Intestinal Tissue Engineering

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Abstract

Tissue engineering (TE) principles have been successfully clinically applied to treat disease affecting specific organs (e.g. trachea) but developments in some organs has lagged behind. The inability to repair or replace significantly damaged intestinal tissue remains a barrier to improving patient outcomes and the promise of Tissue Engineered Intestine (TEI) that was first made more than 20 years ago, is yet to be realised.

This work explored the potential of TEI and literature review formed a basis for developing a clinically transferrable experimental model. It was hypothesised that, porcine large intestine could be retrieved from pigs and decellularized to create a biological scaffold that demonstrated favourable properties for TE, including potential for vascular perfusion and cell engraftment. Novel experiments were performed in intestinal retrieval and decellularization, resulting in scaffolds characterised by a number of methods (e.g. histology, immunohistochemistry). Assessment of the scaffold's ability to support cell engraftment required development of protocols for isolation and culture of appropriate progenitors, including adipose/ bone marrow derived mesenchymal stromal cells and intestinal organoid units. Finally, in-vitro cultures combining scaffolds and cells were used to assess the ability of scaffolds to promote tissue regeneration.

Perfusion decellularization methods proved effective in creating biological scaffolds that retained radiologically demonstrated vascular perfusion networks, permitting a future route for recellularization and/or transplantation. Scaffolds demonstrated retention of essential extracellular matrix components (e.g. glycosaminoglycans, collagen) and an absence of cell nuclei. Mesenchymal stem cells were isolated, cultured and combined in-vitro with scaffolds in an attempted scaled-down seeding model. Control of culture conditions was challenging and results inconclusive with respect to the scaffold's regenerative potential. The work demonstrates an exciting prospect for biological scaffold development for a clinically transferrable, semi-xenogeneic transplant or drug delivery model but further experiments in scaffold seeding are required to assess the full potential.

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Author's Declaration

I hereby declare that all work represented within this text body was performed by me and where this was not the case it is explicitly stated. During the course of this work, the costs of the research were met by funding from the Northwick Park Institute of Medical Research via charities including CORE, the Bowel Disease Research Foundation and the St. Marks Foundation. No financial or ethical conflicts of interest were applicable and I have no disclosures to make.

List of Abbreviations

Abx – Antibiotics

ALB – Alcian Blue

APTS - Aminopropyltriethoxysilane

AD – Adipose Derived

AV - Arterio-venous

BM - Bone Marrow

BMP - Bone Morphogenic Proteins

BSA - Bovine Serum Albumin

CRC – Colorectal Cancer

CTA - Computed Tomography Angiogram

DAB – Diaminobenzidine

DGF – Delayed Graft Function

DMSO - Dimethyl Sulfoxide

DMEM - Dulbecco's Modified Eagle Medium

DNA - Deoxyribonucleic Acid

DPX - Distyrene Plasticizer Xylene

ECG - Electrocardiogram

ECM - Extra-Cellular Matrix

EDTA - Ethylenediametetraacetic Acid

ESC – Embryonic Stem Cell

ET - Endotracheal

FACS - Fluorescence Activated Cell Sorting

FCS - Fetal Calf Serum

GAGs – Glycosaminoglycans

GTR – Guided Tissue Regeneration

HBSS - Hank's Balanced Salt Solution

HCl – Hydrochloric Acid

HPN – Home Parenteral Nutrition

H&E - Haematoxylin and Eosin

IBD - Inflammatory Bowel Disease

IM - Intramuscular

IMS - Industrial Methylated Spirit

IP – Intraperitoneal

IPAA - Ileal Pouch-Anal Anastomosis

iPSC - Inducible Pluripotent Stem Cell

IR – Intestinal Rehabilitation

IRAD - Intestinal retrieval after death

IRBD - Intestinal retrieval before death

ITS - Insulin Transferase Selenium

IV - Intravenous

IVC - Inferior Vena Cava

KRBH - Krebs-Ringer Bicarbonate Hepes

L - Litre

MEM - Minimum Essential Media

MHC - Major Histocompatibility Complex

MM – Micro-Masses

MSC - Mesenchymal Stromal Cell

NBF - Neutral Buffered Formalin

NPIMR – Northwick Park Institute for Medical Research

N/S - Normal Saline (0.9%)

OB - Osteoblasts

OD - Optical Density

ORO – Oil Red O

OU - Organoid Units

P - Passage

PBMC - Peripheral Blood Mononuclear Cell

PBS - Phosphate Buffered Saline

PFA - Paraformaldehyde

PME - Picrosirius Miller's Elastin

PN – Parenteral Nutrition

PNALD - Parenteral Nutrition-Associated Related Liver Disease

RPM - Revolutions per minute

SC – Subcutaneous

SDS – Sodium Dodecylsulphate

SIS - Small Intestinal Submucosa

SOP - Standard Operating Procedure

SPS - Complex - Intestinal Scaffold/Biopsy Pad/Suture Complex

SVF – Stromal Vascular Fraction

TBE - Tris-Borate-EDTA

TE - Tissue Engineering

TEI - Tissue Engineered Intestine

TEM - Transmission Electron Microscopy

TGF-β - Transforming Growth Factor Beta

TPN - Total Parenteral Nutrition

TNS - Trypsin Neutralising Solution

U - Units

UC - Ulcerative Colitis

UNOS - United Network for Organ Sharing

V - Volts

Chapter 1 General Introduction

Tissue Engineered Intestine (TEI) is a broad term to cover all aspects of tissue engineering (TE) when applied to generation of new intestinal tissue or some component of it. The rationale behind the process lies in the unmet clinical need for replacement intestinal tissue in diseases which cause irreversible damage to the intestine, or necessitate resection resulting in intestinal failure. Research to date in solving this clinical problem has followed a traditional scientific approach, with attention to understanding principles in vitro, developing small animal models and progressing to large, with the intention of taking treatments to the bedside for patient benefit. Despite historical work, TEI remains an experimental basic science topic, with many developments necessary before principles can be applied to deliver care to patients.

This body of research is concerned with novel experimental methods of creating TEI, based on models which apply principles that may be transferred to the clinical setting. By exploring the clinical justification for TEI, the rationale behind methods used, detailing experiments and discussing the results, this work aims to discuss the past, present and future of the field. To permit this, important aspects of TEI are reviewed below. Firstly, clinical problems that remain unsolved and indicate the motivation behind undertaking the project are discussed. A general discussion of tissue engineering follows, to enable understanding of the challenges encountered when applying TE principles to the intestine. A review of the most important and significant studies in the field is then discussed before outlining the format of the thesis and motivation behind experimental work. Finally, the hypothesis and aims of the research project are discussed to complete the chapter and provide rationale for subsequent experimental work.

1.1 Clinical Challenges

A number of clinicians are faced with the challenge of treating patients with established organ failure, either from congenital (e.g. cystic fibrosis causing respiratory failure) or acquired (e.g. hepatitis causing liver failure) conditions. Patients may present early or late in disease stages with treatment options ranging from multiple, to very few. If treatment options are exhausted and no cure/control of disease process can be achieved, a clinical decline may be seen. In some cases, patients face an uncertain prognosis, with an unpredictable decline in organ function (e.g. chronic renal failure) but in others this is rapid, predictable and inevitable. Such patients populate transplant waiting lists, awaiting allocation for a new kidney, liver, heart or intestine. The United Network for Organ Sharing (UNOS) 2014 annual report indicated that 29,532 transplants were performed in the USA alone and current UNOS estimates indicate that one more patient is added to a transplant waiting list every 10 minutes and that 22 deaths occur daily of patients on lists whilst awaiting organ allocation. Similar figures can be extrapolated from the National Health Service Blood and Transplant (NHSBT) 2013/14 annual report (1). Clearly the number of suitable organ donors does not, and possibly may never, meet the demands of the patient population.

TE has been defined as the development of biological substitutes that restore, maintain or improve tissue function and has been used to develop treatments for patients (2). Historically, research has focused on patients with established organ failure. A shortage of organ donors is one driving factor for research into development of replacement organs. However, the ability to produce a tissue engineered, immunologically matched graft may prevent the need for long term immunosuppression and be preferable to allogeneic transplantation, even if a sufficient number of donor organs were available. The possible benefit of production of tissue engineered organs when required might also permit better timing of transplant procedures and these factors, in addition to the potential to address other ethical and cultural issues by avoiding allogeneic transplantation makes the proposition even more attractive.

It should be noted in addition, that there are other disease states which could be addressed by solutions of bioengineered tissue which have received scant attention within the literature. Cancer for example, frequently necessitates excision of malignant tissue and replacement may not be possible. In some cases, patients survive without essential tissue but quality of life may be impaired (e.g. post-colectomy). If tissue engineered organs were sufficiently safe and effective, then applications would not only be limited to life threatening diseases and tissues such as breast, muscle and skin could also be replaced to improve quality of life. Such an expansion in the field could also reduce reliance on synthetic implants (e.g. breast) and reduce risks associated with use of synthetic grafts. Such potential clinical applications, in addition to others (e.g. drug delivery, disease models) should be borne in mind when considering the wider applications of TE.

1.2 Intestinal Disease

Appreciation of the rationale for TEI requires discussion of relevant clinical conditions. The term 'intestinal disease' can be used to account for a plethora of conditions affecting the large and small intestine. Diseases can be classified according to; speed of onset (e.g. acute), anatomical location (e.g. large bowel) and pathological process (e.g. inflammatory). Various disease processes may be important in potential applications of TEI but ultimately, replacement TEI would only be an appropriate for diseases which cause irreversible damage and loss of function. In the absence of these 2 pathological processes, it is likely that a more effective and safer treatment could be utilised at an earlier stage of disease. Due to this, diseases causing irreversible loss of function or necessitating resection will primarily be focused on in this chapter.

1.2.1 Intestinal failure

Recognition of ‘intestinal failure’ as a clinical entity is relatively new in comparison to other clinical organ failure syndromes (e.g. renal/ hepatic). It can be defined simply as a state ‘when there is reduced intestinal absorption so that macronutrient and/or water and electrolyte supplements are needed to maintain health and/or growth’ (3). Various classifications have been

posed and as early as 1995, Irving et al. classified the clinical state according to aetiology, grouping patients into those with; short bowel syndrome, motility disorders, small bowel parenchymal disease or intestinal fistula (4). In later years, a more precise classification was sought based on stage of presentation and this has recently been reviewed, refined and endorsed by the European Society for Clinical Nutrition and Metabolism (ESPEN) in a recommendation manuscript (5). The recommendations classify intestinal failure into 3 types; (I) that of acute onset and which is usually self-limiting, (II) that characterised by an acute prolonged condition lasting weeks or months and requiring complex care and intravenous supplementation and, (III) that of a chronic state in metabolically stable patients requiring intravenous supplementation over months or years. Types I and II are reversible but type III may be reversible or irreversible. In discussing applications of TEI, it is important to note that all types may be curable but Types I and II are more likely to respond to medical and surgical interventions and recover than Type III. In addition, it is worth noting that Type II may progress to Type III. As a result, replacement intestine is unlikely to form a relevant clinical solution to Types I and II as numerous other treatment options exist. When intestinal failure is discussed within this work therefore, it will primarily refer to Type III.

When focussing discussion on Type III intestinal failure, the clinical challenges in treatment are better understood. The precise incidence of the disease is difficult to determine and is best estimated by assessing the number of patients on home parenteral nutrition (HPN). The British Artificial Nutrition Survey (BANS) of 2011 reported a prevalence within the UK of 10 cases per million with the number of new cases per year at 3.66 per million. It is likely that these figures are not 100% accurate, as data for HPN administration is also collected from commercial sources demonstrating a greater prevalence with an estimated shortfall in BANS reporting of 53.5% in the adult population and as much as 89.5% in the paediatric population (6). Despite this, the condition cannot be regarded as ‘common’ from an epidemiological viewpoint. An understanding

of the impact of the clinical state provides a justification for the pursuit of complex treatment and research programs which invariably involve high costs (see clinical limitations section).

1.2.2 Causes of Intestinal Failure

Intestinal failure is typically caused by 5 major conditions; short bowel syndrome, intestinal fistula, intestinal dysmotility, mechanical obstruction and small bowel mucosal disease (5). In practical clinical terms, there is considerable overlap between these conditions and reviewing each in detail as separate clinical entities is not necessary for discussion of applications of TEI. For example, a patient may present with a small bowel mucosal disease (e.g. Crohn's), require extensive bowel resection, develop mechanical obstruction post-operatively and require further surgery resulting in intestinal failure. A basic understanding of the disease processes is however necessary, as bowel surgery carries risks of recurrent disease and complications which are unlikely to be avoided, even with the best TEI treatments.

Both large and small intestine are prone to fistula formation. Primary disease of the small bowel (e.g. Crohn's disease) may be responsible due to inflammatory disease causing entero-enteric fistulae or that of the large bowel (e.g. diverticular disease) causing colo-vesical fistulae. Frequently, post- operative complications following complex abdominal surgery cause fistulating disease and patients with multiple surgeries for Crohn's disease may develop high output entero-cutaneous fistula reducing significantly the enteric surface for absorption and resulting in intestinal failure. The problem of post-operative fistula complications is a concern for any attempted surgical solution of bowel disease and any potential TEI applications must take this into account.

Similarly, bowel obstruction of the small or large bowel can result from numerous disease processes, such as primary mucosal disease (e.g. Crohn's), adhesions secondary to previous surgery and malignant lesions (e.g. colon cancer). Mechanical obstruction may arise secondary to inter, intra or extra luminal disease and may arise in either the large or small bowel. With such a

multitude of causes, accurate estimates of incidence are difficult to determine. The significance of bowel obstruction, particularly of the small bowel is that the majority of cases (74%) are caused by adhesions, and these typically result from previous surgery (7). As with fistulating disease of the bowel, it should be borne in mind that the development of bowel obstruction following surgical intervention is possible and therefore TEI approaches to treatment are at risk of such complications.

Intestinal dysmotility refers to a broad range of disorders including; those resulting from post-operative transient impaired function of the small intestine, dysmotility secondary to common chronic diseases (e.g. Parkinson's) and those secondary to rare primary conditions affecting the intestine such as visceral myopathy (5). The result of these disorders is a failure of propulsion of the gut and inadequate function which prevents adequate transit of nutrients. Such conditions can further be classified as either congenital or acquired but the important patient group to consider in applications of TEI are those patients who progress to Type III intestinal failure with potential for further curative treatment. These patients tend to be those with congenital disorders, as those acquired later in life are more likely to occur secondary to disease processes which already cause significant other morbidity (e.g. diabetes) which may contraindicate extensive and high risk treatments. The notable point with application of TEI in treatment of such diseases is the essential requirement for adequate motile function in any replacement tissue. This is a complex and demanding issue which will be discussed elsewhere in further detail.

Extensive small bowel mucosal disease refers to that in which the mucosal surface is intact but non-functioning, thereby preventing adequate absorption of nutrients. The commonest cause of such diseases in childhood is Crohn's and this accounts for the majority of newly registered HPN cases in the UK (6). Where disease is sufficiently extensive to cause Type III intestinal failure, it is likely that some resection of the small bowel will be necessary for symptom control. This may result in short bowel syndrome with overlap of the 2 clinical entities as previously described.

In considering the above diseases, it should be noted that a *failure in function* is directly responsible for intestinal failure. The ability to provide function of any TEI replacement for treatment is therefore paramount as provision of some function (e.g. mucosal absorption) in the absence of another (e.g. motility) may result in clinical failure. This poses significant challenges in applying basic science solutions clinically as an 'all or nothing' ability of replacement intestine seems necessary.

1.2.3 Short Bowel Syndrome

Short bowel syndrome (SBS) refers to 'the malabsorptive state caused by physiological or functional loss of significant portions of the small intestine' (8). In such cases, there is inadequate function due to the absence of intestine. This clinical situation typically arises as a result of resection for treatment of; Crohn's disease, intestinal infarction and abdominal trauma. Crucially, the length of intestine remaining is the important factor in prognosis as the anatomical length of small intestine in humans is variable with a range in length reported from 2.5 – 8.5m (9). The question of what length of residual bowel is 'short' may be further complicated by varying functionality of remaining bowel secondary to (e.g. Crohn's).

Significant small bowel resections typically result in total loss of ileum with variable degrees of jejunum remaining. The surgical outcome may be either formation of a high jejunostomy, or anastomosis of jejunum to remaining colon. Extensive jejunal resections with anastomosis of remaining jejunum to short lengths of residual ileum ($\geq$ 10cm) may also result in SBS but these are rare and residual ileum typically possesses enough length and function to prevent development of SBS (3). The presence or absence of colon is important, as a high jejunostomy empties high volume enteric content externally and rapidly but exit from the body is slowed by travel through the colon if present instead. There are 2 aspects to this; the first is the demonstration that gastric emptying is increased in the presence of a high jejunostomy and the second that the colon provides a 'brake effect' increasing absorption (10). The result of this is that

retention of colon in continuity with jejunum remains favourable to a high jejunostomy when similar lengths of residual jejunum are compared.

Short bowel syndrome (SBS) can be classified according to lengths of residual intestine, with symptoms experienced in those with $\leq$ 200cm of jejuno-ileal segment remaining (11). Dependence on TPN however, typically occurs when <100cm small bowel remains or <60cm in continuity with colon (12). It should be noted therefore that SBS does not always cause intestinal failure and there is a spectrum of intestinal disease resulting from SBS. This is best demonstrated in the classification of SBS promoted by Jeppesen et al., which divides patients into 2 categories; intestinal insufficiency and intestinal failure (8). Patients with intestinal insufficiency are able to replace enteric losses with oral supplementation whereas those with intestinal failure require IV supplementation, either with TPN or IV fluid. This classification is more useful from a clinical perspective as, not all residual small bowel possesses equal functional capacity in all patients. Following extensive resection, the small intestine demonstrates 'adaptation' and much work has been done to characterize the process whereby residual gut responds to an inadequate surface area for absorption. This has been demonstrated in animal models which demonstrate an upregulation of cell proliferation, in addition to increase in villi length and therefore surface area (13). Evidence to support this process in humans is less convincing but demonstrates that the intestine has the ability to adapt and this is more marked in children (14), but reduced in adults (15). Due to these variations, length of residual intestine should not be considered as a completely reliable indicator of function.

Despite the overlap of clinical causes of intestinal failure and the spectrum of disease, there remains a clearly defined patient population affected by these disease processes and living with dependence on TPN, with a poor quality of life. As discussed previously, this represents a small population but the current clinical limitations of treatment justify exploration into alternative treatment regimes. From a healthcare economics perspective, the considerable morbidity and

mortality experienced by these patients may provide justification for the high cost and complexity of researching such alternative treatments as patients already suffer a prolonged course of ill health requiring high cost medical and surgical treatments.

1.2.4 The Large Intestine

The small intestine dominates the literature in relation to TEI, with few studies focused on the large and the term 'TEI' typically refers to the small intestine only. The likely reason for this is the higher morbidity and mortality associated with absence of small bowel when compared with that of large. Patients can survive with total absence of the colon (as demonstrated by numerous patients following total colectomy for treatment of ulcerative colitis), but those with SBS may be dependent on TPN for survival. Therefore, there is a greater 'need' for replacement small intestine than large. However, this approach fails to address the significant benefits that tissue engineered large intestine could provide. The details of this are discussed elsewhere but important aspects of large intestinal disease are reviewed below.

1.2.5 Colorectal Cancer

Malignant tumours of the large intestine are common with the number of new cases reported by Cancer Research UK in 2011 as 41,581. In 2012, the number of reported deaths from colorectal cancer in the UK were 16,187 (16). By contrast, cancer of the small bowel is rare, with a global incidence of less than 1 in 100,000 per population (17). Tumours of the large intestine are adenocarcinomas in > 90% of cases, with the majority occurring spontaneously and some in connection with familial cancer syndromes (18). Curative treatment of colorectal cancer is sought in all possible cases and typically involves limited colonic resection with, or without covering stoma formation and additional chemo/ radiotherapy. Operations are well tolerated and survival statistics good, particularly when early stage disease is treated (19). Survival statistics do not however, reflect morbidity and whilst right sided resections are well tolerated, low tumours of the left colon and rectum resulting in low colorectal or colo-anal anastomoses can cause patients

considerable morbidity from chronic diarrhoea. Colorectal cancer represents an important epidemiological disease therefore and whilst replacement of resected colon is not essential, tissue engineered colon may provide an option for replacement or method of disease modelling providing wider applications.

1.2.6 Ulcerative Colitis

Ulcerative colitis (UC) is characterized by inflammation of the colon or rectum due to unknown causes (20). It is a form of inflammatory bowel disease (IBD) and has an incidence of 156-291 cases per 100,000 population (21). UC has a range of clinical presentations, the majority of which respond to medical management but when severe attacks are unresponsive to treatment, or when malignant lesions develop, surgical treatment by total colectomy may be indicated. Frequently, the rectum is resected in addition to colon (proctocolectomy), leaving only small intestine which is typically fashioned as an ileostomy. The morbidity associated with an ileostomy may be considerable with 'high-output stoma' necessitating hospital admission and IV fluid administration. In addition, quality of life has been demonstrated as impaired when an ileostomy is present, even with the knowledge of planned reversal (22). One option for such patients is the formation of an ileal pouch-anal anastomosis (IPAA) with surgical fashioning of a 'J- shaped' pouch of ileum to act as a neo-rectal reservoir anastomosed to the anus. This procedure (restorative proctectomy/proctocolectomy) has demonstrated good long-term outcomes but is also associated with significant morbidity, including the development of 'pouchitis' (23). In addition, it may not be a suitable treatment option in all patients given the extent of the surgery required. The very existence and application of the IPAA operation demonstrates the cohort of patients who are suffering with total absence of the colon and rectum and seek further treatment to improve their quality of life. This population alone justifies the interest in tissue engineering of the large intestine to help provide alternative future treatment options.

1.3 Current Clinical Limitations

As described, diseases of the small and large intestine have differing presentations and clinical challenges. Whilst both may present with a spectrum of disease, historically TEI has been seen as a potential solution to the treatment of absent small intestine by proposing a tissue replacement strategy. Treatment of Type III intestinal failure is initially supportive with management of concurrent medical issues (e.g. high output stoma) and supplementation of nutrition and hydration as appropriate with TPN and IV fluids. In addition, electrolyte levels may require specific replacement (e.g. potassium, calcium). As previously discussed, the estimated number of patients requiring such treatments is low in comparison to other diseases and European estimates are at 4 per million per population (24). Current clinical care has advanced and international standards now advocate a multi-disciplinary approach to promote 'Intestinal Rehabilitation (IR)' in patients and gradually reduce the need for parenteral support (25). Advances in the quality of TPN, preparations and administration methods have led to improved outcomes on TPN and the possibility of remaining on home parenteral nutrition (HPN) for a sustained time period (26).

There are however risks associated with sustained TPN and research has demonstrated that patients who remain on HPN have a reduced life expectancy to age matched controls (27). In addition, caring for such patients poses significant economic challenges. Although difficult to determine accurately, estimates have been made in cost of sustaining comprehensive care for SBS (in the paediatric population) and the first year of care has an estimated cost of $505,250 $\pm$ 248,398, with costs reducing thereafter for hospital in-patients but increasing for those in the community on HPN (28). This very fact, in conjunction with the reduced quality of life patients endure due to dependence on TPN has promoted the development of surgical treatments for intestinal failure.

Surgical treatments performed today can be divided into autologous bowel reconstruction surgery and transplantation. Autologous procedures have improved in efficacy over the years and primarily consist of either lengthening or tapering procedures (29). These serve to increase intestinal length, slow transit and increase surface area for absorption. Such procedures tend to be more suitable in the paediatric population and suitability is dependent on length and quality of existing small intestine. In patients who are unsuitable for such procedures, the continued management of SBS by use of TPN is necessary but sustained TPN may lead to development of life threatening complications from central venous catheter related sepsis or from parenteral nutrition-associated related liver disease (PNALD). In such patients, allogenic intestinal transplantation may be possible if patients are deemed fit. Due to the high risks associated with the procedure, the proportion of suitable patients is low and UK National Health Blood and Transplant (NHSBT) registry figures indicate that as of March 2015, there were 9 patients on the UK active intestine transplant list, representing 50% decrease relative to four years earlier, when the list reached its maximum figure. In a given 1 year period (April 2012 –2013), 71% had received a transplant two years post-registration, 18% had died and 12% were still waiting (30). Intestinal transplantation is demonstrating improved outcomes with some centres now claiming an 79.6% and 56.3% one and five year survival rates respectively (31). Patients are however, dependent on immunosuppression for the life of their transplant. The limited number of suitable patients and the morbidity associated with long term immunosuppression suggests that whilst intestinal transplantation represents an impressive clinical treatment for a very sick patient population, better options should still be sought.

As the clinical problems of large bowel disease differ to those of small, so to do the treatments. Patients who undergo proctocolectomy or total colectomy with end ileostomy formation are prone to morbidity of the stoma including high-output. Medical management to treat acute episodes of high-output may be successful but in some cases they are ineffective and patients present cyclically requiring hospital admission for dehydration and electrolyte imbalance. Other

complications, such as para-stomal herniation may occur, promoting episodes of bowel obstruction and whilst surgical repair may be possible, these patients present considerable surgical risk both intra operatively and of recurrent disease. Restorative IPAA post proctocolectomy provides an alternative treatment option to fashioning of an ileostomy but there may be associated morbidity with development of 'pouchitis'. There are no other treatment options for these patients to improve their quality of life and an alternative tissue engineered segment of colon, rectum or pouch would provide one, particularly in young patients whose pouch has ceased to function (32).

Current treatment of colorectal cancer (CRC) is limited by different factors to those discussed above. The problem of absent large bowel and the need for replacement tissue does not apply, as CRC resections usually leave a residual length of large intestine that is sufficient for recovery and good quality of life. The issue of mortality following CRC resection is of greater importance than that of morbidity and the number of deaths is significant as discussed previously (16). Evidence demonstrates that deaths from CRC are primarily due to a direct result of the malignant process more than any other (33). Thus, the major clinical issue is malignancy itself and TE strategies for direct treatment of malignancy are problematic due to issues regarding angiogenesis, immunomodulation and metastasis, owing to the interaction of new cell progenitors and the diseased host (34). Direct TE therapies may therefore actually confer increased risk to recipients and caution should be exercised in any applications. Potential applications for TEI in malignancy may however be present in the field of disease modelling. Therefore, although TEI does not represent a direct method of providing potential treatment for CRC, it may provide opportunity for the development of future treatment strategies.

1.4 Tissue Engineering

Tissue Engineering (TE) was first coined as a term in the late 1980s and later refined in the early 1990s to describe the development of biological substitutes that restore, maintain or improve

tissue function (2). Principles of TE have been used in developing treatments for numerous organ systems including; cardiac, intestinal, liver and renal failure (35). The 25 years or so following initial applications has seen TE grow into a complex multi-disciplinary field encompassing aspects of bioengineering, cell culture, immunology and more recently additive manufacturing. Despite this, clinical application of TE and associated cellular therapies has been limited (36). Important concepts of tissue engineering are discussed below before focussing on specific studies related to the intestine.

1.4.1 Definitions

Within this work, the term 'tissue engineering' will refer to studies which utilize a scaffold, in conjunction with cells to promote regeneration of tissue. The need for clarification arises from the use of multiple terms within the field which are often used interchangeably (37). 'Tissue Engineering' is often used interchangeably with 'regenerative medicine'. 'Regenerative medicine' has various definitions but all describe the principle of repair of damaged tissue – replacement of tissue is not a prerequisite component. Confusingly, the term 'cell based therapy' is also used in relation and is defined as 'any treatment that involves the use of exogenous cells in an attempt to repair diseased or damaged tissue' (36). Both the terms 'cell based therapy' and 'regenerative medicine' therefore may describe the use of cells for treatment in the absence of scaffolds to promote tissue repair or regeneration (e.g. IV administration of stem cells in clinical trials). TE by contrast, requires use of a scaffold for cell seeding. As cell based therapies such as stem cell administration now account for a large number of studies (38), 'cell based therapies' and 'regenerative medicine' cover a breadth of science assessing the interaction between autologous and allogeneic cells with human recipients to treat various diseases and are outside the remit of this work. Of final note, the term 'guided tissue regeneration' must also be clarified and refers to the use of scaffolds to promote cellular regeneration in the absence of additional cell based therapy (39). Guided tissue regeneration (GTR) is not technically 'tissue engineering' therefore as application of cells is absent.

1.4.2 Scaffolds

Scaffolds in relation to TE are structures composed of materials that provide a porous structure to enable tissue regeneration, either from engraftment of recipient cells or proliferation and expansion of seeded cells. The choice of material for use as a scaffold in the field of biomaterials is limitless but certain properties are desirable including adequate mechanical strength, the absence of immunogenicity, the ability to permit cell engraftment and the absence of toxicity. Scaffolds should be 'tailored' to the function of the tissue in need of replacement, thus approaches to hard tissue engineering (e.g. bone) are more likely to use scaffolds which are mechanically stiffer than those required for soft tissues (e.g. liver). The many desired properties of the ideal scaffold inevitably lead to a necessary compromise in selection of material. For example, structures with increased mechanical strength tend to have less porosity, limiting cell engraftment. When balancing all the requirements in practice, the choice of which scaffold to use for a particular application of TE may not be straightforward.

Scaffolds can be classified broadly into synthetic and non-synthetic. Non-synthetic scaffolds may be derived from a variety of tissues and are collectively referred to as 'biological'. Synthetic scaffolds may be produced by various methods but have the advantage of reproducibility with minimal variation controlled by machine processing and the ability to adjust specific properties such as porosity and tensile strength. The ability to control production conditions in addition, permits particular attention to be paid to other considerations such as sterility and biocompatibility. Synthetic scaffolds were amongst the first used in early TE studies in the 1990's and Langer et al. utilized polyesters such as polylactic acid (PLA), polyglycolic acid (PGA) and associated copolymers (PLGA) for seeding with chondrocytes (40). Such polymers were initially used due to known safety records with synthetic sutures used during surgery. Production methods have inevitably become more refined over the years and more complex structures such as Polyhedral oligomeric silsesquioxane poly(carbonate-urea) urethane (POSS-PCU) have been developed. POSS-PCU is a nanocomposite polymer developed by an extrusion-phase-inversion

method and has been trialled in a variety of TE applications (41). Such applications indicate the potential of synthetic scaffolds and their role in state of the art TE. Synthetic scaffolds do however have disadvantages and are currently not suitable for all TE applications. The trachea for example, has a less complex structure when compared to soft tissue organs such as the intestine and synthetic design of an intestinal scaffold poses a significant challenge. In addition, synthetic materials remain inert, with no cellular signalling mechanisms or bio-active molecules present unless they are implanted into the scaffold. As increasing evidence and understanding regarding cell niches occurs, the importance of interaction with cells and their environment becomes more apparent (42). This importance of the niche has led to the combination of synthetic scaffolds with bio-active molecules or the search for alternative scaffolds where appropriate.

Biological scaffolds are those derived from naturally occurring substances and may be synthetically produced or derived from animal tissue. In both cases, the resultant scaffold is primarily composed of extracellular matrix (ECM) and the term 'matrix' is often used interchangeably for 'scaffold' in TE. Where biological scaffolds are synthetically produced, naturally occurring compounds such as alginates and collagens may be processed to form sponges or gels with an ECM-like structure to serve as a matrix for cellular engraftment and regeneration as required (39). The ECM is largely preserved amongst species and consists primarily of a mixture of components such as collagen and glycosaminoglycans (GAGs) (43). This preservation across species enables the translation of ECM from one species to another with minimal immunological insult. In the absence of cellular translation, this form of transplantation has been labelled 'semi-xenotransplantation' and forms the basis of many TE models (44). Such ECM scaffolds are typically produced by a process of decellularization, which can be defined as the removal of cells from an organ or tissue to leave the remaining structural and functional proteins that comprise the ECM (45). The resultant ECM then serves as a scaffold for cell seeding and translation. Use of deceased donor human tissue for decellularization has been clinically successful in production of a tissue engineered airway (46). Human intestinal tissue has also been

decellularized using similar methods and resultant scaffolds have been shown to retain angiogenic factors (e.g. VEGF, EGF) and it is the retention of such factors that is believed to be a key benefit of biological scaffolds over synthetic due to their role in cell regeneration (47). A range of alternative projects are currently in progress worldwide utilizing principles of decellularization on a range of animal tissues to create scaffolds for TE with varying degrees of success (35).

The process of decellularization utilizes a number of methods typically including chemical treatment, physical agitation and temperature control (45). A combination of methods is usually required to render tissue void of cells, yet retain the ECM architecture as intact as possible. Decellularization methods typically cause some damage to the ECM and a fine balance between the removal of cells and preservation of ECM must be struck. The process may be time consuming and pose significant challenges in maintaining sterility, toxicity and structural integrity but the perceived benefits in cellular regeneration over synthetic scaffolds are deemed a worthy cause. This is best appreciated when considering the structure and function of the ECM in more detail. More than 20 years ago, work was done to demonstrate that ECM proteins and signalling molecules may demonstrate considerable activity when encountering cells in a 'dynamic reciprocity' (48). Since then, considerable work has been undertaken characterising ECM ultrastructure, its components (e.g. proteoglycans) and assessing the relationship between ECM and relevant cells. There are a number of mechanisms at work therefore in the relationship between ECM and seeded cells which may involve cell signalling, scaffold degradation and remodelling and stimulation of proliferation. These aspects of interaction between cells and ECM are the primary benefit of using biological over synthetic scaffolds and will be discussed further in chapter 3.

1.4.3 Cells

Tissue engineering is distinguished from Guided Tissue Regeneration (GTR) by the application of cell based therapy. Scaffolds may be seeded with cells by various methods in-vivo or in-vitro and subjected to a range of conditions to stimulate cell proliferation. Significant challenges arise in; selection of cell type, mode of seeding, provision of appropriate conditions for proliferation and minimization/ prevention of immunological reaction. In the same manner in which progress in clinical transplantation became largely limited by rejection in a short time period, progress in TE remains stalled by challenges to successful cell seeding.

The choice of cell for seeding seems obvious in that direct replacement of a single cell type would permit optimum regeneration. The first issue with such an approach is the scale up of cells in cultures to sufficient quantity. Primary cell cultures of differentiated cells may be difficult to expand in vitro and even small scaffolds such as a decellularized vein have required cell numbers in the region of $7.5 \times 10^4/cm^2$ for successful application (49). Using this as a guide, the number of cells required to seed a large human organ (e.g. liver) would be extremely large. This poses significant challenges even when using cell types that expand with ease (e.g. mesenchymal stem cells).

Choice of cell is further complicated by complexity of tissue in question. Whilst the vein poses a simple structure, intestine by contrast is composed of many cell types including myocytes and endothelial cells in a niche containing specialized stem and signalling cells (50). The question of which cells require seeding within a scaffold and in what proportion remains a major challenge that is applicable to any complex tissue engineered organ (51). Within living tissue, there exists a continuous dynamic relationship between cell types and the concept of the stem cell niche in various body tissues such as intestine, bone marrow and kidney is now well described as an interaction between cells and a microenvironment which provides biomolecular cues to affect cellular behaviour (42, 52, 53). The interaction between cell types remains key to proliferation

and therefore use of stem cells alone may not be successful. A difficult balance may therefore be required between cell types, ratios and role of scaffold.

The source of cells for seeding requires consideration and autologous or allogeneic cells may be used in tissue engineering. Although a key goal of TE is the avoidance of allogeneic tissue and the prevention of need for immunosuppression, a bridge to an 'ultimate' treatment may be necessary and a combination of cell types and ratios may be required as discussed above. In previous clinically successful studies, autologous cells were utilized to treat patients and were harvested pre-operatively from bone marrow aspirates (46, 49). In diseased patients, healthy autologous cells may be scarce and even if present, methods to culture and expand successfully may not (e.g. short bowel syndrome). In such cases allogeneic cells may be necessary in conjunction with appropriate treatment to prevent significant immunological reaction.

