexi. However, there was no preservation of the intestinal smooth muscle layers, which was present in the original neonatal rat “organoid units”. Human TESI was also produced using similar protocols\textsuperscript{192,256}. Although there are physiological advantages of TESI that consists of all layers of the intestine, this methodology proved to be unfeasible for clinical translation as large lengths of donor intestinal tissue is required to generate relatively low numbers of “organoid units” for scaffold seedings, since the maintenance of these cultures were limited to 2 weeks (as discussed earlier).

Subsequent studies in 2015 using these synthetic scaffolds moved away from seeding multicellular “organoid units” and replaced them with hESC-derived intestinal organoids, the main advantage being their pluripotency and unlimited expansion potential\textsuperscript{227}. In these studies, embryonic derived intestinal organoids were seeded on both the PGA/PLLA synthetic scaffolds and porcine derived native
decellularised matrices. The grafts were cultured in vitro for 2 weeks, before being implanted subcutaneously into the omentum or under the kidney capsule. Interestingly, only synthetic scaffolds retained engineered intestinal epithelium with good epithelial morphology and presence of differentiated cells after 13 weeks in vivo. Vimentin positive stromal cells were also identified. However, despite the pluripotent origin of the embryonic derived organoids, no enteric nervous system (ENS) or smooth muscle cells were present in the grafts. In parallel experiments using the same protocols, seedings of hESC-derived intestinal organoids on native porcine produced largely negative results in vivo, the reasons for this remains unclear. No functional analyses data was included in this study. Although human intestinal decellularized scaffolds (derived from a cadaveric donor) were presented in supplementary figure 1 of this paper, no data demonstrating the seedings of embryonic derived organoids on that human decellularized intestinal scaffold was presented, suggesting that these experiments were either unfeasible or unsuccessful.

Finally, most recently in 2019, Ladd et al. published a study where synthetic scaffolds formed of poly glycerol sebacate (PGS) were fabricated using laser ablation to recreate the nanotopography of the villi but not crypts. Here, either CACO-2 cells or mouse GFP-labelled intestinal organoids (termed “enteroids” in the paper) were used for cell seedings. The results showed poor epithelial cell morphology with no signs of columnar monolayers or polarisation shown. The article focussed on the physical characteristics of the scaffold and biodegradability once implanted in omental pouches of mice. Morphology of the graft epithelium did not improve post-transplantation.

Altogether, these summarised published data indicate the great importance of selecting the appropriate intestinal cell source and scaffold type, which has significant influence on determining the eventual structural and functional identity of engineered intestinal grafts.
1.6 Research Aim, Hypotheses and Objectives

The prevailing research aim of this PhD thesis is to advance the recent developments in the field of intestinal tissue engineering towards clinical translation as much as possible for the target IF patient group. My particular focus is reconstruction of the intestinal mucosa. Specific objectives and associated research hypotheses are:

1. To build a “living biobank” of organoids derived from paediatric patients, specifically seeking endoscopic intestinal biopsies from children with established IF or those who are diagnosed with conditions that increase their risk of developing IF in their future.

- **Hypothesis:** The regenerative potential of ISCs is preserved in IF patients and can be exploited through organoid culture for expansion *in vitro*.

2. To generate a library of human intestinal scaffolds derived from paediatric patients undergoing intestinal resections, and perform in-depth characterisation of the biological scaffold matrix composition and microarchitecture.

- **Hypothesis:** Decellularised scaffolds derived from SI and colon are biosimilar in ECM composition.

3. To define the conditions required to effectively reconstruct the innermost layer of the intestine - the mucosa - and demonstrate the morphological and functional characteristics of the engineered intestinal constructs.

- **Hypothesis:** Decellularised SI and colon scaffolds are equally ideal platforms for engineering functional SI mucosal grafts.

4. To test the engineered intestinal mucosal constructs *in vivo*, and generate proof-of-concept data of graft maturation and survival in small animal transplantation models.

- **Hypothesis:** The survival and morphology of intestinal grafts is influenced by the *in vivo* transplantation site.
2. Material and Methods

2.1 Ethical Approval

Ethical approval for the use of human intestinal tissue was obtained from the Bloomsbury NRES committee (REC references 04-Q0508-79 and 18/EE/0150). The Committee was constituted in accordance with the Governance Arrangements for Research Ethics Committees and complied fully with the Standard Operating Procedures for Research Ethics Committees in the UK. Informed consent for the collection and use of human tissue was obtained from all patients, parents or legal guardians at Great Ormond Street Hospital, London.

2.2 Human Tissue

Wherever possible, endoscopic intestinal epithelial biopsies for organoids or fibroblast isolations were primarily sought from paediatric patients known to the intestinal failure multidisciplinary team at Great Ormond Street Hospital. For comparative experiments, tissue was also sought from other paediatric cases who, due to complex clinical backgrounds were at risk of developing intestinal failure. The full list of patient materials collected over the course of this thesis is listed in Appendix 1. Intestinal tissue for scaffold fabrication were sought from a broader clinical background of paediatric patients undergoing intestinal resection related procedures also listed in Appendix 1.

2.3 Animals

Immune deficient NOD-SCID IL-2Rnull (NSG) female mice, aged 9 - 14 weeks old, were used in all experiments (obtained from the Francis Crick Institute Biomedical Research Facility). All mice were housed in the animal facility at the Francis Crick Institute. All experiments were performed with ethical approval under Home Office Project License PPLs 70/8904 and PEF3478B3.

Porcine (Sus scrofa domesticus) SI from the ‘Pietrain’ breed was used to derive piglet SI scaffolds. Piglets up to 3 kg in weight were euthanized following criteria outlined by the JSR veterinary advisors. Once sacrificed, the animals were transported to the lab via courier and the intestine was harvested immediately on arrival (within 6 hours of euthanasia).
2.4 Patient-Derived Organoid and Fibroblasts and HUVEC Culture

Endoscopic biopsy specimens were cut into finer pieces and washed in cold PBS. For organoid isolations, the tissue fragments were incubated in 2mmol/L EDTA cold chelation buffer, consisting of distilled water with 5.6mmol/L Na2HPO4, 8mmol/L KH2PO4, 96.2mmol/L NaCl, 1.6mmol/L KCl, 43.4 mmol/L sucrose, 54.9 mmol/L D-sorbitol, 0.5mmol/L DL-dithiothreitol) for 30 minutes at 4°C as previously reported. Following this incubation period, crypts were released from the fragments by shaking vigorously. The supernatant was centrifuged at 800RPM for 5 minutes at 4°C, to form a pellet of intestinal crypts, which were washed in Advanced Dulbecco’s modified Eagle medium (DMEM) / F12 supplemented with 5% penicillin/streptomycin, 10mmol/L HEPES and 2mM of GlutaMAX. The crypts were then resuspended in Basement Membrane Extract® (BME®) and seeded in a single droplet on pre-heated 48-well plates. The BME® was polymerised for 20 minutes at 37°C, before adding 250μL/well of either human IntestiCult™ Organoid Growth Medium (STEMCELL Technology, #06010) or human organoid basal culture media consisting of conditioned media produced using stably transfected L cells (Wnt 50%; R-spondin 20%; Noggin 10%) and the following growth factors: B27 1X (Invitrogen), Nicotinamide 10mM (Sigma-Aldrich), N-acetyl cysteine 1mM (Sigma-Aldrich), TGF-β type I receptor inhibitor A83-01 500nM (Tocris), P38 inhibitor SB202190 10μM(Sigma-Aldrich), Gastrin 10nM (Sigma-Aldrich), EGF 50ng/ml (Invitrogen). Rho-kinase inhibitor Y-27632 was added to the culture media for the first week in culture at a concentration of 10μM. The media of each well was changed every 2 days. Organoids were expanded by passaging, as previously described. Briefly, the organoid culture medium was replaced with fresh basal culture medium. Organoids and BME® were mechanically disrupted using a P1000 pipette and transferred into a 15-ml falcon tube, and placed on ice. Further mechanical dissociation was achieved using a fire polished Pasteur pipette. Dissociated organoids were washed with 10 ml of basal culture medium and centrifuged at 800RPM for 5 min at 4°C. To achieve single cell dissociation of organoids, 500μl of TrypLE™ (Gibco) was added to the pellet of organoid, mixed gently with a P1000 pipette and incubated at 37°C for 3 minutes. 10% FBS/ADMEM was then added to the suspension to stop trypsinization, before repeating the centrifugation step at
1000RPM for 5 minutes at 4C. The supernatant was discarded, the pellet resuspended with fresh BME® and organoid culture medium was added. To enhance the expansion rate, organoids were cultured with 3µM CHIR99021 (CHIR) added to the alternate day media changes. The addition of CHIR enabled organoids to be passaged at least once a week, with an expansion ratio of 1:10 wells of a 24-well tissue culture plate. Organoids in differentiation phase were cultured in 10µM DAPT for 48 hours. For the isolation of human intestinal fibroblasts, intestinal fragments left over from the chelation step above were washed in PBS and placed on the bottom of tissue culture dishes with DMEM supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) (Sigma-Aldrich), 5% penicillin/streptomycin and 1x insulin-transferrin-selenium solution (both ThermoFisher). Fibroblasts grew from the fragments within 3-4 days. Cells used for seeding experiments were between passages 3-10. HUVECs were cultured in EGM-2 endothelial medium bullet kit (Lonza, cat.no. CC-3162) and were not used beyond passage 15.

2.5 Real time quantitative Reverse Transcription PCR analyses

RNA was extracted according to the manufacturer’s instructions (Qiagen RNAeasy). cDNA was prepared using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4368813). Quantitative PCR detection was performed using PowerUp™ SYBR® Green Master Mix (Applied Biosystems, A25742). Assays for each sample were run in triplicate and were normalised to the housekeeping gene β-actin. Primer sequences are listed in Appendix 2.

2.6 Western Blotting

BME droplets containing organoids in either basal, expansion (3µM CHIR) or differentiation (10µM DAPT) conditions were disrupted using ice cold Advanced Dulbecco’s modified Eagle medium (DMEM)/F12 supplemented with 5% penicillin/streptomycin, 10mmol/L HEPES and 2mM of GlutaMAX, pelleted at 1000RPM for 5 minutes and lysed in ice cold protein lysis buffer (150mmol/L NaCl, 30mmol/L Tris (pH 7.5), 1mmol/L EDTA, 1% Triton X-100, 10% glycerol, 0.1mmol/L phenylmethylsulfonfyl fluoride, 0.5mmol/L dithiothreitol, protease inhibitor cocktail tablets (EDTA-free) (Roche) and phosphatase inhibitor cocktail tablets (Roche)). Lysates were pelleted for 10 minutes at
13000PRM and supernatants stored at -80°C until analysis. Protein quantification was by Bradford assay. 40µg protein was loaded onto sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to membranes, which were blocked using 5% milk in Tris-buffered saline (50mM Tris, 150mmol/L NaCl, pH6) containing 0.1% Tween-20 (TBST) for 1 hour. Primary antibodies (as in Table S5) were added in 3% bovine serum albumin in TBST as follows: lysozyme (1:1000), Sox9 (1:1000), Olfm4 (1:1000), SI (1:200) overnight at 4°C. Membranes were washed in TBST before appropriate HRP-conjugated secondary antibodies were added at 1:5000 in 5% milk in TBST for 1 hour. Antibody binding was detected using chemiluminescence ECL Prime Western Blotting Substrate (GE Healthcare). Membranes were reprobed with anti-β actin peroxidase antibody (Sigma, A3854, 1:25000) for 10 minutes before ECL development. Band intensity was quantified in ImageJ and normalised to βactin.

2.7 Histology

Samples were fixed in 10% Neutralised Buffered Formalin (Sigma) at room temperature overnight, dehydrated in graded alcohols, paraffin embedded and sectioned at 4µm. Tissue slides were stained according to manufacturers’ instructions with Haematoxylin and Eosin (H&E) (Thermo Fisher), Alkaline Phosphatase (Vector Laboratories), Alcian-Blue Periodic Acid Schiff (Sigma). Picrosirius Red (PR), Elastic Van Gieson (EVG) and Alcian Blue (AB) (Thermo Fisher) staining was used to assess retention of collagen, elastin and glycosaminoglycans respectively.

2.8 Immunofluorescence and Immunohistochemistry

For immunofluorescence studies, paraffin embedded slides were rehydrated, permeabilized with 0.3% Triton X100 (Sigma, UK) for 30 minutes at room temperature. Heat mediated antigen retrieval was performed using a sodium citrate buffer (pH6). For whole mount immunostaining of intestinal organoids, cells were fixed with 4% PFA at room temperature for 20 minutes. Primary antibodies were diluted in 1% BSA/PBS/0.01% Triton X-100 and applied overnight at 4°C. Samples were incubated with Alexa Fluor secondary antibodies (Invitrogen) for 45 min at room temperature, washed and mounted with ProLongTM Diamond Antifade Mountant with DAPI (ThermoFisher). EdU staining with the Click-
iT EdU Alexa Fluor 568 Imaging kit (Life Technologies) followed the manufacturer’s protocol. DNA was stained with DAPI (Molecular Probes). Images were acquired using a Leica SP5 confocal microscope. For immunohistochemistry, antigen retrieval was performed using a sodium citrate buffer. Slides were permeabilized using a 0.2% Triton X100 (Sigma, UK) for 30 minutes at room temperature, and blocked with 5% bovine serum albumin (BSA) for 30 minutes. Primary antibodies were detected using peroxidase conjugated secondary antibodies using standard protocols as described previously. Image analysis and capture was performed using a Leica stereomicroscope or an inverted Nikon microscope. All antibodies used are listed in Appendix 3. Images were processed using ImageJ and Adobe Photoshop. Quantifications were performed on raw images however for presentational clarity, adjustments of brightness and contrast were applied equally to all panels of a given figure.

2.9 Scaffold Fabrication

The detergent-enzymatic treatment (DET) for decellularization, previously established on rat small bowel, was adapted for porcine and human intestine samples. One cycle of DET consisted of the following steps: (i) 24 hours of deionised water at 4°C; (ii) 4 hours of 4% sodium deoxycholate (Sigma) at room temperature; (iii) 1 hour of deionised water at room temperature; (iv) 3 hours of 2000kU DNase-1 (Sigma) in 1M NaCl (for human tissue) or 0.15M NaCl and 10mM CaCl (for piglet tissue) at room temperature. After harvesting piglet small intestine, the mesenteric artery, mesenteric vein and lumen were cannulated using a 29-gauge surgical cannula. The lumen of the intestine and the mesenteric artery were perfused with continuous fluid delivery of DET solutions using a Masterflex L/S variable speed roller pump at 3ml/min, for two cycles. Whole sections of human intestinal tissue collected from surgery were washed in cold PBS containing 5% penicillin/streptomycin to remove luminal contents, before starting decellularization by immersion in DET solutions and gentle agitation on a roller. For human scaffolds, two to three cycles of DET were performed, depending on both age of the patient and weight of the tissue received from the theatre. Specifically, age of patients: <6month: 1-2 cycles; 6 months - 1 year: 2-3 cycles; >1year: 3 cycles; weight of tissue: <3g: 1-2 cycles;
3-20g: 2-3 cycles; >20g: 3 cycles. Gamma irradiation at a dose of 16000Gy for 15 hours was applied to sterilise the scaffolds and then preserved at 4°C, in PBS containing 1% penicillin/streptomycin prior to use in cell culture.

2.10 Mechanical Testing

Age-matched SI or colon scaffolds were cut into transverse strips measuring approximately 10mm wide by 15mm long and blotted dry using tissue. The thickness of each sample was measured using electronic callipers. Tape was added to the ends of each sample before they were loaded into the grips of an Instron Tensile Tester 5565, equipped with a 500N load (Instron, High Wycombe, UK). A pressure of 3 bar was applied by the grips to hold both ends of the scaffold in place. Extension was at 5mm/minute until failure across the middle of the scaffold. Any scaffolds that slipped before failure were excluded from analysis. BlueHill 3 software (Instron) was used to generate stress-strain curves and Young’s modulus. Samples were measured in duplicate (with the exception of one colon sample since the dimensions of the tissue obtained was insufficient).

2.11 Electron Microscopy and Micro-CT imaging

For routine SEM imaging, decellularized human scaffold samples were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer and kept at 4°C for 24 hours. Samples were then washed with 0.1 M phosphate buffer and cut into segments of approximately 1 cm in length and cryoprotected in 25% sucrose, 10% glycerol in 0.05 M PBS (pH 7.4) for 2 hours, then fast frozen in nitrogen slush and freeze fractured at -160°C. Samples were then returned to the cryoprotectant solution and allowed to thaw at room temperature. After washing in 0.1 M phosphate buffer (pH 7.4), the material was fixed in 1% OsO4 in 0.1 M phosphate buffer (pH 7.4). After rinsing with distilled water, specimens were dehydrated in a graded ethanol-water series to 100% ethanol, critical point dried from CO2 and mounted onto aluminium stubs using sticky carbon tabs. Samples were coated with a thin layer of Au/Pd using an ion beam coater (Gatan UK). Images were recorded using a Jeol 7401 field emission gun scanning electron microscope.
For routine TEM, MicroCT imaging, serial block face SEM imaging and montage SEM imaging, recellularised scaffold grafts were fixed overnight at room temperature in 10% neutralised buffered formalin (Sigma) followed by a second fixation step in 2.5% glutaraldehyde/4% paraformaldehyde in 0.1 M phosphate buffer (pH7.4). The sample was post-fixed in 2% reduced osmium (4% OSO4, 1.5% K3FE(CN)6) for 60 minutes on ice and then washed in ddH2O.