Improved knowledge and understanding of stem cells has seen an increase in their application and uses but considerable work remains before they are accepted for widespread clinical use (37, 38). As discussed previously, the prospect of a single progenitor differentiating to all required cell types in a complex tissue type seems unlikely. Options for use of stem cells include embryonic stem cells (ESCs), inducible pluripotent stem cells (iPSCs), umbilical stem cells and adult progenitor stem cells amongst others (35). Each type has advantages and disadvantages and these are explored further in chapter 4. At this introductory stage it is noteworthy that no single cell type, stem or otherwise has been demonstrated to possess all the required properties for TE in a reproducible and successful clinical format.

1.4.4 Approaches

Cells and scaffolds form the 'building blocks' of TE but practical applications of tissue engineering requires an interplay between fields of material science, immunology, cell culture and transplantation (54). Selection of an appropriate scaffold has been discussed but the desired purpose of the scaffold must be defined. Some scaffolds may be primarily seeded in vitro, with

this phase deemed necessary to 'grow' tissue resembling that of native before implantation. By contrast, where scaffolds are intended for early remodelling in vivo, emphasis may be placed on associated important factors such as biocompatibility. Early in vivo regeneration of scaffolds has proved more successful for hard tissues such as bone with in early in vitro used more readily for soft tissues (55, 56). The importance of this is discussed further in Chapter 5.

As discussed previously, the types and quantities of cells used for seeding form an important part of the approach to TE. An ideal TE protocol would involve as few cell types as possible, in minimal required seeding densities, that permit full differentiation into all required types in an appropriate 3D matrix and architecture. This remains one of the most challenging aspects of TE projects as no single progenitor is likely to permit differentiation to all required cell types when gained from an autologous source. Experimental studies in animals have demonstrated success with large numbers of cells, but the translation to clinical practice has often remained elusive. The mode of cell seeding may be varied, with options for direct injection into tissues, layering onto mucosal surfaces or delivery via perfusion methods. Direct injection methods have been demonstrated success in animal models and have the advantage of enabling cells to reach the desired destination immediately (57). Delivery may however be difficult in vivo and the disadvantage of increased density at the injection site together with inadequate distribution globally may be present. With the success of decellularization by perfusion, cell seeding or 'recellularization' has been attempted using established perfusion networks with successful outcomes (58). Perfusion delivery of cells may follow the vasculature or, as in the case of the intestine, be simply delivered via the lumen of a hollow viscus. The appropriateness of this method depends greatly on the tissue in question, cells used and required distribution. This method is more suitable for large scaffolds where required cell numbers are high.

The environment in which TE occurs is crucial to the outcome and success of tissue regeneration. Cells seeded onto scaffolds are fragile, prone to a short life span and highly susceptible to

contamination. The ideal conditions in which to promote proliferation involve control of temperature, pH, oxygen delivery and provision of nutrients amongst other parameters. This complex requirement has led to the cross-over of fields of biomedical and tissue engineering with development of purpose built bioreactors for TE. Conditions beyond those associated traditionally with cell culture can also be manipulated in such systems and studies have demonstrated that factors such as mechanical forces, pressure gradients and electrical stimuli may also promote cellular proliferation (59). Aspects of TE approaches will be discussed further in chapter 5.

1.5 Current Status of Tissue Engineered Intestine

The clinical rationale for the pursuit of Tissue Engineered Intestine (TEI) is well described above but discussion of TEI thus far has focused on patients with untreatable small or large bowel disease. Prior to the establishment of intestinal failure as a clinical entity, studies were focussed on treating specific intestinal pathologies. Early work by Kobold et al. attempted to repair duodenal defects with jejunal serosa in dogs (60). The exciting finding that the serosal patch became coated in duodenal epithelium supported the concept of regeneration and recellularization of the transposed serosa. In 1973, Binnington et al. used similar methodology to patch colonic serosa onto native jejunum and characterized the development of novel intestine development in an animal model (61). Following this early phase, studies in TEI have adopted different approaches to desired end points, choice of scaffold and use of cells for seeding.

1.5.1 Endpoints in Intestinal Regeneration

Early studies by Binnington et al. described above indicated that partial regeneration of the intestine was possible. This is likely in part due to the excellent capacity of intestinal tissue to regenerate and supports a guided tissue regeneration (GTR) approach to intestinal regeneration. The development of mucosal tissue alone however represents a single end point in regeneration and does not serve all the functions of intestine. Demonstration of regenerated mucosa alone

does not necessarily correspond to a functional mucosa capable of absorption. In addition, if a mechanical component of intestine were desired (e.g. pouch substitute) then full thickness tissue would be necessary and a simple absorptive surface alone would be viewed as a failure. Equally if whole organ engineering were sought, absorptive and mechanical functions would form part of a range of desired functions and the ability to sustain a blood supply would be required. Historically, TEI has focused on either the development of an absorptive surface to replace function with a patch graft, the development of full thickness tissue (typically in a tubular structure) with the intention of forming an interposition graft and partial/ whole organ engineering (62-64). The approaches adopted for this have included use of synthetic and biological scaffolds, ex vivo and in vivo regeneration and cell progenitors of various forms including primary culture cells and stem cells.

1.5.2 Intestinal Patch Generation

Binnington et al. further explored the potential of intestinal patch grafting in future work demonstrating the development of jejunal 'neomucosa' when colon was used to repair defects in dog jejunum (65). The resultant mucosa demonstrated a histological architecture similar to that of normal jejunum and thus the term 'neomucosa' was used. This term is used interchangeably with 'neointestine' to describe the development of a new intestinal mucosal surface resembling that of native tissue. Results prompted further work to investigate the functionality and this demonstrated absorptive and digestive capacity of the neointestine (66). This work stimulated interest in the potential of regeneration of synthetic scaffolds and in 1988, Vacanti et al. used a synthetic polymer disc seeded in vitro with primary cultures of intestinal foetal cells before in vivo implantation intra-peritoneally (67). Of the 23 rats implanted with intestinal seeded scaffolds, one developed a cyst which, on histological cross section demonstrated the formation of intestinal mucosa.

Patch type grafts have also been attempted using small intestinal submucosa (SIS). SIS refers to a biological scaffold that is derived from animal small intestine in a process that extracts the submucosa (68). Early work following that of Binnington et al. concluded that full thickness intestine alone did not form a suitable scaffold due to high immunogenicity and through a process of trial and error using different intestinal layers, the extraction and treatment of SIS was refined for effective use. SIS has been used in the experimental treatment and repair of various body tissues including intestine, bladder and vascular grafts. In 2001, Chen et al. reported the first use of porcine SIS in the treatment of intestinal defects in dogs (62). Results proved good with 20 of the 23 dogs surviving with good evidence of repair of defects. Similar results were reported by Demirbilek et al. in 2003 when using porcine SIS to patch repair jejunal defects in rabbits (69). Other biological scaffolds that have been used for patch repair include collagen sponges for treatment of gastric defects (70).

At the time of writing, no synthetic or biological scaffold, seeded or unseeded has successfully reached full clinical application for patch repair treatment of perforated intestinal lesions. The clinical utility of such a treatment would most likely be limited to emergency patch repair of perforations in the upper gastrointestinal tract (e.g. oesophagus and duodenum). Perforations of the distal small and large bowel are typically and successfully treated with resection of appropriate segment rather than simple closure. Perforations high in the upper GI tract carry considerable morbidity and mortality which arises partly from leaks following primary closure and/or omental patch repair. The limitation of use of a patch in treating the more common longer diseased segments of intestine drives the need for alternative TEI approaches.

1.5.3 Tubular Scaffolds and Intestinal Segment Interposition

The requirement for the ability to replace a segment of intestine using TE led to the development of cylindrical scaffold tubes. Direct replacement of absent intestine by interposition of tissue engineered grafts has been attempted by different approaches. The development of tubular

scaffolds for regeneration has been pioneered by Vacanti et al. and has focused on use of PGA synthetic scaffolds seeded with cells and implanted in vivo to promote regeneration, typically in an intra-peritoneal environment such as omentum (71-73). These studies primarily used intestinal organoid units (OU) for scaffold seeding, which consist of a rim of epithelial cells surrounding a mesenchymal core. Original experiments by Evans et al. described OU as cell clusters which could not be maintained in culture for prolonged periods due to a tendency to disassociate and suspensions of such OU have been used extensively for TEI applications (74). The original OU described by Evans et al. have since been superseded with work by various groups including Clevers et al. who have successfully cultured intestinal epithelial '*mini-gut'* structures from intestinal stem cells to produce cysts with a central lumen flanked by highly polarized villous epithelium (75). The importance of OU is discussed further in chapter 4.

In Vacanti et al's early studies, OU were used for seeding tubular scaffolds in small animals before in vivo implantation, followed by explantation after a period of days to reveal development of cyst like structures which contained neointestine on histological cross section (similar to previous studies performed by Vacanti et al. when using disc shaped polymer scaffolds). Such seeded tubular scaffolds were later tested by Kaihara et al. when they were explanted from omentum and anastomosed end to end with small bowel. An anastomotic patency rate of 78% was quoted from the study indicating the potential of these synthetic scaffolds (76). More recently Grikscheit et al. have explored use of similar scaffolds in regeneration of the large intestine with some success (77, 78). The same group produced a landmark study in the field in 2009, as a large animal porcine model was used successfully for the first time with PGA constructs seeded with autologous organoid units before intraperitoneal implantation to generate neointestine (79). Synthetically produced scaffolds have also been repopulated with progenitors in-vitro and Chen et al. describe the development of a 3D porous, protein scaffold system which was seeded by epithelial cells in-vitro in a 3D system (80).

Use of biological scaffolds for interposition and development of full thickness intestine has been less successful. Early use of Small Intestinal Submucosa (SIS) as an interposition graft was a failure when performed as part of a previously described study by Chen et al. The dogs with a SIS interposition graft anastomosed all demonstrated poor survival rates and whilst the SIS used was not seeded, problems were thought to have arisen from mechanical failure as a surgical conduit rather than a failure to regenerate intestine (62). Other uses of SIS in interposition have provided more promising results and in 2003 Wang et al. used rat derived SIS in a tubular structure with a plastic inner stent anastomosed end to end with defunctioned rat small intestine. The authors reported no stenosis or adhesions and the development of neointestinal mucosa and seromuscular layers (81).

The focus on tubular scaffolds is driven by the aim of creating TEI which is structurally similar to that of native tissue. For this purpose, a lumen is necessary and tubular structure essential if the most basic function of sustaining enteric contents without leak is to be maintained. It is noteworthy, that the majority of such synthetic studies demonstrate TEI when tubular scaffolds are implanted in-vivo in omentum and few demonstrate successfully direct interposition and replacement of tissue by comparison. In addition, these studies often do not demonstrate the preservation of a luminal structure post implantation and instead form cystic structures. Similar limitations are noted when comparing biological scaffolds and in the absence of a luminal stent, scaffolds tend to collapse and lose a cylindrical structure. This limitation, in conjunction with the strong reliance on small animals and organoid units suggests that alternative avenues for TEI should be pursued as the clinical utility of such an approach is limited.

1.5.4 Partial and Whole Intestinal Engineering

Success in landmark studies of whole organ engineering have revealed the potential of the approach (35, 46, 57, 58). The benefit of creating a fully engineered organ is obvious as total replacement by transplantation becomes possible. Where desired organs can be separated into

functional segmental components (e.g. intestine, liver, trachea), partial organ engineering may be embarked on and as discussed previously, this has led to clinical success in the case of the airway using a biological scaffold (46). The airway however, has a functionally simpler structure when compared to some soft tissues and synthetic production of a scaffold remains a more viable option than it may for other soft tissues. Decellularization has been described previously in the production of ECM scaffolds for implantation and has the potential to create complex scaffolds for soft tissue such as the intestine.

To date, very few studies have aimed to decellularize intestine in its entirety when compared to the number of studies performed using synthetic and other biological scaffolds such as Small Intestinal Submucosa (SIS). The earliest published work reflecting this approach was by Mertsching et al. in 2005, where porcine small intestine was decellularized using perfusion methods to yield a scaffold capable of cellular engraftment (82). Crucially however, this process stripped the small bowel mucosa mechanically before beginning the decellularization process. This work was further advanced in 2009 by the same group and the scaffold was characterised, seeded with human endothelial cells and implanted into a human for 1 week (83). This particular study stands out amongst others in application of TEI due its large animal model, seeding of cells, use of perfusion decellularization and clinical application. Other studies from our group have focused on perfusion decellularization of the small intestine and successfully characterized an ECM scaffold with potential for perfusion (84). The importance of perfusion as a method of decellularization is that it permits not only an option for transplantation but a route for recellularization and cell seeding either in-vitro or in-vivo which may be analogous to the physiological state (85). In addition, a perfusion network enables adequate delivery of oxygen and nutrients to cells and ECM. Further perfusion decellularization of the whole small intestine has been performed with success in a rat model by Totonelli et al. (64). As alluded to previously, a recent significant study in the decellularization of small bowel has been performed by Patil et al., who successfully decellularized human small intestine and recellularized the ECM scaffold with

human bone marrow stem cells demonstrating definitive cell proliferation and regeneration (47). Whilst the authors acknowledged this method does not provide a route for perfusion of the scaffold, it demonstrates well the application of the principles of TE to regeneration of human small intestine. Further studies have sought to compare decellularized and synthetic scaffolds and Finkbeiner et al. compared a decellularized porcine small intestinal scaffold with a synthetic polymer, both seeded with human stem cells and assessed for their ability to undergo cellular proliferation both in-vitro and in-vivo (86).

1.5.5 Cells in Intestinal Tissue Engineering

Discussion of approaches to TEI thus far have focused on use of scaffolds, modes of tissue engineering and brief discussion of cells used for seeding. Some argue that the selection of cells for seeding in bioengineering is the 'first hurdle' and indeed without appropriate cells source, regeneration is limited to that allowed by Guided Tissue Regeneration (GTR) only (87). The generic challenges of selecting cells for TE apply to the intestine. The first important question to pose, is whether cell seeding is genuinely required for regeneration of intestinal scaffolds. Evidence reviewed thus far clearly indicates that the prospect of whole or partial intestinal organ regeneration in the absence of seeded cells seems extremely unlikely. Detailed discussion of cell progenitors, primary culture methods and technical aspects are discussed in chapter 4, with basic introduction only provided below.

Primary culture cells from intestinal tissue have effectively been harvested as organoid units (OU). These were first described when harvested from rats by Evans et al. in 1992 (74). Since then, protocols have been altered and adjusted a number of times and OU have been utilized successfully in a number of protocols for TEI, typically in conjunction with synthetic scaffolds used by the Vacanti group (71, 76). Culture protocols yield an aggregate of polarized epithelial cells surrounding a core of mesenchymal cells. These large cell clusters are a combination of cells and the precise mechanism by which they aid/ stimulate regeneration is unknown. Studies have

demonstrated however that when the cell clusters disperse, their ability to promote cellular proliferation is reduced both in vitro and in vivo (74, 88). This, together with increased understanding of the intestinal stem cell niche suggests the interaction between the cells within clusters plays an important role in the remodelling process.

The disadvantages associated with primary cultures resides with the source of cells, yield and ability for expansion. As discussed previously, autologous sources of intestinal cells are not present in the patients who need them the most and the amount of tissue required using current methods of extraction and culture with associated yields negates the use of even allogeneic possibilities in humans. To provide perspective, Grikscheit et al. sacrificed 40 neonatal rats and performed complete intestinal primary cell culture to yield enough organoid units to populate just 22cm of intestinal scaffolds at a density of 100,000 OU/scaffold (77). Whilst it has been acknowledged that methods of culture and implantation have improved, this still constitutes a major limitation to an OU approach to TEI (89).

Much work has been done to characterize and more clearly define the intestinal stem cell, its associated niche and demonstrate its activity in vitro. The characterization of intestinal stem cell markers such as Lgr5 has enabled study of the regeneration of intestinal epithelium and the cell signalling pathways important in differentiation (50). Whereas much was previously written regarding the inability to culture intestinal stem cells and OU for prolonged periods, this has now been demonstrated in a landmark proof of principle study with robust data (90). More work is required to demonstrate these techniques can be effectively applied in conjunction with scaffolds to promote regeneration of tissue in a TE format.

The utilization of alternative cell progenitors in TEI arose as a result of identification of mesenchymal stem cells (MSCs) in the process of regeneration of damaged intestinal tissue (91). The many functions of MSCs are becoming better characterized over time, and increasingly they are being used for administration to patients in clinical trials and in tissue engineering projects,

often as an addition to other cell types and progenitors (92-96). Use of autologous human MSCs in a decellularized intestinal scaffold has demonstrated presence of epithelial, endothelial and muscle cells in a recellularized scaffold, proving the efficacy of the method (47). The precise mechanism of action in relation to intestine and other satellite tissues remains unclear but the potential for easy autologous harvest, ability to expand in large numbers and robust properties justify exploration for further use both independently and as an adjunct to other sources for cell seeding.

1.6 Thesis Overview

This research aims to develop a model for tissue engineered intestine in a large animal with future clinical utility in principle and practice. Specific aims involve; (i) producing a large intestinal porcine biological scaffold by perfusion decellularization, (ii) demonstrating appropriate characteristics for TE of the ECM scaffold, (iii) developing protocols for culture of porcine intestinal organoid units and mesenchymal stem cells and (iv) seeding ECM scaffolds with cultured progenitors.

A large animal large intestinal scaffold is sought as this has not previously been documented in the literature and may have a number of potential applications, including; a model for allogeneic use in cadaveric human donors to replace large intestine, a semi-xenogeneic TE graft for future replacement of human large bowel and a mechanism of disease modelling. In addition, it could form the basis for development of an autologous model to treat small intestinal disease in patients who lack small intestine but possess that of large by removal and recellularization. The use of a large animal model allows better translation of methods to the human disease state and the methods adopted for decellularization by preservation of perfusion network permit a means for future revascularization and recellularization. OU have the property of promoting cellular regeneration in intestinal scaffolds; the development of culture protocols in porcine models is a goal of the project to enable demonstration of regeneration of the scaffold. The successful use of

MSCs in recellularizing human intestine drives the aim of developing MSC isolation and culture in a pig model to permit seeding of the scaffold.

The experimental methods, results and discussion of these aims is covered within chapters 2- 5 before general discussion and conclusions. The goal of the project is to test the hypothesis that large animal large intestine can be decellularized using perfusion methods to create an ECM scaffold capable of cellular engraftment and proliferation when seeded with MSCs and OU.

Chapter 2 General Methods and Materials

Standardized and routine experimental methods used are described within this chapter. Where specialized and novel methods were used in experimentation they are described in the relevant chapters.

2.1 Animal Experimentation

All experiments were conducted in accordance with The Animals (Scientific Procedures) Act, 1986. During the course of the study, the act was updated with amendment regulations in 2012 coming into force in January 2013. All regulations were adhered to, including the '3 R's' principles (reduce, refine, replace) and animals from other studies were frequently used for pig experiments. In such cases, only animals which underwent procedures unrelated to the intestine were utilised and where a concern over experimental outcomes in relation to prior procedures was raised, animals were not used.

2.1.1 Rat Experimentation

2.1.1.1 Husbandry

Rats were used in biocompatibility studies detailed in chapter 3. Sprague Dawley rats (*Harlan Laboratories Ltd.,* UK), were sourced commercially. Animals were housed in appropriate conditions with daily attendance and commercially available pelleted feeds used for nutrition. Litter changes were performed twice weekly and temperature, air circulation and light conditions were regulated automatically (12 hour day/ night cycle). Feed and water levels within cages were monitored daily and replenished as required.

2.1.1.2 Anaesthesia and Termination

Scaffold biocompatibility testing detailed in Chapter 3 involved surgery on live rats. For induction of anaesthesia, intramuscular (IM) Hypnorm (0.315mg/ml fentanyl citrate and 10mg/ml fluanisone) was administered followed by 1mg of intraperitoneal (IP) diazepam. This provided

sufficient anaesthesia for the necessary maximum 30 minutes of surgery. Immediately post operatively, subcutaneous (SC) carpofen (8mg/kg) was administered as analgesia. On recovery, rats were assessed daily with husbandry as described above. Termination of rats was achieved by lethal injection of sodium pentobarbitone (25mg/kg) by IP route. Animals were monitored for cessation of respiratory and cardiac activity following administration and additional injection was given where concerns regarding administration/ absorption were present. A 'no touch' period of 5 minutes was applied following termination before tissue was retrieved for analysis.

2.1.2 Pig Experimentation

2.1.2.1 Husbandry

The majority of animal work during the project involved the use of pigs. Crossbred White Landrace pigs were commercially sourced (*Witherick & Sons, Winsley Farm, Bedford,* UK) and housed in appropriate conditions according to the Home Office guidelines. Pigs were fed commercially available pig grow feeds of 1-2kg/ day according to weight. Litter was changed daily and water was supplied on tap using feeding bottles with teats. Pre- operative optimization was ensured by oral nutritional supplementation the night before surgery (*Complan*, *Nutricia Ltd.,* UK), following which no further feeds were given prior to surgery. Fluid balance was maintained peri- operatively by intravenous (IV) supplementation as required and supplied post- operatively ad lib with oral rehydration supplements for electrolyte replenishment (*Lectade, Jurox Pty Ltd.,* AU).

2.1.2.2 Anaesthesia and Termination

Pigs underwent antibiotic and antiparasitic prophylaxis 1 day pre- operatively by IM administration of ampicillin LA (25mg/kg) and SC ivermectin (0.2mg/kg). On the day of operation, initial sedation was achieved by administration of oral diazepam and acetylpromazine (ACP - 10mg/10kg) hidden in an apple. Ketamine (5mg/kg) and xylazine (1mg/kg) were then administered IM. Endotracheal (ET) intubation was achieved by LA xylocaine spray to vocal cords,

followed by direct laryngoscopy and bougie insertion. An ET tube was passed over the bougie using tube sizes ranging from 5.5 – 7mm depending on pig size. Tube position was confirmed by auscultation and tubes connected to isoflurane over nitrous oxide and oxygen. Pig observations were monitored under anaesthetic including; electrocardiogram (ECG), pulse oximetry and temperature. Intravenous access was achieved by cannulation of a visible superficial marginal vein of the ear. Maintenance 0.9% Normal Saline (N/S) was administered IV during anaesthesia at a rate 300ml/ hr (adjusted according to clinical need). The majority of animals used for pig experimentation were terminated but in some cases, bone marrow aspirates were taken (see Chapter 4) and pigs recovered. In such cases, anaesthetic gas administration was ceased once observations were appropriate and pigs extubated. Pigs were observed during the recovery period until conscious.

Termination was achieved where required by IV administration of sodium pentobarbitone (100mg/kg). Following administration, observations were monitored for signs of completed cardiac arrest. A 'no touch' period of 5 minutes was then observed before removing tissue for assessment.

2.2 Surgical Techniques

Surgical techniques were utilised in a number of aspects of the project including; intestinal retrieval, bench preparation of specimens for decellularization, biocompatibility testing and transplantation of vascularised organs. A standard approach to all techniques was used. In cases of live animal surgery (intestinal retrievals, biocompatibility and bone marrow aspiration tests), minimum size incisions were made using standard blades and sharp dissection was favoured in defining anatomy. Where necessary, tissue was cauterized using mono- polar diathermy (coagulation setting at 30- 80W) to minimise blood loss. Larger pedicles and vessels were ligated using silk ligatures. Vascular anastomoses were performed using Prolene sutures and standard vascular surgical techniques. In all cases, tissue handling and blood loss were minimised. In

procedures performed after death, the maximum exposure possible was achieved to improve surgical access and minimize operating time. Tissues were typically cut using dissecting scissors but vessels were ligated where necessary to prevent blood entering the operating field and reducing visibility, or for later identification (see Chapter 3 for further details).

2.3 Tissue Preparation and Storage

2.3.1 Tissue for Decellularization

Tissue retrieved from pigs for decellularization was used as soon as possible when not frozen for storage. This approach was taken to minimise any degradation of extra- cellular matrix (ECM) components occurring during the storage process. Where samples could not be used immediately, intestinal tissue was stored in phosphate buffered saline (PBS) at 4^{o}C for a maximum of 24 hours. The methods of preparing intestinal tissue for decellularization are detailed in Chapter 3 but it should be noted that all bench work of specimens was performed prior to storage. In certain experiments, tissue was frozen at -20^{o}C prior to decellularization and in these cases, the prepared intestinal tissue was wrapped flat in transparent plastic storage bags with clear orientation and then removed for decellularization at an appropriate later date.

2.3.2 Tissue for Histological Processing

Samples for histological stains were fixed in 10% Neutral Buffered Formalin (NBF) (*Genta Medical, UK)* for a minimum of 48 hours before further processing. It was preferred that tissue underwent full cut-up before fixation but in certain experiments this was technically difficult and tissue was partially cut prior to fixation and trimmed to desired size following it and before processing. Machine processing (*Tissue- Tek® VIP 3000, Sakura, Neth.*) was performed according to departmental standard operating procedure (SOP). A copy of the processing protocol can be seen in Appendix 2.1. In all cases, specimens were cut to size and orientated (with biopsy pads where necessary) in processing cassettes (*Thermo Scientific,* UK).

Following automatic tissue processing, specimens were orientated as appropriate and wax embedded using a *Tissue- Tek® III* machine (*Sakura, Neth.*) to create appropriate tissue blocks. Blocks were trimmed at 10µM using a *Thermo- Shandon AS325* microtome (*Thermo Scientific*, *Massachusetts*, USA). Blocks were cooled for 4 hours on ice before sectioning at 5µM onto *SuperFrost®* slides (*Thermo Scientific,* UK).

Slides were dried on a hot plate (*RA Lamb Hotplate, Thermo Scientific, UK*) for 12 hours before preparation for staining. Before staining, slides were de- waxed and rehydrated in paraffin according to standard protocols (Appendix 2.2). Where immunohistochemistry was due to be performed, tissue was fixed, processed and sectioned in the same fashion with the main difference being that tissue blocks were sectioned onto APTS coated slides. The protocol for coating APTS slides can be seen in Appendix 2.3.

2.3.3 Tissue for Molecular Analysis

In a number of experiments, tissue was used for molecular analysis including DNA and collagen quantification. Details of these experiments are given in Chapter 3. In cases of molecular analysis, tissue was used immediately wherever possible, but stored when not feasible. Small samples were favoured for such experiments and tissue pieces, typically less than 1g in weight were cut systematically and inserted into cryogenic vials (*Sarstedt, UK*). These vials were then snap frozen in liquid nitrogen before being stored at -80^{o}C in a cryopreservation tank (*Statebourne Bio 10, Statebourne, UK*). Samples were removed when analysis was performed and defrosted within a few minutes at room temperature.

2.4 Histological Analysis

A number of different histological stains were used. In all cases, tissue fixation and processing took place as previously described. Rationale and use of specific stains is outlined in the relevant chapters. Following staining, all slides were mounted using Distyrene Plasticizer Xylene (DPX) mountant using appropriately sized coverslips (*Leica Biosystems, UK*).

2.4.1 Haematoxylin and Eosin (H&E) Staining

This was used to demonstrate the presence of cells in the process of characterising the biological intestinal scaffold. The protocol for H&E staining was sourced from departmental SOP at NPIMR and can be seen in Appendix 2.4. Universally accepted as a good stain for cells, haematoxylin binds to nuclei demonstrating their presence clearly in deep blue, whilst eosin provides an effective counter stain of the cytoplasm in pink.

2.4.2 Picrosirius Miller's Elastin (PME) Staining

This stain was used to assess the ECM in both scaffolds and control tissue. The protocol can be seen in Appendix. 2.5 and involves a combination of a standard Picrosirius and Miller's elastin (*Leica Biosystems, UK*) stains. Picrosirius staining allows good visualisation of collagen, which stains bright red and green when viewed under polarised light. Collagen is an essential component of the ECM and its demonstration is deemed important in the characterisation of any biological scaffold. Miller's elastin enables clear demonstration of elastin throughout the tissue and notably within blood vessels.

2.4.3 Masson's Trichrome Staining

Masson's Trichrome is a recognised 3 colour stain used in histology used for differentiating cells from connective tissues. It was therefore an ideal stain for use in characterising scaffolds and detecting the desired absence of cells but abundance of ECM. The stain is composed of haematoxylin (responsible for staining cells blue/black) and 3 solutions leading the visualisation of collagen fibres in blue, keratin fibres in red and cytoplasm in pink. A copy of the staining protocol can be seen in Appendix 2.6.

2.4.4 Alcian Blue Staining

The lamina propria of the intestine consists of considerable glycosaminoglycans (GAGs) and Alcian blue (*Alcian Blue 8GX, Surgipath Europe Ltd*., UK) is frequently used in clinical stains of intestinal tissue. GAGs form an important part of the ECM and the stain is able to demonstrate their

presence clearly. The retention of GAGs within the ECM of the decellularized biological scaffold is a desired property and this stain was chosen to provide qualitative data to this effect. A copy of the staining protocol can be seen in Appendix 2.7.

2.5 Immunohistochemistry

Immunohistochemical staining was used to detect the absence or presence of important antigens within the biological scaffold. Details of specific antibodies used and protocols are outlined in Chapter 3. Appropriate tissues were sectioned onto APTS coated slides before drying on a hot-plate as previously detailed. All stains were conducted using *ImmPRESS™ kit* (*Vector Laboratories Ltd.,* UK) and all rinsing of solutions performed using PBS unless otherwise specified. In brief, slides were deparaffinized using Xylene and rehydrated in Industrial Methylated Spirit (IMS). Where necessary, antigen retrieval was then performed before appropriate endogenous block was applied. After an appropriate period, this was rinsed off with PBS followed by addition of non-specific block and primary antibody for an appropriate duration. Slides were then rinsed before addition of *ImmPRESS™ Universal kit*, rinsing with PBS and addition of *ImmPact Diaminobenzidine (DAB) Peroxidase Substrate*. Slides were then rinsed with PBS, washed with distilled water and counterstained. Finally, slides were dehydrated in IMS before cleansing in xylene and mounting.

2.6 Cell Culture

Cell culture techniques were used to isolate and expand different cell types for seeding of scaffolds. Cell types experimented with included; bone marrow derived mesenchymal stromal cells (BM MSC), adipose derived mesenchymal stromal cells (AD MSC), adipocytes, osteoblasts (OB) and organoid units (OU). Specific methods for isolation and culture are referred to in Chapter 4. The following outlines basic common methodology used in all cell culture experiments unless otherwise specified. Details of experimental methods are also applicable to co-culture experiments detailed in Chapter 5.

2.6.1 Culture Hood and Principles

All cultures were conducted in a standard culture hood (*Microflow Class II, Astec Microflow, UK*). The hood was cleaned with 70% ethanol before use and minimal working sterile items placed inside at all times. Serological pipettes were used for transfer (*Stripettor, Costar,* UK) with waste receptacles for fluid placed within the hood and emptied as necessary. Pipette tips (*Sarstedt, UK*) of the smallest possible size were used to promote maximum accuracy when transferring liquids and this was balanced against multiple transfers to minimize contamination. Pipette tips were discarded after single use outside the hood to prevent spread of contamination. Standard principles of cell culture were adhered to, such as preventing pipette tips from touching flask and tube necks and minimising risk of contamination.

2.6.2 Cell Isolation and Culture

Specific primary culture protocols were used to isolate OU, BM MSC, AD MSC, chondrocytes and adipocytes. Details of protocols, culture media and reagents used can be seen in chapter 4. OU were the only cell type not cultured and expanded and details of plating densities for all cell types are provided in chapter 4. Where cells were expanded, use of T75 flasks (*Sarstedt, UK*) was favoured with volumes of 10ml medium to suspend cells unless otherwise specified. Care was taken to minimize contamination and medium was changed every 48 - 72 hours in all cultures unless otherwise specified. Cell flasks and plates were placed in a cell culture incubator (*Jencons Millenium, Jencons, UK*) with independent gas supply providing controlled conditions of CO_2 concentration of 5%. Temperature was regulated at 37.5^{o}C and incubators were maintained according to departmental protocols.

2.6.3 Cell Culture Flask Washing

T75 culture flasks were washed by routine methods as described unless otherwise specified. Flasks were removed from the incubator and viewed under an inverted microscope (*Axiovert 25, Zeiss, Germany)* to assess progress and confirm if washing was necessary. Flasks were then

transferred to a culture hood and medium aspirated and discarded. 3- 5ml warm PBS (*Sigma, UK*) was then pipetted into the flask (taking care to avoid splashing onto flask sides and roof), aspirated within the flask and re-pipetted in a systematic fashion to cover the entire culture surface. The wash fluid was then discarded and the process repeated a second time. Following this, the process was repeated a third time only if debris was still noted within the flask. The flask was then further managed as necessary - either with addition of medium and transfer back into the incubator, or cell dissociation by trypsinization. PBS was the primary wash reagent used for all cell culture experiments. It should be noted that PBS without calcium and magnesium was used when trypsinization was planned as these ions promote adherence of cells to flasks which was to be avoided in the washing process.

2.6.4 Cell Review by Microscopy

All cell lines were reviewed under an inverted microscope every 24- 48 hours. An assessment of cell morphology was made by surveying the flask in a systematic fashion and examining the cell populations. The size, shape and any other notable characteristics of the cell population were noted. Visible communication between cells was noted to assess if populations were dividing appropriately and sufficient cell to cell interactions had occurred. Presence of dead cells was noted by free floating cells within the medium. Confluence was assessed as described below. Finally, the presence of gross contamination or any other significant changes were noted before cells were treated as appropriate.

2.6.5 Cell Dissociation by Trypsinization

Trypsinization was the favoured method of cell dissociation unless otherwise specified. Culture flasks were washed as described above and once wash was removed the second time, the process of trypsinization was commenced. Trypsin EDTA (*Sigma, UK)* was warmed to 37.5^{o}C just prior to use to maintain enzymatic activity and 1.5ml pipetted into the flask. The cap was replaced and the flask gently 'rolled' to permit the liquid to cover the entire flask surface once. The trypsin was

then immediately aspirated and the aspirate pipetted into a 15ml centrifuge tube. A further 1.5ml was added to the flask and the cap replaced. The flask was rolled again to coat the surface and then immediately placed into the cell culture incubator to maintain the temperature.

After 5 minutes, the flask was removed and gently tapped several times in a systematic fashion on the under-surface to release the cells. The flask was then viewed under the inverted microscope to confirm that cells were released before being transferred to the culture hood where >5ml medium containing 10% Fetal Calf Serum (FCS) (*Sigma, UK*) was added to neutralise the activity immediately. After rolling of flasks, flask contents were aspirated into the 15ml centrifuge tube containing the original 1.5ml trypsin EDTA aspirated. The flask was then rinsed with a further 3ml of medium to remove remnant cells and this aspirate was added to the centrifuge tube. The tube was then centrifuged at 1500 g for 5 minutes before being removed and the supernatant removed by pouring off. The remaining pellet was then disrupted before being resuspended. When disrupting pellets, physical agitation by flicking tubes was favoured over pipetting, to reduce trauma to cells. Pellets were agitated until visibly in a liquid phase. The remaining pellet was resuspended in an appropriate volume for the desired action, whether it was freezing, passaging or use in experimentation.

2.6.6 Cell Passaging

Cells were passaged/ subcultured once 70-80% confluent, as higher rates of confluency are associated with genotypic changes in a variety of cell populations. Confluency was assessed by viewing flasks under an inverted microscope and assessing the spread of adherent cells within the visual field. Once 70-80% confluency was confirmed, cells were deemed appropriate for passage (P). Cells were trypsinized as previously described. In primary culture protocols (e.g. BM MSC), the initial plating from crude tissue was labelled as passage 0 (P0). Where cells were passaged following this, passage numbers were increased incrementally by 1 each time as per cell culture conventions (P1, P2 etc.).

When cells were re-plated during passaging, flask contents were typically 'split' and cell pellets resuspended in a volume which was then re-plated in 2 or more flasks. The number of flasks used depended on the number of cells within the pellet i.e. the number released from the original flask and cells were plated at an appropriate density (cells/ml) to permit expansion (see specific protocols in chapter 4). The optimum plating density was established by performing a series of primary cell cultures through passages 1-3 and establishing the minimum cell density on re-plating that was sufficient to permit the cells to reach 70-80% confluence. Once sufficient experience was gained with a cell line (n>5 primary cultures), such that consistent cell counts were noted at 70-80% confluence each time, cell counting was no longer performed following trypsinization and flasks were split at a ratio which was known to have previously been successful (typically 1:2). The exception to this was when the confluence was doubted or the trypsinization process atypical and in these cases, a cell count was performed before splitting flasks to confirm ratios were appropriate.

The method of re-plating was systematic in all cultures. Following trypsinization, cell pellets were disrupted and resuspended in 4ml of appropriate culture medium. Cell counts were then performed as necessary and the suspension volume divided into a number of 15ml centrifuge tubes corresponding to the number of flasks required for passaging. Each tube volume was then made up to 4ml with appropriate medium before being transferred to a new pre-warmed T75 flask. The centrifuge tube was then rinsed twice with 3ml medium which was added to the T75 flask to ensure no cells remained. The entire flask contents were then pipetted gently to mix the cells and medium and the flask placed flat and the liquid allowed to coat the entire surface by gentle rolling. The flask was then appropriately labelled and placed in the cell culture incubator. The process was repeated for each tube such that the desired number of flasks for passaging were plated. Cells were passaged and expanded in numbers until ready for use or storage. Greater passage numbers obviously corresponded to a greater number of cells which was favoured for experimentation if possible. However, evidence to suggest cells undergo genotypic

and phenotypic changes with progressive passaging was considered and an attempt made to avoid using cells beyond P3- P5 wherever possible.

2.6.7 Cell Counting

Cells or OU were counted using a traditional Neubauer Chamber haemocytometer which was a multiple use device requiring washing and re-application of coverslips each time. Later, disposable haemocytometers were used (*C-Chip Neubauer Improved, Cronus tech., USA*). In each device, standard Neubauer Chamber size was present with depth of 100µm and chamber volume of 10µL. Cells were suspended in a noted volume and 10µL of suspension diluted 1:1 with 10µL of trypan blue dye (*Sigma, UK*). 10µL of dyed cell suspension was then loaded into the Neubauer Chamber and cells counted in all large squares (1µL volume) of the chamber. Standard universal protocols were applied and cells bordering left and upper borders were included in count whereas those on the right and lower borders were not. This total cell number was divided by 9 to give the number of cells in the total chamber volume. This number was then multiplied by 2 for the dilution factor of trypan blue and by 10,000 to give the number of cells within 1ml.This product was then multiplied by the initial suspension volume to give the total number of cells within the cell suspension.

2.6.8 Cell Storage

Cells were stored in freezing medium for later use when appropriate. Freezing medium consisted of 10% Dimethyl Sulfoxide (DMSO) (*Sigma, UK*) in FCS, mixed in bulk and stored in multiple aliquots of 5ml at -20^{o} for use when required. Cells were suspended in volumes of < 1.5ml freezing medium at a count of < 1,000,000/ml. Freezing medium cell suspensions were transferred immediately to cryogenic vials and then transferred to a freezing container (*Mr Frosty™, Thermo Scientific, UK*) which was stored at room temperature according to manufacturer's guidelines. Once samples were loaded, the container was placed in a -80^{o}C freezer where it permitted progressive cooling of cells at a rate of -1 ^{o}C/ min. After 24 hours,

cryogenic vials were transferred to a liquid nitrogen storage tank where cells were stored in vapour indefinitely. When removed for use, vials were taken from the tank and the cap loosened initially then re-screwed to prevent fracture on warming. Vials were then swirled in warm water (38 - 40^{o}C) for 2-3 minutes, or until in liquid phase. Vials were dried and then transferred to the cell culture hood for use under normal working conditions. On transfer, vial contents were aspirated using a micro- pipette and transferred to a 15ml centrifuge tube. The vial was then rinsed with appropriate medium to remove any remnants of cells and the liquid transferred to the same tube. After addition of 4ml medium, tubes were centrifuged at appropriate speed to remove DMSO as soon as possible. Supernatants were removed before resuspending cells for further use.

Chapter 3 - Large Intestinal Scaffold Production and Characterisation

3.1 Background

Synthetic scaffolds confer the advantage of large scale production and its control, enabling specific criteria to be applied for development of mechanical properties, sterility and porosity. However, this may result in production of inert scaffolds and machine production may be ineffective in manufacturing the necessary complex structures. Biological scaffolds not only have the benefit of preserving naturally occurring extracellular matrix (ECM) structure and components but critically, may retain biomolecules important in guiding the process of tissue regeneration. A brief review of the subjects of ECM and decellularization is necessary in planning an approach.

3.1.1 Extracellular Matrix (ECM)

ECM refers to the intricate network of macromolecules that occupy the extracellular space between cells. Considerable variability in ECM occurs in different tissues throughout the body and the arrangement of macromolecules is closely related to the function of the specific tissue. This is an important aspect of tissue engineering, as it is postulated that the ECM of a particular tissue forms the ideal conditions in which cells are to thrive; this therefore, justifies the use of biological scaffolds. The 'building blocks' of the ECM are essentially polysaccharide chain type molecules (e.g. glycosaminoglycans) and fibrous proteins such as collagen, elastin and laminin. The proportion of these macromolecules differs in different tissue types.

Collagen is a protein which accounts for the highest proportion of ECM composition. Originally described in a number of 'types' ranging from I-V, now more than 20 types of collagen are described with variations in structure (97). The notable property of collagen is its ability to arrange in fibrillar structures which provide structural and mechanical support to tissues. Fibronectin is a glycoprotein within the ECM that plays a role in assembly of the ECM following its

binding to cell surface receptors (98). Laminins are glycoproteins within the ECM that form a part of basement membranes throughout the body and play a key role in embryonic development - supporting their importance in the regeneration process. The biological effects of laminins arise as a result of their interaction with receptors such as those of the integrin family (e.g. α1β1) (99). Glycosaminoglycans (GAGs) are well known for their role within the ECM and their role in cartilage structure and function is well described. GAGs form part of the structure of proteoglycan chain molecules which display a range of functions within the ECM including; permitting epithelial cell migration, acting as receptors for protease inhibitors and acting as endolytic receptors for bound ligands (100).

Considerable work exists detailing the structure and function of the ECM but discussion of composite macromolecules gives the impression of the ECM as a structure in place that serves a set of discrete functions according to the components. This is however, a misrepresentation and the ECM is in a constant flux state of; interaction with cells, degradation and regeneration. It is best thought of as a functioning aspect of the body in its own right and this helps understand its postulated role in tissue remodelling and TE. The ECM is itself, a product of the resident cell population and is believed to form a template for organ generation and regeneration. It is noteworthy that, whilst cells are orientated in relation to ECM components, evidence also exists to suggest the configuration of ECM and its interaction with cells affects the behaviour of cells in terms of phenotypic differentiation (101). This is supported by evidence indicating the ECM can affect the pathway of differentiating embryonic stem cells (102).

Whilst the ECM may play a role as a template for cells to interact with and form a platform for regeneration, the ECM is not a 'fixed' structure and undergoes degradation and regeneration in relation to surrounding cells. This process may also be important to TE as the degradation of ECM components results in their secretion, after which they may play a role as signalling molecules to promote cellular regeneration and guide tissue growth (103). The technical aspects of such

mechanisms are poorly understood in both physiological circumstances and in relation to implanted scaffolds but the complex nature of the ECM, its multi-functional role and its close interaction with cells supports the pursuit of biological scaffolds for TE.

3.1.2 Decellularization

Decellularization refers to the process of systematic removal of cells from tissues or organs. The resultant ECM can then be used as a biological scaffold for TE, either by seeding or by guided tissue regeneration. The rationale is outlined in chapter 1 but the process of decellularization may vary substantially in approach and is typically guided by the desired function of scaffold sought. Decellularization protocols may utilize chemical or physical methods, frequently in combination, to obtain an ECM scaffold. Methods to remove cells are damaging to tissue structure in general and, thus, the process of cell removal comes at the cost of collateral damage to the ECM. The balance struck between the removal of cells and retention of ECM forms the main challenge in the decellularization process.

Comprehensive reviews of decellularization techniques have been successfully completed (45, 55) and only important aspects will be briefly discussed to frame experimental work. Chemicals frequently used for decellularization include detergents, acids, alkalis and hypo/ hypertonic solutions. Mechanisms of action may vary and include disrupting protein- protein interactions and solubilizing cellular membranes (45). Enzymatic decellularization may utilize trypsin to cleave peptide bonds and therefore remove cells. Physical methods of decellularization may involve freezing or simple mechanical force or agitation to cause direct cell lysis. In addition to desired effects on cells, collateral damage to ECM may occur by mechanisms such as fracture and removal of important molecules such as proteoglycans (104).

The approach to decellularization may be a simple process, for example simple sheet like structures (e.g. skin) may be bathed in reagents which are periodically changed to permit full removal of cells (105). By contrast, complex structures may require an approach involving a

careful combination of chemical and mechanical methods. For example, in a landmark study in a rat heart by Ott et al., decellularization agents were delivered via the aorta in a retrograde perfusion circuit at a specific pressure (57). This approach permitted not only retention of the architecture of important structures in the organ but also a route for recellularization by retaining essential vascular channels. Therefore, the decellularization approach chosen for a specific organ or tissue, is dependent on requirements of the biological scaffold and the characteristics of the tissue and in some cases the goal may be decellularization of a subunit (e.g. liver segment) and not the whole organ.

3.1.3 Assessing Biological Scaffolds

The process of decellularization is well described and has been used in industry for a number of years to create a variety of products including small intestinal submucosa (SIS), Permacol™ and Strattice™. Many of these products are sold commercially for clinical use and have been used successfully. There does therefore, already exist a 'quality control' element to the use of biological scaffolds and many are licensed for use in UK or have FDA approval within the USA. The primary concern in such circumstances is related to scaffold safety for patients and assessment of the risks posed by use. In this respect, the most important risk is immunological. Scaffolds derived from animal tissue should ideally contain only ECM and no cells or molecules which may pose an immunological target. An example of such important molecules can be seen in the α(1-3) galactose (α-Gal) epitope, which is a discaccharide porcine specific molecule responsible for hyper-acute rejection of porcine tissue in humans on xenotransplantation due to naturally occurring human anti-pig antibodies activated in the classical complement pathway (106). The role of α-Gal in TE engineering has been discussed in various studies, with some focussing directly on demonstrating effective removal (107). Demonstration of such desirable scaffold properties is therefore an important consideration in any biological scaffold and particularly those that are porcine in origin. In clinical products therefore, the balance between cellular removal and retention of ECM is likely to be tipped towards aggressive cellular removal at the cost of ECM

integrity. Indeed, the stimulation of a strong immune reaction following implantation of a scaffold would negate a key benefit of TE. Another major risk from scaffold implantation is that of infection transfer. Industrial protocols therefore have rigorous sterilization methods, such as gamma irradiation. Standard safety assessments such as staining for bacteria may also form part of scaffold assessment. Whilst immunological and microbiological concerns may not be problematic in experimental models, failure to address these issues will limit the applicability of any such study.

In experimental models, more preliminary testing of scaffolds may be necessary. Basic assessments of success of decellularization may be tested by staining for cells, quantification of DNA or immunohistochemistry to detect presence of antigens of concern. Assessment of ECM integrity is also required to ascertain that decellularization protocols have retained sufficient macromolecules to permit regeneration of tissue. Standard ECM assessment may include histological and molecular analyses, amongst other methods. Biocompatibility can be assessed by in vivo implantation experiments to check feasibility of animal models from an immunological, microbiological and functional perspective.

The specific requirement of a biological scaffold is important in assessing its suitability. If the desired goal is to permit cell engraftment then this should be specifically assessed. If however, the scaffold was tasked with a primarily mechanical role in guided tissue regeneration then mechanical testing of tensile strength or elastic modulus may play a pivotal role in assessing function. This distinction is important, as the challenges and requirements of a complex large animal scaffold for whole organ regeneration (e.g. intestine) are different to those of a more simple structure (e.g. skin) and decellularization protocols and assessment should reflect this.

3.2 Aims

This experimental work initially aimed to use decellularization by perfusion to create a biological scaffold from porcine large intestine. A secondary aim was to demonstrate that this would result

in the production of an optimal scaffold with minimal immunogenicity and adequate retention of ECM, whilst retaining the capacity for vascular perfusion which could be utilised later for both cell seeding and transplantation. Finally, decellularization of other intestinal segments was attempted to assess the feasibility of a decellularizing a colonic pouch and to evaluate previously published protocols for decellularization of the small intestine.

3.3 Methods

3.3.1 Large Intestinal Scaffold Production

3.3.1.1 Intestinal Retrieval Under Anaesthesia (IRUA)

Pigs were housed as described in chapter 2. Initially, a suitable segment of large intestine for decellularization was identified by exploratory laparotomy after death, using pigs discarded from other studies without prior abdominal procedures (n=2). The porcine colon distal to the spiral portion was identified as suitable given its accessible nature, predictable anatomy and easily identifiable vascular pedicle. Initial retrieval approach was based on previously published work performed within the same institute on the small intestine (84). Pigs (n=11) underwent general anaesthetic as described in chapter 2 and retrieval procedure was initiated by laparotomy. Small intestine and spiral colon were retraced to the left and the appropriate segment of colon identified. Peritoneal covering was incised on the right side of the colon and a plane developed between the inferior vena cava (IVC) and aorta from the level of the aortic bifurcation and proximally 10cm. The origin of the inferior mesenteric artery was noted and the pedicle protected. The colon was then retracted to the right side and a parallel plane developed on the left side of the aorta. The inferior mesenteric vein was identified and slung using a 3.0 silk ligature. The aorta was then dissected posteriorly as far as possible while preserving the posterior lumbar branches. Iliac vessels were identified, completing visualisation of all important structures in the warm (circulation intact) dissection phase. The surgical dissection to this point was performed with careful attention to minimise blood loss.

Pigs were then euthanised (protocol described in chapter 2) whilst the specimen was simultaneously removed. To achieve this, intestine was transected distally at a level 5cm below the aortic bifurcation and approximately 25cm proximal to this. The transection was extended posteriorly along the mesentery to the vascular root. A large crushing clamp was then placed across the aorta parallel to the site of proximal intestinal transection (to prevent excessive blood entering the operating field) and aorta was transected below the clamp and lifted off the posterior abdominal wall whilst dividing the posterior attachments and lumbar branches as far posteriorly as possible. The entire en-bloc specimen was then reflected forwards and inferiorly to demonstrate the aorta bifurcating into the pelvis. Iliac arterial branches were then divided as inferiorly as possible and the entire specimen removed to begin decellularization immediately.

3.3.1.1.1 Bench Preparation of Specimen Following IRUA

Prior to beginning decellularization, considerable bench preparation of the specimen was necessary. The intestinal lumen was washed with warm water (37^{o}C) and the entire specimen rinsed to remove residual faeces. The specimen was transferred to a working tray where the intestinal and vascular lumens were prepared for the decellularization process. The proximal intestinal lumen was cannulated using 10cm silicon tubing of 3.6mm diameter (*Watson- Marlow, UK*) inserted into the lumen (2cm depth) and secured by a 2.0 silk ligature. An identical tube was then inserted into the distal end and secured in the same fashion. This provided 1 'circuit' for the decellularization, referred to as the intestinal circuit.

The vascular circuit required a number of stages of preparation. Initially, a 12G intravenous (IV) cannula was obtained and the introducing needle removed. The cannula was shortened to a length of 3cm and inserted into the proximal aortic trunk and secured using a 2.0 silk ligature. At the distal end of the aortic trunk, the iliac branches were ligated using 2.0 silk sutures, as were any lumbar branches arising from the posterior surface. The inflow aortic cannula was then connected to a standard IV infusion set supplying 1 litre of Normal Saline containing 25,000U

heparin which was permitted to perfuse through by gravity. During this process, the aim was to create an arterio-venous (AV) flow circuit such that inflow from the artery would exit via the main draining vein without leaks throughout the system. Any obvious leaks were identified at this stage and ligated using 3.0 silk ligatures. To assist in this process, dye injection was used. A separate solution of 1:10 betadine in N. Saline was delivered by the IV infusion set via the arterial inflow. Leaking branches were ligated appropriately.

The venous outflow was identifiable from the suture sling placed during the dissection phase. The vessel was distinguished from lymphatics and a 6.0 Prolene suture placed through the wall and tied in a loop to make identification easier during the decellularization process. The entire specimen was then perfused with a further 2 litres of N. Saline with heparin as described above and the decellularization process was commenced. An illustration of the vascular stages of the bench preparation can be seen in Fig. 3.1.

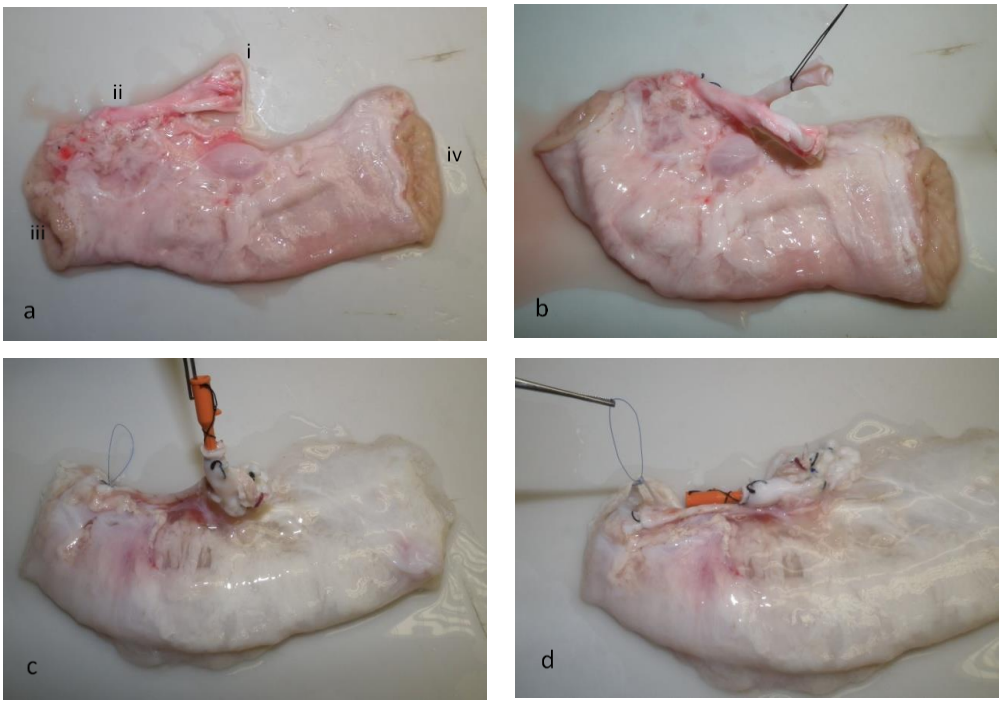

Figure 3-1 Illustration of Bench Preparation of Large Intestine for Decellularization

(a) *Note the appearance of the en- bloc intestinal specimen with external iliac artery branches visible at 'i', proximal aortic trunk at 'ii', proximal intestinal lumen at 'iii' and distal lumen at 'iv'.* ***(b)*** *The iliac vessels are ligated.* ***(c)*** *Cannula positioned into the proximal end of aorta.* ***(c)*** *Draining inferior mesenteric vein marked with a suture.*

3.3.1.2 Intestinal Retrieval After Death (IRAD)

Following a series of retrieval procedures as described above (n=11) use of intestine from pigs after death was explored to increase the pool of specimens for experimentation (n=12). Pigs terminated from other studies were subjected to laparotomy as soon as possible after death. The surgical procedure was modified from that described in 3.3.1.1. A large cruciate incision was made using a post- mortem knife, with the central point at the umbilicus to provide maximal access to the appropriate intestinal segment. The approach to dissection was similar to that previously described but vessels were ligated in-vivo before specimen removal. The entire

specimen was then removed as previously described following transaction of the intestine, aorta superiorly, iliac arteries inferiorly and other attachments.

3.3.1.2.1 Bench Preparation Following IRAD

The specimen required the same preparation to that described in section 3.2.1.2. Specimens were rinsed and the colonic lumen lavaged as described. Minimal vascular preparation was required however as vessels had been ligated in vivo and only the identification suture on the venous outflow required placing. In later experiments, venous outflow was cannulated directly using an IV cannula of smaller bore (10G) prepared in the same way as for arterial and cut to a length of 1.5cm. The cannula was secured using a 4.0 silk ligature. Specimens were perfused with 3 litres of heparinised N. Saline as previously described.

3.3.1.3 Specimen Storage

Two decellularization experiments took place following specimen storage at -20^{o}C. In these cases, bench preparation of specimens was performed as described and vascular cannulas inserted and secured before freezing, as identification at later stages was difficult. Once all appropriate branches were tied, wet specimens were wrapped in plastic bags and placed immediately in -20^{o}C storage. When ready for use, specimens were thawed gently in a warm water bath and intestinal lumen cannulas inserted before beginning decellularization.

3.3.2 Large Intestinal Decellularization

A number of protocols were used to determine the optimum decellularization process. The process consisted of adjusting numerous variables including; (i) types of reagents used, (ii) reagent concentration, (iii) method of delivery and (iv) duration. The method of decellularization deliberately utilised both the vascular and intestinal lumens in order to obtain a fully decellularized specimen with associated vascular pedicle as per the working hypothesis. This involved delivering decellularization reagents via both circuits described above and collecting the discarded effluent from a reservoir for use again. It was anticipated, that after a sufficient

duration, reagents would need to be changed for 'fresh' solutions as biochemical activity would have reduced. Such methodology has been demonstrated in a variety of decellularization protocols as effective in different tissues (57, 64, 108). The method used is schematically illustrated below in Fig. 3.2.

This method was used to form the basis of a decellularization protocol which required adaption as experiments progressed. Previously published protocols used on the small intestine at the same institute were used to trial feasibility of specimen decellularization (n=1) (84). Different reagents were utilised to optimise the protocol and were selected based on mechanism of action. Concentrations of reagents were altered based on outcomes of the decellularization with the intention of ensuring adequate cell removal but preserving ECM as intact as possible. After a series of experiments, (n=4) it was noted that decellularization was markedly affected by the duration of use of reagents and the process was optimised such that reagents were used for the minimum duration. In addition, it was noted that cellular removal frequently left residual nuclear debris within the scaffold, which could be removed by washing of the specimen using phosphate buffered saline (PBS). The duration and frequency of these washes was also optimised to establish a protocol. Finally, the volume of reagent used was varied, as although differing concentrations of reagents could be administered in the same volume of liquid, the quantity of liquid required to sufficiently perfuse the entire specimen and ensure complete immersion in perfusion reagents was not known at the beginning of the experimental process.

By adjusting the variables described above, decellularization was undertaken in a step- wise approach by fixing variables and altering as few as possible in order that any changes in outcome could have an identifiable cause. Initial characterisation of scaffolds was performed during these step-wise experiments and this process is described below. The data acquired from this was used to adjust and optimise the decellularization process as described above.

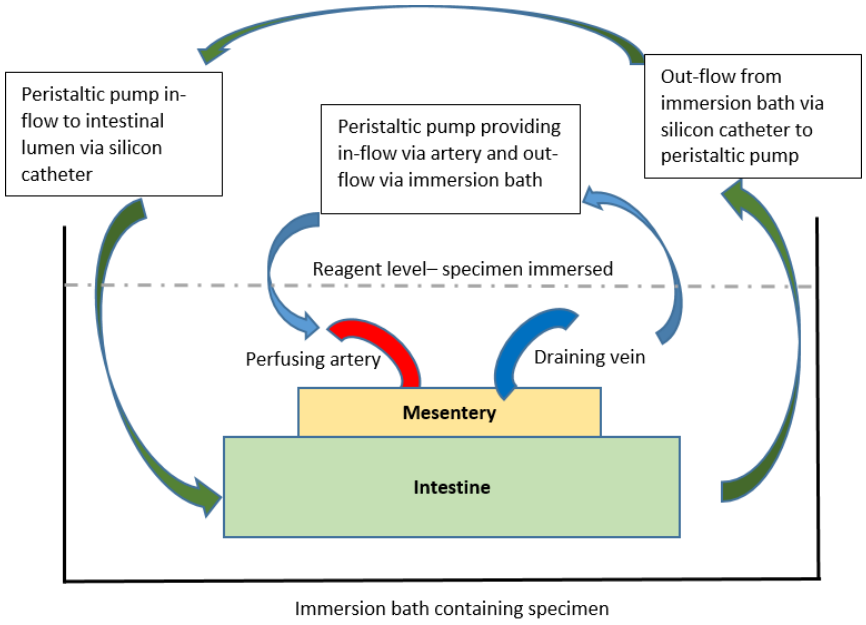

Figure 3-2 - Schematic Illustration of Decellularization Perfusion Circuit

Note double perfusion circuit illustrating intestinal (green) and vascular (blue) circuits. Pump tubing connected directly to cannulas into the intestine and aorta but venous and intestinal outflow cannulas drained into the immersion bath where reagents were suctioned back into pumps before redistribution to tissues.

3.3.3 Scaffold Characterisation

3.3.3.1 Division for Analysis

For the purposes of analysis, the large intestinal specimen harvested for decellularization was divided into 5 x 4cm segments, numbered 1- 5 from the proximal to distal intestinal lumen. This allowed any differences in the level of decellularization throughout the specimen to be identified. It also permitted a more accurate delineation and consistency of sampling as the majority of characterisation tests require very little tissue for analysis and a consistent approach is therefore necessary to ensure validity of results. In all cases, samples were compared to controls sited from the same anatomical location in pigs.

3.3.3.2 Preliminary Characterisation

In determining the optimum protocol for decellularization, multiple variables were experimented with as described above. The effect of altering these was assessed both during and following experiments, to determine if cells were being adequately removed from tissue and ECM was retained appropriately. A panel of 2 tests was used initially as described below. Once suitable decellularization had been noted in preliminary characterisation tests, definitive characterisation was undertaken as described later.

3.3.3.2.1 Haematoxylin and Eosin (H&E) Staining

Samples from segments were excised from the specimen before fixation and staining as described in Chapter 2. The use of H&E staining enabled assessment of efficacy of the decellularization process by assessing for absence of stained nuclei. Decellularized tissue was sampled in segments 1-5 and this approach also determined if tissue was preferentially decellularized based on anatomy of perfusion. Vascular segments could not be assessed in the same fashion during decellularization as this would have breached the perfusion circuit and therefore could only be assessed on completion.

3.3.3.2.2 Picrosirius Miller's Elastin (PME) Staining

PME staining was used to assess the ECM using the approach to sampling described above. PME staining demonstrated the presence of collagen (staining brightly red) and elastin (staining deep blue), the former considered to be an important component of the ECM within biological scaffolds.

3.3.3.3 Definitive Characterisation

Following the full process of decellularization, definitive characterisation of the specimen was undertaken. This allowed a more precise and detailed analysis of specific aspects of the scaffold which were deemed important in later determining what factors were responsible for success or failure in future cell seeding experiments.

3.3.3.3.1 Analysis of Cellularity

Absence of intact cells is considered a prerequisite for decellularization and this was assessed histologically as described in 'initial characterisation' by H&E staining. Assessment of intact cells alone was not deemed sufficient as evidence suggests residual DNA can contribute significantly to the inflammatory response on implantation of scaffolds (109). As a result, DNA analysis was also undertaken as described below.

3.3.3.3.1.1 DNA Extraction

Following decellularization, segment samples were snap frozen in liquid nitrogen and preserved at -80^{o}C for future analysis to prevent degradation of DNA. DNA was extracted using a commercially available kit (*GenElute™ Mammalian Genomic DNA Miniprep Kit, Sigma Aldrich, UK*) according to the manufacturer's guidelines which are summarised in Appendix 3.1. In brief, 25mg samples underwent; mechanical disruption, dissolution in lysis buffer and extraction in buffer before precipitation in ethanol. Samples were either processed immediately or frozen at -20^{o}C for a maximum of 5 days if immediate processing was not possible.

3.3.3.3.1.2 DNA Quantification

DNA was quantified by spectrophotometry, initially using a standard spectrophotometer (*Helios Alpha Unicam, Thermo Electron Corp., UK*). Cuvettes were loaded with 50µL of DNA extractant and wavelengths measured at 230, 260 and 280nm and ratios were used to determine the purity of samples. In later experiments, spectrophotometry was performed using *NanoDrop ND1000* (*Thermo Scientific, UK*) to reduce the required quantity of samples for analysis.

3.3.3.3.1.3 DNA Gel Electrophoresis

Assessment of quality of residual DNA within scaffolds was performed by gel electrophoresis. A number of different protocols were attempted using different percentage concentration agarose gels (0.8 - 1.5%) and different commercially available marking ladders. In addition, the voltage

was varied to optimise the protocol between the ranges 50-100 millivolts (mV). A series of experiments (n=6) were performed before the optimum protocol was devised. The optimum gel was found to be at 1% agarose concentration in Tris-Borate-EDTA (TBE) buffer (*Sigma- Aldrich, UK*) run at 70mV. The optimum ladder marker used displayed a range from 100 – 10,000 base pairs (*Q- Step 4, Yorkshire Biosciences, UK*), with use of bromophenol blue loading buffer (*Sigma-Aldrich, UK*). This protocol was run using a medium sized gel tank (*10x 7cm Flowgen Bioscience Ltd., UK*). Gels were removed and reviewed using an ultraviolet light box with images taken using a standard digital camera.

3.3.3.3.2 Analysis of Extracellular Matrix

Initial characterisation enabled some qualitative assessment of ECM by PME staining but further analysis was undertaken as described below.

3.3.3.3.2.1 Analysis of Collagen in Extracellular Matrix

3.3.3.3.2.1.1 Masson's Trichrome

PME staining was performed to assess collagen as previously described but Masson's Trichrome staining provided an alternative qualitative assessment with good visualisation of other connective tissue structures. Samples were prepared and sectioned as previously described and routine staining protocols were used as described in Chapter 2.

3.3.3.3.2.1.2 Collagen Extraction and Quantification

Collagen was quantified using a commercially available kit (*Sircol Assay, Biocolor, UK*) and extracted according to manufacturer's protocols. A summary of this protocol can be seen in Appendix 3.2. In brief, 50mg samples were weighed before acid pepsin digestion overnight. The following day, the reaction was neutralised with supplied reagents and alternative reagents were used to isolate and concentrate collagen within specimens for a further 24-hour period. Collagen was then released into solution which was aspirated and treated before samples were

centrifuged to pellet extract collagen and supernatants were discarded. Collagen present in tubes was then bound to a supplied dye reagent and quantified by measuring optical density (OD) using a standard microplate reader at wavelength of 555nm (*Versamax Tunable Microplate Reader, Molecular Devices, UK*).

3.3.3.3.2.2 Analysis of Glycosaminoglycans (GAGs) in Extracellular Matrix

3.3.3.3.2.2.1 Alcian Blue Staining

GAGs form an important part of the ECM, particularly in the intestine where they are an integral part of the lamina propria. Demonstration of GAGs was therefore important and GAGs were initially assessed qualitatively using Alcian Blue staining using routine protocols described in Chapter 2.

3.3.3.3.2.2.2 Glycosaminoglycan Extraction and Quantification

A commercially available kit (*Blyscan, Biocolor, UK*) was used to quantify GAGs (see Appendix 3.3). In brief, 50mg samples were digested in a papain extraction buffer, before dilution and addition of specific dye with the affinity of binding to GAGs. Samples were centrifuged to remove unbound dye and bound dye was eventually released to represent GAG quantification. Samples were assessed by measurement of OD at 656nm using a microplate reader.

3.3.3.3.2.3 Transmission Electron Microscopy (TEM)

Ultrastructure of scaffolds was assessed by TEM. Scaffold and control samples were initially fixed in 3% glutaraldehyde in 0.1M sodium phosphate buffer (pH7.4) at room temperature. Subsequent fixation for 1 hour was performed using 1 % osmium tetroxide in PBS (pH7.4). Samples were then washed and block stained in 2% uranyl acetate (*Agar Scientific, UK*) for 2–4h. Following this, samples were washed with distilled water and gradually dehydrated using an acetone to araldite resin (*Agar Scientific, UK*) gradient (1:1 ratio). The ultra-thin sections (100nm thick; using a *Reichert–Jung Ultracut E microtome*) were collected on 200 mesh copper grids, stained with

Reynold's Lead citrate and carbon coated. Specimens were viewed using a *Jeol JEM-1200 EX* electron microscope (*Tokyo, Japan*).

3.3.3.3.3 Analysis of Immunogenicity

Assessing the immunogenicity of the biological scaffold was a key aspect of the characterisation process. The ideal biological scaffold confers no immunological risk on implantation and the potential risk was assessed by two principal methods described below.

3.3.3.3.3.1 Major Histocompatibility Complex (MHC) Class II Immunohistochemical Staining

MHC II staining was performed using standard techniques described in Chapter 2. No existing protocol for paraffin embedded sections was available and a number of protocols were attempted utilising different primary antibody dilutions, incubation times and methods of antigen retrieval. The finalised full staining protocol can be seen in Appendix 3.4. In brief, antigen retrieval was necessary using Citrate Buffer (10mM) at 95^{o}C for 15 minutes and staining was performed using *ImmPRESS™ kit* (*Vector Labs, UK*). Following endogenous and non- specific blocks, primary antibody using mouse anti- pig MHC II (*VMRD, UK*) in 1:100 dilution was incubated with test slides at room temperature for 2 hours. ImmPRESS kit™ was then added according to the manufacturers guidelines. Slides were counterstained with Harris' haematoxylin.

3.3.3.3.4 Analysis of Biocompatibility

Biocompatibility testing was undertaken to determine the effect of the biological scaffold on the host following implantation. A xenogeneic model was developed, involving implantation of scaffolds into rats. This small animal model enabled a greater number of implants and animals to be used and therefore more data collected. Segments of decellularized scaffolds previously described were cut into 1cm^2 sheets and stored in PBS (supplemented with 1% penicillin-streptomycin antibiotic) until ready for use. In addition, aortic rings from the scaffolds were also used for testing. Just prior to implantation, samples were sterilised by removal from PBS and

immersion in sterile peracetic acid (1%) for 2 hours. Following this, samples were washed in sterile PBS within a culture hood before being placed on a mechanical agitator at 100rpm for a further 1 hour. Samples were finally removed and rinsed 3 times with sterile PBS before implantation.

Rats (n=10) were divided randomly into 2 groups; 2 and 4 weeks. 1 rat from each group was randomly allocated to have vascular tissue implanted, with the remainder being used for intestinal tissue. The abdominal wall was schematically divided into 4 quadrants and each rat was allocated 4 tissue samples to be implanted and the location was allocated by number generation. In each animal, 2 control (non decellularized intestine/ aorta) and 2 test (decellularized tissue) samples were implanted.

Rats were anaesthetised (refer to Chapter 2 for detailed protocols) and abdomen sterile prepared and draped. A 5cm midline abdominal skin incision was made and the subcutaneous (SC) plane developed bilaterally. Sufficient room was created to implant the 4 tissue specimens in this plane in 4 quadrants as described. Samples were inserted and secured to the abdominal wall muscle using a 3.0 Prolene suture for marking purposes. The skin was then closed using a subcutaneous absorbable Monocryl 3.0 suture and the animal recovered. All surgery was performed on the same day. Rats were assessed daily post operatively and administered SC analgesia on day 0 and day 1. Standard husbandry was otherwise performed in the post- operative period as described in Chapter 2.