To check the orientation of cells on recellularised scaffolds, the sample was embedded in CYGELTM (BioStatus, Leicestershire, UK) in an Eppendorf tube and a microCT scan was performed at 4kV/3W, with no filter, 1601 projections and a pixel size of 7.3379 µm using an Xradia 510 Versa (Zeiss). The data was automatically reconstructed using Scout-and-ScanTM Control System Reconstructor software (Zeiss) and viewed in TXM3DViewer software (Zeiss). With the presence of cells confirmed, the sample was immersed in ice, the CYGELTM washed off the sample with ddH2O and the sample trimmed to approximately 1 mm³ blocks. The trimmed blocks were then incubated in 1% aqueous thiocarbohydrazide at room temperature for 20 minutes then washed in ddH2O. The blocks were incubated in 2% aqueous osmium tetroxide for 30 minutes at room temperature and washed in ddH2O. This was followed by a further incubation in 1% aqueous uranyl acetate at 4°C overnight. The blocks were washed in ddH2O and then incubated in Walton’s lead aspartate for 30 minutes at 60°C before being dehydrated through a graded series of ethanol, infiltrated with Durcupan resin (Sigma-Aldrich) and polymerised for 48 hours at 60°C.

For routine TEM, 70 nm sections were cut on a UC6 ultramicrotome (Leica), picked up on formvar-coated slot grids and imaged in a Tecnai G2 Spirit Biotwin (ThermoFisher) with an Orius CCD camera (Gatan UK). For Serial Block Face SEM, Samples were trimmed to the region of interest, mounted on an aluminium pin (Leica Microsystems) and sputter coated with 2 nm of platinum. See Supplementary Table 6 for serial block face SEM imaging conditions. For SEM montage images, after the orientation of the cells within the block was determined by microCT, 200 nm sections were cut on a Powertome ultramicrotome (RMC) and picked up on ITO-coated coverslips. The coverslips were mounted on SEM
pin stubs (Agar Scientific) using a sticky carbon tab and sputter coated with 0.5 nm platinum. Sections were viewed in a Quanta FEG scanning electron microscope (ThermoFisher) using a backscattered electron (BSE) and large montage images acquired using MAPS 1.1 software (ThermoFisher). See Supplementary Table 7 for SEM montage imaging conditions. The montaged image in figure 5.5 was generated by stitching together individual images using TrakEM2, a plug-in of the FIJI framework. The montaged images in figure 6.5 were generated by stitching together individual images using MAPS 1.1 software (ThermoFisher).

2.12 Raman Spectroscopy

Raman imaging was conducted using a Renishaw RA816 Biological Analyser coupled to a 785 nm laser excitation source that is reshaped using cylindrical lenses to produce a line illumination (Renishaw plc, Wotton-under-edge, UK). A total laser intensity of approximately 158mW was focused onto the sample through a 50x/NA 0.8 objective. A 1500 lines/mm grating was used to disperse the laser light providing a spectral range of 0 to 2100 cm\(^{-1}\) in the low wavenumber range. The RA816 series undergoes a fully automated calibration and optimization sequence to ensure optimal performance, including calibration to the 520.5 cm\(^{-1}\) silicon peak. Raman imaging was conducted on colonic and small intestine sections previously embedded in paraffin wax and cut at 8 µm. Sections were mounted onto stainless steel slides, deparaffinised in xylene and rehydrated in graded alcohol and distilled water prior to Raman analysis. A total of thirty single point spectra were acquired from each histological region of both the large and small intestine (mucosa, submucosa and muscularis propria) using a 15 seconds integration time. Large Raman maps were acquired using the Renishaw StreamLine™ mode using a 4.4 µm step size and 1.1 seconds integration time per pixel. A total of 8,544 spectra were acquired across the colon map and 22,825 spectra across the small intestine map. Prior to any analysis all spectra were pre-processed to remove all non-chemical effects of the data acquisition process. All spectra were truncated between 400 - 1800 cm\(^{-1}\) which encompasses the fingerprint region where the majority of all biological signal lies. Cosmic ray removal was conducted using the width of feature and nearest neighbour methods in Renishaw’s WiRE 5 software. Spectra
were then imported into MATLAB R2017a (MathWorks, Natick, MA, USA) where baseline correction to a third order polynomial was conducted using the modified polyfit method\(^\text{37}\). Spectra were vector normalised and then analysed in both WiRE 5 and MATLAB R2017a.

Point spectra obtained from each distinct histological site in the colon and small intestine (mucosa, submucosa and muscularis propria) before and after decellularization were averaged and plotted on the same axis. The most obvious differences pre- and post-decellularization were highlighted. Principal component analysis (PCA) was then carried out to ascertain whether the biochemical difference between each distinct intestinal layer post-decellularization could be observed. This is an unsupervised multivariate analysis technique that allows an effective reduction in the dimensionality of the spectral dataset and hence facilitates the identification of combinations of highly correlated variables that best describe the variance in the data. Both of these procedures were carried out in MATLAB R2017a. The large high spatial resolution Raman maps were analysed in WiRE 5 (Renishaw plc, Wotton-under-Edge, UK) using unsupervised Multivariate Curve Resolution - Alternating Least Squares (MCR-ALS) approach for initial exploratory analysis to determine the global composition of each specimen. Using the reconstructed component curves, it was possible to identify some of the components abundant within each histological layer. Direct Classical Least Squares Component analysis was then used to acquire component images of a number of known biomolecules using previously acquired reference spectra. This enabled the mapping of the spatial distribution of known biomolecules within the full thickness of the intestinal wall.

2.13 Mass Spectrometry

Four biological replicates of both decellularized colon and SI ECM scaffolds were used for mass spectrometry analysis. Samples were prepared for analysis as previously reported\(^\text{260}\). Briefly, 1mg of lyophilised decellularized tissue was solubilised in 8M urea containing 10mM dithiothreitol. Iodoacetamide was added to a final concentration of 55mM and incubated for 30 minutes at room temperature protected from light. Proteins were treated with PNGaseF (25,000 units/mg) overnight. An initial protein digest using Lys-C (10 µg/mg for 4 hours) was followed up with two successive tryptic
digests (20 µg/mg overnight; 10 µg/mg for 4 hours). All enzyme reactions were performed at 37°C.

Four biological replicates each of colon and intestine scaffolds were processed. Peptide material was cleaned up using C18 Sep-Pak columns (Waters 50 mg sorbent, WAT054955). Eluates were dried in a speedvac concentrator. Dried peptides were solubilised in 0.1 % trifluoroacetic acid (TFA) to a concentration of approx. 5 µg/µl, then diluted to 0.25 µg/µl in a glass auto-sampler vial immediately prior to analysis. Each of the eight samples were analysed in technical triplicate (approx. 1 µg per injection) using a ThermoFisher Scientific QExactive mass spectrometer coupled to an UltiMate 3000 HPLC system for on-line liquid chromatographic separation. Each sample was initially loaded onto a C18 trap column (ThermoFisher Scientific Acclaim PepMap 100; 5 mm length, 300 µm inner diameter) then transferred onto a C18 reversed phase column (ThermoFisher Scientific Acclaim PepMap 100; 50 cm length, 75 µm inner diameter). Peptides were eluted at a flow rate of 250nL/min with a stepped gradient of 5-25% buffer B (80% acetonitrile, 0.1% formic acid, 5% DMSO) for 60 minutes followed by 25-40% for 20 minutes. Higher energy Collisional Dissociation (HCD) was used for MS/MS peptide fragmentation. Singly-charged and unknown charge state precursor ions were not analysed. Full MS spectra were acquired in the orbitrap (m/z 300–1800; resolution 70k; AGC target value 1E6) with the MS/MS spectra of the ten most abundant precursors from the preceding MS survey scan then acquired (resolution 17.5k, AGC target value 1E5; normalised collision energy 28 eV; minimum AGC target 1E2). Selected precursors were dynamically-excluded for 15 s.

Raw data files were processed using MaxQuant software (version 1.6.0.13) for protein identification and quantification using intensity based absolute quantification (iBAQ). iBAQ values were calculated for colon and SI by combining technical and biological replicates. A SwissProt Homo sapiens protein database (downloaded July 2017 containing 26,389 reviewed sequences) was searched with a fixed carbamidomethylation of cysteine modification and variable oxidation of methionine and protein acetylation (N-term) modifications. Protein and peptide false discovery rates were set at 1 %. The MaxQuant protein groups output file was imported into Perseus software (version 1.4.0.2) for further statistical analysis and data visualisation. Common contaminant proteins and reverse sequences were
removed. Intensity values were log2 transformed and Gene Ontology cellular compartment (GOCC) descriptions were added by Perseus. Protein detection was called when it was detected in at least 3 out of the 4 biological replicates of either colon or SI scaffolds. This resulted in 377 total proteins detected in these intestinal scaffolds (Appendix 4 and 5).

2.14 Scaffold Seeding Protocol
Sterilised sections of acellular intestinal scaffolds were immobilised in custom made mini platforms and placed at the bottom of 12 well tissue culture plates. When seeding scaffolds were intended for transplantation, human jejunal fibroblasts were trypsinised and resuspended in DMEM before seeding by multiple microinjections via 26g cannulae (Terumo SKU:SR+DU2619PX) into the lateral walls of the scaffolds, at a density of 1x10^6 cells/cm^2 and cultured in static conditions for 3 days. Human jejunal organoids were trypsinised and seeded onto the mucosal surface of the scaffolds at a density of 1x10^6 cells/cm^2. The cells were allowed to engraft for a period of 30 minutes at 37°C, before covering the whole scaffold with basal human intestinal organoid culture media. The scaffolds were maintained for another 4 days in static culture conditions, before transferring the scaffolds into perfusion plates (Amsbio #AMS.AVP-KIT-5) and connecting this to a bioreactor circuit. For in vitro functional and histology analyses, graft cultures were maintained for 14 days in dynamic culture conditions, with media circulating at a rate of 3ml/min. For in vivo transplantation, dynamic culture times were reduced to 7 days. For vascularisation experiments, HUVECs were injected at a density of 1x10^6 cells/cm^2 into the lateral walls of the grafts submucosally, 24 hours before transplantation into mice. Piglet scaffold seedings were seeding in a similar manner, without pre-injections of fibroblasts or HUVECs due to the lack of accuracy of injection in the thin scaffold walls.

2.15 Perfusion Bioreactor
The bioreactor circuit consisted of a media reservoir (custom made by Chem Glassware UK Manufacturers Ltd, London) with a 0.22μm air filter, inlet and outlet tubing (Cole Parmer cat.no. 224-2081), a peristaltic pump (Cole Parmer cat.no. 224-1505) and 3-way stopcocks (Becton Dickinson UK Ltd cat.no. 394601) for media sampling at both inlet and outlet points of the circuit.
2.16 Citrulline Quantification Assay

Graft culture supernatants were collected and citrulline levels quantified by spectrophotometry according to methods reported previously\textsuperscript{261}. Briefly, 20µl of each test sample was added to 20µl of water and 10µl of working urease solution, then incubated at 37°C for 30 minutes. 150µl of chromogenic reagent was then added to the solution and incubated for a further 60 minutes at 100°C to allow colour development. Absorbance was read at 520nm in a 96 well microtitre plate using a Tecan microplate reader (Infinite® M1000 PRO). Concentration was determined by comparison to a citrulline standard curve.

2.17 Disaccharidase Functional Assay

Grafts in dynamic culture were transferred from the bioreactor circuit into 6 well plates. The scaffolds were washed in PBS three times then incubated with a solution of 56mM sucrose in PBS (or PBS alone in control wells) for 60 minutes. Aliquots of the solution were then sampled for glucose detection using the Amplex\textsuperscript{TM} Red glucose/glucose oxidase assay kit (ThermoFisher cat.no. A22189) according to manufacturer’s protocol. Briefly, 50µl of the reaction working solution was added to 50µl of the test samples in a 96 well black flat bottom microtitre plate in triplicates, and incubated in the dark for 30 minutes at room temperature. The fluorescence (excitation 535nm, emission 590nm) was measured using a Tecan microplate reader (Infinite® M1000 PRO). Concentration was determined by comparison to a glucose standard curve.

2.18 β-Ala-Lys-AMCA Peptide Uptake Assay

Grafts in culture (or unseeded scaffolds as controls) were transferred from the bioreactor circuit into a 6 well plate and rinsed several times with PBS. They were then incubated with fresh human organoid culture media containing 25µM β-Ala-Lys-AMCA for 2 hours at 37°C. After incubation the media was removed and the graft was rinsed in cold PBS three times. The grafts (or unseeded scaffolds as controls) were fixed in 4% paraformaldehyde for 30 minutes at room temperature. Whole mount immunostaining was performed as described above, with Alexa Fluor 568 phalloidin staining to mark
cellular boundaries. The samples were then imaged using a Leica SP5 inverted confocal microscope. The fluorescence signal of β-Ala-Lys-AMCA was acquired using the UV laser.

2.19 Dipeptidyl peptidase IV assay
Grafts in culture (or unseeded controls) were removed from culture and washed in PBS three times. Gly-Pro p-nitroanilide hydrochloride (Sigma cat.no. G0513) was dissolved in PBS at a concentration of 1.5mM and added to the grafts for 1 hour at 37°C before supernatant was collected. Optical density (415nm) was measured in duplicate and concentration determined by comparison to a 4-nitroaniline (Sigma cat.no. 185310) standard curve.

2.20 FITC-Dextran barrier function assay
Piglet scaffolds were mounted into CellCrowns (Sigma cat.no. Z742381) (instead of onto custom made mini-platforms) before seeding with organoids and culture as described above. Grafts in culture (or unseeded controls) were removed from culture and washed in PBS three times before 100µl of 500µg/ml FITC-Dextran (Sigma cat.no. 46944-100MG-F) in media was added to the apical chamber of the CellCrown. 900µl of media was added to the basal chamber. Samples were incubated for 2 hours at 37°C. Media was then collected from the basal chamber into individual wells of a flat-bottomed, black-walled 96-well plate in duplicate. Presence of FITC-Dextran was determined by measuring fluorescence (excitation 490nm, emission 520nm). Percentage permeability of cellular grafts was calculated by comparison to unseeded scaffolds.

2.21 Lentiviral Preparation and Human Organoid Labelling
The lentiviral vector pHIV-LUC-ZsGreen (Addgene Inc. MA, USA, Plasmid #39196, kind gift from Dr Bryan Welm, Department of Surgery, University of Utah) was used to generate a lentivirus containing both ZsGreen fluorescent protein and firefly luciferase from an EF1-alpha promoter. Human jejunal organoids were labelled by lentiviral transduction as previously reported. Briefly, LUC-ZsGreen lentivirus was produced by co-transfecting 293T cells with the above plasmid along with packaging vectors PAX2 and VSV-G envelope plasmid (kind gifts from Dr Hans Clevers). Transfection was performed according to manufacturer’s instructions for 8 hours at 37°C. The medium (Opti-MEM®)
was exchanged for virus collection. After 24 hours, the virus-containing medium was purified by centrifugation at 2500 rpm (4 °C) and filtered through a 0.45μm membrane and ¼ volume of PEG-itTM was added to the filtered supernatant before ultracentrifugation at 2300 x g for 30 mins at 4°C (SW28 rotor, Optima LE80K Ultracentrifuge, Beckman). The viral pellet was resuspended in 1ml of pre-cooled Opti-MEM® (Gibco), aliquoted and stored at -80 °C. Human organoids were dissociated into single cells and cultured in the presence of 250μl 2x organoid culture media and 250μl viral particles and 2.5μl of TransDux™. Transduction efficacy was determined measured as the proportion of cells expressing the fluorescent protein ZsGreen 72 h after transduction.

2.22 In Vivo Transplantation Studies

NSG mice were anaesthetized with a 2-5% isoflurane: oxygen gas mix for induction and maintenance. The dorsum of each animal was shaved and the skin cleansed with 70% ethanol and povidone-iodine solution. For kidney capsule transplantation (n=3), each seeded graft (cultured as 1cm² patches) was carefully cut in half and gently folded to maintain the epithelial surface internally, before immediately inserting the graft under the capsule of the kidney. For subcutaneous transplantation (n=12), closed blunt scissors were used to create subcutaneous pockets bilaterally in the dorsum of each mice and one folded graft segment was inserted in each pocket. Subcutaneous Teduglutide or vehicle (PBS) was administered at a dose of 0.2 mg/kg/daily after implantation. The mice were sacrificed at 7 or 14 days for analysis. 2 hours prior to culling, each mouse was administered a dose of EdU (3ul/g of a stock solution of 10mg/ml).