Animals were terminated at 2 and 4 weeks by lethal injection (see chapter 2). On termination, the midline subcutaneous incision was opened and the skin reflected to permit full view of the samples. Images were taken and a $2cm^2$ area of the abdominal wall musculature on which the implants rested was removed. These tissue samples were then fixed for histological processing. Samples were stained for routine H&E staining to determine the cellular infiltrate and extent of scaffold degradation.

3.3.3.3.5 Analysis of Scaffold Perfusion Properties

An overarching principle of this project involved the retention of vascular arcades during decellularization. This enabled the potential for future vascularised recellularization/ implantation of the biological scaffold. Characterisation of the perfusion properties of the scaffold was therefore essential and was achieved by a number of methods described below.

3.3.3.3.5.1 Analysis of Intestinal Luminal Patency

Intestinal luminal patency of the scaffold post decellularization was a pre- requisite but assessment by the naked eye remained difficult due to the appearance of the specimen. To assess patency, existing cannulas in then lumen were used and distal cannula was occluded using a surgical clamp and the proximal end infused with Betadine in N. Saline solution (1:10). This dye was readily visible within the intestinal lumen and leaks could be easily identified. The procedure was performed until the intestinal lumen was distended with dye such that all surfaces were adequately assessed for leaks.

3.3.3.3.5.2 Dye Injection Analysis of Vascular Perfusion Network

The vascular component of the scaffold was injected with Betadine in N.Saline (1:10) solution by hand and the scaffold observed. This demonstrated any gross leaks in the arterial vascular network. Following this, the perfusion pattern throughout the entire specimen was viewed with the aim of demonstrating an arterio-venous (AV) flow throughout the scaffold. Draining contrast exited the system via the venous outflow or through any leaks that were present. An identical dye injection study was performed using a Trypan Blue solution diluted 1:10 with distilled water to provide alternative images.

3.3.3.3.5.3 Computed Tomography Angiogram (CTA) Analysis of Scaffold Perfusion

A more precise imaging modality to demonstrate perfusion properties of microvasculature of the scaffold was sought. Potential options included labelling of perfusion liquids (e.g. radio-labelled

cell suspensions), laser Doppler and photoacoustic imaging. CT angiography was favoured instead to enable a clinical type correlation and a protocol was developed for scaffold imaging. Vascular cannulas were connected to 3 way taps with connecting IV tubing to permit intra-arterial contrast injection. The specimen was placed in a plastic radio-lucent dish and the distal intestinal luminal cannula was clamped and the lumen filled from the proximal end with 1 litre of water to distend the specimen and improve visualisation. 10mls of *Visipaque 270* (*GE Healthcare, USA*) contrast diluted 1:10 in distilled water volume was then injected intra-arterially and images taken using a *Philips INGENUITY (128-slice)* scanner at 80kV and 20mA (pitch of 1), equating to 20mAs with a Field of View (FoV) of 441mm.

3.3.3.3.5.4 Analysis of Haematogenous Perfusion

The decellularization process sought to create a biological scaffold capable of perfusion for the purposes of cell seeding or in vivo transplantation. In vivo transplantation properties were therefore directly assessed. A scaffold prepared by routine decellularization protocols was sterilised in 1% peracetic acid by immersion for 2 hours just prior to transplantation. Following sterilisation, the specimen was rinsed with sterile PBS and left immersed in it. Immediately prior to implantation, the specimen was loaded with an anti- thrombolytic solution of heparin dissolved in N. Saline (25,000 U in 1L) via the arterial inflow and the venous exit occluded.

An adult pig was placed under general anaesthetic by standard protocols. A midline laparotomy incision was made and small intestine retracted to the left side of the abdomen. The right renal artery and vein were dissected out, exposed and mobilised for maximum length and then slung using silk ligatures. Renal artery and vein calibre were compared with the scaffold and the scaffold vessels were prepared for transplantation. The right kidney was then ligated at the renal pelvis and the scaffold transferred to the operating field. Vascular clamps were placed on the renal artery and vein which were then anastomosed to scaffold artery and vein using interrupted, 6.0 prolene sutures. On completion of anastomoses, IV heparin (5000 U) was administered

before clamps were removed and reperfusion assessed. Following a period of reperfusion and macroscopic assessment, the scaffold was removed with vascular anastomoses and fixed in 1% NBF.

3.3.4 Small Intestinal Scaffold Production

Existing departmental protocols for small intestinal scaffold production were trialled to; assess reproducibility of protocols and create scaffolds for later co-culture experiments. Small intestinal scaffolds served as an alternative test group to compare to the principal large intestine sample group. Scaffold retrieval and production methods are described within the literature and were adhered to with some minor modifications in the surgical retrieval procedure (84). In brief, small bowel mesentery was examined at laparotomy and root vessels traced by eye to identify a good calibre feeding artery and vein to a distributing vascular arcade within the ileal region. These principal vessels were dissected free and slung before the corresponding perfused segment of intestine was identified and mesenteric lymph nodes ligated for a 30cm length. The principal feeding artery was then ligated proximally, cannulated and perfused with heparinised saline (25,000U/L) and perfusion distribution noted. The blanched perfused segment of intestine was then resected using standard small bowel resection techniques to minimise bleeding and retain vascular arcades intact for decellularization.

3.3.4.1 Small Intestinal Decellularization

The intestinal specimen was decellularized using published methods and techniques previously described. A summary of the protocol can be seen in appendix 3.5. Notable differences from the large intestinal model was the direct drainage of the venous outflow and intestinal luminal outflow into tubing and back into the pump without drainage into the immersion bath. Once decellularization was complete, specimens were stored as previously described for later experimental use.

3.3.5 Production of a Large Intestinal Decellularized Pouch

The feasibility of production of a large intestinal decellularized pouch was assessed. Large intestine was retrieved and bench prepared as described above. The distal lumen was then occluded by continuous Prolene suture. The distal end of the specimen was incised laterally to the anti- mesenteric border for an approximate 8cm distance. The specimen was then folded superiorly at the mid-point of the incision and the lumens joined by a 'side to side' anastomosis technique. Finally, the blind stump at the base of the specimen was incised open to fashion the pouch. The specimen was then decellularized according to protocols described above for the large intestine.

3.4 Results

3.4.1 Large Intestinal Specimen Retrieval

Retrieval of the large intestine was performed successfully both under anaesthetic (n=12) and after death (n= 12). 'Success' of the surgical procedure was judged by the ability to complete the retrieval and proceed with decellularization. Surgical injuries did occur, for example cutting of lumbar arteries flush with aorta and thus creating a defect in the perfusion circuit. Serosal tears were the most common injury but had little effect on the decellularization process. The majority of injuries could usually be overcome by suturing defects. Significant injury to the inferior mesenteric artery precluded completion of the decellularization and this was documented during retrieval under anaesthetic (n=1). The transfer to the intestinal retrieval after death (IRAD) model was attempted to increase the pool of animals for experimentation and was successful, with no significant injuries during retrieval following introduction of IRAD.

3.4.2 Development of Decellularization Protocol

Decellularization of the large intestine was successfully achieved but multiple changes to protocol were necessary in optimisation. In the initial stages, preliminary characterisation panel of testing

(H&E and PME staining) was not undertaken during decellularization and only performed once the entire process was complete. All specimens first underwent histological staining before proceeding to other tests and full characterisation as described above was not performed for all specimens. Methods described above relay the full panel of characterisation which developed over the course of experiments and as knowledge of the decellularization process progressed. The results are provided below in a series of phases which led to establishment of the final protocol.

3.4.2.1 Decellularization Phase I – Determining Important Variables

Multiple decellularization experiments of intestine took place (n=15), initially with protocols based on previously published work from the small intestine. During this period, macroscopic assessment of scaffolds was combined with H&E assessment to assess success of protocols. The results of these experiments can be viewed in appendix 3.6 and a summary is provided below. An initial analogue roller pump was used for experimentation but the inability to control rotation speeds accurately meant it was later substituted for the device described above. An initial volume of 2L of reagents was used to pump through specimens but volumes were noted to be excessive for the size of the specimens and not practical to work with, so the volume was reduced to 1 litre. Pump speeds were initially set at 60 rpm but later reduced to 30 rpm as higher speeds proved traumatic to the ECM and caused obvious macroscopic damage following decellularization, such as rupture of small vessels. Longer durations of washes between reagents was initially trialled but this was later altered as considerable cellular debris was noted on H&E staining. Various other methods were trialled including decellularizing via the vasculature only. The results of phase I studies identified the important criteria to fix certain variables before proceeding with further decellularization experiments.

3.4.2.2 Decellularization Phase II – Establishing Decellularization Cycles

Following Phase I experiments, it became apparent that macroscopic assessments of scaffolds was unreliable and more objective methods of assessment were sought. Early poor perfusion quality was thought to be secondary to residual thrombi and anticoagulation protocol was increased to that described in 3.3.1.1.1 during the bench preparation stage. As 'decellularized' specimens were found to be inadequate and predominantly demonstrated minimal ECM damage with less aggressive protocols, washes were changed from PBS to distilled water. This continued to enable rigorous washing to remove cellular debris but in addition, provided an additional gentle decellularization effect due to the increased osmotic activity of water when compared to PBS. Sodium dodecylsulphate (SDS) was found to be effective in promoting cellular removal but was noted to cause occlusion of small vessels and therefore concentration was reduced from 0.075% to 0.05%. Reagent concentration, volume, frequency of washes and rpm of the pump were then fixed and a 'cycle' developed which can be seen in table 3.1. Specimens were subjected to cycles before analysis which included macroscopic imaging, preliminary characterisation panel histology and dye perfusion studies. In total, n=8 further decellularization experiments were performed to establish an optimum protocol of 3 cycles of decellularization.

Table 3-1– Single Decellularization Cycle

Note that prior to first decellularization cycle 'step 2' was performed. All reagent volumes were 1litre.

SINGLE DECELLULARIZATION CYCLE		
STEP	**AGENT**	**TIME**
1	**Trypsin (0.05%) and EDTA (0.05%)**	30 mins
2	Distilled Water Wash x 3	15 mins each
3	**DNAse I**	30 mins
4	Distilled Water Wash x 3	15 mins each
5	**SDS (0.05%)**	30 mins
6	Distilled Water Wash x 3	15 mins each

Following each cycle of decellularization, cells were progressively removed from the intestinal tissue as anticipated (Fig. 3.3). Following 3 cycles, cells appeared to be absent indicating complete decellularization. ECM was simultaneously analysed and a progressive reduction in ECM was noted as decellularization progressed. Following 3 cycles however, significant architecture of ECM remained in place as illustrated in Fig. 3.4. Macroscopically, scaffolds began to take on a translucent, gelatinous like appearance after a single cycle of decellularization which progressed (Fig. 3.5). Perfusion dye injection studies indicated small vessel perfusion remained within scaffolds, with no evidence of small leaks (Fig. 3.6).

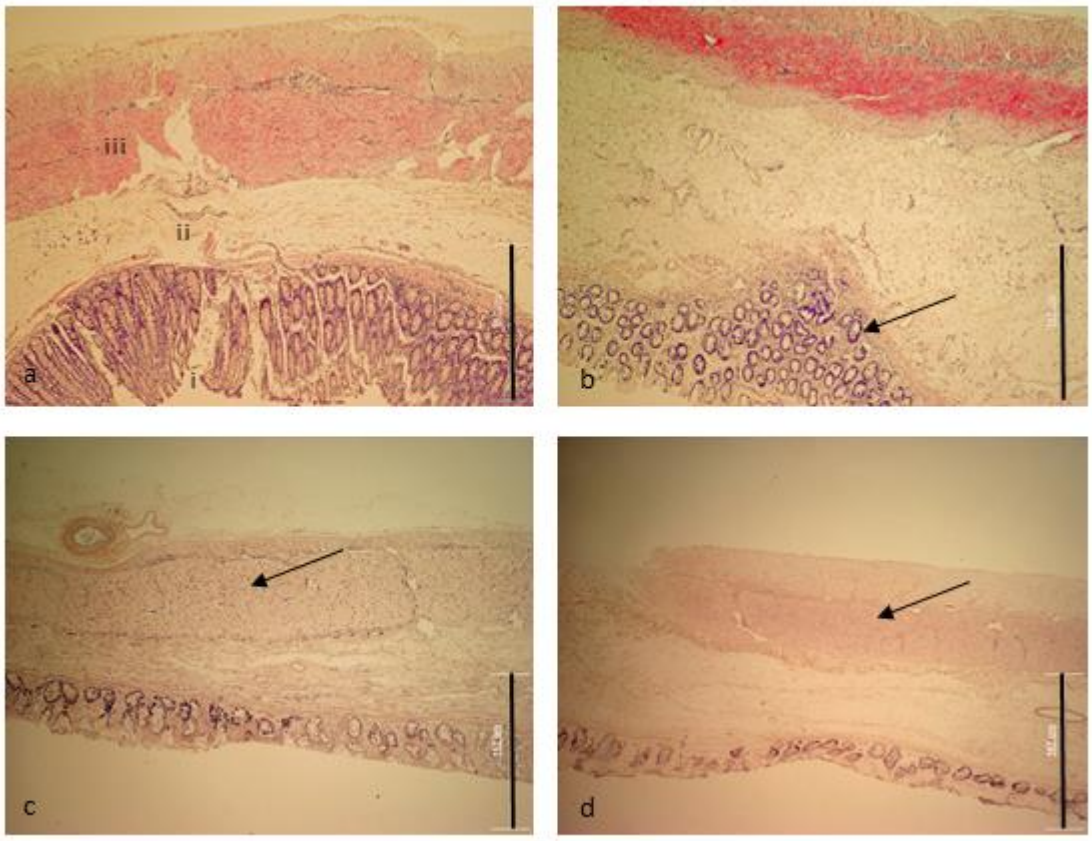

Figure 3-3 – H&E Staining of Intestinal Scaffolds Following Decellularization Cycles
(a) *Control intestinal tissue indicating layers of colon; (i) mucosa, (ii) submucosa and (iii) muscularis mucosa.* ***(b)*** *Intestine following 1 cycle of decellularization, note general reduction in cells and particularly in mucosa denoted by arrow.* ***(c)*** *Following 2 cycles of decellularization, cells still remain present in muscularis layer indicated by nuclei in region denoted by arrow.* ***(d)*** *Following 3 cycles, cells are no longer present in layers with the absence of nuclei in region of arrow. (All scale bars correspond to 167µm)*

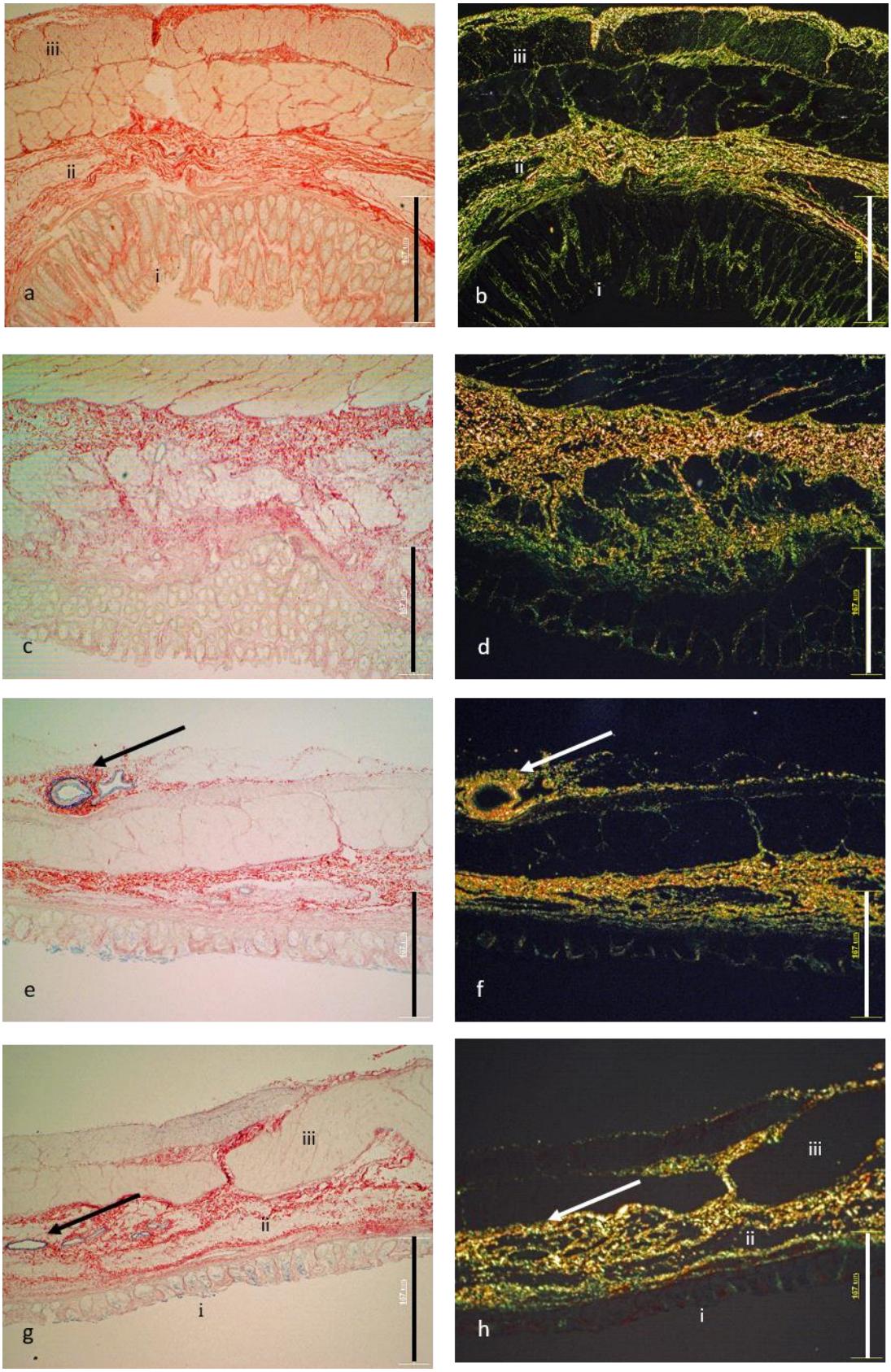

Figure 3-4 - PME Staining of Intestinal Scaffolds Following Decellularization Cycles

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*…….**(a, b)** Control intestinal tissue indicating layers of colon; (i) mucosa, (ii) submucosa and (iii) muscularis mucosa. (b) Illustrates same image viewed under polarised light to assess collaged integrity. **(c, d)** Intestine following 1 cycle of decellularization, note the initial loss of ECM architecture. **(e, f)** Following 2 cycles of decellularization, the layers of ECM are intact and a vessel indicated by the arrow remains clearly visible. **(g, h)** Following 3 cycles, all layers of the ECM corresponding to those in (a, b) are still identifiable and another vessel indicated by the arrow remains intact with internal elastic lamella staining blue from the Miller's aspect of PME staining. (All scale bars correspond to 167µm)*

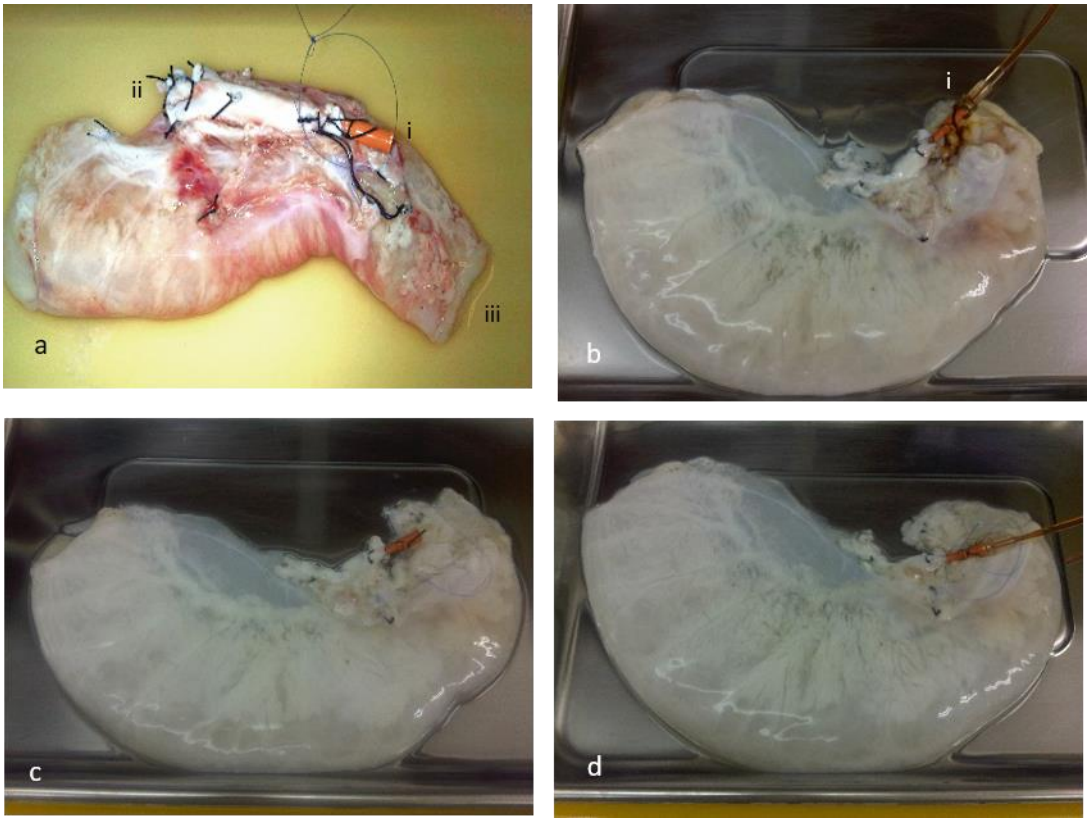

Figure 3-5 – Appearance of Intestinal Scaffold Following Decellularization Cycles
(a) *Porcine colon before decellularization process is commenced, note cannula in aorta at 'i', tied iliac branches at bifurcation of aorta at 'ii' and proximal intestinal lumen at 'iii'.* ***(b)*** *Following 1 cycle of decellularization, marked change of the scaffold is noted as it takes on a translucent appearance, orientation remains the same as for (a) with cannula in aorta at 'i'.* ***(c)*** *Scaffold following 2 cycles of decellularization.* ***(c)*** *Following 3 cycles of decellularization the scaffold remains similar in appearance with minimal difference to the naked eye.*

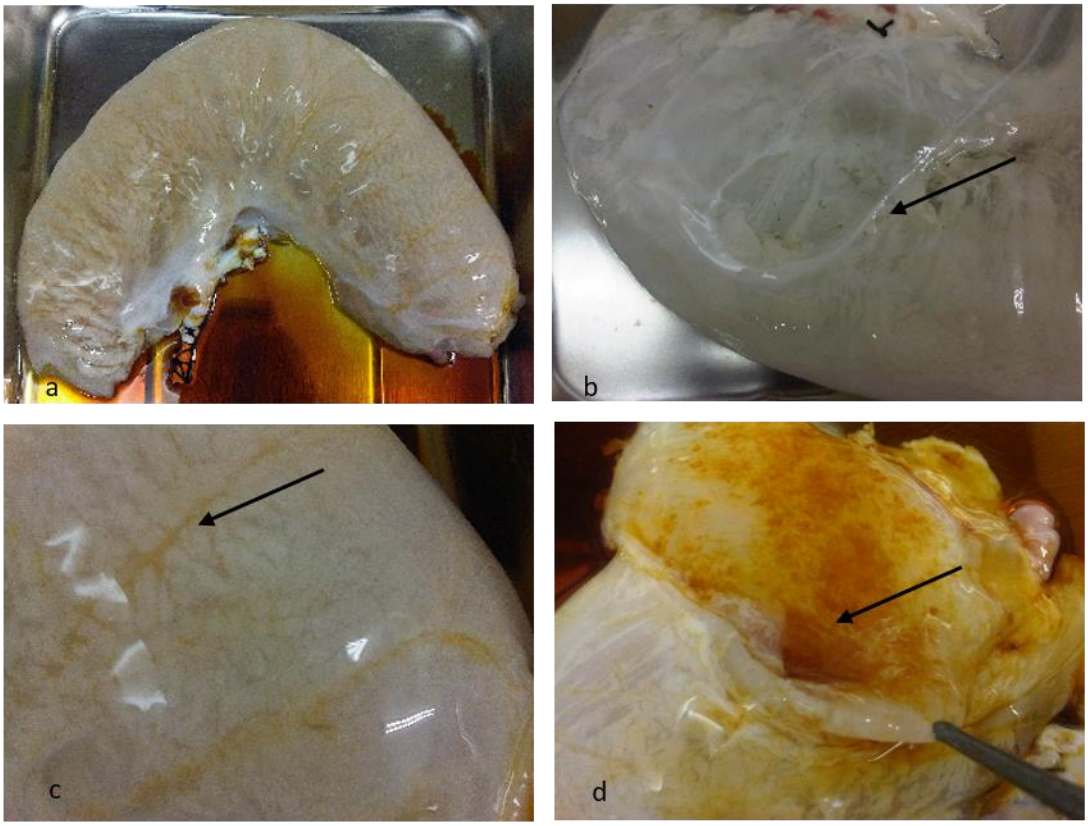

Figure 3-6 – Phase II Decellularization Dye Injection Studies
(a) *Scaffold appearance with 1:10 Betadine in water injected intra-arterially demonstrating perfusion through vessel network following 1st cycle.* ***(b)*** *Scaffold following second cycle with no contrast injection. Fine network of vessels is apparent. Arrow indicates serosal injury.* ***(c)*** *Magnified image of scaffold wall following second cycle demonstrating contrast flow through fine vessel indicated by arrow.* ***(d)*** *Following 3rd cycle in this specimen, considerable contrast has leaked intra-luminally suggesting vessel leak.*

3.4.2.3 Decellularization Phase III – Establishing Working Protocol

A series of further decellularization experiments (n=8) indicated that following completion of 3 cycles as described in Table 3.1, the porcine colon could be decellularized histologically on H&E staining, retain ECM architecture and demonstrate perfusion properties on dye injections studies. No gross difference in appearance of the scaffold was noted histologically along its length from segments 1 – 5. Further characterisation was therefore undertaken by biocompatibility testing and immunohistochemical staining as described above.

MHC II immunohistochemical staining was completed according to protocol described above. A number of different protocols were trialled using different antigen retrieval techniques before the protocol described above was successful. Fig. 3.7 below demonstrates positive membrane bound staining of control colonic tissue with no cells identified on scaffold tissue and therefore no positive staining.

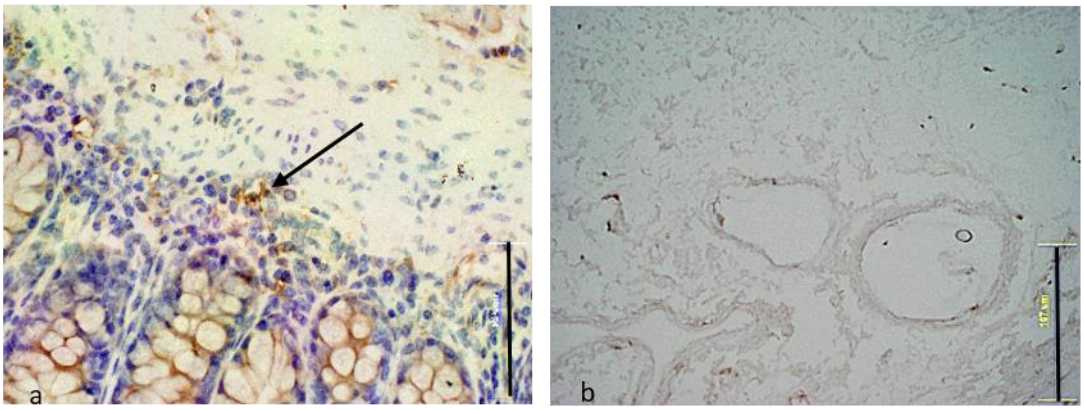

Figure 3-7 – MHC II Immunohistochemical Staining of Decellularized Scaffold
(a) *Control colon tissue stained with MHC II demonstrating membrane bound staining of cell indicated by arrow in lamina propria.* ***(b)*** *Decellularized scaffold subjected to the same staining protocol with an absence of cells and no positive staining as a result. (Scale bars correspond to length of 83.6µm and 40X magnification).*

Biocompatibility testing was successfully completed, with all rats (n=10) surviving the experimental period. In rats that underwent 2-week implantation, a macroscopic inflammatory reaction was noted and can be viewed in Fig. 3.8. No morbidity was reported in the rat group but additional lesions were noted on termination of which no clinical cause could be identified and were presumed secondary to immunological reaction (see Fig. 3.8). Implanted aortic rings demonstrated absence of any macroscopic immunological reaction but interpretation was limited by the number (n=1 in each time group). Histological images demonstrated an inflammatory type cell infiltrate (most likely macrophages) into both scaffolds and control implants, as illustrated in Fig. 3.9.

After 4 weeks, evidence of persistent inflammatory reaction was minimal macroscopically and all intestinal scaffolds and control tissues were degraded almost entirely (Fig. 3.10). Vascular control and scaffold implants remained in situ but no localised inflammation was apparent. Similar lesions were noted to those after 2-week implantation described in Fig. 3.8d. Histological images demonstrated a considerable reduction of volume in scaffold and control tissue size when compared to 2 week animals suggesting a degradation of samples.

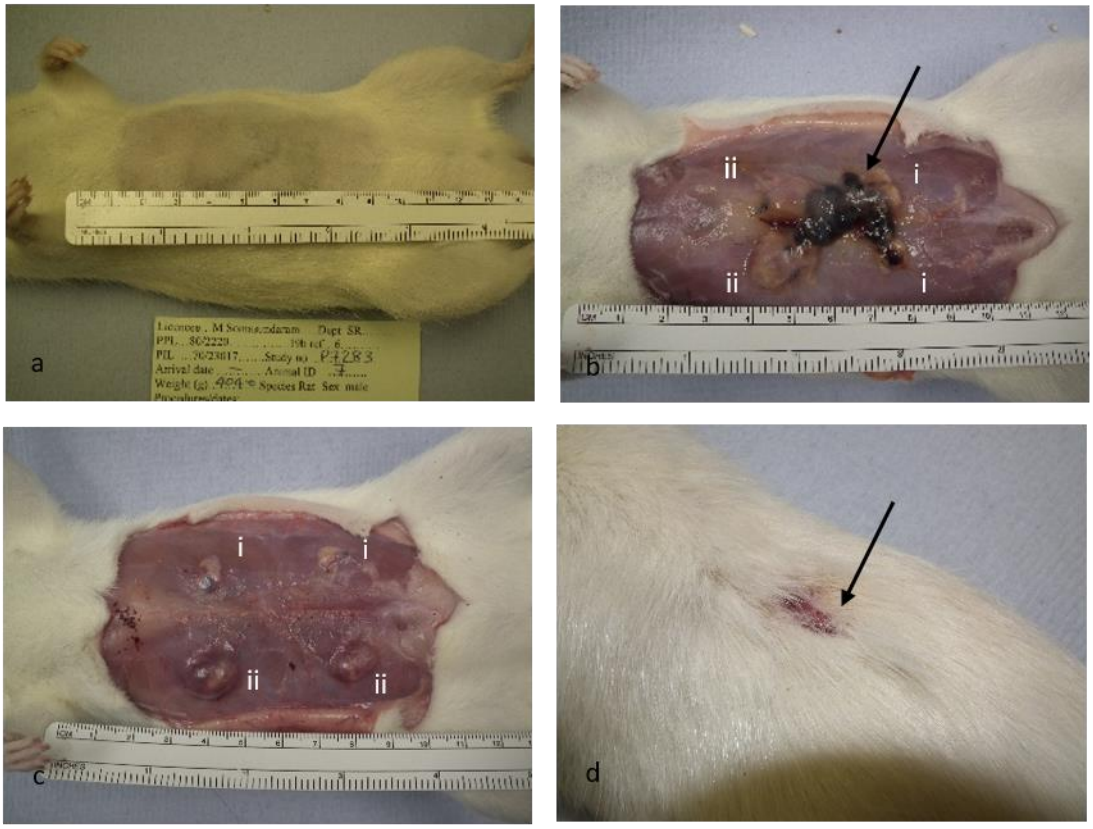

Figure 3-8 – Macroscopic Biocompatibility 2 Week Studies
(a) *Anterior abdominal wall of rat post implantation indicating orientation.* ***(b)*** *Cutaneous layer removed, scaffold implants noted at regions 'i', control samples at 'ii'. Note inflammatory haematoma indicated by arrow.* ***(c)*** *In another rat, inflammatory capsules surrounded control tissue implants at 'ii' but not scaffolds at 'i'.* ***(d)*** *Lesion noted on dorsal aspect of rat with a crusted erythematous appearance.*

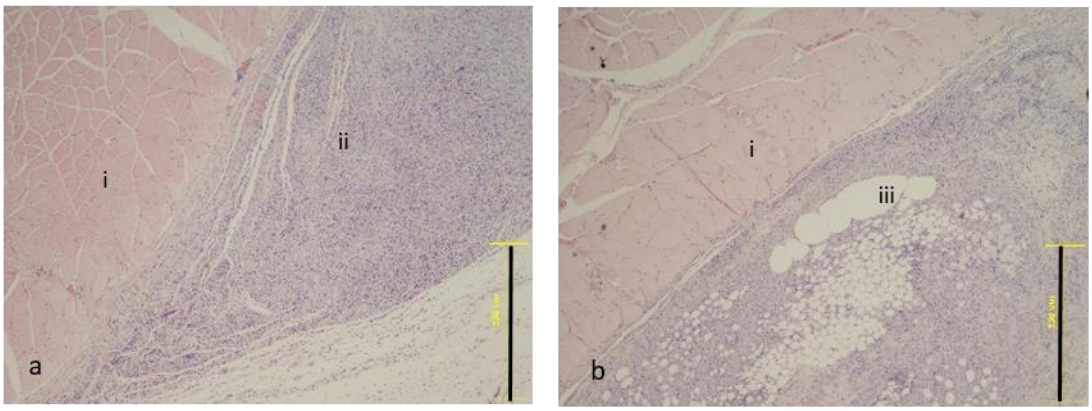

Figure 3-9 – H&E Staining of Biocompatibility Studies at 2 weeks
(a) *Image of control tissue following explantation at 2 weeks. Note native rat abdominal wall muscle layer at 'i' and control porcine tissue on surface at 'ii' with mass cellular infiltrate which is likely of macrophage origin.* ***(b)*** *Scaffold implant at 'iii' over native abdominal wall indicated by 'i'. The scaffold has a more porous appearance but demonstrates a similar large-scale cellular infiltrate. (Scale bars correspond to a length of 836μm).*

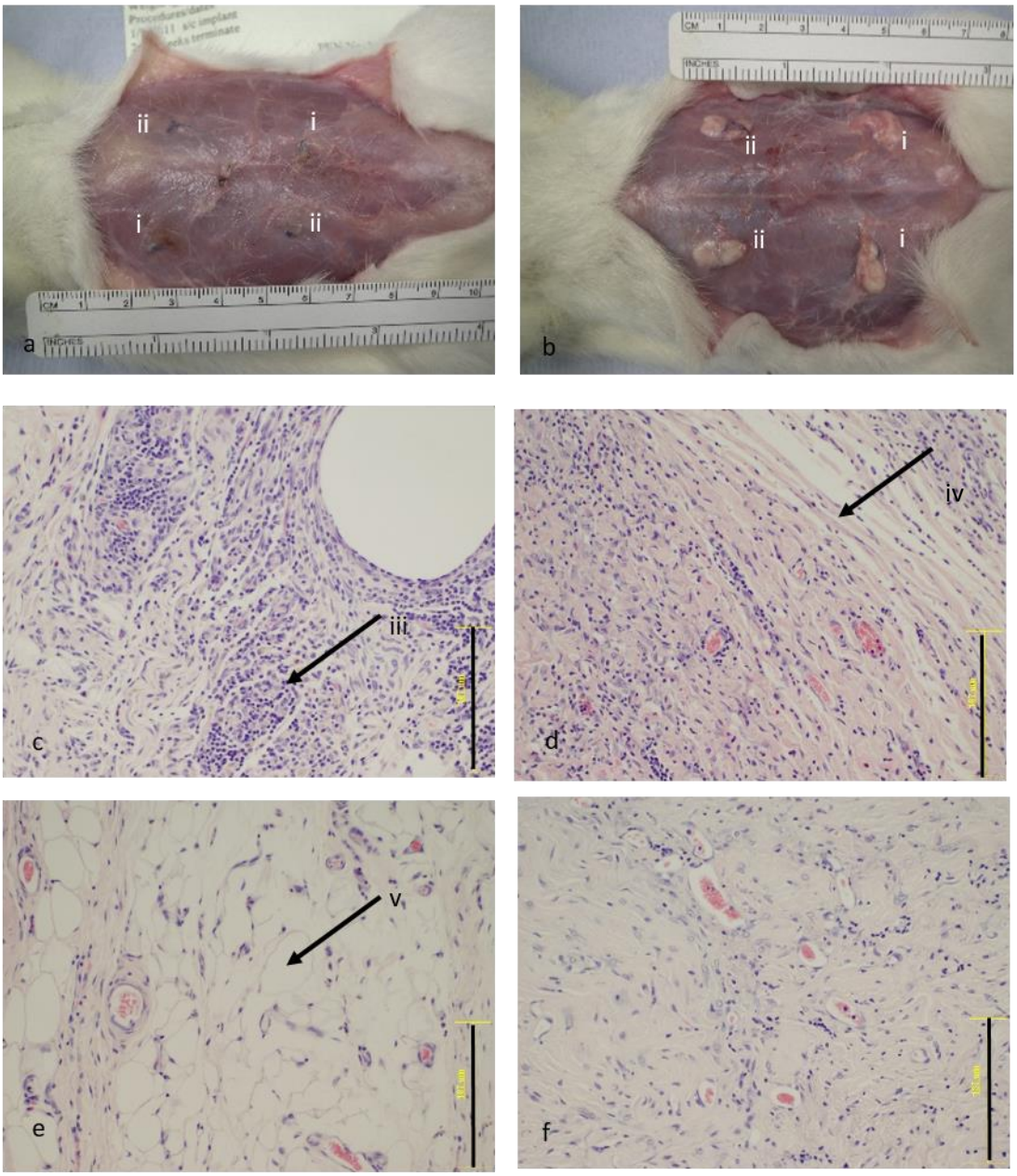

Figure 3-10 – Biocompatibility 4 Week Studies….
….(a) *The abdominal muscle layer wall of rat after 4 weeks. Note that both control intestinal tissue at regions 'i' have been degraded as have scaffolds which were implanted at region 'ii'. Images below (a) correspond to histological images with control intestinal tissue noted in image* ***(c)*** *with arrow at 'iii' demonstrating cellular infiltrate. Image* ***(e)*** *demonstrates implanted intestinal scaffold after 4 weeks with vacuous appearance demonstrated by arrow at 'v' and ongoing cellular infiltrate of a different pattern.*

(b) *Abdominal wall of rat in which aortic rings were implanted, both control vascular tissue at regions 'i' and scaffolds at 'ii' both remain in situ with no gross immunological reaction visible. Images below (b) correspond to*

*histological images with control aortic tissue noted in image **(d)** with arrow at 'iv' demonstrating layers of adventitial wall. Image **(f)** demonstrates implanted aortic tissue scaffold after 4 weeks with ongoing cellular infiltrate of a different pattern from control tissue.*

(All scale bars correspond to a length of 167µm).