2.23 Bioluminescence Imaging (BLI)

BLI was performed using an IVIS Spectrum in vivo imaging system (PerkinElmer, Waltham, MA, USA) and Living Image 4.3.1 software (PerkinElmer). Mice were injected intraperitoneal with 150 mg/kg D-luciferin (PerkinElmer) twenty minutes prior to imaging. All images were taken at field of view C or D, with automatic exposure time, pixel binning set to 8, f-stop 1 and open emission filter. This generated pseudo-coloured scaled images overlaid on grey scale photographs, providing 2-dimensional
localization of the source of light emission. All images were analysed using Living Image 4.3.1 software (PerkinElmer). Regions of interest (ROI) were drawn manually and the light emission was quantified in photons s⁻¹. ROI shapes were kept constant between images within each experiment.

2.24 Three-Dimension Volume Rendering of Transplantation Data
Serial sections were cut of the paraffin embedded sample. Odd numbered slides were stained with H&E while even numbered slides were kept unstained for further immunostaining analyses. Odd numbered H&E slides were then serially scanned (Olympus VS120 slide scanner) and the region of interest was aligned manually using Amira Software (ThermoFisher). Using the aligned slices, the kidney, scaffold, epithelial ring and lumen were segmented manually and saved as four separate label fields before generating 3D surfaces. A movie was created using Amira Animation Director.

2.25 Quantification of In Vivo data - Lumens and Vessels
Odd numbered H&E slides were scanned and images opened in QuPath software (University of Edinburgh). Each slide was checked for presence of epithelial lumen and blood vessels and this was quantified as a percentage across all sections.

2.26 Statistical Analyses
Statistical analysis was conducted on data from three or more biologically independent experimental or biological replicates wherever possible, as stated in the figure legends. Given the small sample sizes, the Shapiro-Wilk test of normality was performed. This indicated data distribution to be normal for the data presented in figures 3.2; 3.3; 4.5. Data in figures 6.5 and 6.6 did not pass the Shapiro-Wilk test of normality. Results are expressed as the mean ± s.e.m. Statistical significance was analyzed using unpaired Student’s t tests (two sided) or Mann Whitney U tests, for comparisons between two different groups. For analysis among more than two groups, statistical significance was analyzed using one-way ANOVA with Dunnett’s or Tukey’s post hoc multiple-comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 were considered significant. All attempts at replication were successful. Statistical analysis was carried out using Prism 8 (GraphPad Software).
3. Results I - Expansion of Patient Derived Intestinal Cells

3.1 Introduction

The physiological characteristics of the intestinal epithelium varies greatly along the cephalo-caudal axis of the GI tract\textsuperscript{50}. The SI is divided into three parts, based on anatomical, histological and functional specialisations: duodenum, jejunum and ileum. The duodenum receives gastric contents, bile, and digestive enzymes from the pancreas. Here, absorption of iron, calcium, and water-soluble vitamins takes place\textsuperscript{50}. The jejunum has the largest surface area of the small intestine whereby the majority of dietary lipids, carbohydrates and proteins are digested and absorbed\textsuperscript{50}. The ileum absorbs residual nutrients and mediates transport of bile acids and vitamin B12\textsuperscript{50,264}. Therefore, biological geography within the longitudinal axis of the SI is an important factor in determining the functional identity of epithelial cells once transplanted onto scaffolds. A previous study confirmed that intestinal region specific gene expressions are intrinsically programmed and are independent of extracellular and stromal cell signals\textsuperscript{265}. This indicates that in order to engineer a mucosal graft with jejunal function, organoids must be derived from biopsies of the jejunum, which is more challenging to access endoscopically and less frequently operated on surgically. Engineering a graft with jejunal function was prioritised in this thesis, as restoration of jejunal function is more likely to be associated with clinical improvements in nutritional autonomy and reducing PN requirements. This is because 90% of nutrient absorption and digestion occurs in the proximal 100-150cm of jejunum\textsuperscript{266,267}.

Considering the significant ethical and translational hindrances to the use of embryonic and induced pluripotent cells in clinical trials, discussed in the introduction chapter, an early decision was taken to focus on the use of primary ISCs resident in the human gut for this thesis. The main advantage of primary sources of ISCs are their high expandability, lineage restriction and genetic stability, when cultured using organoid methods. Therefore, this source is currently the safest option for use in tissue engineering of the gut. However, one potential disadvantage of the primary human intestinal organoid
technology is that it is a purely epithelial culture system\textsuperscript{202}. This means that, unlike iPSC or embryonic derived intestinal cultures, only epithelial cell populations are readily expanded \textit{in vitro} for each patient from endoscopic biopsies. Although the epithelium is the major functional component of the intestinal mucosa, non-epithelial cells such as fibroblasts, neural cells, muscle cells and endothelial cells have important niche roles. Isolating these important non-epithelial cell populations remains an important challenge in the effort to generate autologously sourced engineered grafts.

Finally, intestinal biopsies for the study were specifically sought from children with IF. This task may be complicated by the underlying cause of IF in the patient. For example, it is well recognised that chronic inflammation and tissue damage eventually depletes the regenerative capacity of many tissues\textsuperscript{268}. Therefore, isolating and expanding intestinal epithelial stem cells from patients with conditions such as IBD may prove more challenging. In addition, the efficiency of forming primary intestinal organoids from patients with IF, who have limited functional native gut \textit{in situ}, has not been demonstrated to date. Therefore, in order to apply this biotechnology to patients with IF specifically, it is necessary to optimise the organoid culture conditions for this specific patient group. This points the focus towards expanding the intestinal cells from each patient rapidly \textit{in vitro}, whilst preserving multilineage differentiation potential. This objective is guided by a previous study which demonstrated that the use of two small molecules, CHIR99021 and valproic acid, can increase to colony-forming efficiency of intestinal organoids by about 100 fold\textsuperscript{269}.

\subsection*{3.2 Isolation and expansion of patient derived intestinal organoids from endoscopic biopsies.}

To achieve maximal clinical relevance, I initiated a restriction of using a maximum of two endoscopic intestinal epithelial biopsies that were about 2mm in size, from each paediatric patient involved in the study (Fig. 3.1 a). In total, patient derived organoids (PDOs) were established from 12 children who had IF or are at risk of developing IF due to co-morbidities or complex surgical histories (Appendix 1). On average, three to five organoids were established by week four in culture following isolation. This was further expanded to over ten million cells by week eight of culture, passaging at a
ratio of roughly 1:8 to 1:10, depending on the density of organoids per well (Fig. 3.1 a-b). Once established in culture (i.e. after the 4-week point), the expansion efficiency was similar in all three regions (duodenum, jejunum or ileum) of the small intestine (Fig. 3.1 c), and did not vary across different clinical backgrounds. However, I noted that it took more time in culture to establish organoids derived from Crohn’s disease materials with (Patients 6 and 7 - appendix 1). However, once the organoids were established, the proliferation and differentiation potential of these organoids were indistinguishable from other PDOs, suggesting that in our culture conditions, the clinical background of these patients did not affect PDO production.

Figure 3.1 - Isolation and expansion of patient derived organoids from endoscopic biopsies: (a) Schematic overview demonstrating the expansion timeline after harvesting intestinal crypts endoscopically from paediatric patients. Each patient biopsy sample yields ~3-5 organoids by week 4
and over 10 million cells by week 8 after successive passaging as indicated by a representative phase
contrast image of organoids in culture at week 8. (b) Phase contrast images of human intestinal
organoids cultured in BME® [patient 6] established from isolation at the indicated time points. Original
magnifications: X20 (days 0); X10 (days 3, 8); X5 (days 15, 18, 28). (c) Representation phase contrast
images of first passage expansion cultures of duodenal [patient 9] (left), jejunal [patient 2] (middle)
and ileal [patient 3] organoids (right), all cultured in BME®. Scale bars represent 200μm. All images (b,
c) are representative of 3 experiments.

3.3 Intestinal cell location specificity is retained following extended passaging in vitro.

Among the 12 PDOs established, there were 3 duodenal, 3 jejunal and 6 ileal organoids
(Appendix 1). Previously study showed that the SI exhibits regionality in nutrient digestive and
absorptive function\(^{265}\). Quantitative reverse transcription-PCR (qRT-PCR) confirmed that PDOs
generated from epithelial biopsies expressed region-specific functional markers appropriately.
Duodenal organoids expressed the apical brush border enzyme cytochrome b reductase 1 (CYBRD1)
and the iron transporter solute carrier family 40 member 1 (SLC40A1); jejunal organoids express the
brush border digestive enzymes sucrase isomaltase (SI) and lactase (LCT); and ileal organoids
expressed the apical bile acid transporter (SLC10A2) and the basolateral organic solute transporter
(OSTB) (Fig. 3.2 a). Next, I focussed on the expansion of PDOs derived from the jejunum of 3 patients
[patients 2, 7 and 8 - appendix 1], since 90% of digestion and absorption occurs in the proximal 100–
150 cm of the jejunum. To reach cell numbers in the order of >10 million cells for each patient,
organoids were passaged rapidly in culture. The expression of jejunal specific markers SI and LCT were
verified after significant passaging time (P > 25), showing that the intrinsically programmed regional
identity of intestinal cells is maintained despite long-term culture in vitro (Fig. 3.2 b).

3.4 Differentiation potential of PDOs following extensive expansion

In order to expand the organoids in the shortest amount of time possible, PDOs were cultured
in the previously published human intestinal organoid media\(^{205}\), with the addition of the GSK3β
inhibitor (CHIR99021) to boost Wnt signalling and promote proliferation\(^{269}\). qRT-PCR analysis showed
that stem cell markers (OLFM4 (olfactomedin 4) and LGR5 (leucine rich repeat-containing G-protein-
coupled receptor 5)) and Paneth cell (LYZ (lysozyme)) genes were significantly upregulated in these conditions (Fig. 3.3 a). This was confirmed at protein level, by expression of EdU (ethynyl-2'-deoxyuridine) and KI67, indicating proliferation, and crypt compartment markers LGR5, SOX9 and LYZ.

Figure 3.2 - Intestinal region specific characterisation of organoids in culture: (a) Quantitative RT-qPCR analysis of human duodenal [patient 10], jejunal [patient 2] and ileal [patient 14] organoids for functional duodenal markers (CYBRD1; SLC40A1), jejunal markers (SI; LCT) and ileal markers (SLC10A2; OSTB). (b) Quantitative RT-qPCR analysis of human jejunal organoids [patient 2, 7, 8] at passages 5, 15 and 25 for jejunal specific markers SI and LCT. Quantitative data shown in represents mean ± s.e.m. of n = 3 experimental replicates (a) or of n = 3 biologically distinct replicates (b). Differences were analyzed by one-way ANOVA with Dunnett’s multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 were considered significant; n.s., not significant.

Data acquired with Isobel Massie and Nikos Angelis (Crick)
Next, the differentiation capacity of these jejunal PDOs were tested after prolonged expansion culture conditions. This was done by the addition of the gamma-secretase inhibitor (DAPT) to inhibit Notch signalling. qRT-PCR analysis showed that differentiation genes (MUC2 (mucin 2), ALPI (alkaline phophatase) and CHGA (chromogranin A)) were upregulated upon DAPT-treatment, alongside a loss of stem and Paneth cell markers (Fig. 3.3 a). This was confirmed at protein level by immunostaining for differentiation markers UEA-1, ALPI and CHGA (Fig. 3.3 c (lower panel)).

**Figure 3.3 - Differentiation potential of patient derived jejunal organoids:** (a) Quantitative RT-qPCR analysis of human jejunal organoids [patients 2, 7, 8] cultured in basal media culture conditions as indicated in the method, treated with the GSK3β inhibitor CHIR99021 (CHIR), or the Notch inhibitor DAPT. (b), Representative stainings for EdU and DAPI of human jejunal organoids [patient 2] in basal culture conditions and expansion conditions (+CHIR). Scale bars represent 30μm. (c) Representative immunostaining of human jejunal organoids [patient 2] cultured in expansion conditions (+CHIR) or
differentiation conditions (+DAPT) using the indicated antibodies to mark proliferating cells (Ki67), stem cells (LGR5 and SOX9), Paneth cells (LYZ), goblet cells (UEA-1), epithelial cells (E-cad), enterocytes (alkaline phosphatase) and enteroendocrine cells (CHGA). Scale bars represent 100μm. Images are representative of 2 (b) or 3 (c) experiments. Quantitative data shown in (a) represents mean ± s.e.m. of n = 3 biologically distinct replicates. Differences were analyzed by one-way ANOVA with Tukey’s multiple comparisons. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 were considered significant; n.s., not significant.

3.5 Isolation and expansion of human intestinal fibroblasts

I further generated primary human intestinal fibroblasts isolated from the same intestinal tissues used to derive organoids or SI scaffolds (Appendix 1). Primary human intestinal fibroblasts could be cultured and expanded for up to 10 passages while retaining strong expression of typical stromal matrix markers fibronectin (F-NEC), vimentin, fibroblast surface protein marker-1 (FSP-1), laminin α5 (LAMAS5) and with scattered weaker co-expression of alpha-smooth muscle actin (αSMA) (Fig. 3.4). This indicates a mixed population of intestinal fibroblasts and myofibroblasts, which are both essential stromal niche for the survival and maintenance of human ISCs. These cells were used for seedings of jejunal grafts intended for in vivo transplantation experiments, discussed further in chapters 5 and 6.

![Image of immunofluorescent images of primary human jejunal fibroblasts showing fibronectin (F-NEC, yellow), vimentin (green), fibroblasts surface protein marker-1 (FSP-1, magenta), laminin alpha 5 (LAMAS5, cyan) and alpha-smooth muscle actin (α-SMA, red).]
3.6 Discussion

All together, these results demonstrate that PDOs can be generated robustly children with IF, from as little as two 2mm sized epithelial biopsies. The organoids multiply rapidly \textit{ex vivo} under expansion media (+CHIR) while maintaining their original intestinal regional identity and differentiation potential upon Notch inhibition (+DAPT). This data is in keeping with results from previously published studies using human and mouse intestinal organoids\textsuperscript{265,269}. Using the same tissue samples, human SI fibroblasts could also be isolated and expanded from the IF patient group.

Clinically, the small bowel length in a term neonate measures just above 200cm and there is a correlation between those with $\geq 10\%$ of their predicted small-bowel length and their ability to wean off parenteral nutrition (PN)\textsuperscript{266}. Therefore, in order for these methods of intestinal organoid expansion to be relevant to the most severe patient cases (i.e. a baby who has lost their entire small intestine), the methods described would need to be scaled up to provide enough cells to engineer a 20cm length graft. This current work focuses on engineering 1cm$^2$ sized grafts, for which I need $1 \times 10^6$ intestinal epithelial cells. Consequently, in order to scale up to a tubular construct, since [Area = $2\pi rl$], assuming a neonatal small intestine luminal radius (r) of 1cm and length (l) of 20cm, the area to be repopulated is approximately 125cm$^2$. Since $1 \times 10^6$ cells are required to repopulate 1cm$^2$ of scaffold, the number of cells required to repopulate a 20cm tubular neonatal scaffold will be 125 million.

Using methods outlined here, the conditions for robust epithelial cell expansion has been optimised whereby 10 million cells can be obtained by 8 weeks. Once the organoids are established at this point, they can quadruple every 7-9 days under expansion condition. Based on these parameters, I estimate that it would require around 10 weeks in total from a starting material of 2 endoscopic epithelial biopsies to achieve 125 million cells for reconstruction of a 20cm jejunal graft. Increasing the volume of starting biopsy material may further shorten the time required to expand enough number of cells for scaffold seeding. I therefore conclude this timescale to be achievable when extrapolating to hypothetical clinical scenarios.
4. Results II - Fabrication and characterisation of decellularised human intestinal scaffolds

4.1 Introduction

The ECM acts as a support network and serves as functional scaffolding to cells, as well as providing key biological signals for cell proliferation and differentiation. Resident cells of the matrix secrete the structural and organ specific functional molecules that impact epithelial cell behaviour\textsuperscript{143,238,258,270,271}. For these unique advantages, an early decision was taken to focus exclusively on using biological ECM human intestinal scaffolds generated by decellularisation methods in this thesis. From here on, the term “scaffold” refers to decellularized human or piglet ECM intestinal materials.

Previous work from the De Coppi lab at UCL described decellularization protocols to generate rodent or piglet small intestinal (SI) ECM scaffolds\textsuperscript{230,258}, named the “Detergent-Enzymatic Treatment” (DET). DET involves 3 steps: Firstly, the tissue is washed in deionised water, which results in cell lysis by osmotic shock. Next, the tissue is washed in a detergent solution of 4% sodium deoxycholate, to solubilise cell and nucleic membranes. In the final step, the tissue is washed in 2000kU DNase-I to catalyse the hydrolysis of deoxyribonucleotide chains. Other groups have also generated decellularised porcine and human intestinal matrices using different decellularisation protocols\textsuperscript{193,227,249}. Results of cell seeding on decellularised scaffolds can be highly dependent on the method of decellularization. For example, a recent study fabricated human SI ECM decellularized scaffolds but did not include any characterisation data of the decellularized scaffolds in the publication. Furthermore, the human decellularized scaffolds were not used to demonstrate successful seedings or transplantation with these scaffolds\textsuperscript{227}. Indeed, in the same study suboptimal results were achieved when seeding intestinal cells onto piglet ECM decellularized scaffolds over PGA/PLLA synthetic scaffolds for transplantation studies. This may indicate to problems with the decellularization technique, or possible residual detergents on the scaffolds post-decellularization.
Hence, decellularization of tissue is a precise skill that must ensure reproducibility of the scaffold product in order to generate the desired tissue engineered results.

In this chapter, I apply the DET protocol to optimise the decellularization of both human SI and colonic scaffolds acquired from paediatric patients undergoing intestinal resection surgeries (listed in Appendix 1). I proceed to fully characterise the structural and biophysical features of the scaffolds, using electron microscopy, Raman spectroscopy and mass spectrometry. Whilst electron microscopy is a standard tool used to assess scaffold microarchitecture in this field of study, Raman spectroscopy and mass spectrometry are less widely employed to characterise decellularized scaffold composition. Raman spectroscopy is a vibrational light scattering technique that provides a biochemical fingerprint of tissue composition. It is a valuable tool that enables the extraction of a wealth of quantitative biomolecular information regarding the specific biochemical conformation of proteins, carbohydrates, lipids and nucleic acids with submicrometer spatial resolution. Combining Raman spectroscopy with mass spectrometry, provides valuable insight into the spatial distribution and global composition of decellularized scaffolds generated for tissue engineering.

4.2 Scaffold decellularisation and microarchitecture preservation

To increase the translational impact of this research, I fabricated decellularized scaffolds from native SI and colon tissue that was collected from paediatric patients undergoing intestinal resection (Appendix 1). Due to the nature of the surgeries, excess tissues is often collected without intact mesentery, thus cannot be decellularized via perfusion. Instead, I optimised the decellularisation of the native intestinal tissues using the DET protocol previously published. The main adjustment of protocols was in performing the decellularization through a series of immersion and agitation in the 3 main steps, rather than by perfusion. The number of cycles required to achieve decellularization whilst maintaining crypt-villus architecture ranged from one to three cycles, depending on the age of the patient and the weight of tissue acquired. By age of patient: < 6 months required 1-2 cycles; 6 months to 1 year required 2-3 cycles; > 1 year required 3 cycles. By weight of tissue: < 3g required 1-2 cycles;
3-20g required 2-3 cycles; >20g required 3 cycles. Judgement on the number of cycles required was based on histological analysis by H&E and DAPI staining of the scaffold, rather than DNA quantification.

I first show that applying the DET protocol in this way resulted in full removal of cellular and nuclear material as revealed histologically by H&E and DAPI staining in the decellularized scaffolds (Fig 4.1a-b top and lower panel). Furthermore, the intestinal crypt-villus axes were well preserved (Fig. 4.1a-b middle panel). Immunofluorescent staining confirmed the presence of the key ECM protein collagen in both SI and colonic decellularized scaffolds (Fig. 4.1a-b lower panel). The ultrastructure of the ECM scaffolds was examined using scanning electron microscopy (SEM), showing remarkable preservation of the microarchitecture of mucosa, submucosa and muscularis layers. Importantly, intact crypt-villus axis of the SI scaffold and crypts of the colonic scaffold in the mucosal layer was clearly identified after decellularization (Fig. 4.1c), which offer ideal natural nanotopography for intestinal graft construction.

Several patients were undergoing intestinal resections for IBD (Crohn’s disease) strictures. When scaffolds were generated from these samples and examined with SEM, significant distortion was seen in all 3 layers of the intestine, for both SI and colon samples (Fig 4.2). Therefore, these decellularized scaffolds were excluded from further studies in tissue engineering grafts.
Figure 4.1 - Fabrication of human decellularised intestinal scaffolds: (a, b) Representative images of SI [patient 1] (a) and colon [patient 18] (b) samples before (native) and after decellularization. Top, macroscopic images; middle, H&E histological images; bottom, immunofluorescent staining using the indicated antibodies. Scale bars: top, 1cm; middle, 200μm; bottom, 100μm. (c) Representative scanning electron micrographs of decellularized SI [patient 1] and colon [patient 18] scaffolds highlighting the microarchitecture of the mucosa (Mu) submucosa (S) and muscularis (M). Yellow arrowheads indicate intestinal crypts. The red arrow head indicates villi structure present on the SI scaffold. Scale bars: top, 100μm; bottom, 10μm.

Electron microscopy data acquired with Elizabeth Hirst (NIMR)
Figure 4.2 – Microarchitectural intestinal ECM changes in Inflammatory bowel disease (IBD): Scanning electron micrographs showing a comparison of healthy (Control – patients 1 and 18) versus diseased scaffolds (IBD – patients 29 and 30) derived from the small intestinal and colon. Mucosa (Mu) shown in the top row. Submucosa (S) shown in the middle row. Muscularis (M) shown in the bottom row. Scale bars 100 micron (top and middle rows) and 30 microns (bottom row).

Electron microscopy data acquired with Julia König (Crick)
To conserve the use of human decellularized scaffolds, piglet decellularized SI scaffolds were used in optimisation experiments of seedings. These scaffolds were provided by kind collaboration from members of Prof De Coppi’s lab at UCL (characterisation data shown in Appendix 4). Importantly, the decellularized tissue did not differ substantially from the native porcine intestine in ECM structure. The major difference when compared to the paediatric human intestinal decellularized scaffolds, was in scaffold wall thickness.

4.3 Raman spectral analyses

In order to establish the effect of DET processing on the biomolecular profile of human SI and colon scaffolds, I used Raman microspectroscopy to compare the average spectra of the distinct microstructural layers of native intestinal tissue and their corresponding decellularized scaffolds.

In native SI and colon tissue, regions with dense cellular populations such as the mucosa exhibit distinct peaks at 726 cm\(^{-1}\) and 780 cm\(^{-1}\), which are characteristic of ring breathing modes of nucleotides contained within DNA (Fig. 4.3a). This is indicative of the high density of cell populations in this region. These nucleic acid signals however were not captured from the muscle cells within the muscularis propria. This likely reflects the sparse distribution of nuclei in stretched out muscle cells, compared to the tightly packed columnar cells and their nuclei of the mucosa.

The characteristic lipid peaks at 1078cm\(^{-1}\) and 1303cm\(^{-1}\), indicative of the v(C-C) skeletal of acyl backbone of lipids and methylene bending mode, was also noted to be more intense in both native mucosa and muscle spectra, which could be associated with the lipid-rich cellular membranes of these two cell-enriched layers. This feature is also characteristic of regions containing dense cell populations as it is absent from the submucosa known to be predominantly composed of connective tissue, where cells are more sparsely distributed. The native SI is noted to have weaker lipid signals than observed in the native colon. These combined spectral features disappear from the decellularized tissue supporting the conclusion that DET processing successfully removes all cellular material without having a significant impact on the remaining spectral profile of the intestinal ECM.
One of the most notable spectral features in the decellularized tissue of both SI and colon decellularized scaffolds were peaks at 570 cm\(^{-1}\) and 1418 cm\(^{-1}\), these peaks were also more obvious in the submucosa of native SI and colon, known to be primarily composed of connective tissue. The spectral peak at 570 cm\(^{-1}\) has previously been attributed to CO\(_2\) rocking and the S-S bridge of cysteine, proline and tryptophan, and is therefore likely to be characteristic of the ECM\(^{273}\). The prominence of the 1418 cm\(^{-1}\) peak, attributed to CH\(_2\) bending mode of proteins and lipids, within the submucosa is indicative of its dominance in the structural matrix. The enrichment of these two peaks in the decellularized scaffolds highlights the unique properties of the structural ECM scaffolds that lack a dense cell population.

Interestingly, minimal difference in peak intensity of the spectra profile was observed between native and decellularized scaffolds in the submucosal region that contains the least cellular density, suggesting that the main difference between the native and decellularized tissues is likely due to the removal of the cellular mass rather than the DET process itself. Furthermore, the overall spectra profiles between SI and colonic decellularized scaffolds were highly analogous, suggesting that the biochemical composition is largely conserved between SI and colonic decellularized scaffolds.

Next, spectral data of SI and colon decellularized scaffolds with similar profiles were grouped to enable a visualisation of their spatial localisation with heatmaps (Fig. 4.3b). In particular, it is noted that collagen was mainly localised to the submucosal layer, while phenylalanine - indicating most proteinaceous regions - was most abundant in the muscularis propria. Meanwhile GAGs, indicated by glucosamines spectra, were highly enriched in the mucosal layer. Consistent to the spectra profiling, the ECM component distributions were also highly similar between SI and colonic decellularized scaffolds. The results highlight the distinct biomolecular compositions for each histological layer of the scaffolds after decellularization processing, which offer essential structural and biochemical cues for cellular reconstitution.
Figure 4.3 - Compositional analyses of the ECM of intestinal scaffolds: (a) Raman spectral analysis of comparable histological regions (mucosa (Mu), submucosa (S) and muscularis (M)) of the native tissue (blue lines) and decellularized scaffolds (red lines) of SI [patient 1] (left) and colon [patient 18] (right) samples. Peaks at 726 and 780 cm$^{-1}$ were assigned to ring breathing vibrations of nucleic acids whilst peaks at 1078 and 1303 cm$^{-1}$ were assigned to δ(CH2) and ν(C-C)/ν(C-O) modes of lipids. (b) False coloured heat maps representing direct classical least squares component analysis of Raman maps using previously acquired reference spectra of purified biomolecules. Distinct spatial distribution of Phenylalanine (PHE), Collagen (COL) and Glycosaminoglycans (GAGs) in SI [patient 1] and colon [patient 18] scaffolds is shown. Scale bars represent 50μm. Images representative of 2 experiments. (c) Top - Score plot from principal component analysis differentiates the distinct Raman biochemical spectral profiles of each distinct histological layer of the SI [patient 1] (blue) and colon [patient 18] scaffolds (red). Bottom - PC1 loading plot associated with the PC1 vs PC2 scores plot.

Raman spectroscopy data acquired with Riana Gaifulina, (UCL)
Principal component analysis of the spectra profiles readily segregated the spectra into distinct clusters based on their layer identities (Fig. 4.3c). The intensity of the corresponding peaks found within the loadings plot indicate their influence on the separation in the scores plotted along the associated axis. Remarkably, the mucosal spectra of both SI and colonic decellularized scaffold was tightly clustered together, which was distinct from the submucosal and muscularis spectra which are positively characterised (Fig. 4.3c). The data suggest that the biochemical composition of the mucosal layer from both SI and colon decellularized scaffolds are more similar to each other than their corresponding deeper histological layers.

4.4 Proteomic profiling

To further characterise the biomolecular profiles, mass spectrometry was used to generate a global proteomic profile of the decellularized SI and colonic ECM scaffolds (n=4 each). This revealed a total of 377 proteins detected in the decellularized scaffolds, among which 126 were ECM proteins (Appendices 5 and 6). Strikingly, majority of the proteins were detected in both SI and colonic scaffolds, while only 11/377 total proteins and 2/126 ECM proteins were detectable in either SI or colonic scaffolds (Fig. 4.4a). These included 17 collagen subtypes and 5 Laminin subtypes (Fig 4.4 and Appendix 5). Only 11 of 377 total proteins were detected in either SI or colon scaffolds (Appendix 6). Among the 2 ECM proteins, Defensin-SA (DEFAS) was detectable only in SI scaffold while Thrombospondin-4 (THBS4) was detectably only in colonic scaffolds. Immunohistochemical staining confirmed the restricted expression of DEFAS in native SI but not native colon tissue, which is in keeping with this protein being known to be secreted by SI-specific Paneth cells (Fig 4.4b). THBS4, on the other hand, was found to be expressed in both native SI and native colon tissue (Fig. 4.4c), suggesting that the difference noted in mass spectrometry might be due to the detection limit in the SI ECM decellularized scaffolds rather than actual expression difference.
Figure 4.4 - Proteomic profiles of SI and colon scaffolds: (a) Venn diagrams showing total and extracellular proteins detected in the SI [patients 4, 11, 12, 13] and colon [patients 2, 15, 16, 17] scaffolds by mass spectrometry. Proteomics data represents samples from 4 biologically independent patient samples in each group. (b, c) Representative immunohistochemical staining of the native paediatric SI [patient 1] and colon [patient 18] tissue using the indicated antibodies, n = 2 biologically distinct patient tissue samples. Scale bars represent 100μm.

Proteomic data acquired with Peter Faull, (Crick)
Altogether, comprehensive analysis of the proteomic profiles of the decellularized intestinal scaffolds demonstrate the vastly similar biochemical composition between human SI and colonic matrix, implying that both can potentially be used for jejunal graft reconstruction.

### 4.5 Mechanical testing

Finally, the mechanical properties of human SI and colon scaffolds were assessed, given the importance of this characteristic in future clinical translation studies, when surgically anastomosing grafts to the native intestine. When tensile stress and strain across the transverse axis of age-matched scaffolds (n=3) was quantified, no significant differences in Young’s modulus was found (Fig 4.5a-b). However, SI scaffolds tended to have higher break points on the stress-strain curves (Fig. 4.5a).

![Figure 4.5 – Mechanical properties of human SI and colon scaffolds](image)

**Figure 4.5 – Mechanical properties of human SI and colon scaffolds:** (a) Stress-strain curves of human colon [patients 19, 20, 21] and small intestine [patients 21, 23, 24] scaffolds. (b) Corresponding calculated Young’s modulus of the same scaffolds in (a). Data represent mean ± s.e.m. of n=3 biologically distinct patient samples. No significant differences identified between human SI and colon samples; unpaired t test, p = 0.5871. This experiment was performed once.

*Data acquired with Lucinda Tullie and Isobel Massie (Crick)*
4.6 Discussion

Developing and standardising methods to assess each scaffold produced by decellularization is an essential step when setting up a quality assurance process and ensuring reproducibility, prior to scaling up and using these biomaterials in future human clinical trials. In this chapter, I used several modalities to characterise the biophysical characteristics of the intestinal scaffolds derived from human tissue: Histological analyses, SEM, Raman spectroscopy, mass spectrometry and tensile testing. To conserve the numbers of human scaffolds available for seeding experiments, homogenising scaffolds in order to quantify DNA before and after decellularization was not performed in these experiments. There is no international consensus on the definition of decellularization, and the FDA does not currently establish tissue decellularization standards. In future experiments with human intestinal decellularized scaffolds, the additional confirmation of a reduction in scaffold DNA to less than 50ng per mg of dry weight, or a reduction of DNA fragments to less than 200 base pairs will be important to meet the 3-point criteria for defining decellularized tissue, set by prominent previous publications\textsuperscript{274,275}.

SEM is the gold standard method for assessing the nanotopography and architecture of biomaterials. It was important to assess whether the crypt villus architecture was maintained, since it is known that physical stimuli and topography significantly impact stem cell behaviour\textsuperscript{276,277}. Although decellularisation via perfusion of the vasculature of the organ is best practice\textsuperscript{278}, I show that the DET protocol can be applied to segments of intestinal tissue without intact vasculature and good microarchitectural results. The advantage this represents is that it doesn’t limit the sole use of human intestinal tissue resected with an intact vascular supply, thereby increasing the opportunity for perioperative tissue that may be accessed for decellularisation. Since fibrosis and scarring of the matrix can impact cell behaviour through cellular mechanosensing, tissue from children undergoing surgical resections for fibrotic IBD were found to have distorted matrix architecture after decellularization, and were therefore excluded from this study.
Conventionally, the techniques used to characterise the composition of collagen and GAGs in scaffolds often include biochemical assays and histological tissue staining\(^\text{179}\). However, biochemical assays only yield the bulk concentration of matrix constituents in the tissue, whilst histological staining provides a limited qualitative representation of spatial distribution. I therefore used Raman spectroscopy to study the human SI and colon scaffolds, comparing them to their native counterparts as well as to each other. The key advantage is that it is a non-invasive, label-free technique that provides both quantification and localisation of biomolecular constituents within tissue. To my knowledge, Raman spectroscopy has not been used in the assessment of intestinal decellularized scaffolds before. The data show that aside from delineating features specific to the loss of cells, the spectral profiles between the native and decellularized samples are largely conserved. When comparing SI and colon scaffolds against each other, I observed that the spectral profiles and distribution of ECM components on Raman heat-maps are highly analogous. Indeed, the biochemical composition of the mucosal layer of both scaffold types are more similar to each other than their corresponding deeper histological layers (submucosa and muscularis). This indicates that both may be equally as effective as each other, when used as a platform for jejunal graft engineering. Hence both scaffold types are used in subsequent seeding experiments, explained further in chapter 5. Overall, this data supports the use of Raman Spectroscopy in tissue engineering, in analysing scaffolds and constructs.