Molecular quantification techniques described above were used in an attempt to quantify collagen and GAGs. Samples were processed in duplicates and n=3 scaffolds tested. n=2 sample sets demonstrated errors in final data were therefore excluded. The single scaffold tested demonstrated results which can be viewed in Figs. 3.11- 12. These graphs demonstrate collagen and GAGs quantifications. A progressive reduction in ECM components was noted following three cycles of decellularization. As the study progressed, the value of the *Sircol* collagen assay for analytical purposes in this work was questioned and no further assays completed. Further GAGs quantifications were performed and are discussed in section 3.4.2.4.1.

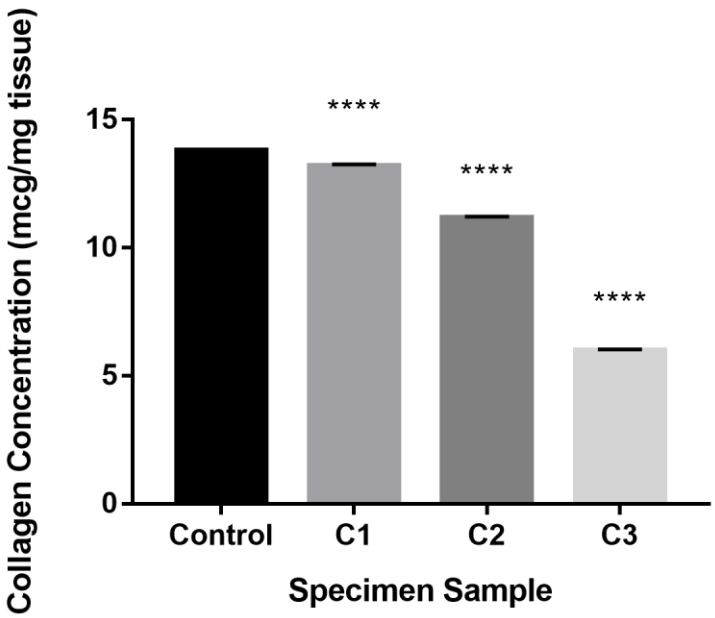

Figure 3-11 – Phase III Collagen Quantification During the Process of Decellularization
'CON' *refers to control tissue and C1-3 refer to the decellularized scaffold following each corresponding decellularization cycle. Note that collagen concentration progressively reduces with an apparent increased rate as decellularization progresses. Error bars denote standard error and statistical ANOVA testing performed revealed significant difference (P<0.0001) between decellularized samples and control tissue at all stages.*

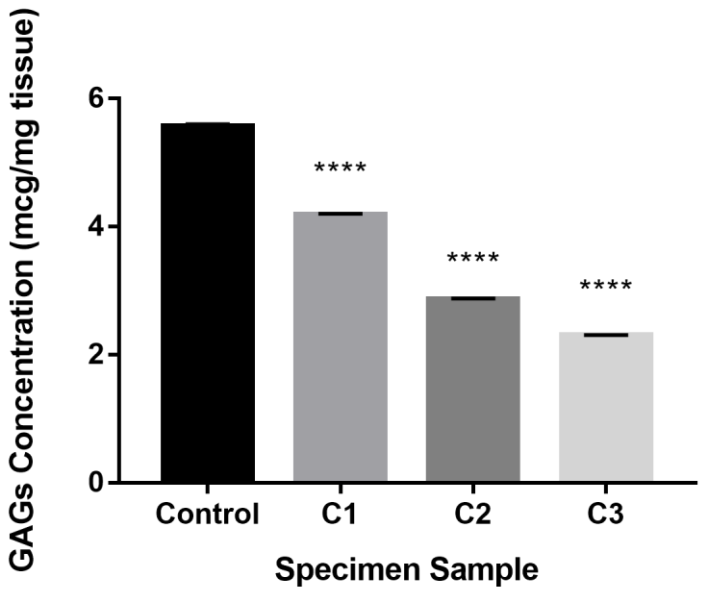

Figure 3-12 - Phase III GAGs Quantification During the Process of Decellularization
'CON' *refers to control tissue and C1-3 refer to the decellularized scaffold following each corresponding decellularization cycle. Note that GAGs concentration progressively reduces with each cycle of decellularization. Error bars denote standard error and statistical ANOVA testing performed revealed significant difference (P<0.0001) between decellularized samples and control tissue at all stages.*

Completion of establishing a decellularization working protocol was achieved by DNA quantification. To assess for consistency in the level of decellularization throughout the specimen, samples from segments 1 to 5 of decellularized scaffold were assessed alongside intestinal control tissue (n=1). The results can be seen in Fig. 3.13 indicating substantial quantities of DNA remaining within decellularized scaffolds ranging from 68ng/mg – 573 ng/mg within the same specimen and a standard deviation of 180 ng/mg. This indicated that despite the absence of cells on histology, considerably more DNA remained within scaffolds than was expected or desired. Optimisation of DNA gel electrophoresis as described above was completed with initial gel analysis performed as indicated in Fig. 3.14. The quantity and nature of DNA found following decellularization of 3 cycles by molecular analysis and gel electrophoresis initiated alteration in the protocol.

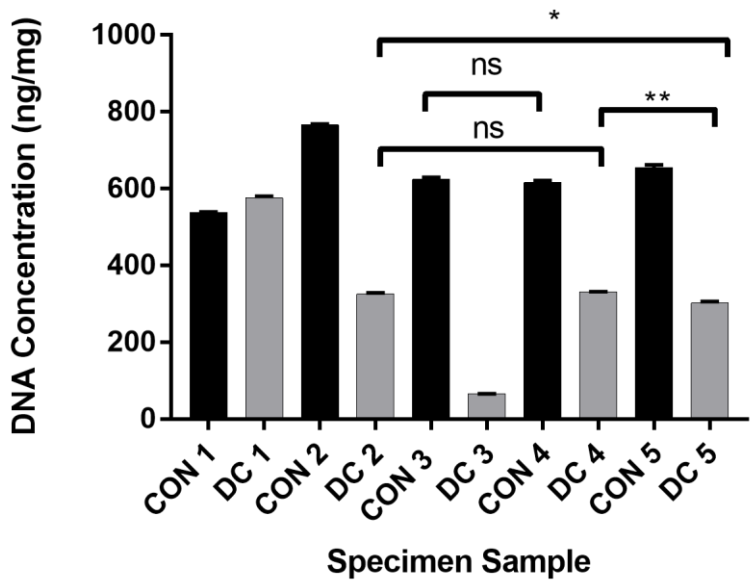

Figure 3-13 – Quantified DNA in Segments of Scaffold Following 3 Cycles of Decellularization

'CON' denotes control tissue and 'DC' indicates decellularized samples. Each scaffold segment (1-5) is displayed alongside its corresponding segment in control tissue indicating the difference in DNA quantity throughout a single scaffold and demonstrating an indication of the inconsistency of decellularization within a single scaffold along its entire length. Error bars denote standard error and statistical analysis was performed using one-way ANOVA testing. A significant difference was noted between all groups ($p < 0.001$) unless otherwise specified within the graph.

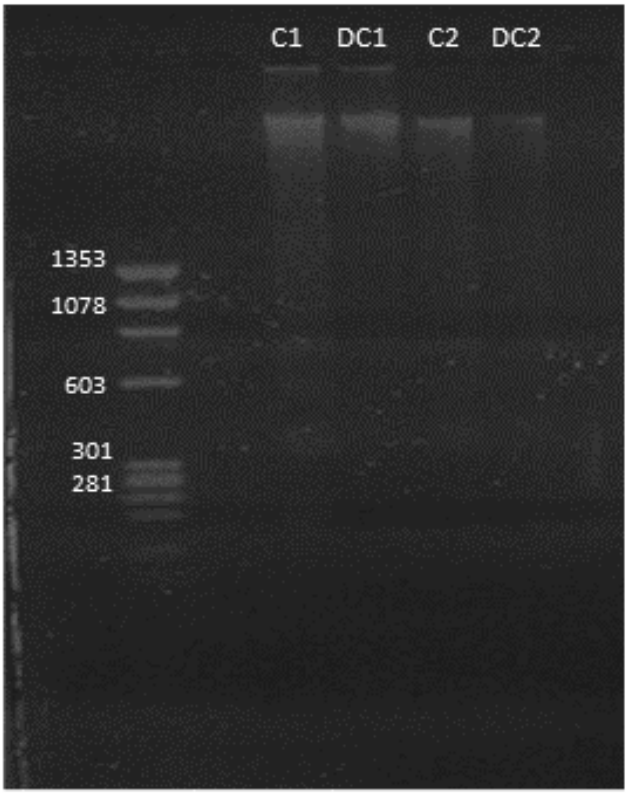

Figure 3-14 – DNA Gel Electrophoresis in Segments of Scaffold Following 3 Cycles of Decellularization
'C' *denotes control intestinal tissue in 2 separate samples.* ***'DC'*** *corresponds to decellularized scaffolds in 2 separate samples. DNA marker ladder is noted on the left with base pair bands labelled. Both control and decellularized scaffold samples display gel band smears consistent with genomic DNA indicating presence of large base pairs residing within scaffolds.*

3.4.2.4 Decellularization Phase IV – Establishment of Final Protocol

Residual quantities of DNA remaining in scaffold were deemed too great to proceed with the intestinal scaffold created after 3 cycles of decellularization and a result a further 2 cycles were added to the protocol. In addition to lengthening the protocol, the effect of freezing samples was assessed by comparing scaffolds decellularized immediately following retrieval, with those which had been frozen for 3 and 6 months before decellularization. Characterisation of this final 5 cycle protocol was expanded using methods previously described.

3.4.2.4.1 Final ECM Analysis of Scaffold

Histological analysis can be seen in Fig. 3.15 including Alcian Blue and Masson's Trichrome staining which demonstrated retention of important ECM molecules such as GAGs and collagen further. ECM was further analysed by use of TEM and images can be seen in Fig. 3.17. Decellularized scaffolds demonstrated islands of fibre like structures consistent with collagen but loss of cells and considerable change to architecture of tissue.

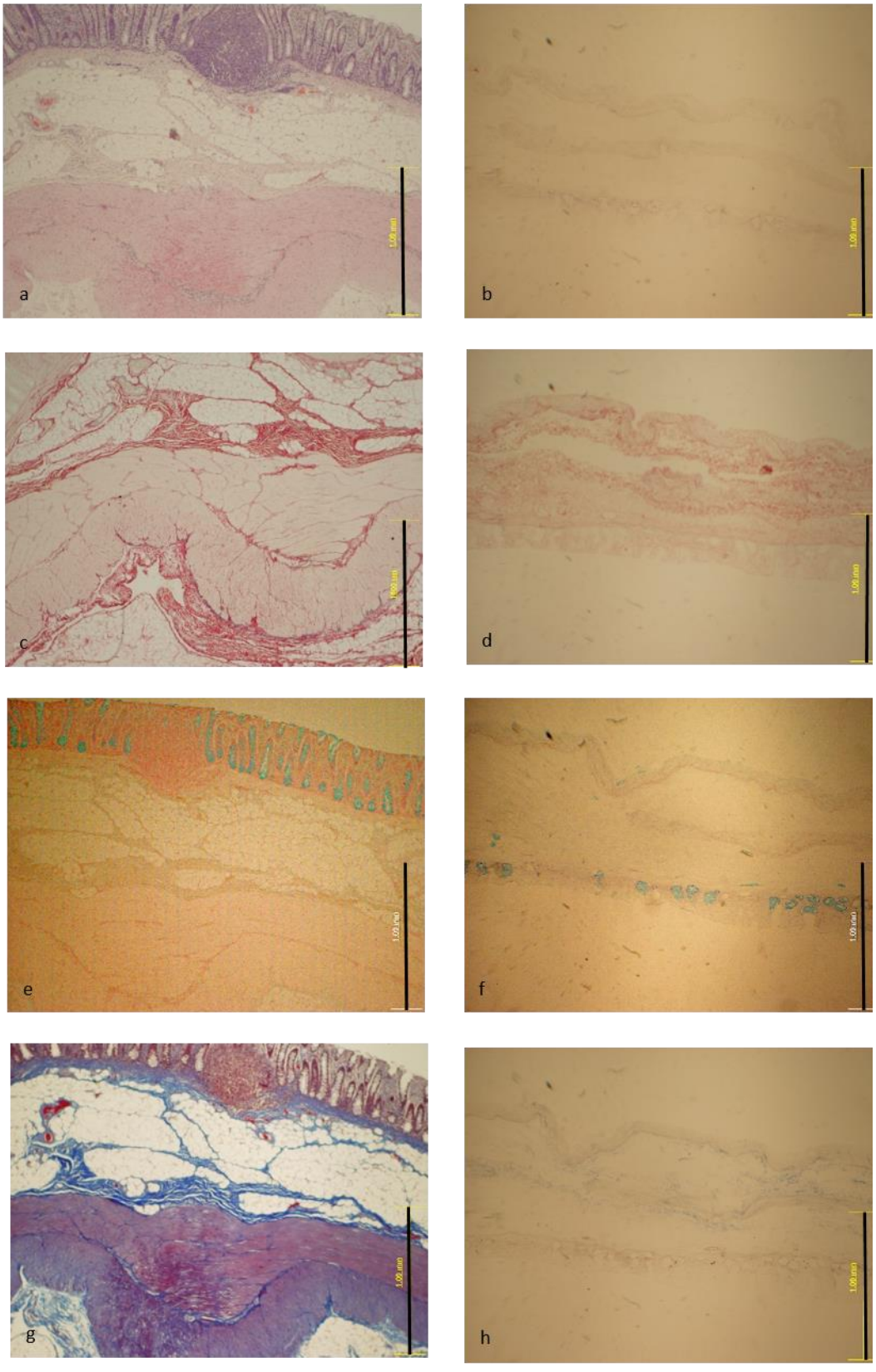
a
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Figure 3-15- Histological Staining of Scaffold Tissue Following 5 Cycles of decellularization
(a) *Control H&E stain of colon to compare with,* ***(b)*** *H&E staining following 5 decellularization cycles demonstrating preservation of layers of scaffold but absence of cells. Images* ***(c)*** *and* ***(d)*** *correspond to Picrosirius Miller's Elastin stains of control and scaffold tissue respectively. Similarly, images* ***(e)*** *and* ***(f)*** *illustrate Alcian Blue staining and* ***(g)*** *and* ***(h)*** *that of Masson's Trichrome. Right sided images of decellularized scaffolds demonstrate the retention of ECM and clear scaffold tissue layers despite decellularization. (All images at 4x magnification with scale bars corresponding to 1mm.*

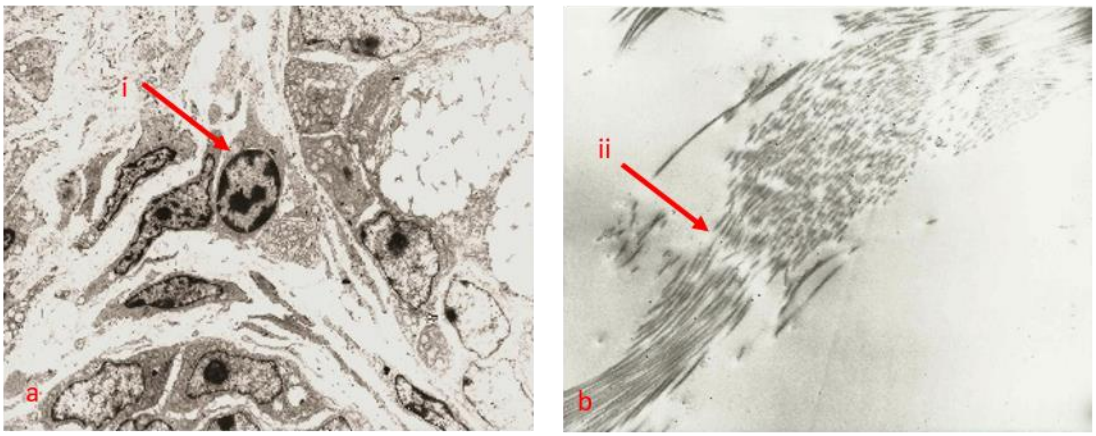

Figure 3-16 – TEM Image of Control Colon Tissue and Scaffold
(a) *Image (x 6,900 magnification) demonstrates ECM image control colon tissue with cell visible at 'i' and surrounding structures clearly visible.* ***(b)*** *Image (x 27,600 magnification) demonstrating decellularized scaffold with no visible cells and fibres of collagen like nature arranged in sheets at 'ii'.*

3.4.2.4.2 Final Cellular Analysis of Scaffold

Removal of cells and nuclei from intestinal tissue within scaffolds was demonstrated clearly by H&E staining following 3 cycles of decellularization but further assessment of individual vessels was performed. Fig. 3.18 demonstrates the progressive loss of nuclei within the muscular tissue of the aortic feeding vessel of the specimen which was the most resistant to decellularization. Due to the considerable variability of quantified DNA in phase III experiments, quantification of

DNA within a series of scaffolds (n=5) was completed in addition to assessment of the effect of freezing before decellularization on DNA quantity. The results can be noted in Fig. 3.19-20.

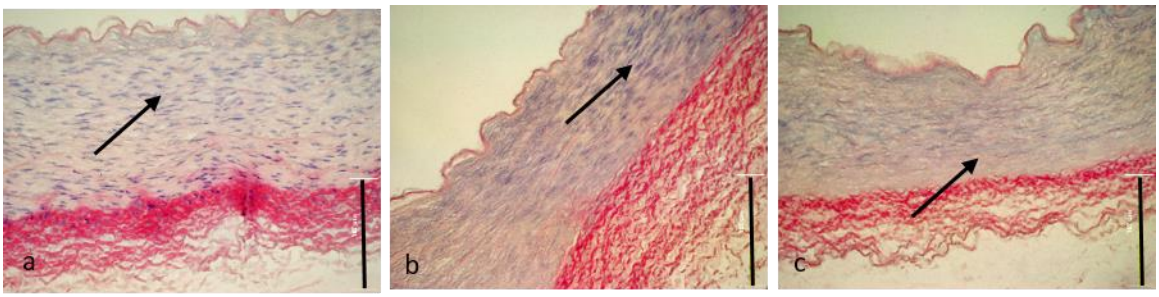

Figure 3-17 – Effect of Decellularization Process on Aortic Wall
(a) *Image of aortic vessel wall with arrow within tunica media layer and indicating intact cells following 1 cycle of decellularization.* ***(b)*** *Aortic lumen can be seen in top left corner of image with arrow within tunica media indicating residual cell nuclei following 3 cycles of decellularization.* ***(c)*** *After 5 cycles of decellularization, nuclei are no longer clearly delineated but residual nuclear material indicated by arrow remains within vessel wall.*

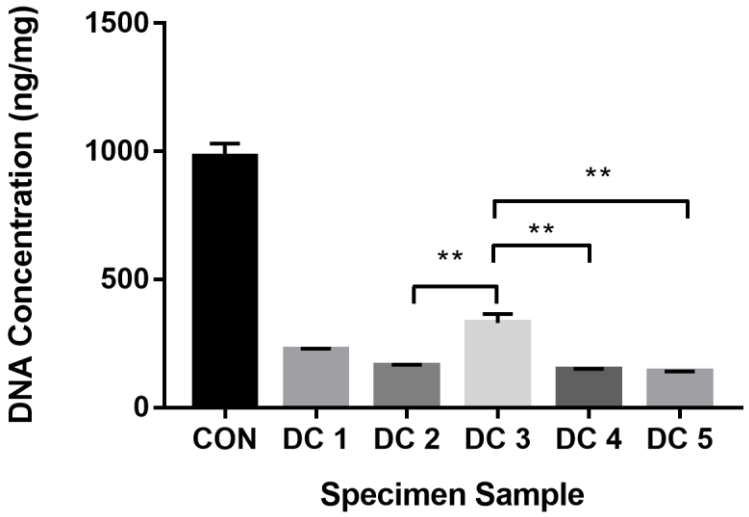

Figure 3-18 – DNA Quantification in Scaffolds Following 5 Cycles of Decellularization
An anticipated reduction in DNA takes place in all scaffolds but residual DNA within scaffolds remains > 100ng/mg in all samples despite the increased decellularization. All error bars denote standard error and all decellularized samples demonstrated significant difference ($p<0.0001$) when compared to control tissue. A single decellularized sample (DC 3) demonstrated significant difference to others as indicated above ($p\leq0.001$) despite use of identical decellularization protocol. All statistical tests were performed using one-way ANOVA calculations.

3.4.2.4.3 Perfusion Analysis of Scaffold

Perfusion was assessed during the decellularization process as described above. The lumen of the scaffold remained water tight with no leaks present as illustrated in Fig. 3.21. Further dye injection of trypan blue into the vasculature was performed which indicated small vessel perfusion but likely leak of dye from small vessels. This was confirmed on CT angiography illustrated in Fig. 3.22. Vascular transplantation of the scaffold was performed as described above to further assess perfusion properties. The implanted scaffold demonstrated arteriovenous perfusion with micro-vascular leaks present and perfusion ceased 17 minutes after transplantation due to venous thrombosis within the specimen. An illustration of the transplant process can be seen in Fig. 3.23.

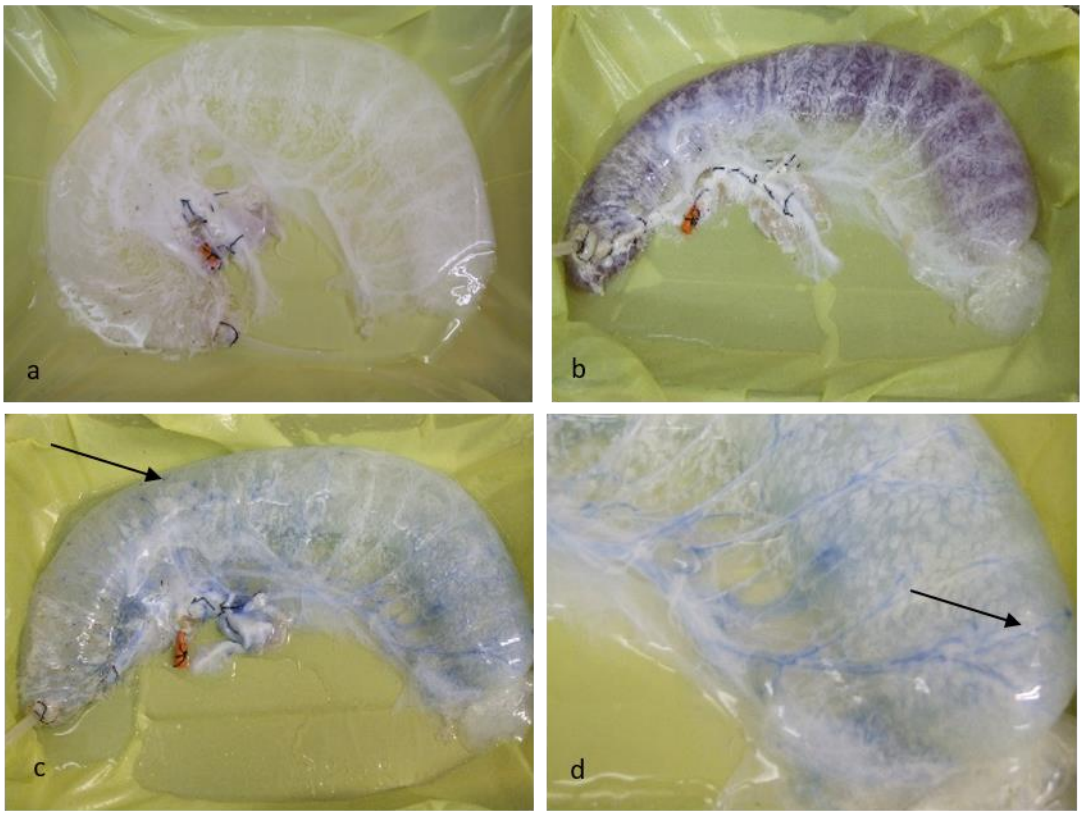

Figure 3-19 – Dye Injection Studies of Large Intestinal Scaffold
(a) *Image of final decellularized scaffold after 5 cycles for comparison.* ***(b)*** *Scaffold following intra- luminal injection of Betadine solution demonstrating no leaks from intestinal wall.* ***(c)*** *Trypan blue dye injection studies demonstrating perfusion throughout specimen but blushing areas noted at arrow tip consistent with micro- vessel leaks.* ***(d)*** *Higher magnification of image 'c' with arrow indicating small vessel passage of injected dye.*

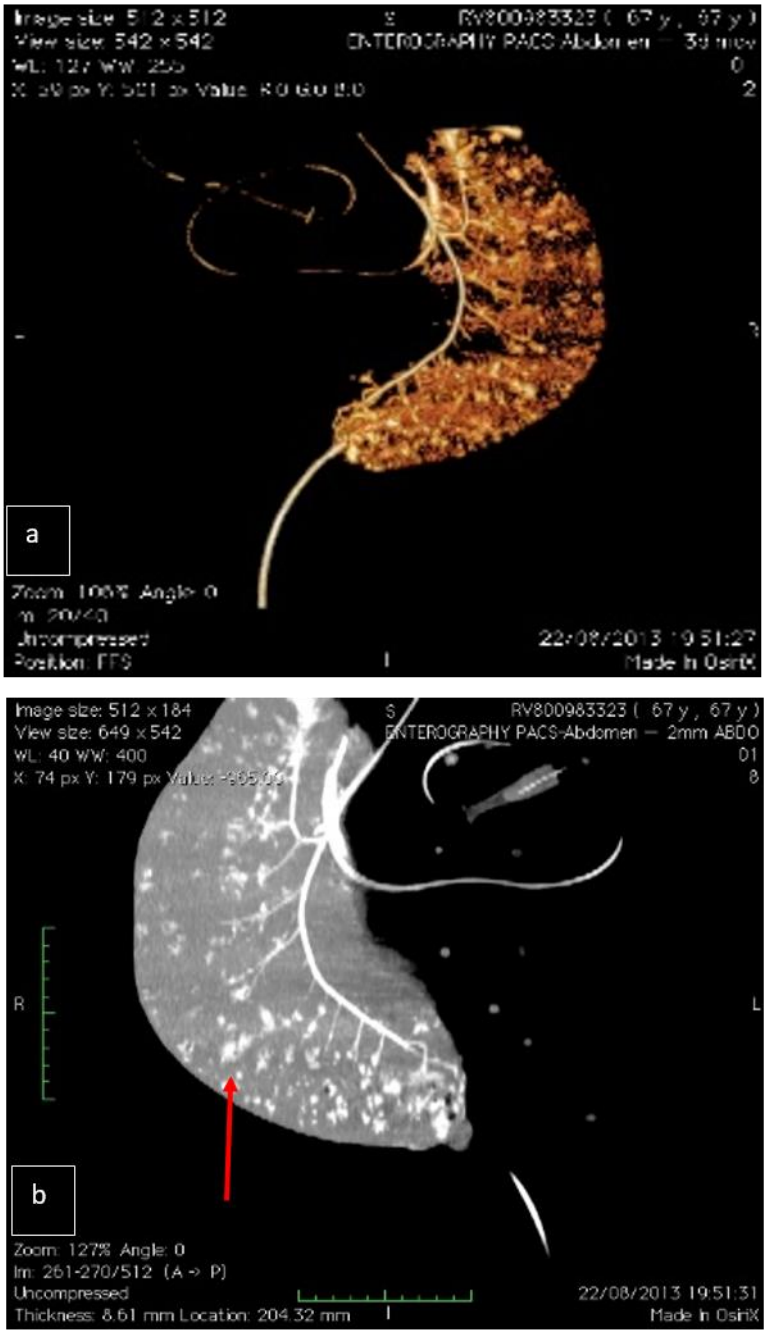

Figure 3-20 – CT Angiographic Images of Scaffold Perfusion
(a) *Image displays 3D reconstruction of scaffold with demonstration of contrast flowing through highly rich and dense network of fine vessels.* ***(b)*** *Standard contrast injection and imaging provides evidence of intact vessels branching from main feeding artery but numerous 'blush' type lesions indicated by the arrow which are consistent with small vessel leaks.*

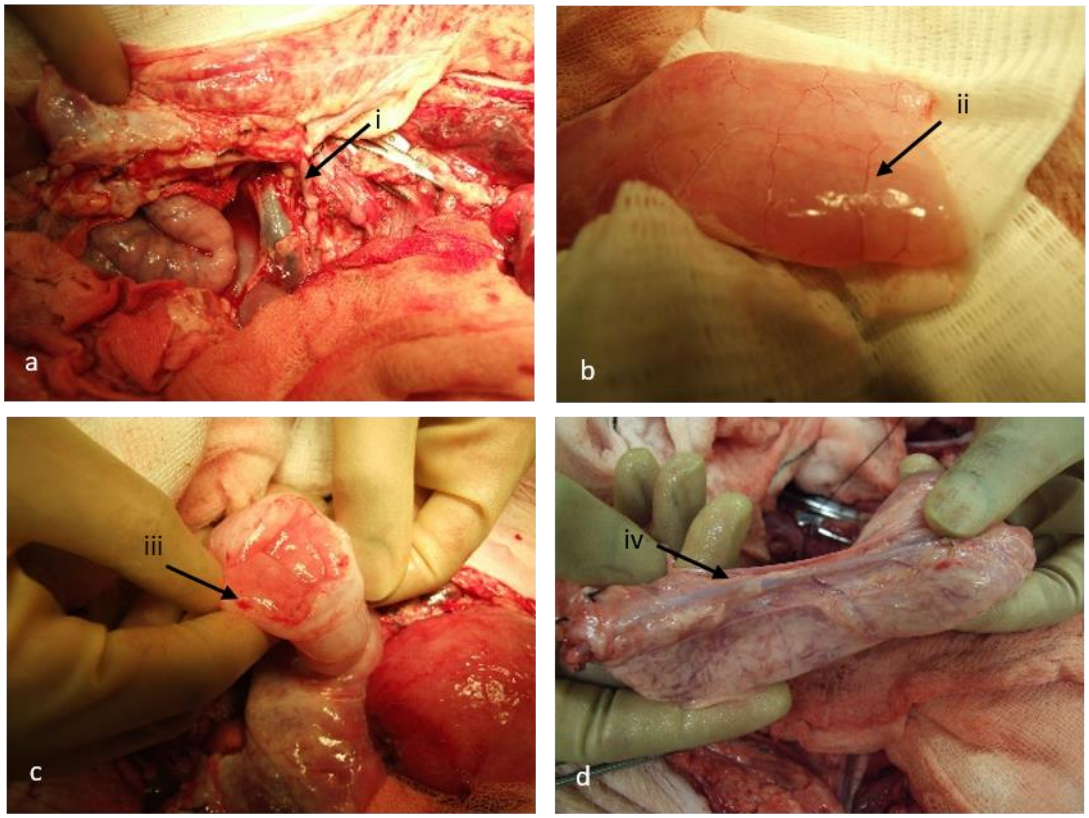

Figure 3-21 – Vascular Transplantation of Scaffold
(a) *Scaffold immediately post implantation, held up manually with arterial anastomosis noted at 'i'.* ***(b)*** *Perfused scaffold laid on swab with vessel at 'ii' demonstrating small vessel perfusion.* ***(c)*** *Despite perfusion, intraluminal and mucosal bleeding suggested vascular compromise.* ***(d)*** *Following a sustained period of perfusion thrombosis occurred with 'iv' indicating thrombosed inferior mesenteric vein.*

3.4.3 Small Intestine and Pouch Decellularization

Protocols for small intestinal decellularization were adhered to and specimens decellularized successfully. Detailed characterisation was not undertaken but the challenges and problems encountered were almost identical to those of the large intestine. Such challenges included retrieval damage and specimen damage during the decellularization process. A large intestinal pouch was fashioned as described and decellularization performed. Pouch images can be seen in Fig. 3.24. Pouches were increasingly fragile as the mechanical integrity of the scaffold reduced strength of suture lines and cause leaks and fracture.