Mass spectrometry was used to confirm the Raman spectral findings, and also to map the global proteomic profile of the scaffolds. This challenging task required several months of optimisation, since the ECM is primarily formed of fibrillar components such as collagen and elastin that are resistant to solubilisation. Mass spectrometry pipelines require proteins to be solubilised and digested into peptides. Therefore, comprehensive and accurate scaffold proteome analyses is dependent on reproducibly generating peptides from the entirety of proteins present in the scaffolds. Initial experiments failed to provide a representative global proteomic profile of the scaffolds as solubilisation of the scaffolds was visibly incomplete using standard sample preparation protocols\(^\text{279}\). I therefore adopted a method whereby the scaffolds were snap frozen and grinded into a power with
a pestle and mortar in a bath of liquid nitrogen first, prior to lyophilisation. Samples were then denatured, reduced and alkylated, deglycosylated and digested by proteases LysC and trypsin, based on the methods described in a previous publication\textsuperscript{260}. This reproducible method provided interesting insights into the ECM signature, or “matrisome”, of the SI and colon showing that there is significant overlap between them. Attempts at gaining a more quantitative approach, for example to assess the relative amounts of specific proteins between SI and colon scaffold samples were unsuccessful due to the small sample sizes (4 biological replicates only) and concern about variation between experimental replicates. Nevertheless, the mass spectrometry data acquired provides important qualitative insights into the similarities between SI and colon scaffolds, which is a new contribution to the research field but has a lot of scope for further development. For example, one previous study used filter associated sample preparation (FASP) on decellularized rat livers and human lungs to identify remnant cellular immunogenic proteins that may result in tissue rejection upon transplantation\textsuperscript{275,280,281}.

Next, the mechanical properties of both SI and colon decellularized scaffolds were tested. This was important on two levels. Firstly, on a practical level this information is relevant to the surgical integrity of these biomaterials when anastomosed to the native intestine in future translational studies. The desired mechanical properties of surgical grafts depend on the site of organ transplantation, and must take into consideration factors such as permeability, porosity, pore size, elasticity and biodegradability\textsuperscript{282-284}. When considering the intestine, scaffolds that are prone to disintegration would be poor candidates for intestinal transplantation, due to the risk of deformation, enteric wound dehiscence and anastomotic leaks leading to intra-abdominal sepsis. Secondly, I was particularly interested to know if scaffolds derived from colonic tissue, that propel more solid material through the lumen, had significantly different properties to scaffolds derived from SI tissue, that propel more liquid material. In our work, only tensile testing was employed, whereby deformation of the intestinal scaffolds was measured with gradual increases in the force applied to the samples. Although this is quite a basic mechanical testing method, the data did not demonstrate any significant differences between the two decellularized scaffold types, however SI scaffolds tended to have higher break
points. This may be due to the presence of villi in SI scaffolds resulting in higher tenacity, which may be a surgical advantage in vivo but would require further testing in orthotopic transplantation models. Unfortunately, the mechanical data set presented in this thesis lacks comparison to native gut, as fresh tissue it was not accessible at the time of mechanical testing experiments. However, our findings may be considered alongside data previously published on tensile testing in native human SI and colon, where maximal tensile stress values were reported to be 0.5MPa and 0.9MPa respectively. When progressing to orthotopic transplantation models, it will be important to test mechanical properties in both axial and transverse directions. End-to-end anastomoses of the graft to native tissue may be further assessed by suture testing in future work.
5. Results III - *In vitro* intestinal graft engineering

5.1 Introduction

The major burden of nutritional digestion and absorption is taken on by the proximal 100-150cm of jejunum. Hence, the focus of this chapter was to engineering intestinal mucosal grafts with jejunal specific function. In this way, even modest increases in jejunal surface area could restore nutritional autonomy in a child. Since data from the previous chapter indicates SI and colon scaffolds to be unanimous in biophysical features, both are used in this chapter for jejunal organoid seedings. This raises the important research question: What are the contributing roles of the origin of ISCs versus origin of the decellularised matrices, in determining the eventual functional identity of the final engineering graft?

In addition, several studies have previously reported techniques to develop physiological *in vitro* intestinal models, useful for drug discovery and disease modelling. Porous silk protein scaffolds were used in combination with commercially available human colon cancer cell lines (Caco-2 and HT-29-MTX) and myofibroblasts (H-InMyoFibs)\(^1\). In this *ex vivo* static culture system, the epithelial layer retained a functional double mucus barrier and modelling interactions with the intestinal pathogenic bacterium *Yersinia pseudotuberculosis* was demonstrated. However, the main limitation to this system was that function of the model decreased over time in culture. A second *in vitro* modelling approach, developed by Professor Allbritton’s research group, uses human SI or colon organoids for seeding onto rat tail collagen based hydrogels\(^2\). This creates a 2D monolayer of epithelial cells that is expandable by mechanical and chemical breakage of the 2D layer and re-seeding onto new collagen hydrogels. The 2D layers are converted into 3D models by using PDMS stamps to recapitulate the crypt-villus axes on a transwell set-up. Using biochemical gradients across the epithelial layer, they show to proliferation is restricted to the crypt compartments whilst differentiation is present outside of the crypt regions. A third example is from a study in 2016, where human intestinal organoids were expanded and seeded as single cells on to biological scaffolds derived from porcine SIS scaffold\(^3\).
Cultures were maintained in Transwell-like settings. After 7 days the presence of differentiated cells (goblet and enteroendocrine) were identified on the scaffold. Furthermore, dynamic culture conditions using a perfusion bioreactor had beneficial effects on epithelial morphology. Whilst all these studies are not directly translational for clinical transplantation purposes, the methods described provide important insights into the optimal in vitro conditions to achieve barrier function and differentiation on engineered jejunal grafts prior to transplantation.

5.2 Static culture conditions

To assess conditions required for cell engraftment, PDOs were expanded and trypsinised for seeding onto human colon scaffolds at a density of $1 \times 10^6$ cells/cm$^2$ of scaffold, where the scaffold was fully submerged in organoid culture media (Fig 5.1). A lower seeding density resulted in patchy cell engraftment with significant areas of the scaffolds left blank. Similarly, a higher seeding density also resulted in patchy cell engraftment with significant blank areas of the scaffold. Excessive cell debris and cell death was also noted in the scaffold culture supernatant. This is likely due to increased cellular demands on a fixed volume of media covering the tissue culture well, where the scaffolds are maintained.

At a density of $1 \times 10^6$ cells/cm$^2$, intestinal epithelial cells were noted to have good epithelial coverage of the scaffold (Fig. 5.1a), including the crypt compartments (Fig 5.1b) after 10 days in culture. This indicated conditions were adequate for cellular engraftment. However, the morphology of the epithelial layer was suboptimal. Although a brush border was noted at higher magnification (Fig 5.1b), cells were largely vacuolised and unpolarised. This led me to explore a period of dynamic culture in the subsequent experiments to improve epithelial morphology.
Figure 5.1 - Morphology of the intestinal epithelial layer in submerged static culture conditions: (a) Representative H&E staining of human duodenal graft after 10 days in static culture on human colon scaffolds. (b) High magnification of vacuolised and unpolarised cells covering the crypt compartments. All organoids in this figure originate from patient 10. Scaffolds originate from patient 18. Scale bars represent 50 microns.
5.3 Bioreactor design and dynamic culture conditions

To establish dynamic conditions, primary human jejunal organoids were seeded onto the luminal surface of scaffolds which were individually mounted on a customised scaffold holder (Fig 5.2a-b). The mounted grafts were submerged in media and maintained under static conditions for 4 days, after which, they were transferred to dynamic culture conditions using a perfusion bioreactor (Fig. 5.2a-c). The grafts remained submerged and were maintained under dynamic conditions for at least 14 days before performing histological analyses.

For initial optimisations, human jejunal organoids were seeded on piglet SI scaffolds in order to conserve more limited human intestinal scaffolds. Micro-CT imaging was performed on the seeded scaffolds as the first step of analyses. This enabled a quick assessment of the volume and distribution of epithelial cells on the scaffold. I show that surface area coverage by cells on the scaffolds remained high after introducing a dynamic culture period (Fig 5.3).
Figure 5.2 - Bioreactor set-up for in vitro submerged graft culture: (a) Schematic outline of the scaffold seeding strategies using a bioreactor circuit. The timeline shows seeding of each cellular component onto the scaffolds and the time periods in static and dynamic cultures (top). The bioreactor circuit design and all individual components are indicated (bottom). (b) Representative images showing a blank scaffold (left), after stromal cell injection (middle) and during organoid cell seeding (right). (c) Photographs showing the media reservoir (left), the peristaltic pump (middle) and the perfusion plate (right) holding 4 individually mounted grafts.
After a period of static culture followed by dynamic culture, histological analyses of the jejunal grafts using piglet SI scaffolds showed an improved morphology, expected of the intestinal epithelium. An organised monolayer of columnar cells extending to cover the crypt compartments was noted (Fig 5.4 a-b). However, variability in morphology was noted as regions of collagen-positive new matrix deposition were detected, with structures resembling new villi (Fig 5.4 b-c). This variability may be due to the impact of an uneven flow of media across the scaffolds, using the perfusion plate system.

In addition, all intestinal cell types were readily detected in the graft, including lysozyme-positive Paneth cells, AB-PAS-positive goblet cells and alkaline phosphatase-positive enterocytes (Fig. 5.4 d-f). Proliferation marker Ki67 was also identified in the graft (Fig 5.4g). Importantly, the jejunal-specific enzyme, sucrase isomaltase, was also widely detected on the brush border of the epithelial cells (Fig 5.4h). This indicates that jejunal region-specific identity was preserved in the engineered grafts using piglet SI scaffolds.
Figure 5.4 - Characterisation of engineered jejunal grafts cultured *in vitro* using piglet SI scaffolds: (a) Representative H&E staining of a jejunal graft showing epithelial lining with invaginated crypts (black arrowheads). (b) H&E staining of a jejunal graft showing regions of columnar epithelial monolayers (bottom right) as well as regions of new matrix deposition (black asterisks, top and bottom left). (c) New matrix deposition indicated by black asterisk in H&E image (top) stains positive for collagen-1 (magenta, white asterisk, bottom). (d-h) Representative immunohistochemistry images of engineered jejunal grafts using the indicated antibodies. Paneth-like cells are indicated with red arrowheads in (d) and goblet-like cells are indicated with yellow arrowheads in (e). Enterocytes are indicated by brush border staining with alkaline phosphatase (ALK-PHOS) (f) and sucrase isomaltase (SI) (h). Proliferating nuclei are indicated by Ki67 staining (g). All scale bars represent 50μm unless specified otherwise. Images are representative of 3 independently cultured grafts. All organoids in this figure originate from patient 2.
Next, I examined engineered graft epithelial morphology when seeding human jejunal organoids onto either human SI or human colonic scaffolds.

Similar to piglet scaffold data, histological analyses revealed a continuous monolayer of columnar epithelial cells distributed evenly along the decellularized human SI scaffold surfaces (Fig. 5.5a). Immunostaining confirmed that proliferation (as indicated by PCNA) and differentiation (ALPI to mark enterocytes and AB-PAS to mark goblet cells) potential was maintained in the reconstructed grafts. These findings were also observed when jejunal graft reconstruction was performed using identical methods onto human colon scaffolds, where proliferation and differentiation were all detected (Fig. 5.5b). Interestingly, immunofluorescent staining of collagen showed new matrix deposition in multiple regions underneath the epithelial cells, suggesting ECM remodelling mediated by epithelial cells on both scaffold types (Fig. 5.5 a-b immunofluorescent images). This pattern of matrix deposition was also seen in earlier optimisation experiments with piglet SI scaffolds (Fig 5.4c). This is indicative of active ECM remodelling driven by the intestinal epithelial cells on all scaffolds types. The expression of tight junctions (as marked by ZO1) and polarity (indicated by Na+/K+/ATPase) was assessed in these areas of matrix remodelling, on sections of the graft where new matrix deposition was identified(Fig. 5.5c). This indicates abnormal polarity of cells involved in matrix deposition.

Electron microscopy analysis further demonstrated the presence of microvilli (brush boarder of enterocytes); basement membrane (as indicated by basal lamina and reticular lamina features between the scaffold and the epithelial cells); goblet cell mucous vesicles and Paneth cell secretory vesicles (Fig. 5.5d-e). The presence of both basement membrane microstructure, composed of basal lamina and reticular lamina, as well as tight junction markers is encouraging, as both are essential components for epithelial barrier function of the intestine.
Figure 5.5 - Characterisation of engineered jejunal grafts cultured in vitro using human scaffolds. (a-b) Representative histological and immunostaining images of jejunal grafts reconstructed using human SI scaffolds [patient 13] (a) or human colon scaffolds [patient 16] (b) at day 18. New matrix deposition is shown by newly synthesized collagen (white asterisks). Black arrowheads indicate AB-PAS-positive goblet cells. Scale bars represent 50μm. (c) Representative immunofluorescent images of a jejunal graft seeded on human SI scaffolds, in a region of new matrix deposition, [patient 5] using the indicated antibodies. Scale bars represent 50μm. (d) Representative electron micrographs of a jejunal construct [SI scaffold - patient 5] showing microvilli (MV) (left and second from the left); basement membrane with basal lamina (BL) and reticular lamina (RL) at the scaffold (Sc) border (middle); Goblet cell (G) with mucous vesicles indicated by the orange arrow head (second from the right) and Paneth cell (P) with secretory vesicles indicated by the green arrow head (right). Scale bars represent 5μm. (e) Electron micrograph showing a monolayer of epithelial cells on a graft [SI scaffold – patient 5]. Scale bar represents 50μm. All organoids in this figure originate from patient 2. Images are representative of at least 3 independent graft cultures.

Electron microscopy data acquired with Anne Weston (Crick)
5.4 Functional analysis

Next, the absorptive and digestive function of the bioengineered grafts at various time points of \textit{in vitro} culture was examined (Fig. 5.6a). First, the absorptive capacity of the reconstructed jejunal grafts was tested at day 18 of culture by incubating the grafts with fluorescently labelled peptide β-Ala-Lys-AMCA. Immunofluorescent staining confirmed the presence of β-AMCA peptides incorporated within the boundaries of intestinal epithelial cells on the grafts (Fig. 5.6b). This indicates that the engineered graft possesses peptide absorptive function and the presence of active peptide transporters on the graft epithelium.

Digestive capacity was assessed by demonstrating the function of the jejunal enterocyte brush border enzyme, sucrase isomaltase. Here, the jejunal constructs were challenged with sucrose and measuring the subsequent glucose (one of the metabolic products) production at various time point of culture. This method tests the disaccharidase activity, since sucrase isomaltase hydrolyses sucrose into two glucose molecules. Remarkably, glucose production was detected as early as day 4 culture in all three scaffold types, indicating that the enzyme sucrase isomaltase is functional throughout the \textit{in vivo} culture timeline (Fig. 5.6c).

Plasma citrulline levels positively correlate to enterocyte mass and absorptive function, and are used as a clinical biomarker of intestinal failure\textsuperscript{290}. Therefore, graft culture supernatants were sampled to quantify the citrulline concentration as a surrogate biomarker of enterocyte mass on the scaffolds. This was done at days 0, 11 and 25 of the culture. The data shows an increase in citrulline levels from day 0 to day 25 in the jejunal grafts regenerated from all three scaffold types. This further indicates the formation of healthy functional enterocytes in all constructs (Fig. 5.6d).

Digestive capacity was also detected by assessing the activity of the enzyme dipeptidyl peptidase IV. This was achieved by incubating the grafts with the compound Gly-Pro p-nitroanilide hydrochloride to and measuring the release of nitroaniline (Fig. 5.6e). This indicates cleavage activity of dipeptidyl peptidase IV on the engineered grafts.
Figure 5.6 - Functional assessment constructed jejunal grafts *in vitro*: (a) Timeline indicating the experimental sampling (marked by each coloured symbol) of jejunal grafts for functional analyses. (b) Immunofluorescent staining showing uptake of β-AMCA peptide (red) on a jejunal graft (piglet SI scaffold). Phalloidin staining (green) indicates epithelial cell boundaries. Scale bars, 30μm. Images representative of two experiments. (c-f) Functional analysis using jejunal grafts seeded on human colon scaffolds (green lines), human SI scaffolds (red lines), piglet scaffold (blue lines) or unseeded
blank scaffolds (black lines). (c) Disaccharidase enzyme activity, showing sucrose (solid lines) or PBS control (dashed lines). (d) Graft supernatant citrulline concentration over time. (e) Nitroaniline concentration from jejunal grafts over time. (f) Barrier function as indicated by percentage leakage on jejunal grafts over time. Blank scaffolds show an average of 61% baseline leakage (dashed line). All organoids used in this figure originated from patient 2. Data represent the mean ± s.e.m. of three independently cultured jejunal grafts.

Data acquired with Isobel Massie (Crick) and Simon Eaton (UCL)

Finally, barrier function was assessed by measuring the percentage leak of FITC-labelled dextran through jejunal grafts constructed with piglet SI scaffolds, which is more permeable than human scaffolds. This was expressed as a measurement from the ‘luminal side’ to the ‘serosal side’. Blank piglet SI scaffolds showed an average baseline leakage of 61% (as indicated by the dashed line) and this was reduced to 45% by day 18 of graft culture (Fig. 5.6 f).

In summary, the results from functional analyses indicate that the engineered jejunal grafts regenerated using either SI or colon decellularized scaffolds, exhibit some aspects of physiological jejunal function, namely (i) peptide absorption, (ii) peptide digestion, (iii) sucrose digestion and (iv) partial improvement in barrier formation over time.

5.5 Discussion

In this chapter, the culture conditions required for epithelial regeneration on decellularized scaffolds were investigated. It is widely accepted that two-dimensional in vitro static culture conditions differ significantly from true in vivo conditions. Even three-dimensional organoid cultures that are perceived as more biomimetic are still maintained in static conditions, depriving the cells of mechanical stimulation such as shear stress, stretching, compression, contraction and the resulting variability of matrix stiffness. Therefore, the organoid culture system does not adequately recapitulate mechanically induced cell signalling responses which represents an important limitation.

When culturing jejunal grafts in submerged static culture conditions, I found that cells lacked polarity and organisation expected of the intestinal epithelial monolayer. In addition, cells appeared
vacuolised which histopathologically is often associated with cell death. Indeed, longer periods of culture in static conditions were more difficult to maintain and resulted in highly variable results in epithelial survival and morphology. Experimental attempts at creating an air-liquid interface culture using the human scaffolds were largely unsuccessful. This may be attributed to the significant thickness of the human scaffolds, and the relative impermeable properties of it. Air-liquid interface culture set ups require cells to take up nutrients from the media compartment via their basolateral membrane. The thickness of the human scaffolds represented a barrier to this process, resulting in poor cell survival. Alternative approaches to achieving good air-liquid cultures would be to reduce the thickness of the intestinal scaffold by dissecting away the muscle layer and using only the mucosa/submucosal layers of the scaffolds. However, this resulted in damage to the mucosal layer and overall was not in keeping with the prevailing research aim of the thesis, which was to translate the use human biomaterials into clinical practice for intestinal tissue engineering. The translational strategy taken from the outset of the project was to adjust my experimental protocols to suit the human biomaterial properties (rather than converse of altering human biomaterial properties to suit the experimental aims). Therefore, I moved on to exploring dynamic culture conditions to improve morphology of the intestinal epithelial cells on the human decellularised intestinal scaffolds. In progressing to dynamic culture conditions, a simple bioreactor circuit consisting of a peristaltic pump and perfusion culture plates produced much improved results in epithelial cell survival and morphology on all scaffold types. Here, some physiological conditions of the native intestine such as shear stress and turbulent fluid pressures, experienced during intestinal peristalsis, are partially recreated.

Interestingly, the data suggest that epithelial cells were remodelling the scaffold matrix as indicated by the deposition of new hyaline matrix on the surface of the scaffolds. This data supports findings already published in the field by Kitano et. al., who also show evidence of collagen IV deposition by the transplanted epithelial cells was also included in supplementary data. Although definite crypt villus axis was present on the human scaffolds, a clear organisation of cells along the crypt villus axis
was not seen in either this study or that of Kitano and colleagues (i.e. proliferative stem cells at the base of the crypts and differentiated cells at the villi). Compartmentalisation of proliferative and differentiated cells was achieved in earlier publications on transwell set ups where the crypt villus contour had been created using molds.\textsuperscript{286,289} In this study, the new matrix deposition may be indicative of early matrix remodelling towards new crypt-villus axis formation that might have been limited by suboptimal dynamic culture conditions. Future experiments allowing longer culture periods would be useful to study the impact of matrix remodelling on compartmentalisation of stem and differentiated cell populations.

The majority of differentiated intestinal cell types were detected on the engineered grafts, including Paneth cells, goblet cells and enterocytes. Jejunal specific sucrase isomaltase expression was also widely detected on the brush border, signifying maintenance of region-specific identity of the engineered grafts. It is important to note that expression of chromogranin A was not detectable, indicating a lack of terminally differentiated enteroendocrine cells. In some of the human scaffold seedings, lysozyme expression was not detectable, however in-depth electron microscopy analysis did confirm the presence of cells containing multiple secretory vesicles were also identified, which may represent early immature Paneth cells. These findings are comparable to previous studies using hiPSC derived intestinal organoids on rat decellularized scaffolds, which also did not find the full complement of differentiated intestinal cell subtypes of native gut\textsuperscript{193}. Specifically, fully differentiated goblet and Paneth cells were not found \textit{in vitro}. However, after transplantation goblet and Paneth cells were abundantly present, suggesting further maturation \textit{in vivo}\textsuperscript{193}. To achieve a more global profile of cellular and matrix composition of the graft, future experiments could also employ methodologies such as flow cytometry, RNA extraction for single cell RNA sequencing, imaging mass spectrometry, and other spatial resolution approaches such as MERFISH and imaging CyTOF.

A particular advantage of our bioreactor culture and seeding methods is that we optimise our protocols to the shortest \textit{in vitro} culture times possible (i.e. 14 days) whilst methods described in
previously published studies require longer in vitro culture times (28 days)\textsuperscript{193}. Shorter culture period (both in the patients’ cell expansion phase as well as the bioreactor culture phase) is likely to be advantageous for future clinical translation, particularly when constructing a patient-specific graft for children with intestinal failure.

Most importantly, I show that engineered grafts possessed jejunal specific functions such as nutrient digestion and absorption, regardless of the origin of the scaffolds. Several publications reporting on tissue engineered intestine do not include studies of functional assays of digestion, absorption or barrier integrity at all\textsuperscript{194,227,251,253,254}. Whilst one more recent publication the transfer of glucose and medium chain fatty acids from the lumen of the construct to the scaffold vasculature\textsuperscript{193}. The concern with their experimental set up is that it does not demonstrate digestive capacity alongside absorptive capacity. This chapter of my thesis contributes new in vitro functional assays to the research field, by demonstrating the activity of digestive disaccharidase and peptidase enzymes alongside absorption of peptides into the jejunal grafts. In addition to this, I show that the measurement of citrulline levels in the jejunal graft culture supernatants is a novel application of a non-destructive biomarker method for tracking enterocyte growth on scaffolds. This has important implications in future upscaling of the jejunal graft engineering process. Finally, although evidence of barrier structure was seen on immunostaining and electron microscopy, the FITC-dextran assay performed produced more variable data. This is likely due to micro-tears in the thinner piglet scaffold wall after decellularisation. The functional assay was not performed on the human scaffolds since they are significantly thicker in dimension, and highly impermeable to the FITC-dextran compound. Overall, since the expression of brush border enzymes (sucrase isomaltase and alkaline phosphatase) were detected on engineered grafts that were formed using all scaffold types, I conclude that either SI or colon ECM scaffolds were suitable to proceed with in vivo transplantation studies.
6. Results IV - *In vivo* graft transplantation models

6.1 Introduction

Due to the limitations of *in vitro* culture conditions, discussed in chapter 5, the engineered jejunal grafts were tested *in vivo*, to assess the potential for graft survival and further epithelial maturation. Animal transplantation experiments are an essential step when testing the safety and efficacy of tissue engineered organs. There are two types of organ transplantation models: orthotopic (in the correct anatomical position) or heterotopic (in an abnormal position).

Older studies of tissue engineered intestine using synthetic scaffolds were tested orthotopically in rat models of SBS, reported improved survival rates of rats receiving the graft compared to controls\(^{254}\). These experiments used full thickness cellular sources, contain mucosa, submucosa and muscle layers of the intestine (first described by Evans et al in 1992)\(^{199}\). More recent studies have utilised heterotopic models, whereby the vascularised engineered graft (using rat scaffolds) was anastomosed to the carotid arteries and jugular veins of immunodeficient rats\(^{193}\). The grafts constructed over the course of this thesis were purely mucosal and did not have intact vasculature, therefore orthotopic transplantation models were not possible. The options for heterotopic transplantation with these engineered grafts include omental wrapping, insertion under the kidney capsule or subcutaneous transplantation\(^{291,292}\). Here, I adopted two approaches to assess the engineered grafts: the kidney capsule model and the subcutaneous model, since these were less invasive and better tolerated by the NSG mice in my experience.

6.2 Kidney capsule transplantation

Jejunal grafts were engineered for all in vivo transplantation experiments as previously described in the previous chapter, using the experimental timeline illustrated in figure 5.2a. In the kidney capsule model, grafts engineered using human scaffolds were more difficult to insert under the tight capsule, due to dimensional limitations. Therefore the jejunal grafts formed using piglet scaffolds were
exclusively transplanted in this model. To recap, no fibroblasts were injected into the piglet scaffolds. Organoids were seeded on day 0 and maintained in static culture conditions for 4 days, before converting the culture to the dynamic system for a further 7 days. At Day 11 on the _in vitro_ timeline, grafts were implanted into the animals. Graft epithelial morphology at the point of implantation was equivalent to the data presented in figure 5.4 (i.e. including the presence of differentiated cells). After 7 days _in vivo_, the kidneys were harvested and macroscopic evidence of neovascularisation was noted on the graft (Fig 6.1a).

**Figure 6.1 - In vivo** kidney capsule transplantation and characterisation of jejunal grafts: (a) Macroscopic image of the kidney collected after implantation of a jejunal graft in the kidney capsule. (b-c) Histology of a transplanted jejunal graft as analyzed by H&E and human nucleoli staining. (d) 3D volume-rendered model of the jejunal graft structure after transplantation under the kidney capsule. (e-g) Serial H&E staining of a jejunal graft 1 week after transplantation in vivo under the kidney capsule. L indicates graft lumen. (h-i) Histological staining to show the epithelial ring of jejunal grafts are negative for goblet cell (Alcian Blue - Periodic Acid Schiff, AB-PAS) and enterocyte marker (Alkaline Phosphatase, Alk-phos). (j-l) Representative immunofluorescence images of transplanted jejunal grafts obtained using antibodies to the indicated markers.

_Surgery performed by Paola Bonftanti (Crick)_)
Serial histological sectioning along the length of the scaffold demonstrated the presence of continuous rings of intestinal epithelium that were positive for human nucleoli staining (Fig. 6.1b-c, e-g). 3D volume reconstruction of the serial histological section data revealed segments of continuous tubular structures maintained throughout the graft, indicating formation of intestinal lumens (Fig. 6.1d). Unexpectedly, immunostaining of AB-PAS and ALPI was largely negative in the graft, suggesting a lack of goblet cell and enterocyte differentiation in the engineered graft after engraftment under the kidney capsule (Fig. 6.1h-i). No evidence of intestinal cell differentiation was ever seen in any of the experimental replicates grafts that were harvested from the kidney capsule transplantation experiments. However, a large population of cells co-expressing vimentin and αSMA was noted in the stroma surrounding the epithelial rings, indicating that there is a strong infiltration of host myofibroblasts from the kidney into the scaffold (Fig. 6.1j). Immunofluorescent staining confirmed the epithelial cells forming lumen were composed of undifferentiated intestinal cells that demonstrated high expression of stem cell markers OLFM4 and SOX9 (Fig. 6.1k-l).

6.3 Subcutaneous transplantation

In order to enable live in vivo tracking of cellular growth on the scaffolds, jejunal PDOs were labelled with a GFP-luciferase reporter prior to seeding on scaffolds (Fig. 6.2a). The GFP reporter in the plasmid construct enabled monitoring of epithelial growth on scaffolds whilst in the in vitro culture period (Fig 6.2b). Meanwhile, the luciferase reporter in the plasmid construct enabled the live tracking of cellular growth after transplantation in vivo with live bioluminescence imaging (Fig 6.2c).
Figure 6.2 - Live tracking of intestinal epithelial cell growth in vitro and in vivo: (a) Schematic representation of the luciferase-GFP reporter plasmid (top) used to label jejunal organoids (bottom). (b) Live imaging of piglet grafts in culture from days 2 to 12 prior to transplantation. Scale bars represent 1000µm. (c) Left, luminescence scale bar showing bioluminescence signal intensity; Right, representative image of live bioluminescence imaging in the subcutaneous transplantation model.

These engineered grafts were then transplanted in subcutaneous pockets of NSG mice, an approach that allows engraftment of larger dimension constructs. Initial experiments were performed using jejunal grafts formed on piglet scaffolds to allow direct comparison of data with the kidney capsule model. Similar to kidney capsule model, lumens of human nucleoli-positive intestinal epithelial cells were readily detected as early as 7 days after implantation (Fig. 6.3a). However, in contrast to the kidney capsule data, ALPI⁺ enterocytes and AB-PAS⁺ goblet cells were detected in the grafts (Fig. 6.3b-c). It is also important to note that there were significantly less stromal cells surrounding the epithelial rings of the grafts analysed, when compared to grafts in the kidney capsule transplantation.
Figure 6.3 - In vivo subcutaneous transplantation of jejunal grafts formed using piglet scaffolds: (a) Left, representative image of haematoxylin and eosin staining of the jejunal graft epithelium. Right, representative image of human nucleoli immunostaining marking the jejunal graft epithelium. (b-c) Histological staining with alkaline phophatase (Alk-phos) marking the enterocyte brush border, and alcian blue - periodic acid schiff (AB-PAS) marking goblet cells.

Next, experiments progressed towards transplantation of larger dimensions of jejunal grafts formed on human SI or colon scaffolds. However initial histological analyses yielded largely poor results, with no intestinal epithelial rings seen on serial sectioning of the grafts and minimal stromal cell infiltration from the host.

In order to enhance the survival of transplanted jejunal grafts forming using human scaffolds in this model, the role of Teduglutide was investigated for tissue engineering applications. Teduglutide is a clinically licenced drug used in patients with intestinal failure to promote intestinal adaptation by increasing villus height and crypt depth\textsuperscript{59,60}. The receptor (GLP2R) of Teduglutide was found to be expressed in both human jejunal fibroblasts and in human jejunal organoids (Fig. 6.4a). Therefore, primary human jejunal fibroblasts (characterisation data shown in section 3.3) were expanded and
injected into human SI and colon scaffolds intended for *in vivo* subcutaneous transplantation. In these experiments, fibroblasts were first injected into the scaffolds at the mucosal-submucosal boundary, and maintained in static culture for 3 days to recreate the native microenvironment prior organoids seeding (Fig. 5.2a-b). Histological analysis of these grafts showed full coverage by columnar epithelial cells on the scaffold surface with visible crypt units (Fig. 6.4b). Co-staining of the grafts indicated abundant fibroblasts that were predominantly vimentin* and αSMA*, located subepithelially before transplantation (Fig. 6.4c).
Co-cultured grafts were transplanted subcutaneously into NSG mice which were administered either a daily subcutaneous dose of teduglutide (0.2 mg/kg) or vehicle (PBS) after transplantation. Two weeks after transplantation, serial sectioning showed intestinal lumens populated with epithelial cells in 10.4% ± 0.7% of sections for all grafts receiving teduglutide, in comparison to only 0.28% ± 0.6% of sections for all grafts in the control group (P = 0.0271; Fig. 6.5a). Grafts were significantly thicker than earlier analysed piglet scaffolds, where the three histological layers (mucosa, submucosa and muscularis) of the graft were clearly preserved after transplantation (Fig. 6.5b). A distinctive monolayer of human intestinal epithelial cells was formed on the mucosal luminal surface of the implanted grafts. Proliferating cells (indicated by PCNA and EdU staining) were detected in both epithelial and pericryptal stromal cells of the scaffold (Fig. 6.5c). Electron microscopy analysis identified mucous granules within the graft epithelial cells, suggesting the presence of early goblet cell differentiation (Fig. 6.5d). Immunofluorescent staining for pancytokeratin and e-cadherin confirmed the epithelial identity of the epithelial monolayer (Fig. 6.5 e-f). Expression of stromal marker vimentin further confirmed the survival of human intestinal fibroblasts in the injected grafts after transplantation (Fig. 6.5e). Importantly, the jejunal-specific brush border enzyme sucrase isomaltase was broadly expressed in majority of the jejunal graft (Fig. 6.5f), indicating that human ECM scaffold supports and maintains intestinal epithelial cell differentiation towards enterocyte lineage in vivo. Similar to piglet scaffold data, co-cultured jejunal grafts formed using human scaffolds were also able to form luminal structures after transplantation (Fig 6.6a) with histological evidence of vascularisation within the graft (Fig. 6.6b).
Figure 6.5 - Teduglutide treatment enhances in vivo survival of engineered human jejunal grafts: (a) Quantification of the formation of lumens within jejunal grafts following subcutaneous implantation in teduglutide-treated versus control mice. Data represent mean ± s.e.m. for n = 4 independently transplanted jejunal grafts after 2 weeks in vivo. Mann Whitney U Test, *P = 0.0286. (b) Representative histology of graft indicating the mucosa, submucosa and muscularis structure. Arrowhead indicates polarized columnar jejunal cells. (c) Immunostaining analyses of cell proliferation as indicated by PCNA (top) and EdU (bottom). Arrowhead indicates the jejunal epithelium. (d) Electron microscopy analysis identifying mucous granules of goblet cells in the native intestine (top left) and a jejunal construct (bottom and top right). (e-f) Representative immunostaining of a jejunal graft using antibodies to epithelial markers (pancytokeratin and e-cadherin), stromal marker (vimentin) and jejunal brush border enzyme (sucrase isomaltase). Grafts in (a) were formed using human colon scaffolds from patient 2. Grafts in (b-f) were formed using human SI scaffolds from patient 1. All organoids and fibroblasts used in this figure originated from patient 2. Scale bars represent 50μm unless specified.