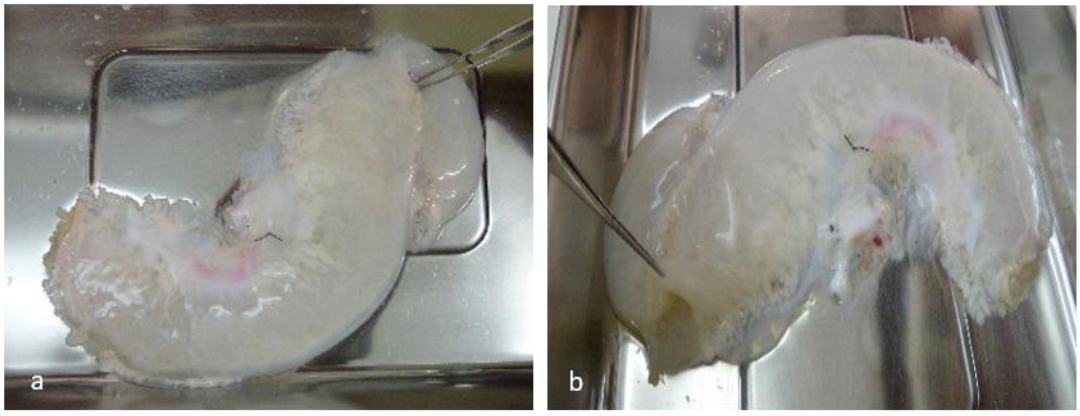

Figure 3-22 – Images of Large Intestinal Decellularized Pouch
(a) *Image of decellularized pouch with forceps pointing to suture line apex at distal end.* ***(b)*** *Specimen viewed from other side and forceps lifting lumen to illustrate pouch entry.*

3.5 Discussion

The results demonstrate a successful and reproducible model for retrieving porcine colon for the process of decellularization. In addition, significant characterisation of this specimen took place alongside decellularization of the small intestine and a large intestinal pouch thus achieving the chapter aims. During this process, numerous experimental and practical challenges were noted in the technical aspects of scaffold production which have received scant attention in the literature historically.

The use of the porcine colon distal to the spiral portion enabled an easily identifiable anatomical structure to be exposed and removed with limited surgical experience. The ability to remove a small vascularised segment allows the application of TE principles applicable to 'whole organ' engineering, without the need for the entire organ. This enables an understanding of the challenges of establishing such a model in a large animal when compared to small, yet permits a graduated approach. Such steps are important when considering if and how such a model could ever be developed for clinical benefit.

The initial model of intestinal retrieval under anaesthesia (IRUA) was established previously as it was not deemed possible to maintain blood vessel integrity for perfusion if intestine was retrieved after death. The procedure was however, time consuming and costly. Full anaesthetic was required for the animal and completion of the procedure in the warm phase in the operating theatre was inevitably slower than the retrieval after death procedure. Costs were incurred from staff availability for anaesthesia, theatre time, animal husbandry pre-operatively, associated consumable costs and live pig purchase costs. A conservative estimate of total cost is £350-500, which was likely a 10-fold increase on costs using the IRAD model. For any healthcare system to justify the increase in cost would require a substantial demonstrable benefit of IRUA over IRAD which was not present.

The change from the IRUA model to IRAD was prompted by the need to expand the pool of available animals for experimentation in addition. Using principles of human organ retrieval and adjustment of anticoagulation techniques, this model was successful and proved more effective with better ability to prepare specimen for perfusion. The application of this model following cardiac death provides valuable information. No study to date has assessed how long after death tissue can be decellularized and yet still create an effective scaffold for tissue regeneration. Whilst this study does not attempt to answer this question, the knowledge that large animal complex vascularised structures such as the intestine can be removed following cardiac death and undergo successful decellularization allows us to extrapolate that this model could be used on human cadavers successfully. The benefit of an after-death model permits a larger pool of potential tissue donors which could promote increased production of scaffolds – either allogeneic or xenogeneic.

An additional point regarding the pool of donor tissue relates to the results of using frozen tissue for scaffold production. Clinical transplantation is limited by the need to use an organ within a given time period to limit the 'cold ischaemic time'. As a result, the possibility of using frozen

tissue would allow stored tissue to be used on demand. Freezing is a known method of decellularization and this suggests the period would have a marked effect on scaffold properties (45). Results demonstrated that it was feasible to retrieve, prepare and freeze complex tissues at -20^{o}C for 6 months before completing decellularization. Beyond this however, it is difficult to draw specific conclusions regarding direct effects on tissue due the low number of scaffolds used and data was not robust to support any relation between duration of freezing and DNA or GAG content post decellularization. Although the effect of freezing on decellularization has been investigated, this has typically been done in musculoskeletal tissues and the intended use of such tissue is different from that of a large vascular structure intended for perfusion (110). Any relevant studies related to the ability to freeze and later, use tissue for decellularization is likely to be specific for the tissue in question. This may be important in future applications to permit complex tissue structures to be stored for long periods of time, before decellularization and use, thus permitting a true 'off the shelf' type approach to tissue replacement.

The choice of decellularization agents was based on a rationale. Sodium dodecylsulphate has been used successfully for decellularization in a variety of tissues and has been shown to remove nuclear remnants from tissues. Given the highly cellular nature of the intestinal tissue, it was deemed a suitable agent for use. DNase enzyme causes degradation of DNA and trypsin cleaves peptide bonds on the C- side of arginine and lysine (used in combination with EDTA). The use of multiple reagents was favoured over a single reagent for 2 main reasons. Firstly, it is well demonstrated that all decellularization reagents cause some disruption to the ECM and the repeated use of a single agent is likely to cause systematic targeted damage and lead to specific scaffold inadequacies which may be detrimental to the recellularization process. Secondly, the cycled use of reagents will promote cellular removal by a variety of different mechanisms and this was perceived to be more effective in performing the decellularization process. In practice, determining the true effects and difference between chosen reagents on the intestinal scaffold was difficult and would require detailed study and analysis of the effect of reagents on tissue with

a number of variables controlled. As so many variables effect the outcome of use of reagents, such studies would need to be extensive and it is not certain this would provide great benefit to a full decellularization protocol.

Decellularization protocols are widely reported in the literature for a number of tissues including heart, lung, liver, kidney and trachea (46, 57, 58, 111, 112). The majority of groups have used a range of methods to characterize their scaffolds including; molecular assays, immunohistochemistry, biomechanics and histology. The absence of cells on H&E staining seems to be a prerequisite for the production of a successful scaffold for TE purposes, although no universally accepted definition of decellularization exists. Within this study, cells were absent from scaffolds after only 3 cycles of decellularization. DNA quantification was used to characterise the large intestinal scaffold in this study and has been used in a number of studies within the field. In our experience, successful removal of cells on histological staining does not necessarily translate to minimal quantities of DNA in a number of different decellularized animal tissues. Indeed, following 3 cycles of decellularization, large quantities of DNA remained in intestinal scaffolds necessitating a further 2 cycles. It has been suggested in the literature that a threshold for acceptable quantities of residual DNA within scaffolds is < 50 ng/mg (55). However, the cited evidence for this is not specific to tissue engineering and the figure is therefore open to debate. This is supported by published literature that has questioned the reliance on removal of cells from biological scaffolds as the most important indicator of immunogenicity, citing the importance of antigens which are not bound to cell membranes (113). The quantity of DNA found in the decellularized scaffold was 202± 79.6 ng/mg, with a reduction noted when samples were frozen before decellularization. DNA gel electrophoresis of remaining DNA demonstrated a pattern of smearing consistent with genomic DNA indicating that, whilst cell nuclei may be destroyed, residual base pair fragment lengths may be high. The importance of this quantity and quality of DNA remains a matter of conjecture, as little work has been done to measure the immune response of a recipient in relation to quantities of residual DNA within implanted scaffolds (114).

It is also likely that such measurements will vary greatly based on; scaffold and host species, the nature of residual DNA and the type of implanted cells on scaffolds which may confer immunomodulatory properties.

The decellularization protocol applied in this study enabled the production of a scaffold with some desired properties but, whilst the results were reproducible, considerable variability was noted. As discussed above, DNA quantity varied despite using the same protocols and this also applied to retention of ECM components. Little is discussed in the literature regarding the reproducibility of decellularization protocols but the majority of studies suggest applying a single standardised protocol to a given organ or tissue remains a successful approach, often without providing the number of successful outcomes (115). Some studies however state the number of animals tested before establishment of a successful decellularization protocol. For example, Ott et al. required 140 decellularization experiments to establish a protocol for a rat heart scaffold (57). This is significant in the fact that, if the scaffold of a small animal required this many trials for optimisation, a complex large animal scaffold with vascular pedicle may require considerably more. However, our experience with this large animal intestinal scaffold was that application of the same protocol for decellularization resulted in substantially different quantities of DNA and retention of ECM products. It is postulated that this is likely secondary to multiple factors including specimen size, mass, anatomical variability and quality of perfusion affecting decellularization. This finding is important in considering the model for future use as it is likely that decellularization protocols may need to be tailored to specific tissue samples.

The importance of the extent of required preservation of ECM architecture should be further debated. Retained ECM components are believed to confer one of the main benefits of biological scaffolds over synthetic by guiding tissue regeneration by a series of poorly understood mechanisms. The threshold of retained ECM components necessary to confer this benefit is not known but it is postulated that the more ECM retained, the better the regenerative properties of

the scaffold. This study demonstrated an approximately 3-fold decrease in GAGs following the decellularization process. Of note, the proportion of control intestinal tissue containing GAGs was low and this is consistent with similar tissue engineering studies (64). The significance of this reduction in a component of tissue that comprises such a quantifiably small percentage is uncertain. There exists a significant body of work in TE attempting to characterise and define the relationship between retained ECM components and cellular regeneration. Despite this however, there is a lack of an evidence based and clearly defined quantitative relationship that permits even a 'roadmap' of how to rationalise the retention of ECM components such as GAGs when creating a scaffold. In any case, the likely benefit of such components will be dependent on a multitude of factors utilised during cell seeding including, sterilization methods, cell progenitors used and the provision of appropriate conditions for cell proliferation and regeneration, be they chemical, mechanical or other forms. To convincingly demonstrate this, studies involving cell seeding, in vivo implantation and regeneration are necessary to determine important thresholds which may promote, or hinder tissue regeneration. This is likely to be species and host specific and is beyond the scope of this work but it seems that the ECM within scaffolds should be thought of as a dynamic system interacting with seeded cells and host rather than a vehicle to be characterized independently of cell seeding.

Characterisation of scaffolds is reported using similar methods within the literature relating to a variety of different scaffolds, but the practical aspects of characterisation are often not discussed in detail. Histological and molecular analyses require very small amounts of tissue to produce data and results are taken as representative of the entire specimen. In some cases, methods of assessment are clarified. In Totonelli et al's study of decellularized rat intestine, entire specimens were homogenized or minced before assessment indicating that results of tested samples were a good representation of the entire specimen (64). In the case of this work however, specimens were large and 50mg was not representative of the entire specimen. Homogenization of the entire specimen would not reflect the difference between the vascular and intestinal tissues. The

characterisation methods of larger, complex scaffolds should be clearly documented if the data is truly believed to be relevant to the efficacy of the scaffold. Acknowledgement of this is important in developing better methods to assess scaffolds in future work.

Biocompatibility studies illustrated that scaffolds promoted a reduced localized immune response when compared to control tissue after 2 weeks. This was visible both on macroscopic imaging following explantation and microscopy, where a dense cell inflammatory infiltrate was visible. No further analysis of cell populations was attempted, but these likely represented macrophages as part of the late phase of acute inflammation. The precise nature of these cells in relation to the scaffold may be relevant, as evidence suggests that they may provide an inflammatory/ destructive (M1) or regenerative/ remodelling role (M2) (116). Further testing by immunohistochemistry may provide useful information in this regard. After 4 weeks, both scaffolds and control intestinal samples were resorbed and no longer macroscopically visible. The information gained from this experiment can be considered from different perspectives. From the immunological point of view, it is difficult to make conclusions regarding this result as the immune burden on the rats tested arose from the implantation of 4 samples of tissue; 2 control and 2 scaffold. Despite this, the absence of significant morbidity and mortality in rats suggests that the implantation of scaffolds had no added negative immunological effect. The noted lesions were likely a systemic clinical response and therefore may have been caused by either scaffold or control tissue. The absence of microscopic infection or other adverse events suggests that sterilization methods were appropriate and non-toxic to test animals. The resorption of scaffolds demonstrates clearly that, without the appropriate conditions and cell progenitors, scaffold tissue will not regenerate. True biocompatibility testing requires assessment of the appropriate bio-implant as required for eventual purpose. With knowledge that implanted cell progenitors are required for tissue regeneration of intestinal scaffold, further tests should assess implantation of seeded scaffolds to map future hurdles to translation.

Scaffold resorption following implantation is consistent with the literature and supports the premise of the study that a method of appropriate delivery of oxygen and nutrients under suitable conditions is necessary for cellular regeneration. The preservation of the vascular arcade of the scaffold in this work is one of the key features of the scaffold and perhaps the most significant findings of this study involves the properties of scaffold perfusion. Decellularization by perfusion has been demonstrated in a number of tissues and organs and our group has used a similar model to decellularize the small intestine with associated vascular arcades (84). This remains the first study in which the process has been completed using large intestine. Despite a number of studies utilising perfusion decellularization, frequently this has not been performed in an arterio-venous, physiological fashion and has been documented in other studies using an alternative vascular approach (57, 58). It is not possible to discern if this approach provides an advantage or disadvantage to the process and further work should address this as considerably more work is required to persist with a physiological arteriovenous approach.

This work is the first known case of use of CT angiography to assess perfusion properties of a scaffold. This enables an assessment of genuine patency of perfusion and directly illustrates that, patency of vasculature is not maintained sufficiently for direct revascularisation. The use of such imaging also enables contextualisation of results in a clinical sense enabling clearer conclusions to be drawn. The limitations of the perfusion properties of the scaffold were further demonstrated by in vivo transplantation which resulted in thrombosis of scaffolds within 17 minutes. The absence of a cellular endothelium is likely to be responsible for this pro-thrombotic state. Such experiments enable the clinician to visualise the limitations of biological scaffolds despite careful attention to attempting to preserve vasculature during the decellularization process. CT angiographic images demonstrate perfusion throughout the scaffolds but small vessels do not maintain patency. This suggests that whilst the decellularized vascular network provides a means to distribute liquid throughout the scaffold, leaks will be present and the vascular system will

contain breaches. Whilst perfusion is maintained, the degree to which it is limited suggests that further stages of ex vivo scaffold preparation are necessary before in vivo application.

The results gained from this chapter demonstrate that it is possible to create a large intestinal biological scaffold by perfusion decellularization. This supports the work done on the small intestine, the protocol of which was reproducible. Fashioning of a decellularized pouch was unsuccessful and if a tissue engineered intestinal reservoir is sought, the results argue that decellularization principles should be used to create a biological rectum as a first choice scaffold rather than attempt the fashioning of a substitute decellularized colonic pouch. Results indicate tremendous variability; in the decellularization process, the nature of tissues and their response to decellularization and the validity of methods of scaffold characterisation and their importance. This variability suggests that scaffold optimisation is a vast area and should be done in conjunction with a genuine assessment of the benefit in changes to protocols. The best method of assessing scaffold success is the ability to support cells and promote regeneration as this is the primary goal of any scaffold. In view of these findings, the project proceeded to isolate, culture and characterise appropriate cells for this purpose and this is detailed in chapter 4.

Chapter 4 Cell Isolation and Culture for Scaffold Seeding

4.1 Background

Studies have demonstrated that the most successful regeneration of intestinal tissue occurs when scaffolds have been used in combination with seeded cells (47, 79). The most appropriate cell source for use remains a matter for debate (117). Knowledge of the cellular composition of the small and large intestine, in addition to their histological structure, functions and patterns of proliferation is essential in planning a preliminary intestinal tissue engineering strategy.

4.1.1 Histology of the Small and Large Intestine

The small intestine mucosal surface is highly folded to increase surface area and arranged into villi and microvilli. The epithelium of the villi is composed of columnar cells known as enterocytes and mucin secreting 'goblet cells'. Between the villi, the crypts of Lieberkuhn extend to the muscularis mucosa and contain 'Paneth cells' which are eosinophilic and aid in immune function. Enteroendocrine and stem cells are also located within the crypts. Histologically, the highly vascular lamina propria layer lies beneath the epithelium and superior to the muscularis mucosae. The submucosa forms the layer between muscularis mucosa and muscularis externa. The large intestine differs significantly in that villi and Paneth cells are absent. The differences in structure between large and small intestine is not relevant in relation to cell culture and will not be discussed further.

4.1.2 Intestinal Proliferation and Regeneration

The differentiated cells within intestinal epithelium are enterocytes, enteroendocrine, Paneth and goblet cells. Stem cells within crypts give rise to transit-amplifying cells which reside in crypts for 48 hours, undergo 4-5 divisions and then differentiate terminally into epithelial cells types. All of these cells except Paneth cells undergo migration upward toward the tips of villi before undergoing apoptosis and being shed into the intestinal lumen (50). The pathway of cell

proliferation within the intestine is now well defined, characterised and seems shared amongst species (118). 2 distinct pathways; 'Wnt' and 'Notch' commit cells to a secretory (Paneth/ Goblet/ Enteroendocrine cells) or absorptive (enterocytes) lineage respectively. The Wnt pathway predominates and is responsible for the process of proliferation, whist Notch is concerned with maintenance of proliferation and specific differentiation (50).

Intestinal stem cells themselves may also be referred to as crypt base columnar (CBC) cells and are multipotent cells, with potential for differentiation into all intestinal epithelial cells (50). Markers of these stem cells have been reported and 'Lgr5' seems the most robust and reliable described. Lgr5 is a G protein-coupled receptor that has been demonstrated on the surface of intestinal stem cells, is specific to them and appears to have greater specificity than the previously described putative marker 'Musashi-1' (119). A stem cell niche can be defined as the supporting microenvironment in which stem cells are found and within the intestine, the subepithelial myofibroblasts seem to support this function (50). Proteins expressed by fibroblasts, such as bone morphogenic proteins (BMP) have a demonstrated effect on epithelial cell proliferation, supporting the concept of the intestinal stem cell niche. The intestinal mesenchyme comprises the physical bulk of the intestine accounting for the lamina propria, muscularis mucosa, smooth muscle and comprises numerous cell types including fibroblasts and pericytes. There is a bi-directional interaction between the epithelial and mesenchymal cells of the intestine that is likely involved in regeneration and proliferation of both layers. Some mechanisms are well demonstrated in this respect, whilst others remain poorly characterised (120). This close relationship between the 2 germ layer derivatives supports the investigation of mesenchymal stem cells (MSC) in TEI.

4.1.3 Organoid Units (OU)

First described by Evans et al. in 1992, OU consist of a rim of epithelial cells surrounding a core of mesenchymal cells (74). A number of studies have used this landmark work in the development of

OU isolation protocols and have frequently used OU for implantation in scaffolds for TEI. The original method described in 1992 involved mincing intestine into pieces approximately 1mm in size, digestion in enzyme solution, agitation and separation, before extraction of OU. Studies have progressed from this, adapting protocols with modifications to the original method, altering enzymatic digestion protocols, agitation speed and extraction processes (72, 121, 122).

Historically, OU were limited by their low yield (as described in chapter 1), fragility and difficulty in expansion. Original experiments by Evans et al. demonstrated difficulty in sustaining in vitro culture owing to the fact that mesenchymal cells adhered to plastic but epithelial cells did not, thus disassociating OU. The precise composition of OU was not clear and as they exist in clusters of cells, examination proved difficult without disassociation. This previous work has been superseded by more recent studies in rats, which has refined culture methods to permit isolation of OU and extraction of the mesenchymal component. The resultant epithelial cells have then been sustained in culture for greater than 6 months. In addition, these cells have stained positively for Lgr5, indicating the presence of intestinal stem cells (90).

The work of Clevers et al. described above is seminal in its achievement and stands alone when compared to other works. In the absence of such methodology, OU consist of an unknown proportion of epithelial, mesenchymal and stem cells. The approximate number stem cells within the total small intestine is not known but estimates are that 4-6 cells reside within each crypt, though this estimate pre-dates the discovery and application of the Lgr5 marker (123). However, it is clear that intestinal stem cells are not present in great abundance and there is no guarantee that a particular isolated OU will contain 1 or more intestinal stem cells.

4.1.4 Mesenchymal Stromal Cells (MSCs)

Mesenchymal stromal/ stem cells have been discussed in their application in TEI in chapter 1. Although clinical interest in the regenerative properties may have driven use of MSCs for TEI, the importance of the mesenchyme in regeneration of intestine was alluded to as early as 1992 by

Evans et al. (74). As discussed above, the precise mechanisms of action of MSCs in relation to intestinal epithelium remain unclear but it appears there is a co dependant relationship in regeneration. Given the natural regenerative properties of MSCs it is likely they play a pivotal role in this relationship. This theory is supported by the multiple studies that demonstrate regeneration of damaged intestinal tissue in response to administration of MSCs in both small animals and humans (124-126).

MSCs are stromal cells that contribute to the regeneration of mesenchymal tissues such as bone, fat, muscle and cartilage. Originally described arising from bone marrow (127), they have since been isolated from other tissues including adipose tissue, periosteum and amniotic fluid (128). Suggested properties necessary to define MSCs (in humans) include; (i) plastic adherence, (ii) ability to differentiate into appropriate mesenchymal cells (adipocytes/ chondrocytes/ osteoblasts) and (iii) expression of appropriate surface markers. According to The International Society for Cytotherapy, these markers include expression of CD105, CD90 and CD73 (in >95% culture) and absence of CD34, CD45 and CD14 (129). A much smaller volume of literature is available on use of MSCs from large animals when compared to humans and there is a lack of species-specific markers. The majority of studies in large animals have used anti-human antibodies which do not always cross react appropriately (130). The standards described therefore, cannot be applied directly in pigs and evidence demonstrates that MSCs from pigs may stain positively for additional surface markers and less intensely than humans for those listed (130).

The immunological characteristics of MSCs require special consideration. The phenotypes they express are MHC I positive but MHC II negative. As a result, they are regarded as non-immunogenic. The presence of MHC Class I molecules alone suggests that although T cells may undergo stimulation in response to MSCs, full activation in the absence of costimulatory molecules is unlikely (131). This supports the use of allogenic MSCs in transplantation and has led

to their use in clinical trials. The potential applications for use in TEI is therefore broad. Evidence also exists to suggest that MSCs possess some immunomodulatory properties and postulated mechanisms include inhibition of B cell proliferation in vitro but evidence remains inconclusive and the mechanisms involved are a matter of debate (128). The evidence that MSCs have; led to intestinal regeneration in TEI studies, play a role in epithelial regeneration physiologically and possess no MHC Class II and yet have a role in immunomodulation on transplantation, fully supports their use in this project.

4.2 Aims

The successful use of OU in TEI suggests they form a good cell source to assess the regenerative properties of an intestinal biological scaffold and may be able to serve as a 'positive control' population for scaffold seeding. The first key aim was therefore to develop a protocol for the successful isolation of OU from pigs for the purpose of seeding. The second key aim was to develop an autologous model for the extraction and culture of MSCs from bone marrow in pigs and to characterize this cell population as MSCs. This required additional cell cultures and experimental techniques detailed below. Finally, to expand the pool of possible cells for use in TEI, MSCs derived from adipose tissue were also sought and characterisation attempted. All these experiments were conducted with the intention of utilizing the cell populations for scaffold seeding.

4.3 Methods

4.3.1 Intestinal Organoid Unit (OU) Isolation

Organoid unit (OU) isolation in pigs was attempted using a number of protocols based on previously published work in small animal models. A number of variables were experimented with including: concentration of enzymatic digestion solution, duration of digestion and cell washing

method. The methods below describe the finalised culture protocol but the results shown demonstrate data from different protocols attempted during optimisation.

4.3.1.1 Intestinal Tissue Retrieval and Preparation

10cm of Ileum was resected from living pigs approximately 2 feet proximal to the ileo-caecal valve. The specimen was squeezed to remove faecal contents and transferred to a warmed sterile 0.9% N. Saline bath. The specimen was gently agitated and the washed 3 times to remove gross faecal residues. The specimen was then transferred to a working surface where peritoneal attachments and coverings were dissected off before cutting the specimen into 4x 2.5cm long segments. Each segment was then opened by incision along the lumen, creating a sheet of intestine which was then rinsed again rapidly a further 3 times in warm N. Saline. Resultant tissue sheets were sliced (mucosal surface facing upwards) manually using a 158mm blade (*Swann-Morton, UK*) into 1- 2mm pieces by an alternating horizontal and vertical pattern. Care was taken not to mince tissue too fine. Each separate volume of minced tissue from a corresponding sheet was then transferred into 4x 5cm diameter petri dishes and the 4 dishes transferred to the cell culture hood. Individual petri dish contents were then transferred into 4 corresponding T25 tissue culture flasks for washing to remove smaller faecal residues. 20ml of warmed (37-40^{o}C) Hank's Balanced Salt Solution (HBSS) was pipetted into flasks and manual agitation performed. After 30-60 seconds, intestinal tissue settled at the base of the flask leaving a distinct supernatant above. This was removed using a pipette and discarded. The process was repeated 3 times to ensure adequate cleansing of tissue under sterile conditions and prevent contamination.

4.3.1.2 Collagenase Digestion

Enzymatic digestion was the primary culture method used to release OU from intestinal tissue. A pre-prepared 200ml solution consisting of dispase (0.25mg/ml) and collagenase (800U/ml) in HBSS was warmed to 37.5^{o}C ready for use. 25ml of digestion solution was pipetted into each flask in the culture hood and flasks sealed before transferring to a mechanical agitator (*ZHWY-*

100D, SciQuip, UK) and laid flat with rotation speed set to 100rpm and temperature maintained at 38°C. Flasks were agitated for 30 minutes and then transferred to the cell culture hood. 20ml of the supernatant fluid within each flask was then collected and transferred into 4 separate 50 ml centrifuge tubes (*Sarstedt, UK*). The collected supernatant then underwent OU isolation as described in the next section. Prior to beginning this, a second phase of enzymatic digestion was undertaken and a further 25ml of enzymatic solution was added to each T25 flask containing the intestinal tissue. The flasks were then replaced in identical fashion into the mechanical agitator and removed after a further 30 minutes. The entire supernatant was then collected as previously described and isolation performed as below.

4.3.1.3 Organoid Unit Isolation

Centrifuge tubes containing supernatants from enzymatic digestion were immediately treated to cease enzymatic activity, permit the process of isolation and prevent disruption of the OU within the cell suspension. This was achieved by addition of 20ml of Dulbecco's Modified Eagle Medium (DMEM) containing 4% Sorbitol and 10% Fetal Calf Serum (FCS). Tubes were gently shaken and centrifuged (*Harrier 15/80, MSE, UK*) at 100G for 5 minutes at room temperature. Low speeds were used to avoid disruption and disassociation of OU. Tubes were removed and supernatant carefully extracted as pellets were extremely fragile. Pellets from 2 separate 50ml centrifuge tubes were then resuspended in 2.5ml of standard DMEM and combined in a single 15ml centrifuge tube for easier visualisation of pellets. Empty 50ml tubes were rinsed with 5ml of DMEM to remove any residual cells and added to new working tubes. After completing this process for all samples, 4 x 15ml centrifuge tubes were present, each containing a cell suspension in 10ml DMEM. Tubes were centrifuged again to wash OU at 100G for 5 minutes and supernatants removed as previously described. This wash step was repeated again using 10ml of DMEM per tube before cells were suspended in a volume of 2ml for the purposes of cell counting. Care was taken at every stage not to cause disruption to pellets as this may have caused OU to

disassociate. Whenever OU pellets were resuspended therefore, gentle shaking of tubes was favoured to dislodge the pellet.

4.3.1.4 Organoid Unit Identification and Quantification

Suspensions of OU were identified and counted to give yield by using standard haemocytometry techniques (see chapter 2). Neubauer chamber grid lines were used to estimate the size of cell clusters and in order to be deemed OU, they were required to display appropriate size (<300 µm length) with the classical appearance of an epithelial rim encasing a core of mesenchymal cells. Quantification of OU was performed using low volume cell suspensions (2-5ml) and standard haemocytometry calculations.

4.3.2 Bone Marrow Derived Mesenchymal Stromal Cell (BM MSC) Isolation and Culture

Cells for biological scaffold seeding should contribute to appropriate tissue regeneration but also be easily isolated and expanded. MSCs have advantages in isolation and expansion as previously described. A protocol for the harvest of bone marrow aspirate and plating, primary culture and expansion of the mesenchymal stromal population was developed and is described below.

4.3.2.1 Bone Marrow Aspiration

A variety of sites of were attempted to gain a good yield of bone marrow aspirate from pigs under anaesthesia. Attempted access sites included the iliac bone, tibia, sternum and femur. The femur was found to give the greatest volume aspirate and a reproducible, effective protocol was developed which was eventually used safely in a recovery model. Pigs under anaesthetic as described in Chapter 2 were laid supine. Anatomical landmarks were identified and the region of the right iliac fossa, femur and pelvis was sterile prepared and draped as per standard methods. Under sterile technique, the leg was bent at the knee joint and the hip externally rotated to bring the femur to superficial prominence. A transverse 1cm skin incision was made 5cm proximal to the distal end of the femur and the blade used to divide the underlying muscle fascia. The muscle

layers were then spread using an artery clip by blunt dissection under vision as far as possible and the instrument advanced blindly onto femoral bone until the resistance was felt. The clip jaws were then spread to dilate the passage and the instrument removed.

An 11G bone marrow biopsy needle (*Ranfac, USA*) was favoured in early experiments due to its large bore (allowing minimal disruption to aspirate on removal) and strength as the pig bone marrow cortex in older pigs was extremely strong. The needle was passed in the path created by the dissection, perpendicular to the femur and onto the bone where it was inserted by continuous pressure applied while twisting the instrument in a corkscrew motion. A clear 'give' in resistance was felt in older pigs as the cortex was breached but in younger animals this was less apparent and aspiration was attempted if it was believed the needle had breached the cortex. The introducing needle was removed from the stylet and a 20ml syringe preloaded with 25,000U heparin in 5ml was used to aspirate a total volume of 15ml. The process was repeated until no further aspirate was gained. Syringes were then transferred to the cell culture hood for processing as soon as possible. The animal was terminated by standard methods (see chapter 2).

In later experiments, the procedure was performed in a recovery model. In such cases, the procedure followed the same protocol for aspiration but the volume aspirated was reduced to prevent morbidity. A 10ml total volume of aspirate was withdrawn in a 20ml syringe preloaded with 2.5ml of heparin [25,000U/ml]. Following aspiration, the stylet was withdrawn slowly and the wound assessed for haemostasis. The skin was closed with interrupted 3.0 prolene sutures and pressure placed on the entry site with a resting 1L IV fluid bag for 10 minutes. Pigs were reviewed 4 hours after the procedure for any complications and sutures were removed after 7 days.

4.3.2.2 Marrow Aspirate Gradient Separation and Cell Monolayer Extraction

Marrow aspirate syringes were transferred into 50ml centrifuge tubes within the culture hood. Tubes were gently agitated and the separation process performed as rapidly as possible as

otherwise aspirates clotted which prevented the procedure continuing. The mononuclear cell population was separated from the marrow aspirate by centrifuge gradient separation using a Ficoll-Paque preparation (*Histopaque 1077, Sigma, UK*). Histopaque was allowed to reach room temperature prior to use and 6ml was transferred into a 15ml centrifuge tube. An equal volume of bone marrow aspirate was then layered on the surface in a 1:1 ratio, taking care not to mix the 2 liquids by gently pipetting the aspirate onto the surface. The process was repeated in separate tubes until the entire aspirate was used. Tubes were then left to stand within the culture hood for 30 minutes, after which time, preliminary separation of layers within the tube was noted. Tubes were then centrifuged at room temperature for 45 minutes at a speed of 400g.

On removal, separation of layers of the bone marrow aspirate was noted with red cells pelleted at the base, a layer of Histopaque seen superiorly and a layer of plasma superior to this (see Fig. 4.1). At the interface between the plasma and Histopaque, a cloudy layer of variable visibility was noted. When centrifuging blood by gradient separation, this layer is known to contain peripheral blood mononuclear cells (PBMC) and is reported to contain the same population in bone marrow aspirates. This layer was aspirated using a standard P100 pipette (*Gilson Pipetman, Gilson, USA*) by hand for added control. The aspirated volume was transferred into pre-warmed Phosphate buffered saline (PBS) solution in a 1:4 ratio. The mononuclear cell population from 2 separate tubes was combined in a single 15ml centrifuge tube at this stage thus halving the total number of tubes.

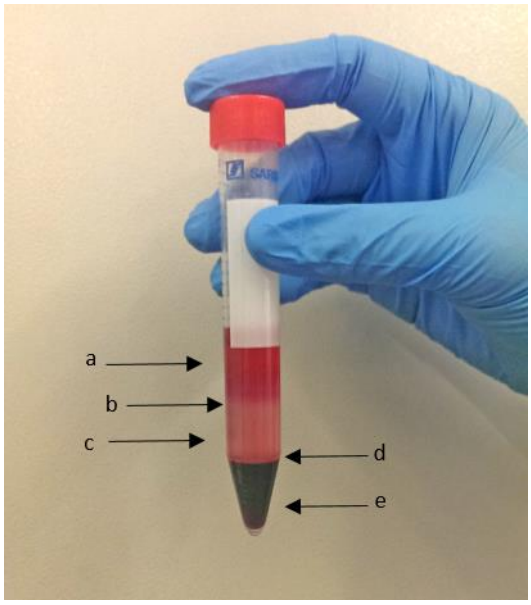

Figure 4-1 – Ficoll-Paque Separation of Bone Marrow Aspirate
Layered separation of crude bone marrow aspirates following Histopaque separation. ***(a)*** *Plasma layer,* ***(b)*** *Interface between layers where mononuclear cell population resides and site of aspiration,* ***(c)*** *Histopaque reagent layer,* ***(c)*** *Granulocyte layer,* ***(e)*** *Erythrocytes at base.*

4.3.2.3 Mononuclear Cell Population Washing and Plating

Mononuclear cell populations in PBS were washed by centrifugation at 400 g for 10 minutes. Supernatants were aspirated before disrupting pellets and repeating 2 further washes. Finally, pellets from 2 separate tubes were combined in a single 15ml centrifuge tube and resuspended in Minimum Essential Media Alpha (MEMα) (*Invitrogen, UK*). Cell suspensions from each 15ml centrifuge tube was then plated in a T75 flask and transferred to a cell culture incubator maintained at 37^{o}C with 5% CO_2 concentration. After 48 hours, flasks were washed with PBS and medium changed using standard methods as described in Chapter 2. Flasks were then reviewed every 24- 48hours using an inverted microscope.

A single 20ml volume of bone marrow aspirate (including anticoagulant volume) therefore yielded a mononuclear cell population that was plated in a single T75 flask. This strategy was employed initially to provide manageable working number of flasks and assess success of

expansion protocol before determining appropriate mononuclear cell population plating densities for culture. Following success of cultures using this approach, detailed information of initial plating density was later sought. A similar calculation strategy was applied to assessing appropriate plating density for passaging. Confluent T75 flasks were passaged in 1:2 ratios which successfully prolonged cultures.

4.3.2.4 Cell Passaging

On microscopy and flask review, if large amounts of dead cells, or debris were noted, cells were washed with PBS and medium replaced. Once 70-80% confluence was achieved, cells were trypsinized and counted using methods described in chapter 2. After n=5 primary cultures, cells were no longer counted following trypsinization unless specifically required or doubt regarding confluence existed. Flasks were divided in a 1:2 ratio once trypsinized. Cell lines were passaged typically up to P3 and then frozen for storage in vials (500,000 - 1,000,000 cells/ vial).