*Surgery performed by Paola Bonftanti and Sara Camponiti (Crick)*
Figure 6.6 - Engineered jejunal graft vascularisation after *in vivo* subcutaneous transplantation (a) H&E staining of luminal ring (L) of intestinal epithelium present on grafts harvested following 2 weeks of subcutaneous transplantation from teduglutide treated mice. (b) Immunostaining of CD31 (green) in the same graft. (c) Macroscopically visible neovascularization (red arrowhead) on a transplanted graft (white outline) formed of human SI scaffold [patient 24]. (d) Quantification of jejunal graft vessel and lumen formation (e) per grafts injected with HUVECs or control (fibroblasts alone). N=6 grafts, [SI scaffold - patient 1 & 24; colon scaffold - patient 2]. Grafts containing vessels are indicated by red points. Data represent mean ± s.e.m. Mann Whitney U test: vessels, p=0.8333; lumen, p=0.2857. (f) Representative image indicating the proximity of blood vessels (red arrowheads) to the epithelial lumen (L) grafts injected with HUVECs. (g) Representative histology of grafts injected with HUVECs, using the indicated antibodies/stainings. L indicates lumen. Intestinal epithelial cells. All scale bars represent 50μm unless specified otherwise.

*Data acquired with help from Isobel Massie and Lucinda Tullie (Crick)*
Finally, the effect of increased vascularization of the grafts was investigated by introducing an additional injection of human umbilical vein endothelial cells (HUVECs) into human scaffolds 1 day before subcutaneous transplantation. On harvesting grafts from the mice at 2 weeks post transplantation, macroscopically visible vessels were present (Fig. 6.6c). From the six grafts injected with HUVECs, two contained vessels microscopically, and these same two scaffolds had visible lumens on 33% of the serial sections (Fig. 6.6 d-e). Conversely, in the remaining four scaffolds without vessels, lumens were detected in only 1.4 ± 3% of the serial sections (Fig. 6.6 d-e). Blood vessels were often observed in close proximity to the lumens (Fig. 6.6f), suggesting that improved vascularization enhances epithelial cell survival. Histological analysis confirmed the presence of goblet cells and sucrase isomaltase+ enterocytes in epithelial lumens surrounded by a mixed population of vimentin+ or SMA+ fibroblasts (Fig 6.6g). CD31+ staining indicates the presence of endothelial cells within the graft (Fig. 6.6g).

6.4 Discussion

In this chapter, two models of in vivo models of ectopic transplantation were used to test the potential for survival of the engineered jejunal grafts: [1] under the kidney capsule and [2] in subcutaneous pockets. In contrast, other research groups have commonly adopted ectopic models of omental wrapping transplantation, to test their tissue engineered constructs251,293. More recently, in 2017 researchers used a cervical heterotopic transplantation rat model to assess both function and morphology of hiPSC organoid derived grafts formed on rat decellularised SI scaffolds193. This particular model has the advantage of being able to connect the vascular supply of the constructs to the carotid artery and jugular vein, whilst standard ectopic transplantation models (omentum, kidney capsule and subcutaneous) do not directly connect to the host vascular system. This heterotopic transplantation approach enables more sophisticated methods of in vivo functional testing of the graft by systemic venous sampling and positron emission tomography scanning to confirm uptake of labelled glucose and fatty acids193.
Neither of the two chosen transplantation models in my studies were completely however both offered distinct *in vivo* microenvironments with regards to stromal cell infiltration, vascularisation and impact on epithelial cell identity. Therefore, both models represented opportunities to learn more about the potential behaviour of the jejunal grafts *in vivo*, informing future studies. Significant differences were noted in epithelial morphology and identity on harvesting and analysing grafts after transplantation. In the kidney capsule transplantation model, the intestinal epithelium of grafts remained in a predominantly undifferentiated state. This may be due to high levels of host myofibroblast infiltration into the graft, that recapitulates the stem cell niche of the intestinal crypt compartment. In contrast, lower levels of stromal cell infiltration seen in the subcutaneous transplantation model seemed to promote more differentiation of the graft epithelium. Although the subcutaneous transplantation models allow more space for larger dimension grafts that are formed using human SI or colon scaffolds, co-culture of fibroblasts with epithelial cells, and the use of teduglutide, were required in order to enhance graft epithelial survival and maturation *in vivo*. This may be due to the increased thickness of these jejunal grafts impeding the infiltration of host endothelial or stromal cells required for epithelial survival. Using an amended *in vitro* culture timeline, jejunal epithelial and stromal cell populations were successfully co-cultured within the scaffolds prior to transplantation. Reduced angiogenesis within tissue-engineered grafts can limit the survival of cells on the graft. HUVECs were therefore injected carefully into seeded grafts one day prior to transplantation to study the impact of this on vessel formation within the human scaffolds, and also lumen formation in grafts. Overall, jejunal specific identity of the grafts was maintained in engineered patient-derived grafts after transplantation *in vivo*.

The most ideal *in vivo* transplantation model would be one where the grafts can be transplanted orthotopically in continuum with native small intestine, however this surgical technique is complex and was beyond the scope of this thesis. These orthotopic experiments have been performed in published studies from 2004, in rats following massive small bowel resection when assessing TESI constructs (discussed earlier in section 1.5.3), with excellent results. The greatest advantage of
orthotopic transplantation models is exposure of the tissue engineered grafts to the native microenvironment and signalling pathways of the gut.

Attempts to increase the length of jejunal graft survival in vivo past the 2-week time point using HUVECs were not successful as few luminal rings were seen when grafts were harvested at later time points. This is a particular disadvantage of transplanting large grafts in ectopic sites in small animal studies. Kitano et al., demonstrated an elegant solution to the problem of vascularisation in vivo, by using HUVECs to revascularise intestinal grafts in vitro, prior to seeding hiPSC derived epithelial cells in vitro (in a 4-week long bioreactor seeding protocol). When transplanted heterotopically grafts survived for up to 4 weeks. In future studies, replacing HUVECs with primary patient-derived endothelial cells are immensely important for improving the translational value of these experiments for patients in the clinic.
7. Conclusions and Future Perspectives

In this progressive work of engineering transplantable human jejunal mucosal grafts, I sought to derive cells autologously from children with IF, or predisposing conditions, in order to repopulate human decellularised scaffolds. I establish clinically relevant protocols for timely intestinal graft reconstruction using patient-derived materials of post-natal adult somatic intestinal stem cells and human biological intestinal decellularized scaffolds. In doing so, the data presented in this thesis demonstrates the feasibility and proximity of translating this approach in clinical practice in the future, and encourages further studies into bringing tissue engineering technologies using patients’ cells and patients’ biological scaffolds for children with IF.

Firstly, autologous primary somatic ISC s were derived from relatively small pieces of endoscopically acquired tissue, showing the applicability of this method for children with minimal lengths of residual functional SI in situ. The organoids can be efficiently expanded in vitro without losing their intrinsic location specific functional identity and differentiation potential. This data would also support proposals to introduce the practice of isolating and banking of intestinal epithelial organoids and stromal cells as a new clinical standard at the point of intestinal resections for at risk children, for example in cases of necrotising enterocolitis in the neonatal period. This would give such a child the prospect of a patient-customised graft being constructed should the clinical need arise in the future.

Other recent studies that opted to use iPSC derived intestinal organoids to reseed rat decellularized scaffolds for tissue engineering, showed excellent in vitro and in vivo morphology of the engineered epithelial layer together with endothelialisation of the rat scaffolds. Whilst iPSC-sourced intestinal cells have the advantage of being applied to children who have lost their entire small intestine (although rare clinically), autologous sources of primary somatic stem cells remain preferable to induced pluripotent or embryonic stem sources for therapeutic use in children, due to their safety.
profile. Importantly, the PDOs and fibroblasts collected in the biobank survived well after repeated freeze-thawing cycles throughout this project.

This data also confirms that under expansion conditions ISCs do not lose their location specific functional identity and differentiation capacity, following significant expansion culture periods in vitro. The significance of fibroblasts in the reconstruction of the ISC niche is also demonstrated in vitro and in vivo, providing support to the epithelial layer of the graft. The organoid characterisation data presented in this thesis on location specification of intestinal organoids expanded from the duodenum, jejunum and ileum confirms the results of previously published studies. This raises an important question on the applicability of this tissue engineering strategy on children who have lost their entire jejunum. Since the data demonstrates that organoids derived from the duodenum have relatively lower expression brush border digestive enzymes sucrase isomaltase, this suggests that in order the form a jejunal graft for a patient, they must have at least a few centimetres of jejunum left in situ. This is an important limitation of this data. It is currently unknown whether it is possible to manipulate ISCs-derived from the duodenum or ileum in vitro, in order to promote the expression of jejunal specific enterocytes. This would be a significant advantage for patients who have lost their entire jejunum.

Biological scaffolds, derived from human decellularized SI and colon, were used in this study that focussed on engineering transplantable grafts with jejunal-specific nutritional function. This represents a particular novelty of this study, as no other publications have investigated the use of human intestinal decellularized scaffolds for transplantation purposes. One publication extensively investigated the use of human colon decellularized scaffolds for modelling colon cancer, leading to the identification of 38 candidate invasion-driver genes. Another brief publication reported the use human SI decellularized scaffolds with intestinal myofibroblasts, for modelling intestinal fibrosis in vitro. Other studies that are focussed on transplantation purposes, rather than in vitro modelling purposes, use synthetic scaffold sources or animal biological scaffold sources.
An important limitation to the source of human tissue used in these studies, is not having been able to decellularize the tissue through perfusion of the vascular tree supplying the intestinal explants. However, SEM was used to demonstrate perfect suitability of DET decellularization by gentle immersion and agitation for maintaining the ultrastructure features of human intestinal tissues. This enabled the fabrication of immunologically tolerogenic scaffolds for regenerating the human intestine\(^{230}\). The ability of decellularised scaffolds to retain their biological and structural features after cryopreservation has previously been demonstrated, enhancing the potential of these scaffolds to become “off-shelf” clinical products\(^{179}\).

Previous studies have illustrated the preservation of biological ECM molecules in rodent scaffolds using mainly histological and immunohistochemical evaluations\(^{230}\). While these approaches are useful in assessing the spatial distribution of major ECM components, they remain mostly qualitative in their nature. Biochemical quantification of collagens and glycosaminoglycans (GAGs) extracted from digested scaffold material is limited to providing bulk information only. Here, I adopted a three-pronged approach to improving the depth of understanding of the biological properties retained in human derived intestinal scaffolds - namely electron microscopy, Raman spectroscopy and mass spectrometry. Raman spectroscopy is a vibrational laser scattering technique that provides a biochemical fingerprint of tissue composition. It is a valuable tool that provides data on the biochemical conformation of proteins, carbohydrates, lipids and nucleic acids with submicrometer spatial resolution\(^{272}\). Raman spectroscopy was employed to provide comparative insights into the complexities of the native human intestinal tissue and their resultant decellularised scaffold biomaterials. The results illustrated the overall conservation of biomolecular cues between the histological layers of the native and scaffold mucosa, submucosa and muscularis histological layers. I then applied Raman imaging approach to compare the spectral signatures arising from the human small intestine and colon scaffolds. Quite remarkably, the data illustrated there to be significant spectral commonality specific to the mucosal layers of both SI and colon scaffolds. Mass spectrometry
comparing the small intestine and colon scaffolds supported the Raman spectroscopy data, confirming overall conservation in the global and matrix protein profiles of the two scaffold products.

The scaffold characterisation data presented here provided an in-depth insight into the biochemical features of the human decellularized intestinal scaffolds, which had not been investigated in previous publications using human SI and colon scaffolds for in vitro cancer or fibrosis modelling studies\textsuperscript{249,250}. This non-biased approach to exploring the scaffolds’ biological characteristics led to the concept of investigating the potential of “recycling” colon scaffolds, utilising them to reconstruct functional SI grafts, by allowing the jejunal derived organoids to drive remodelling of the acellular colon matrix. Undeniably, with good patient education, empowerment resources and appropriate stoma nurse support, patients can lead a relatively good quality of life following significant resection of the colon. This is in stark contrast to the detrimental quality of life significant resections of SI bring, due to the malnutrition and recurrent hospitalisations that ensue.

Hence, I sought to collect both human SI and colon scaffolds for the purpose of in vitro seedings with human jejunal organoids. In practice, I found that I was able to retrieve more paediatric colon surgical samples than SI, since resections of colon in children are a more commonly planned occurrence than those of the SI. The concept of recycling colonic biomaterial could be applied in clinical practice to derive a bank of allogeneic donor scaffolds, as an alternative source to cadaveric tissue for the derivation of decellularised scaffolds. As yet, unaddressed research questions remain such as: to what extent is disease held in the structural matrix versus the cellular matrix? Therefore, further studies using Raman spectroscopy, electron microscopy, mass spectrometry, as well as other modalities such as imaging mass spectroscopy, RNA-sequencing and spatial transcriptomics on the recellularised scaffolds, would be useful to characterise the scaffolds of intestinal tissue derived from resections due to dysmotility for example.

Moving forwards, I demonstrate that human jejunal organoids are able to engraft, polarise, proliferate and differentiate to express jejunal specific brush border enzymes, on both SI and colon scaffolds.
Despite preservation of the crypt-villus axis on the human decellularised scaffolds, after re-seeding experiments, compartmentalisation of proliferative cells within the crypt domains or differentiated cells within the villus domains, was not seen, as previously published in-vitro modelling studies (also discussed in the introduction chapter)\textsuperscript{250,286,289}. The epithelial morphology data presented here is most comparable to previous studies that seeded iPSC-derived intestinal organoids onto rat decellularized intestinal scaffolds, where new matrix deposition was noted to be driven by seeded epithelial cells\textsuperscript{193}.

Intestinal stromal cells (fibroblasts and myofibroblasts) are important sustainers controlling the assembly and turnover of the structural ECM, secreting fibronectins, laminins, collagens, proteolytic enzymes and inhibitors\textsuperscript{96}. They also provide growth factors and ligands essential for the intestinal stem cell niche\textsuperscript{96}. Intestinal derived fibroblasts were therefore seeded into both human SI and colon scaffolds to aid the survival of the epithelial graft. These findings support the innovative concept of using colon scaffolds for SI graft reconstruction. There are strong clinical implications of this data for two main reasons. Firstly, colon derived from cadaveric donors or resected in children affected by conditions such as Hirschsprung’s disease could be decellularized, stored, and donated for therapy. Secondly, in conditions such as midgut volvulus, in which typically the large bowel is preserved, there is the potential to convert the IF patient’s own colon to SI by replacing the mucosal layer with jejunal organoids as an alternative treatment solution.

The engineered jejunal grafts proved to be structurally and functionally competent platforms encouraging cell engraftment and vascularisation when transplanted in vivo. However, the physiological behaviour of the intestinal grafts differed depending on the site of implantation. In the kidney capsule transplantation model, I observed a greater amount of host myofibroblast (vimentin\(^+\), \(\alpha\)SMA\(^+\)) infiltration into the scaffold surrounding the rings of human intestinal epithelium. This mimics the crypt environment of the ISC niche and resulted in less differentiated epithelial cells. Instead, the cells remained highly expressive of stem cell markers OLF4, SOX9. In contrast, I found less infiltration of host myofibroblasts in the subcutaneous transplantation model. The in vitro seedings of stromal
cells was an essential step prior to epithelial cell seeding, to allow me to assess the survival of the graft in this model. Teduglutide (a clinically approved drug for use in human trials of IF, and licenced in the EU) was also administered to the NSG mice. I found this approach aided the survival of the ectopically implanted intestinal grafts, where less host infiltration of the scaffolds occurs, however more studies investigating the use of the drug during recellularisation steps are important. Overall this indicates the dual importance of a robust pre-implantation scaffold seeding protocol and appropriate host cell infiltration, in the survival of engineered intestinal grafts in vivo.