4.3.2.5 Cell Characterisation

By definition, MSCs must demonstrate; (i) plastic adherence, (ii) expression of appropriate surface markers and (iii) appropriate differentiation potential. Plastic adherence was confirmed by progressing cells through the stages of primary cell culture but remaining properties were demonstrated as below. MSCs demonstrate tri-potency and therefore differentiation into chondrocytes, adipocytes and osteoblasts was sought in vitro by various methods. The presence of appropriate surface markers was assessed using flow cytometry.

4.3.2.5.1 Cell Differentiation

4.3.2.5.1.1 Differentiation into Adipocytes

Differentiation into adipocytes was performed using a commercially available kit (*StemPro®, Invitrogen* USA). 12- well adherent tissue culture plates (*Sartstedt, UK*) were pre- warmed in a cell culture incubator before addition of 1ml working volume MEMα per well and return to incubator

in preparation for plating. BM MSC at P1 were trypsinized and resuspended in a small working volume of 1ml MEMα at a high concentration (1,000,000 cells/ml). The pre prepared 12-well tissue culture plate was removed from the incubator and each well containing medium pipetted with cells to reach a plating density of 10,000 cells/cm^2 (38µL in 3.8cm^2 well). Plates were then returned to the cell culture incubator and allowed to reach 80- 90% confluence before proceeding with differentiation.

Cells reached 80% confluence after 24 hours and medium was aspirated before a gentle wash with PBS. Differentiation was induced by addition of *StemPro® Adipogenesis Medium*. Wells were allocated different test purposes; 2 consisted of negative controls with cells and proliferation medium (MEMα) only and 2 consisted of 'test' samples containing cells and differentiation medium. Following washing of the wells, appropriate medium was added gently and the lid replaced before the culture plate was returned to the incubator. The plate was reviewed every 48 hours, after which medium was aspirated and replenished in each well. After 14 days the experiment was terminated and the wells stained with Oil Red O as described below.

4.3.2.5.1.1.1 Oil Red O Staining

Oil Red O (ORO) staining was performed to demonstrate the presence of adipocyte like properties within BM MSC cells that had undergone differentiation. Published protocols were used to perform staining and protocol can be seen in Appendix 4.1. Plates were removed from the incubator on day 14 and medium discarded. Wells were washed using Dulbecco's PBS taking care not to disrupt the cellular mono-layer. Cells were fixed by adding 1ml of 10% NBF and plates incubated at room temperature for 45 minutes. Fixation buffer was aspirated from the wells and they were washed with distilled water before adding 60% isopropanol to cover the monolayer. Plates were then incubated at room temperature before isopropanol was aspirated. 1ml of ORO working solution was added to each well and plates incubated at room temperature for 15 minutes. ORO solution was aspirated and wells washed several times with distilled water. Plates

were then dried by inversion and blotting on an absorptive surface. Wells were counterstained by Harris haematoxylin before washing 5 times. Wash was aspirated and wells reloaded with PBS for viewing under an inverted microscope. Positive controls were established by staining adipocytes from primary culture. Due to the different culture conditions required by adipocytes, staining could not be performed within the same plate as BM MSC but was performed in tandem using the same methodology. Details of adipocyte primary culture and plating for this purpose are described in section 4.3.3.1.

4.3.2.5.1.2 Differentiation into Chondrocytes

Differentiation of BM MSCs into chondrocytes was induced by use of specific differentiation media and cell culture in micro-masses (MM). Primary culture of chondrocytes was performed as described in section 4.3.3.2. Differentiation was induced in a pre-warmed 24-well culture plate as this was the appropriate size to enable construction of MM of sufficient number for the experiment. BM MSC were trypsinized at P1 and resuspended in a high density pellet at a concentration of 2.5 x 10^7 cells/ml. Cells were then manually carefully pipetted in the centre of the culture well in a volume of 20µL. This constituted the 'micro-mass'. The process was repeated using the same method for chondrocytes which were also trypsinized from P1. The final plate contained 8 micro-masses; 4 of BM MSC and 4 of chondrocytes. The lid of the culture plate was closed and the plate returned to the culture incubator without addition of medium to enable MM to acclimatize and adhere. After 3 hours, appropriate medium was added to each well. Serum-free, Phenol-red free Dulbecco's Modified Eagle's Medium (DMEM) was used to maintain cultures and 2 chondrocyte and 2 BM MSC micro-masses were supplemented with DMEM proliferation medium only. The remaining 4 wells consisted of 'test' wells and BM MSC differentiation into chondrocytes was induced by supplementing DMEM with Transforming Growth Factor Beta1 (TGF-β1) at 10ng/ml. MM were monitored for disassociation and medium changed every 48 hours.

4.3.2.5.1.2.1 Alcian Blue Staining

Micro masses were stained with Alcian Blue (ALB) dye (*Carl Roth, Germany)*. Medium was removed and wells were rinsed twice with PBS and fixed with 4% glutaraldehyde solution for 15 minutes at room temperature. Glutaraldehyde solution was removed before washing of wells with 200µL of 1M HCl solution in distilled water. Wells were then covered with 1% ALB dye in 0.1M HCL (pH <1) at room temperature and left in the dark for 30 minutes. Wells were then carefully but rigorously washed prior to imaging. Staining protocol can be seen in Appendix 4.2.

4.3.2.5.1.2.2 Alcian Blue Quantification by Colorimetry Assay

Quantification of ALB staining was sought by use of previously published protocols (132). ALB stained cultures were extracted by addition of 200µL of 6M guanidine hydrochloride (*Sigma-Aldrich, UK*) overnight. Extracted dye was transferred to cuvettes and optical density (OD) measured at 630nm using a spectrophotometer (*Spectronic 2000, Bausch & Lomb, USA*). OD values were interpolated with an ALB standard curve and cartilage-specific sulfated glycosaminoglycans (GAGs) protein content of each sample determined using Pierce™ BCA Protein Assay Kit (*Thermo Scientific, U.S.A*) according to manufacturer's guidelines. GAG content determined was normalized with extracted ALB and expressed as ALB/Protein (µg/ µg).

To reflect ALB/Protein concentrations in relation to cells, DNA was quantified in samples in addition. Measurement was performed by fluorescence using SYBR Green I dye (*Invitrogen, UK*). Samples were diluted 1:50 and homogenized (*PreCellys®24, Bertin Technologies,* USA). Homogenate was then diluted 1:20 in an assay solution containing 10mM Tris HCL, 1mM EDTA and 1% SYBR Green. Finally, OD was measured at 485/535nm using a spectrophotometer (*TECAN-M200; Tecan, Switzerland*) and interpreted with a standard curve which was plotted using 'dsDNA for Standard Curve-Lambda DNA' (*Invitrogen, UK*). For data presentation, ALB/ Protein content was normalized to DNA content with final reading represented as (µg/µg).

4.3.2.5.1.3 Differentiation into Osteoblasts

MSC from P2 were seeded in 24-well plates at a density of 5,000 cells/cm^2 in 9 wells with MEMα and allowed to adhere for 24 hours. After this period, wells were assigned to triplicates of the following; (i) a negative control population supplemented with MEMα only, (ii) MSCs supplemented with differentiation medium (MEMα solution containing 0.1μM dexamethasone, 10 μM β glycerophosphate and 50 μM ascorbic acid) and (iii) MSCs supplemented with differentiation medium for later planned mineralization. A duplicate plate with identically plated cells was run in tandem without the final 3 mineralization wells. Cultures were reviewed and media changed every 3 days.

After 14 days, the plate containing 6 wells was removed and wells stained with alkaline phosphatase (see below) to assess for differentiation of MSC into osteoblasts (OB). The second 9 well plate was removed and the final 3 wells containing MSCs and differentiation medium were aspirated and replenished with mineralization medium consisting of osteoblast growth medium supplemented with 400nM hydrocortisone-21-hemisuccinate and 10mM β glycerophosphate (full details in section 4.3.3.3.3). Media in the remaining 6 wells was changed as before. The plate was returned to the culture incubator and reviewed every 3 days with medium changes each time. On day 21, it was removed and cells stained for Alizarin red to assess for osteoblast mineralization.

In parallel to the above culture plates, osteoblasts (OB) were used to serve as positive controls. Cells were purchased commercially in human species as porcine lines were unavailable (*Lonza, UK*). Full details of culture and expansion are provided in section 4.3.3.3. 4 wells within a 24 well plate were plated with OB at a density of 3,900 cells/cm^2 (reflecting available supply and recommended density). A duplicate identical plate was plated in parallel. In both plates, all 4 wells were supplemented with OB growth media as per manufacturer's guidelines for 24 hours. After this period, a single plate was allocated for alkaline phosphatase (ALP) staining and the other for mineralization. The ALP staining plate underwent change of medium every 3 days until

14 days after which point it was stained simultaneously with MSC differentiation plates described above to serve as positive controls.

The mineralization plate was removed after 24 hours and 2 wells allocated as negative controls. In these wells, OB growth medium was changed every 3 days until termination of the experiment at 21 days. The remaining 2 wells were allocated for mineralization positive control test wells and mineralization media with hydrocortisone-21-hemisuccinate and β glycerophosphate supplementation as above was changed every 3 days. At 21 days, the plate was stained for Alizarin Red to assess for the presence of calcium deposits consisted with bone mineralization as per protocol below.

4.3.2.5.1.3.1 Alkaline Phosphatase Staining

MSC and OB plates were removed from culture incubators at 14 days as described. Medium was removed from wells and cells washed with PBS. Wells were then fixed with 10% NBF for 60 seconds before removal and immediate washing with specific washing buffer containing 0.05% Tween (Appendix 4.3). Cells were then stained with NBT/BCP solution prepared from commercial tablets (*SigmaFast™ BCIP-NBT; Sigma, UK*) and kept in the dark for 10 minutes. Staining substrate was then removed and wells washed with washing buffer and PBS before review under a microscope.

4.3.2.5.1.3.2 Alizarin Red Staining

Plates dedicated for mineralization studies were removed at 21 days as described. Media were removed and wells washed with Dulbecco's PBS. Wash was removed and cells fixed with 10% NBF for 30 minutes. Fixative was removed and wells washed with PBS before staining with Alizarin Red S in 0.1% ammonium hydroxide (pH 4.1- 4.3) for 45 minutes in the dark. Following this, staining solution was removed before multiple washings with PBS and review under microscope. Full protocol can be seen in Appendix 4.4.

4.3.2.5.2 Flow Cytometry

Demonstration of appropriate surface markers of MSCs is a necessary property, defined by internationally-agreed standards. As discussed previously, no specific standards are available for porcine MSCs and those existing in humans required adaptation for the isolated cell line. An antibody panel was defined following literature review and cells tested to characterise presence or absence of appropriate surface markers.

4.3.2.5.2.1 Antibody Panel

Literature review indicated that porcine MSCs should demonstrate surface CD29 (β1 integrin adhesion molecule), CD44 and CD90 (stromal cell markers) (133-136). These antibodies were sourced labelled with a range of different fluorochromes to enable cells to be tested simultaneously for all antibodies. In addition, the absence of CD14 and CD34 (haematopoietic cell markers) was anticipated based on the literature and these antibodies were therefore also tested. As visible in Table 4.1, the negative markers were sourced with different fluorochromes and this enabled all antibodies to be tested for simultaneously in a flow cytometry system with 5 or more channels.

Table 4-1 – Antibody Panel for Bone Marrow MSC Flow Cytometry

Antibody	Function	Specificity	Conjugate	Emission Spectra (nm)	Isotype Control
CD29 (Anti-ITGB1)	beta1 integrin adhesion molecule	Pig, canine, human	FITC	519	Murine IgG1-FITC
CD44	stromal cells marker	Pig, canine, human	RPE	575	Murine IgG1-PE
CD90	stromal cells marker	Pig, unhuman primates	APC	660	Murine IgG1-APC
CD34	haematopoietic marker	Human	RPE-Cy7	667	Mouse IgG1,K
CD14	haematopoietic marker	Human	APC-Cy7	765	Mouse IgG2b, K

4.3.2.5.2.2 Cell Labelling and Fixation

BM MSC from P2 were thawed from frozen as previously described. Cells were resuspended in Fluorescence Activated Cell Sorting (FACS) buffer solution (1%FCS in PBS) and centrifuged further at 1500g for 5 minutes. Supernatants were poured off and the process repeated, completing 2 cell washes. Cells were resuspended in FACS buffer and counted before transferring to FACS polythene tubes (*BD Biosciences, UK*) at a count of 100,000 cells/tube in a volume of 100µL. Each tube containing 100,000 cells was labelled with a single antibody using a volume of 5µL and pipetted vigorously. The exception to this was CD29- FITC which was labelled using a volume of 10µL (due to the weaker FITC signal). A single tube was labelled with all 5 antibodies and a further 5 tubes were labelled with individual isotype controls for each antibody. In addition, an unlabelled tube was prepared and triplicates of all tubes were left in the dark for 30 minutes before removal. 2ml FACS buffer was then added to each tube and tubes centrifuged for 5 min at 1500g. The process was repeated completing 2 cell washes. Samples were then fixed by adding 200µL of 1% paraformaldehyde (PFA) to each tube. Following fixation, samples were tested within 48 hours.

4.3.2.5.2.3 Flow Cytometry Data Acquisition

Flow cytometry data acquisition was performed using a *CyAn™ ADP Analyzer* (*CY20130, Beckman Coulter, UK*). Cells were labelled as described and samples processed in triplicates from a single animal to develop a protocol for characterization. A minimum of 10,000 live events were collected per sample. Reporting channels corresponded to specific fluorochromes as per system set up; FITC in FL1, PE in FL2, PE-Cy7 in FL5, APC in FL8 and APC-Cy7 in FL9. Data was acquired and processed using *Summit Version 4.3 Software* (*Beckman Coulter, UK*). Samples were initially gated to exclude debris and isolate cell population on forward scatter (FSC)- side scatter (SSC) views. Unstained cells populations and isotype controls were used to detect auto fluorescence of cells and permit compensation in processing as required.

4.3.3 Primary Cell Culture Protocols for Positive Controls

In culturing BM MSC, demonstration of differentiation potential was a desired characteristic. The tri-potent nature of MSCs enables differentiation to adipocytes, chondrocytes and osteoblasts. In order to demonstrate this, positive control populations of cells were required. Primary cell culture protocols were used to provide populations of adipocytes and chondrocytes. Osteoblasts were purchased commercially but expanded as described below.

4.3.3.1 Primary Culture of Adipocytes

4.3.3.1.1 Adipose Tissue Retrieval and Cell Isolation

Pigs were placed under general anaesthetic as previously described. Adipose tissue was excised before death to prevent fat necrosis and maximise cell culture viability. A variety of sites were assessed for tissue sampling including posterior neck fat pad, abdominal wall and lower limb subcutaneous fat. Abdominal wall fat was found to be the preferred sampling site based on success of cultures and ease of sampling. Meticulous sterile technique was applied and a 10x 10cm area of skin was identified on the lateral upper abdominal wall superior to the umbilicus

and skin incised on 3 sides of the square leaving the lateral side intact. The flap of skin was then reflected laterally by sharp dissection. Once fully reflected, exposed fat was excised from the underlying abdominal wall muscle and placed immediately in PBS for preservation. For recovery procedures, adequate haemostasis was achieved before skin was closed using interrupted 2.0 prolene sutures (removed after 10 days).

Tissue was weighed and 8- 15g tissue was transferred to the culture hood and placed in a 50ml centrifuge tube containing 8ml Krebs-Ringer Bicarbonate (KRBH) Buffer supplemented with 200nm adenosine (see Appendix 4.5 for full details of all solutions used). Sterile scissors were inserted into tubes and used to cut the fat into a fine mince of approximately 2mm size pieces. 2ml of pre-prepared, warmed Type I collagenase in KRBH Buffer solution (20mg/ml) was pipetted into the tubes and caps replaced. Tubes were removed and secured lying horizontally in a temperature controlled (37.5^{o}C) mechanical agitator at 100RPM. After 1 hour, tubes were removed and had adopted a creamy like consistency. 20ml of KRBH buffer was then added to the individual tubes. Tubes were gently mixed by hand before being filtered over a 250µM pore sterile cap filter into another 50ml centrifuge tube. A further 30ml KRBH buffer was added to each tube and tubes centrifuged at 200g for 5 minutes. Resultant tubes demonstrated a distinct floating layer with an infranatant which was aspirated and discarded. A further 2 washes were performed using 40ml KRBH buffer, centrifuging tubes at 200g for 5 minutes each time. Tissue was then washed twice using DMEM1 by the same method as before, being resuspended at a cytocrit of 40%. Finally, 2ml of cell resuspension was transferred to a 5cm tissue culture dish using a 200µL pipette. Culture dishes were placed in the cell culture incubator for 1.5 hours before being removed and medium changed to DMEM2 which had a greater concentration of Bovine Serum Albumin than DMEM1.

4.3.3.1.2 Adipocyte Review and Characterisation

Cells were reviewed every 48 hours and medium changed with care as considerable lipid content was seen floating on the surface of the medium and required retention. Images using an inverted microscope were taken at intervals and after 14 days, cultures were terminated. Cells were stained with ORO in tandem with BM MSC using methods previously described above.

4.3.3.2 Primary Culture of Chondrocytes

Pigs were euthanized according to methods previously described and the hip region was prepared and sterile draped from the lower abdomen to the lower limb. A large post-mortem blade was used to dissect onto the hip joint and surrounding attachments divided to expose the femoral head. Using a scalpel, the surface of the femoral head was shaved off in small pieces 2-3mm in thickness and these collected in a sterile receptacle. Care was taken to avoid including mineralized cartilage and subchondral bone in shavings. Once the maximum amount of surface cartilage was removed, shavings were transferred to the culture hood.

Shavings were washed in a high glucose 'complete' medium composed of DMEM/F-12 and *GlutaMax™* in a 1:1 ratio supplemented with 10% FBS, 1mM sodium pyruvate and 2% antibiotic/antimycotic (*Invitrogen, Germany*). Chondrocytes were released by enzymatic digestion using Pronase in 1mg/ml concentration for 30 minutes followed by collagenase P in 1mg/ml concentration overnight under agitation (*Roche, UK*). Chondrocytes were recovered and resuspended in complete media before culturing at a density of 10,000 cells/cm^2.

4.3.3.3 Osteoblast Culture and Expansion

4.3.3.3.1 Initial Plating of Osteoblasts

Osteoblasts (OB) were used as positive controls in demonstrating differentiation of MSCs. Human OB were purchased (*Lonza, UK*) as porcine alternatives were commercially unavailable. Cells were expanded according to manufacturer's guidelines. In brief, cells were received on dry ice and

stored immediately in liquid nitrogen until ready for use. The cryovial containing the cells was warmed to liquid phase as previously described but resuspended within the vial using the commercially supplied medium. Cells were then counted and immediately plated at a density of 5000/cm^2 in an appropriately sized culture vessel (pre- warmed in incubator for 30 minutes and containing medium) before storage in incubator. Medium was changed 24 hours after plating and every 48 hours thereafter. Flasks were reviewed using similar methods previously described.

4.3.3.3.2 Trypsinization and Passaging of Cells

Protocols used for trypsinization were in line with manufacturer's guidelines. Cells were received with recommended reagents including OB growth medium, *'Bulletkit'* (containing necessary medium additives), trypsin-EDTA, trypsin neutralising solution (TNS) and HEPES-BSS. Medium was prepared by adding the necessary *Bulletkit* components to OB regeneration media before thawing cells. Once plated and cells reached 80% confluence, trypsinization was undertaken. 5ml of HEPES-BSS was used to rinse the flasks in place of PBS as previously described. Fluid was discarded and 2ml of Trypsin EDTA was added to flasks and gently rolled over the surface. Flasks were then removed from the hood and viewed under the inverted microscope under timer and the cell dissociation observed. If after 5 minutes, 90% of the cells were not released from adhesion, the flask was gently wrapped every 30 seconds until >90% cells were mobile. Flasks were then transferred to the culture hood and 4ml of TNS immediately added to terminate the reaction. Flask contents were aspirated and transferred to a 15ml centrifuge tube and the vessel rinsed with 2ml HEPES-BSS to collect residual cells. Flasks were examined under an inverted microscope to confirm cell removal. Released cells were centrifuged at 220g for 5 minutes. Supernatant was extracted leaving ≈200µL within the tube and the tube flicked to release the pellet. Cells were then resuspended in 2ml medium before counting using standard techniques. Cells were passaged and re-plated using densities described above. Cells were expanded until P5 before cryogenic storage using methods previously described.

4.3.3.3.3 Mineralization of Osteoblasts

As described in section 4.3.2.5.1.3, the differentiation of MSCs into OB was a goal of experimentation and Alizarin Red stain was used to demonstrate this. This stain demonstrates the presence of calcium deposits within cells which are formed following cell mineralization. To promote this, OB cultures were treated with mineralization media according to manufacturer's guidelines. Media were prepared by adding supplied aliquots of 200µM Hydrocortisone-21-hemisuccinate and 1M β-Glycerophosphate to OB growth media. This was performed in parallel with differentiation experiments as described above.

4.3.4 Adipose Derived MSC (AD MSC) Culture

In order to maximize MSC yield from animals and provide additional cells for seeding, culture of MSCs from adipose tissue was attempted as a secondary goal. Adipose tissue was retrieved as previously described in section 4.3.3.1.1.

4.3.4.1 Adipose Derived Tissue Plating for MSC Culture

Fat tissue was transferred to the culture hood and washed with an equal volume of PBS to remove blood. Tissue was then finely minced using a sterile blade and transferred to a T75 flask. 50ml of 1mg/ml Type I Collagenase (*Sigma-Aldrich,* UK) solution was then added to flask to begin enzymatic digestion. After 90 minutes, reaction was terminated by addition of 25ml of DMEM containing 10% FCS and 1% antibiotic-antimycotic. Flask contents were then divided equally into 2x 50ml centrifuge tubes and spun at 1200g for 10 minutes. Supernatants were discarded and the residual pellet containing stromal vascular fraction (SVF) was resuspended in 10ml of 160mM ammonium chloride for 10 minutes to lyse red blood cells. Tubes were then centrifuged at 1200g for 10 minutes again before addition of DMEM to resuspend pellets. Cell suspensions were filtered through a 100µM pore filter (*Sigma, UK*) into new tubes and made up to 10ml volume of DMEM. Tubes were then each plated in T75 flasks and placed in cell culture incubator. After 24

hours, flasks were washed with PBS and assessed. Flasks were trypsinized and passaged when 60 – 80% confluent and divided in a 1:2 ratio.

4.3.4.2 Characterisation of Adipose Derived MSC

Characterisation was undertaken by flow cytometry using methods listed in 4.3.2.5.2. The primary alteration in protocol was a single change in the antibody panel, in which CD31-FITC was used in place of CD29 in the FL1 channel. This change was initiated based on evidence in the literature (135, 136). Characterisation was more extensive than with BM MSC and cells lines from 3 different pigs, of passages P1-P3 were tested in triplicates, totalling 27 samples. Once required cells were in suspension, the protocol for cell fixation and labelling was identical to that described in section 4.3.2.5.2.

4.4 Results

4.4.1 Organoid Unit Isolation

A total of n=5 pigs underwent organoid unit isolation to optimize the protocol. Using the tissue from these 5 pigs, 12 isolations were performed using 5 different protocols, with the final protocol trialled 4 times. Optimum yield was gained using a collagenase concentration of 800U/ml. This was strongly supported by a failure to achieve any yield when a concentration of 300U/ml collagenase was used in 2 separate samples. Increased agitation speeds were associated with reduced yields and horizontal placement of flasks permitted better agitation (noted visually). OU were identified based on an estimated length (100- 300µm approx.), display of an epithelial rim and a mesenchymal core. A short maximum period of enzyme incubation was favoured (60 minutes) to reduce duration of exposure to damaging enzymatic reagents. A greater number of OU was isolated from the same tissue after 60 minutes when compared to 30 minutes suggesting prolonged collagenase digestion provided greater yield but digestions were not performed beyond 60 minutes. The mean number of OU isolated was 15,000 (+ 35,262) OU/ml obtained

from a length of 2.5cm ileum (6,000 OU/cm ileum). These results are summarised below in Table 4.2 and raw data visible in Appendix 4.6. An image of an OU can be noted in Fig 4.3.

Table 4-2 – Summary of Organoid Unit (OU) Isolation Yields

Collagenase Concentration (U/ml)	**Number of test isolations**	**Total Time of Enzymatic Digestion**	**Organoid Unit Yield (OU/ml)**	**OU Yield/cm ileum**
300	6	1 hour max.	3,125	1,250
800	6	1 hour max.	26,875	10,750
Combined	12	1 hour max.	15,000	6,000

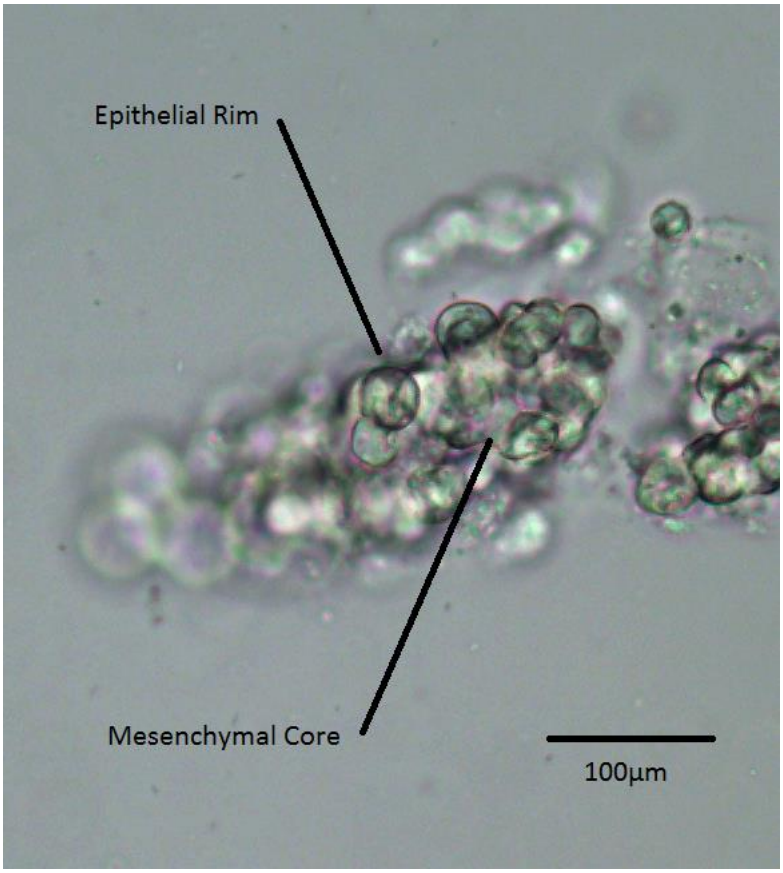

Figure 4-2- Image of an Organoid Unit:

Organoid unit with cluster of cells visible (20x magnification). A rim of epithelial cells can be noted surrounding a core of mesenchymal cells. Size of OU varied but the majority were between 100-200µm. Clusters of cells <50µm or >300µm were not counted but morphology was the most important factor in recognition.

4.4.2 Mesenchymal Stem Cell Culture from Bone Marrow

4.4.2.1 Bone Marrow Aspiration and MSC Culture

Bone marrow aspiration protocols were trialled at various sites detailed below in Table. 4.3. Aspirations were successful at the site of the femur only, with the iliac crest and sternum posing difficult access issues, with poor or no yield. The abdominal route was used on a single occasion in a pig with an already opened abdomen for alternative studies to assess yield from the femur before planning a cutaneous approach. Successful aspiration from a single incision with muscle and skin closure in a recovery model demonstrated the procedure as effective and reproducible.

The procedure was more difficult in older pigs, presumably secondary to greater resistance at the more calcified bony cortex and aspirate volumes tended to be reduced in these animals.

Table 4-3 - Sites of Trialled Porcine Bone Marrow Aspiration

Trials of bone marrow aspiration protocols in pigs revealed that the femur was the location which provided easy access with minimal morbidity demonstrated by successful recovery. Successful aspirations were performed on n>10 occasions and the protocol is now in continuous use within the research group.

Animal Number	**Aspiration Site**	**Outcome**
1	Iliac Crest	Failure to access and gain aspirate effectively
2	Sternum	Minimal aspirate gained (<5ml)
3	Femur (open abdominal approach)	Successful aspiration – unsuitable for recovery model
4	Femur (cutaneous approach)	Successful aspiration with good volumes (repeated with success = n>10)

All successful bone marrow aspirations that proceeded to culture over the duration of the project are detailed in Appendix 4.7. Aspirate volumes were variable and tended to be greater in younger animals, most likely reflecting the more active status of the bone marrow. The mean maximum volume of aspirate gained from the femoral approach was 38.1ml (n=8 and excluding recovery cases where volume was intentionally limited). Successful aspiration did not always proceed to a successful culture and a single total failure was noted and 1 initial culture plating failed to progress after P1. Initial aspiration procedures utilised 1ml heparin anticoagulant (1000U/ml) in a 15ml aspirate volume to prevent anticoagulation but samples frequently developed large clots so this was altered to 5ml (5000U/ml) in 15ml volume. All aspiration volumes listed exclude anticoagulant volume.

Mononuclear population of crude bone marrow aspirate was calculated as 32.9×10^6/ml. Cells were initially plated at a density of 2.2×10^6/ cm^2 (n=1). This led to successful cultures in all but 2 cases (see Appendix 4.7). Counts from a single confluent T75 flask revealed a population of approximately 1×10^6 which when split in a 1:2 ratio, equated to an approximate seeding density

of 6,666/cm^2. Cultures progressing to P3 had an approximate mean total yield of cells of 9.0 x 10^6 of cells for use in further seeding.

Typical images from successful MSC cultures are visible below in Fig. 4.3. Following initial plating and washing of cells, populations appeared scant, with cells free floating, non-adherent and displaying a spherical morphology (Fig 4.3a). Following a period of 3 days, cells began to change morphology to a more elongated shape (Fig. 4.3b). By day 7, initially plated cells had typically reached confluence, displaying a flattened, spindle like, elongated appearance and clear adherence to flask base. Percentages of confluence were reduced in initial plating and due to paucity of cells, confluent 'islands' were present with spaces between them. As cultures continued, those of greater passage displayed greater tendency to adhere more rapidly and cells reached confluence within 2-5 days. An image of a confluent MSC before trypsinization can be seen in Fig. 4.4.

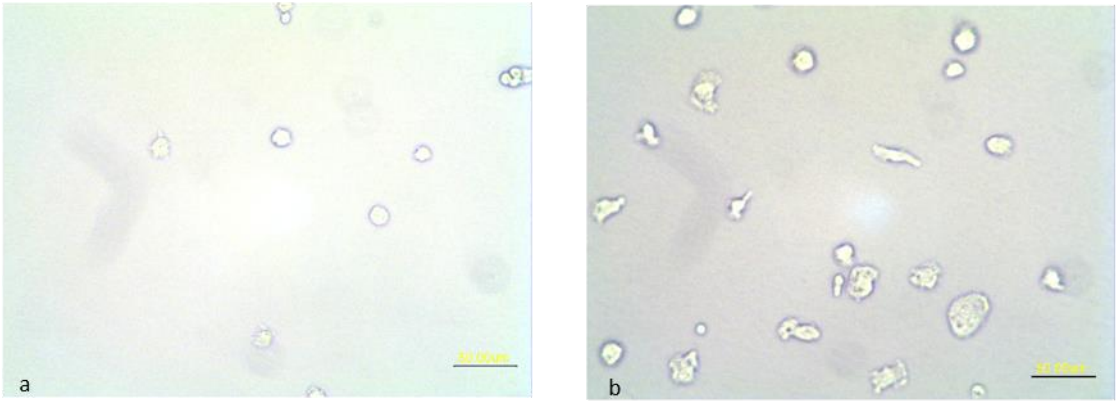

Figure 4-3 - Appearances of initial plated MSCs:

(a) *Cells plated at day 0 demonstrated a scant appearance and rounded morphology,* ***(b)*** *after 3 days, cells began flatten and develop spindle like morphology. (Scale bars correspond to 50µm length).*

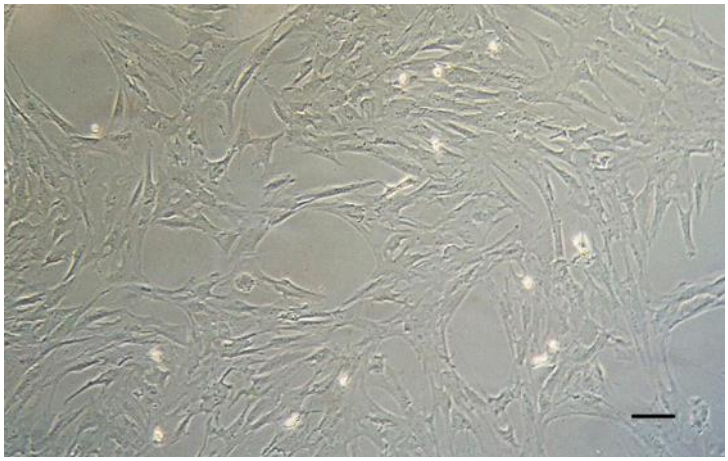

Figure 4-4 - Confluent MSCs at day 7 before trypsinization and passage:

Note cells display a flattened, spindle like appearance and are closely associated. (Scale bar corresponds to length of 50µm).

4.4.2.2 Bone Marrow MSC Differentiation

4.4.2.2.1 Adipocyte Differentiation

To demonstrate adipocyte differentiation, adipocytes from primary culture were used as a control population (see section 4.3.4.1). Initial plated adipocyte cell fluid floated in flasks due to lipid rich content and formed large spherical globules several millimetres in size (Fig. 4.5a). Following several days, vesicles migrated to the flask base and consisted of large lipid vesicles (Fig. 4.5b). Cells then adhered to the flask base, demonstrating multiple small lipid vesicles before forming confluent adherent colonies (Fig. 4.5c). Oil red O staining confirmed cells as adipocytes for the purposes of controls (Fig. 4.5 d,e).

Following protocols described, MSCs were subjected to induction of differentiation into adipocytes using specific media. Oil red O staining in 12 well plates at day 14 revealed demonstration of positive oil red O staining of lipid vesicles in cells supporting the process of differentiation into adipocytes. This is demonstrated in Fig. 4.6 below.

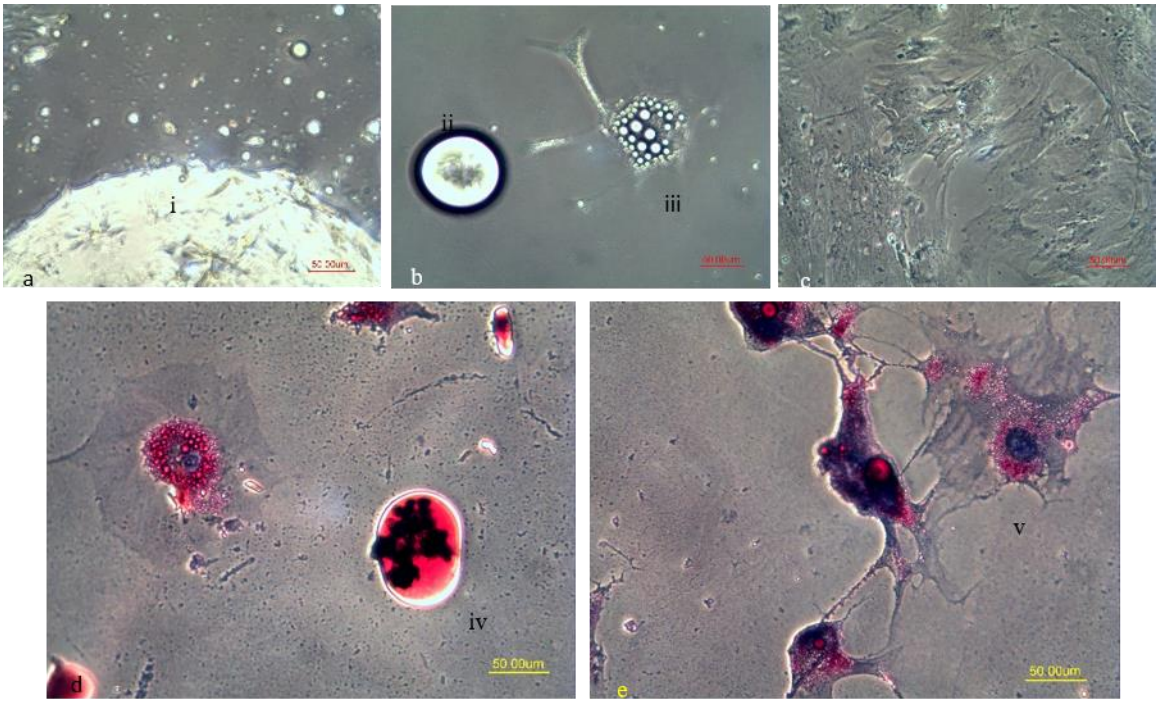

Figure 4-5 - Culture and Oil Red O Staining of Porcine Adipocytes:

(a) *Initial plating of adipose tissue extract formed large floating lipid vesicles at 'i',* ***(b)*** *after several days, lipid vesicles began to adhere to flask floor at 'ii' before attaching and lipid vesicle dispersing at 'iii',* ***(c)*** *cells formed organised culture adherent to flask after 5 days.* ***(d)*** Oil red O staining demonstrated large lipid vesicles brightly staining at 'iv', **(e)** smaller vesicles within adherent cells at 'v'.