In conclusion, the novelty of this work stands in the efforts made to isolate, expand and study jejunal intestinal stem cells and intestinal stromal cells specifically from children with intestinal failure, and use these cells to repopulate human derived intestinal scaffolds and form functional and transplantable mucosal grafts. Although this approach was novel in the research field and important for clinical applicability to the IF patient-population in need, it is important to remember that the causes of IF are heterogeneous. Indeed, sourcing patient ISCs from children with IBD or other conditions that retain epithelial mutations may influence the long-term success of the graft, unless underlying disease pathogenic processes are addressed prior to recellularisation and transplantation. More experiments addressing this specific translational need are required, whereby cells from IBD patients are specifically sought and their long-term cell and matrix phenotype of the engineered grafts are studied in vivo. Original approaches were adopted to analyse the human scaffold products and new rationales for utilising colon scaffolds for jejunal reconstruction are proposed from this work, thereby addressing the problem of native organ donation. The relatively simple methods employed here can easily be scaled up for pre-clinical testing in larger animal models of orthotopic transplantation. A purely mucosal engineered graft will have a particular clinical impact on paediatric patients with primarily epithelial defects. However, this work also represents an important advance towards a staged layer approach to future full tubular jejunal intestinal reconstruction. Unlike previous publications that focused on iPSCs, synthetic scaffolds or rodent ECM matrix, the use of primary human materials (both cells and decellularized ECM matrix) in this study is a highly relevant
step towards translation. Additionally, the experiments with teduglutide indicate novel tissue engineering applications, promoting the survival of grafts in vivo. Whilst this study represents an important conceptual advance towards full thickness intestinal wall construction, significant challenges persist specifically the efficient co-ordinated expansion of human enteric nerves and smooth muscle cells essential for driving peristalsis of the gut. Critical future steps are to focus on capturing data on the global cellular and ECM profile of the engineered graft at both in vitro and in vivo transplantation stages. This may be achieved by combining single-cell transcriptomics with spatial transcriptomics to map the distribution of cells and compare this to native intestinal tissue. Furthermore, neuromuscular wall regeneration and vascularisation of grafts are vital future experimental steps, while scaling up to in vivo pre-clinical testing in orthotopic transplantation models.

Overall, this work represents the first stepwise scalable approach towards using patient cells and biomaterials, for ultimately reconstructing a full thickness intestinal graft for children with irreversible intestinal failure.
8. References


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## Appendix 1 - Patients Demographics Table

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<td>n/a</td>
<td>+</td>
<td>n/a</td>
<td>n/a</td>
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<tr>
<td>3</td>
<td>10</td>
<td>F</td>
<td>Severe neuropathic dysmotility; <strong>Intestinal failure</strong> on parenteral nutrition</td>
<td>Ileum</td>
<td>+</td>
<td>+^</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>M</td>
<td>Intestinal dysmotility; ileostomy; stoma prolapse with necrosis; <strong>Intestinal failure</strong> on parenteral nutrition</td>
<td>Ileum</td>
<td>+^</td>
<td>+</td>
<td>+^</td>
<td>n/a</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>F</td>
<td>Intestinal dysmotility; ileostomy; <strong>Intestinal failure</strong> on parenteral nutrition</td>
<td>Ileum</td>
<td>n/a</td>
<td>+</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>M</td>
<td><strong>Crohn's disease</strong>; Orofacial granulomatosis</td>
<td>Duodenum</td>
<td>+</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>M</td>
<td><strong>Crohn's disease</strong></td>
<td>Jejunum</td>
<td>+</td>
<td>n/a</td>
<td>+</td>
<td>n/a</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>F</td>
<td>PEXB11B deficiency; Gastro-oesophageal reflux disease</td>
<td>Jejunum</td>
<td>+</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
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<tr>
<td>9</td>
<td>11</td>
<td>M</td>
<td>Eosinophilic GI disease, Colitis</td>
<td>Duodenum</td>
<td>+</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
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<tr>
<td>10</td>
<td>7</td>
<td>M</td>
<td>Hemiplegic cerebral palsy</td>
<td>Duodenum</td>
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<td>n/a</td>
<td>n/a</td>
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<td>11</td>
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<td>M</td>
<td>Hypoganglionic bowel dysmotility, mucous fistula</td>
<td>Ileum</td>
<td>n/a</td>
<td>+^</td>
<td>+</td>
<td>n/a</td>
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<tr>
<td>12</td>
<td>1</td>
<td>M</td>
<td>Hirschsprung's disease, enterocolitis</td>
<td>Ileum</td>
<td>n/a</td>
<td>+</td>
<td>+^</td>
<td>n/a</td>
</tr>
<tr>
<td>13</td>
<td>10</td>
<td>F</td>
<td>Chronic intestinal pseudo-obstruction, dysfunctioning PEG-J, gastric and oesophageal dilatation, gastrostomy with GJ extension</td>
<td>Ileum</td>
<td>n/a</td>
<td>+</td>
<td>+^</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Autosomal dominant mutation in MNX1 gene, Curriano syndrome, Ano-rectal malformation, perineal fistula, closure of ileostomy</td>
<td>Ileum</td>
<td>+</td>
<td>+^</td>
<td>+^</td>
<td>n/a</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
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<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
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<tr>
<td>15</td>
<td>13</td>
<td>F</td>
<td>Young-simpson syndrome; Gastro-oesophageal reflux; severe constipation</td>
<td>Colon</td>
<td>n/a</td>
<td>+</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>16</td>
<td>8 months</td>
<td>M</td>
<td><strong>Necrotising enterocolitis</strong> at birth; Left hemicolecotomy and end transverse colostomy; Sigmoid colon perforation; Pulmonary stenosis</td>
<td>Colon</td>
<td>n/a</td>
<td>+</td>
<td>n/a</td>
<td>n/a</td>
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<tr>
<td>17</td>
<td>8 months</td>
<td>M</td>
<td>Hirschsprung’s disease</td>
<td>Colon</td>
<td>n/a</td>
<td>+</td>
<td>+^</td>
<td>n/a</td>
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<tr>
<td>18</td>
<td>10</td>
<td>F</td>
<td>Colonic neuropathy and intestinal dysmotility</td>
<td>Colon</td>
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<td>+</td>
<td>n/a</td>
<td>n/a</td>
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<td>19</td>
<td>5 months</td>
<td>M</td>
<td>Hirschsprung’s disease</td>
<td>Colon</td>
<td>n/a</td>
<td>+</td>
<td>n/a</td>
<td>n/a</td>
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<td>20</td>
<td>4 months</td>
<td>M</td>
<td>Hirschsprung’s disease</td>
<td>Colon</td>
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<td>+</td>
<td>n/a</td>
<td>n/a</td>
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<tr>
<td>21</td>
<td>1</td>
<td>F</td>
<td><strong>Necrotising enterocolitis, Intestinal failure</strong> on parenteral nutrition</td>
<td>Ileum</td>
<td>n/a</td>
<td>+</td>
<td>n/a</td>
<td>+</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Colon</td>
<td>n/a</td>
<td>+</td>
<td>n/a</td>
<td>n/a</td>
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<tr>
<td>22</td>
<td>1</td>
<td>F</td>
<td>Duodenal web</td>
<td>jejunum</td>
<td>n/a</td>
<td>n/a</td>
<td>+^</td>
<td>+</td>
</tr>
<tr>
<td>23</td>
<td>6 months</td>
<td>M</td>
<td>Duplication cyst</td>
<td>ileum</td>
<td>+^</td>
<td>+</td>
<td>+</td>
<td>n/a</td>
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<tr>
<td>24</td>
<td>1</td>
<td>M</td>
<td>Neurodevelopmental delay, abdominal distension and stoma formation</td>
<td>ileum</td>
<td>+^</td>
<td>+</td>
<td>unsuccessful contamination</td>
<td>n/a</td>
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<td>25</td>
<td>12</td>
<td>F</td>
<td>Dysmotile colon with diversion colitis, ileostomy</td>
<td>Ileum</td>
<td>n/a</td>
<td>+^</td>
<td>n/a</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Colon</td>
<td>n/a</td>
<td>+^</td>
<td>n/a</td>
<td>n/a</td>
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<tr>
<td>26</td>
<td>10</td>
<td>M</td>
<td>SCID, previous bone marrow transplant, constipation and failure to thrive</td>
<td>Duodenum</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>+</td>
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<tr>
<td>27</td>
<td>5</td>
<td>M</td>
<td>Investigation of rapid transit diarrhoea. Endoscopy and histology normal.</td>
<td>Duodenum</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td>Jejunum</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>+</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ileum</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>+</td>
</tr>
<tr>
<td>28</td>
<td>10</td>
<td>M</td>
<td>Coeliac disease</td>
<td>Duodenum</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>+</td>
</tr>
<tr>
<td>29</td>
<td>12</td>
<td>F</td>
<td>Crohn’s disease</td>
<td>ileum</td>
<td>n/a</td>
<td>+</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>30</td>
<td>2</td>
<td>F</td>
<td>Early onset Crohn’s disease, unresponsive to maximal medical therapy</td>
<td>colon</td>
<td>n/a</td>
<td>+</td>
<td>n/a</td>
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### Appendix 2 - Primer Sequences for qRT-PCR

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<td>human β-ACTIN</td>
<td>TTCTACAATGAGCTGCCTGTG</td>
<td>GGGGTGGTGAAGGTCTCAAA</td>
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<tr>
<td>human CYBRD1</td>
<td>GCTATTGTATACGTTCTCTCAGG</td>
<td>CAGGATCTCTCAGGGAAAAATC</td>
</tr>
<tr>
<td>human SLC40A1</td>
<td>GATGCTGTGGATTCTCTGCCC</td>
<td>CCACATCCGATCTCCCACCTAGA</td>
</tr>
<tr>
<td>human SI</td>
<td>CATACCATTTGATACACAGGTC</td>
<td>GCAGGGTCAGATGATGAC</td>
</tr>
<tr>
<td>human LCT</td>
<td>TCAGGGCGGAAAAACTCTCTGTTCGTC</td>
<td>CGACCGTGTCCGTGGCAGC</td>
</tr>
<tr>
<td>human SLC10A2</td>
<td>GGCGACATGGACCTGAGGTAGC</td>
<td>CGAGAGAAACCAGGATGTACCTATAG</td>
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<tr>
<td>human OSTB</td>
<td>GGGGCTAAGGGGCTCTAAGG</td>
<td>CAGGGCAAAGGATGAATGA</td>
</tr>
<tr>
<td>human OLFM4</td>
<td>CTCTTCCAAAGTGAGGGAATATGTC</td>
<td>GATGTCATTCCGGAGCAGTTAG</td>
</tr>
<tr>
<td>human LGR5</td>
<td>CAGTGCGATGGTTCCTTCC</td>
<td>AGTGCCAGAACGTCTATG</td>
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<tr>
<td>human LYZ</td>
<td>CATTGTCTGGGCTGTTCGTC</td>
<td>TCATTACACCAGTGGCAGC</td>
</tr>
<tr>
<td>human MUC2</td>
<td>CAACACTCCGAGGTGT</td>
<td>CAAATGTTCCTCAGGTACC</td>
</tr>
<tr>
<td>human ALPI</td>
<td>TCATCATGAGGGGTGGGCTT</td>
<td>TGTAGGCTTGGCTGTTCCT</td>
</tr>
<tr>
<td>human CHGA</td>
<td>GAAGAAGGCCCTCAGTGT</td>
<td>AGTGCCTCTGTTCCTCCT</td>
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### Appendix 3 - Antibodies for immunofluorescence, immunohistochemistry and immunoblotting

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<th>Antibody</th>
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<th>Cat. Number</th>
<th>Dilution</th>
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<tbody>
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<td>Beta-actin</td>
<td>mouse</td>
<td>Sigma Aldrich</td>
<td>A3854</td>
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<td>CD31</td>
<td>mouse</td>
<td>Abcam</td>
<td>Ab9498</td>
<td>1:200</td>
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<tr>
<td>ChgA</td>
<td>goat</td>
<td>Santa Cruz</td>
<td>Sc-393941</td>
<td>1:500</td>
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<tr>
<td>Collagen-1 (human)</td>
<td>goat</td>
<td>Abcam</td>
<td>Ab34710</td>
<td>1:200</td>
</tr>
<tr>
<td>Collagen-1 (porcine)</td>
<td>mouse</td>
<td>Abcam</td>
<td>Ab6308</td>
<td>1:50</td>
</tr>
<tr>
<td>Collagen-4 (porcine)</td>
<td>rabbit</td>
<td>Novabio</td>
<td>NB120-6586</td>
<td>1:200</td>
</tr>
<tr>
<td>Defensin-α5</td>
<td>mouse</td>
<td>Abcam</td>
<td>Ab90802</td>
<td>1:50</td>
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<tr>
<td>E-cadherin</td>
<td>rabbit</td>
<td>Cell signalling</td>
<td>#3195</td>
<td>1:400</td>
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<td>Fibronectin (human)</td>
<td>rabbit</td>
<td>Abcam</td>
<td>Ab23750</td>
<td>1:200</td>
</tr>
<tr>
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<td>Santa Cruz</td>
<td>Sc-59826</td>
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<tr>
<td>FSP1</td>
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<td>Ab11333</td>
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<td>Abcam</td>
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<td>hNucleoli</td>
<td>mouse</td>
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<td>Ab190710</td>
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<td>Ki67</td>
<td>rabbit</td>
<td>Abcam</td>
<td>Ab16667</td>
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<tr>
<td>Laminin-α5 (human)</td>
<td>mouse</td>
<td>Abcam</td>
<td>Ab210957</td>
<td>1:200</td>
</tr>
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<td>Laminin (porcine)</td>
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<td>Ab11575</td>
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<tr>
<td>LGR5</td>
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<td>Miltenyi Biotec</td>
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<td>Lysozyme</td>
<td>rabbit</td>
<td>Dako</td>
<td>a0099</td>
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<td>Ab198367</td>
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<td>#14369</td>
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<td>Invitrogen</td>
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<td>Ab5694</td>
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<td>rabbit</td>
<td>Millipore</td>
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<td>Sucrase Isomaltase</td>
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<td>Santa Cruz</td>
<td>Sc-393424</td>
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<td>Sc-28293</td>
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<td>villin</td>
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<td>Abcam</td>
<td>Ab190085</td>
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<td>Goat</td>
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Appendix 4 - Piglet SI scaffold fabrication and characterisation

Piglet SI scaffold characterisation: (a) Quantification of DNA, glycosaminoglycans (GAGs) and collagen per milligram of wet tissue in native piglet intestine and following one and two cycles of DET. Data represent mean ± s.e.m of n = 3 biologically distinct piglet intestine samples (DNA and GAG quantification) and of n = 2 biologically distinct samples (collagen quantification). One-way ANOVA with Dunnett’s multiple comparisons test. The experiment was performed once. (b) Representative H&E images and DAPI immunostainings of a native piglet intestine and following one and two cycles of DET. Scale bars represent 100µm. (c) Representative histology images of Elastic Van Gieson (EVG) and Alcian blue (AB) stainings confirm preservation of elastin and GAGs respectively following one and two cycles of DET. Masson’s trichrome (MT) and Picro-sirius red (PS) histological stainings confirms maintenance of connective tissue and collagens following one and two cycles of DET. Scale bars represent 200µm. (d) Representative immunohistochemical staining for Collagen I (Col-1), Collagen IV (Col-4), Fibronectin, Laminin indicating the preservation of these ECM proteins in a piglet scaffold following two cycles of DET. Scale bars represent 100µm. Images are representative of 3 biological replicates.

This data in its entirety was acquired by Suzanna Eli, Alfonso Tedeschi and Alessandro Pellegata (UCL)
### Appendix 5 - Global proteomic profile of human SI and Colon scaffolds

<table>
<thead>
<tr>
<th>Protein</th>
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</tr>
<tr>
<td>Band 4.1-like protein 3</td>
<td>Band 4.1-like protein 3, N-terminally processed</td>
</tr>
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<td>Cytochrome c oxidase subunit 7A2, mitochondrial</td>
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</tr>
<tr>
<td>Thioredoxin-dependent peroxide reductase, mitochondrial</td>
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</tr>
<tr>
<td>Cilia- and flagella-associated protein 46</td>
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Extracellular
### Appendix 6 - Protein candidates potentially specific to either SI and Colon scaffolds

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<th>iBAQ Colon_D</th>
<th>iBAQ SI_A</th>
<th>iBAQ SI_B</th>
<th>iBAQ SI_C</th>
<th>iBAQ SI_D</th>
<th>GOCC name</th>
<th>Score</th>
<th>Q-value</th>
<th>iBAQ Colon_A</th>
<th>iBAQ Colon_B</th>
<th>iBAQ Colon_C</th>
<th>iBAQ Colon_D</th>
<th>iBAQ SI_A</th>
<th>iBAQ SI_B</th>
<th>iBAQ SI_C</th>
<th>iBAQ SI_D</th>
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