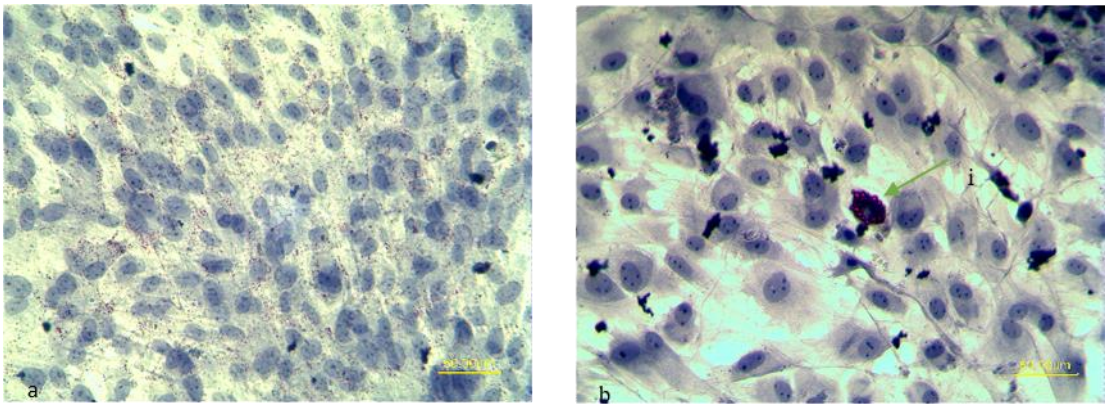

Figure 4-6 - Oil Red O Staining of Bone Marrow MSCs demonstrating Adipocyte Differentiation:

(a) *Negative control MSCs plated with standard growth medium after 14 days,* ***(b)*** *MSC cells supplied with differentiation medium with formation of lipid vesicles positively staining for oil red O at 'i'.(Scale bar corresponds to length of 50µm)*

4.4.2.2.2 Chondrocyte Differentiation

Micro-masses remained stable in structure during culture and staining process. Fig. 4.7 demonstrates staining results with images of a positive control chondrocyte well and MSC seeded wells with and without chondrogenic differentiation medium. Visual assessment demonstrates the bright blue staining of the MSCs with differentiation medium (growth medium supplemented with TGFβ), which appears similar to that of chondrocytes indicating GAG ECM formation and therefore chondrogenic differentiation. By contrast the MSC population without differentiation medium stained only weakly blue. Alcian Blue/DNA quantification is represented in Fig. 4.8. Data represents quantification in n=2 samples. A significant difference between expression of GAG proteins between MSC supplemented with differentiation medium and that without is apparent. Notably differentiated MSCs displayed similar quantities of GAG when compared with control chondrocytes.

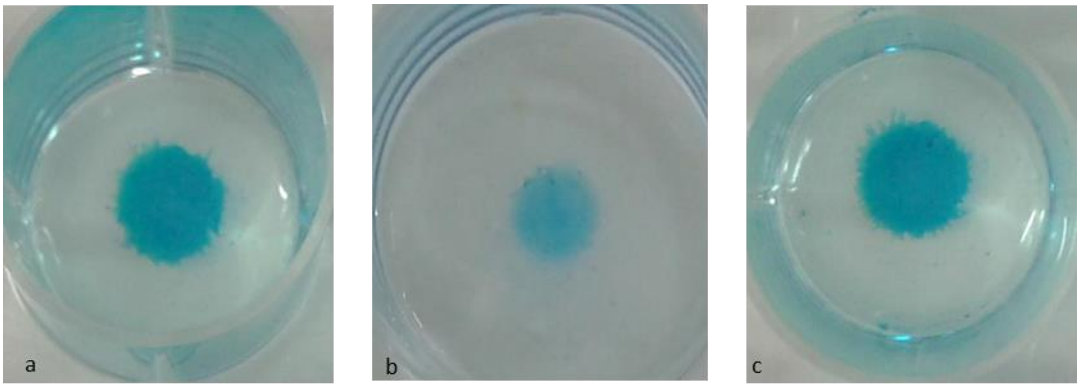

Figure 4-7 – Alcian Blue Staining of MSC demonstrating Chondrocyte Differentiation:

(a) *Chondrocytes seeded served as positive controls and demonstrate bright staining as anticipated,* ***(b)*** *MSC seeded in micro mass and supplemented with standard αMEM proliferation medium demonstrate weak staining with Alcian blue when compared to,* ***(c)*** *MSCs supplemented with chondrogenic differentiation medium displaying staining similar to that of positive control chondrocytes*

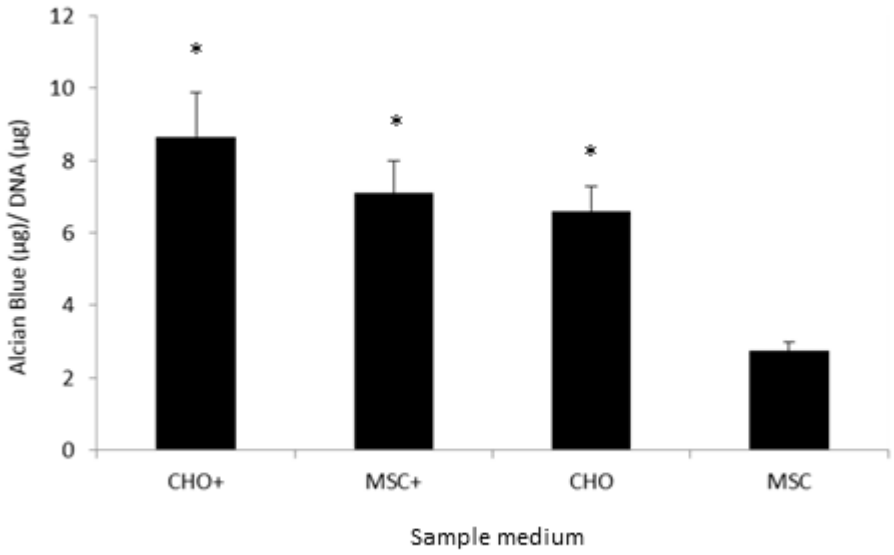

Figure 4-8 - Alcian Blue Quantification as GAG/DNA concentration by Colorimetry:

Chondrocyte micro masses (n=3 per medium type) supplemented with differentiation medium and TGFβ are denoted 'CHO+' and those without as 'CHO'. MSCs supplemented in growth media with TGFβ (MSC+) demonstrated increased quantity of Alcian Blue especially when compared to MSC without and levels were comparable to CHO. Error bars denote standard error. Statistical analysis performed using one-way ANOVA demonstrated significant difference ($p \leq 0.05$) of all chondrocyte cell groups and MSCs in differentiation medium when compared to MSC alone (negative controls).

4.4.2.2.3 Osteogenic Differentiation

Alkaline Phosphatase (ALP) staining demonstrated positive staining of MSCs as illustrated in Fig. 4.9. Negative control MSCs without osteogenic differentiation medium did not stain positively for ALP in contrast to MSCs cultured with osteogenic differentiation medium containing dexamethasone, β glycerophosphate and ascorbic acid (Fig. 4.9b). In contrast to other differentiation experiments, results proved difficult to reproduce and Alizarin red staining to assess for mineralization failed to demonstrate any positive staining in MSCs or even control populations of OB treated with appropriate mineralization medium.

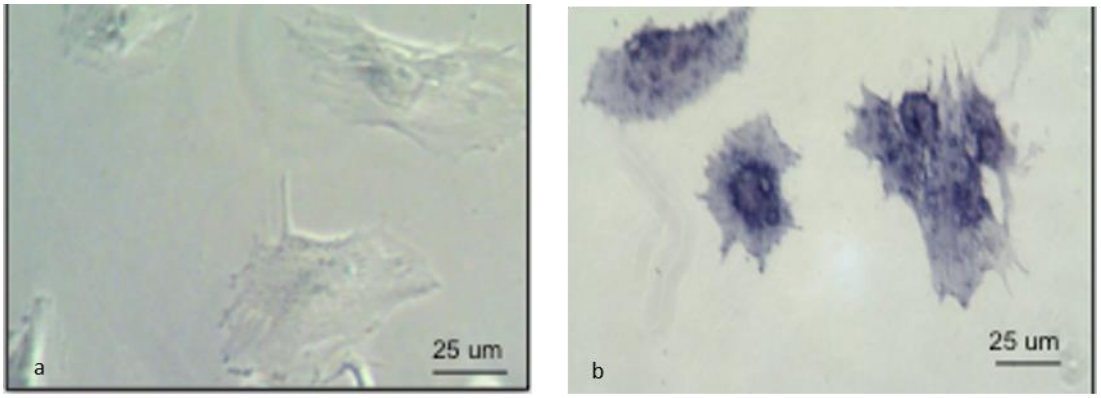

Figure 4-9 – Alkaline Phosphatase Staining of MSCs demonstrating Osteoblastic Differentiation:

(a) *Staining of MSCs cultured with standard αMEM demonstrating negative staining,* ***(b)*** *Positive staining of MSC cultured with osteogenic differentiation medium with deep blue staining consistent with increased ALP activity.*

4.4.2.2.4 Bone Marrow derived MSC Characterisation by Flow Cytometry

The population of tested MSCs was successfully identified using flow cytometry. The mean number of acquisitions per sample was 17,808 at an average rate of 406 events per second (eps) for single antibody labelling. Where cell populations were labelled with all antibodies, the number of acquisitions was significantly greater at 61,550 (280 eps). A single large cluster of cells was identified on FSC-SCC and gated to target 71.2% of acquisitions identified as the cell population of interest (Fig. 4.10a). This population was further gated to demonstrate 87.53% of acquisitions (Fig. 4.10b). Cells demonstrated positive fluorescence for CD29-FITC, CD44-RPE and CD90-APC. Weaker 'negative' signals were detected for CD34-PE-Cy7 and CD14-APC-Cy7. These results are illustrated in histogram and plot data illustrated in Fig. 4.11 - 4.12.

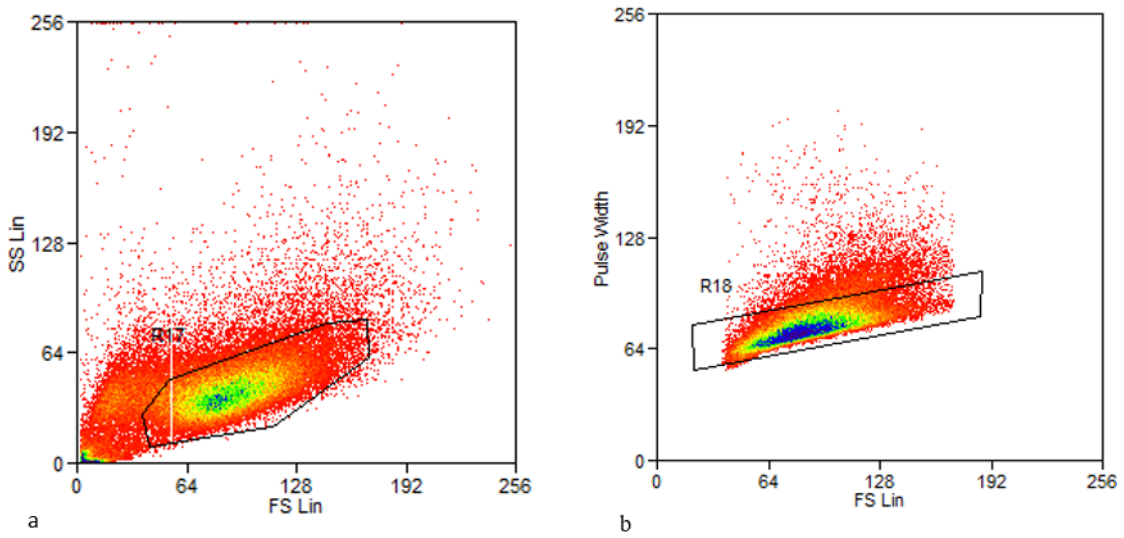

Figure 4-10 – Flow Cytometry Detection of Bone Marrow MSC Population:

(a) *FSC-SSC plot demonstrating single large population of cells with high density area gated at R17 containing 71.1% of acquisitions, the remainder of acquisitions likely represented debris and alternate cell types.* ***(b)*** *FCS-SSC plot of R17 gated cells further gated to R18 isolate cells of interest representing 87.53% of acquisitions*

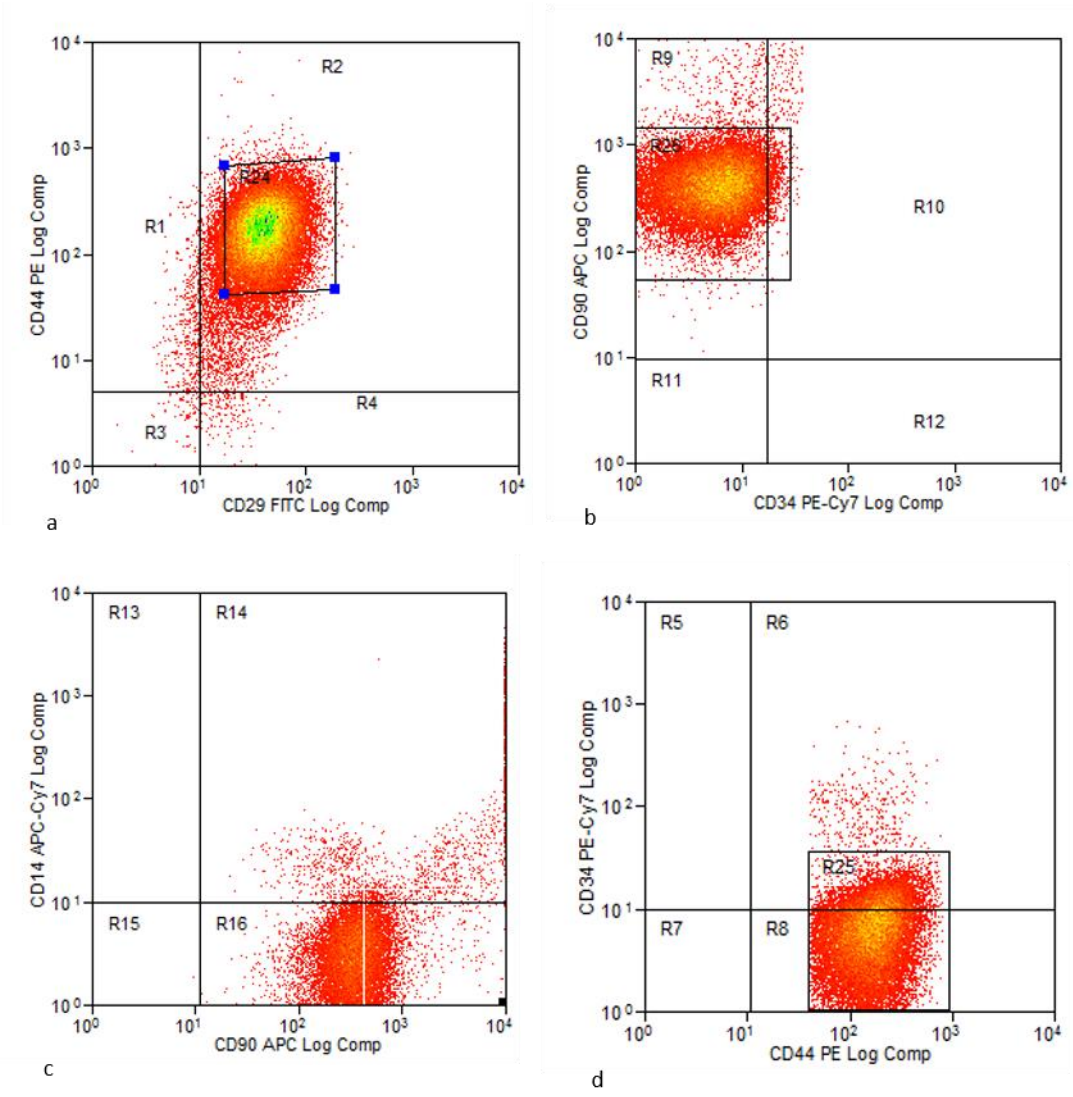

Figure 4-11– Flow Cytometry Plots of Bone Marrow MSCs:

(a) *Cells labelled with CD29-FTIC and CD44-RPE demonstrate positive fluorescence as anticipated in keeping with demonstration of MSC surface markers located in region (R) 2,* ***(b)*** *low emission of fluorescence of cells labelled with CD34-PE-Cy7 but high signal of CD90-APC labelled cells demonstrating cells in R9, cells further gated into R25.* ***(c)*** *Low signal of cells labelled with CD14-APC-Cy7 when compared to CD90-APC which emits high signal as previously demonstrated,* ***(d)*** *cells labelled with CD34-PE-Cy7 plotted against CD44-PE labelled cells with application of gate applied in 4.10b*

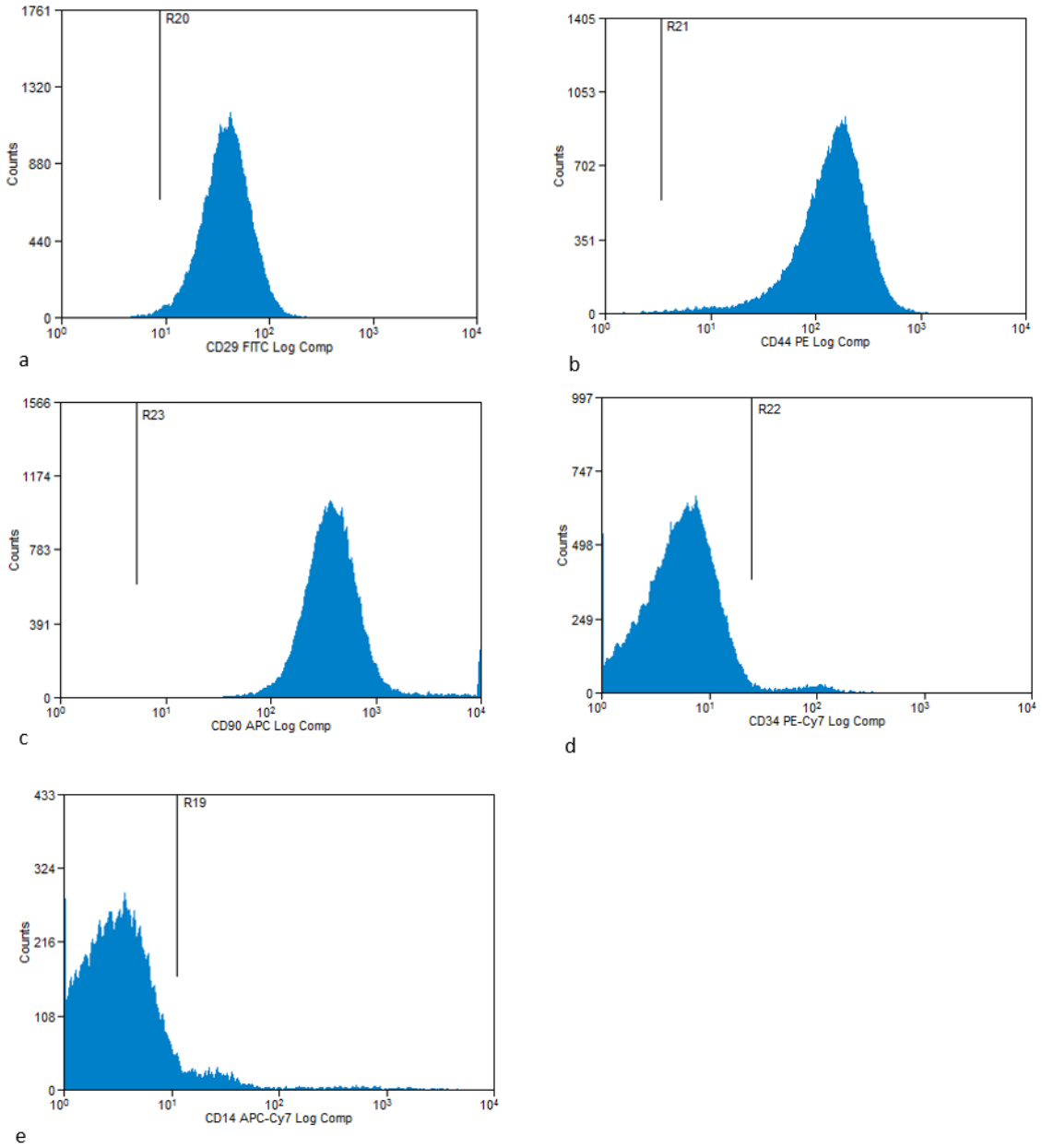

Figure 4-12– Flow Cytometry Histograms of Bone Marrow MSCs:

(a) *Cell counts of CD29-FITC labelled cells demonstrating predominance of surface marker, similar results are demonstrated in the case of* ***(b)*** *CD44-PE labelled cells and* ***(c)*** *CD90-APC labelled cells.* ***(d)*** *Cells labelled with CD34-PE-Cy7 emitted low signals indicating the relative absence of this surface marker and* ***(e)*** *CD-14-APC-Cy7 labelled cells emitted the lowest signal of all.*

4.4.3 Mesenchymal Stromal Cell Culture from Adipose Tissue

Culture of MSCs from adipose tissue was successful. The protocol remains in use within our research group to date. Mononuclear populations isolated from crude fat adhered to flaks within 7 days and displayed a similar appearance to bone marrow derived MSCs though cells appeared smaller. Cells were passaged to P5 and a confluent flask typically yielded 2-4million cells. The protocol was successfully utilized in n>10 cultures.

4.4.3.1 Characterisation of Adipose Derived MSC by Flow Cytometry

Flow cytometry experiments were successful but cell samples contained significant quantities of debris. Gating strategies were effective in isolating the population of interest but in an example initial FSC-SSC plot shown in Fig. 4.13, the R17 gated population only accounts for 21.1% of the cell population. Results were similar in all 27 samples tested with low proportions of cells falling within the gated area of interest despite >100,000 acquisitions in numerous samples. Cells demonstrated positive fluorescence for CD31-FITC, CD44-RPE and CD90-APC. Weaker 'negative' signals were detected for CD34-PE-Cy7 and CD14-APC-Cy7. Sample results demonstrating this are below in plot data and histograms illustrated in Fig. 4.14 - 4.15.

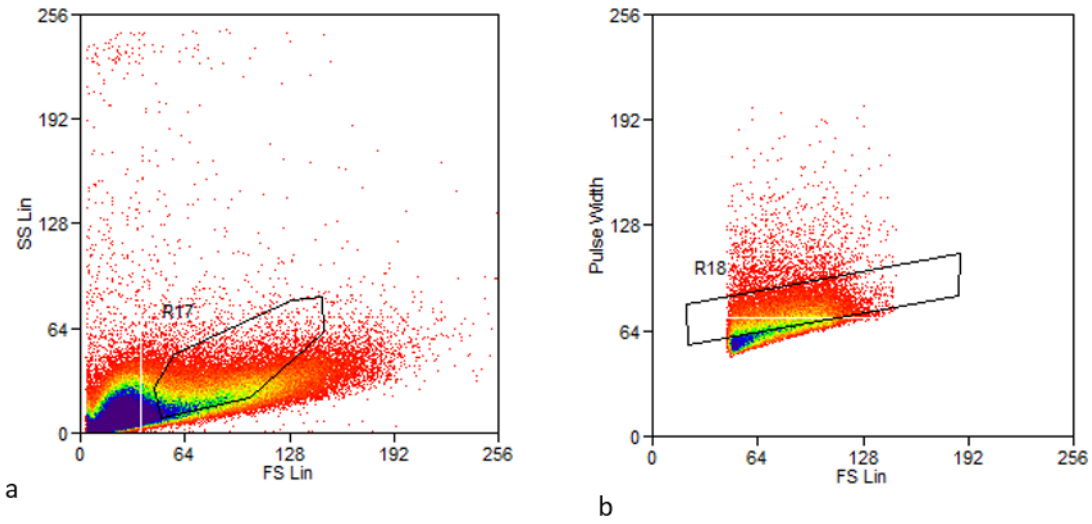

Figure 4-13 – Flow Cytometry Detection of Adipose Derived MSC Population:

(a) *Initial FSC- SSC plot demonstrating considerable debris in sample. Population gates (R17) to exclude debris only accounted for 21.1% of cells.* ***(b)*** *Gated population of cells from R17 further gated to isolate cell population as R18.*

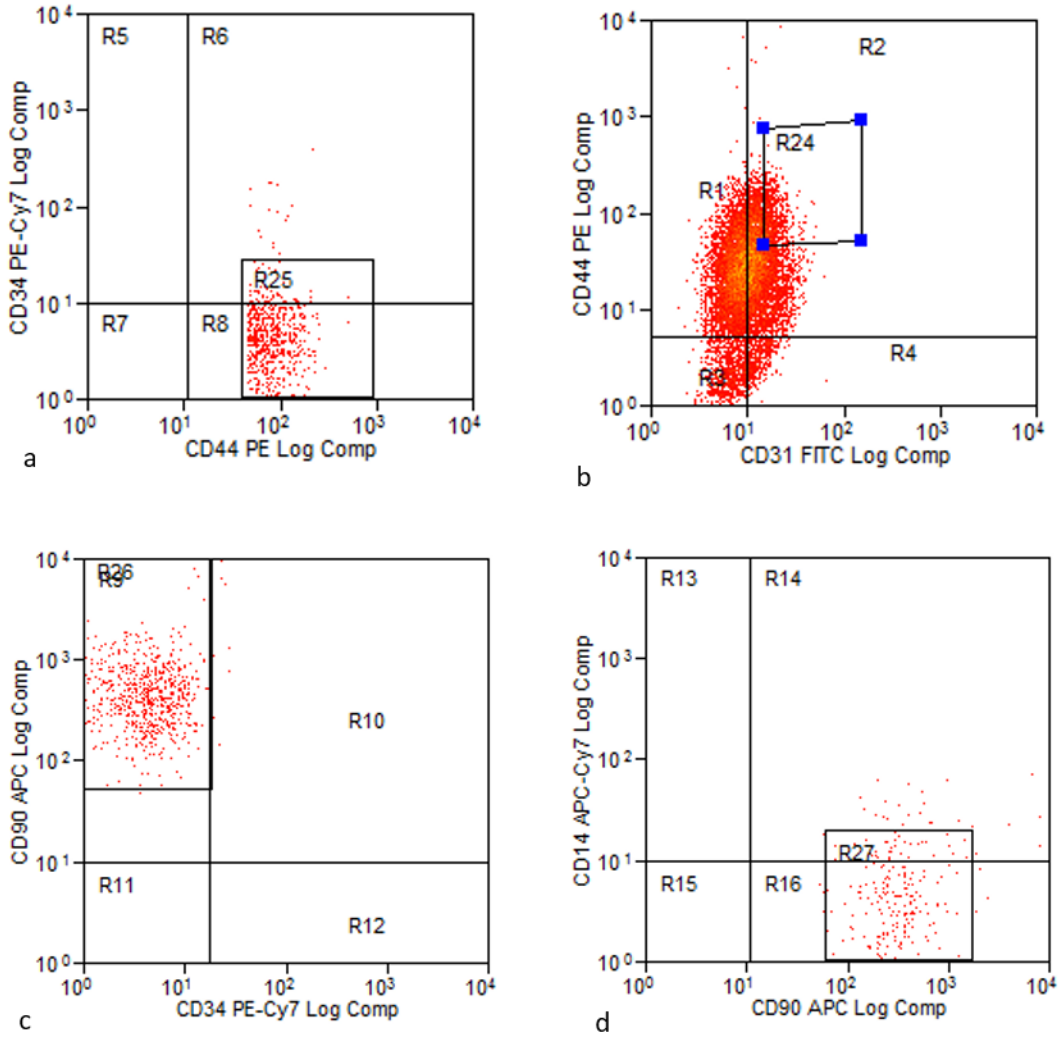

Figure 4-14 - Flow Cytometry Plots of Adipose Derived MSCs:

(a) *Low emission of fluorescence of cells labelled with CD34-PE-Cy7 but high signal of CD44-PE labelled cells demonstrating cells in R8, cells further gated into R25* ***(b)*** *Cells labelled with CD31-FITC and CD44-RPE demonstrate positive fluorescence as anticipated in keeping with demonstration of MSC surface markers but population is not discrete and crosses regions.* ***(c)*** *Low signal of cells labelled with CD34-PE-Cy7 when compared to CD90-APC which emits high signal,* ***(d)*** *cells labelled with CD14-APC-Cy7 plotted against CD90-APC labelled cells demonstrating low signal of CD14-APC-Cy7*

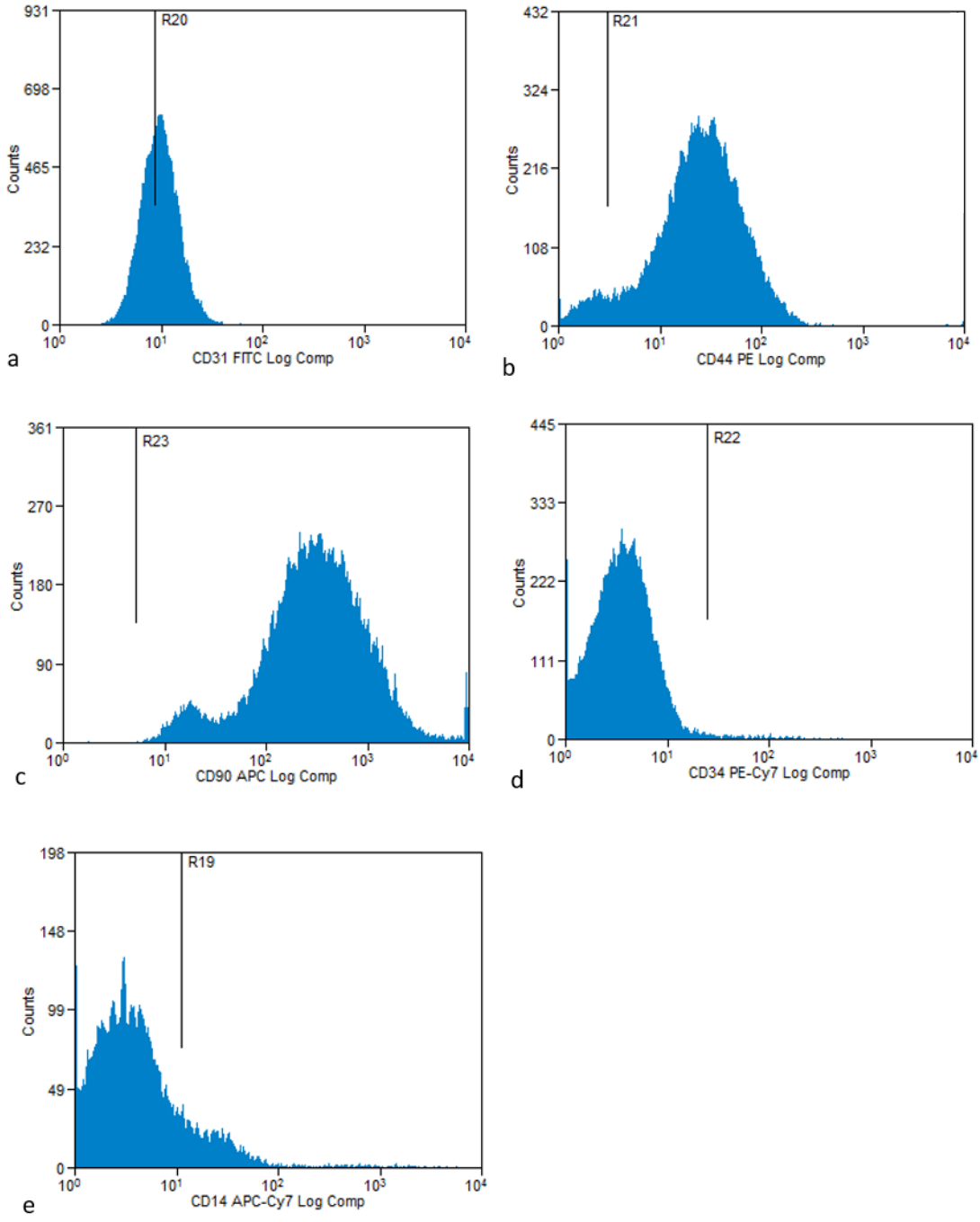

Figure 4-15 - Flow Cytometry Histograms of Adipose Derived MSCs:

(a) *Cell counts of CD31-FITC labelled cells demonstrating predominance of the surface marker, similar results are demonstrated in the case of* ***(b)*** *CD44-PE labelled cells and* ***(c)*** *CD90-APC labelled cells.* ***(d)*** *Cells labelled with CD34-PE-Cy7 emitted low signals indicating the relative absence of this surface marker and* ***(e)*** *CD-14-APC-Cy7 labelled cells emitted the lowest signal of all.*

4.5 Discussion

Organoid units were successfully isolated from porcine intestine in experiments completed in accordance with aims. Historically, the majority of studies in TEI intestine have isolated OU in small animal models (72, 74, 121, 122, 137-139), with more recent studies utilizing large animals (79) and human OU (140). Despite the graduation from small animal models to human studies, the process of OU isolation and use for TEI by many groups has changed very little and these methods were used as a guide to developing a protocol for isolation of OU in pigs. With the exception of Evans. et al's landmark study in 1992, little is documented precisely in methods of OU isolation within the literature. For this reason, numerous variables were adjusted in experiments.

The question of the correct site of intestine from which to isolate OU remains unanswered. Rat experiments typically used full lengths of intestine from which to obtain OU (72, 74, 137). Thus, OU are isolated from the entire jejunum and ileum. The specific location where the majority of OU arise from in these experiments is therefore unknown. Literature suggests that OU can be isolated from any part of the small or large intestine (50) but this poses a difficult question in large animals as to where the optimum location is. The practical challenges of isolating OU from the entire length of intestine in a large animal are substantial and costly. In Sala et al's work, extensive enterectomy was not in keeping with the animal model and OU were isolated from a 2x 4cm piece of stomach tissue (79). In the work of Levin et al., OU were isolated from human small bowel resections peri-operatively which were due for discarding so obtaining more tissue was a clinical impossibility (140). The optimum region of intestine for OU culture was not the purpose of this study, and therefore the question was not addressed. The ileum was chosen due to its established physiological regenerative properties and the location maintained in all experiments. Despite this, it is not known if yield of OU could be significantly improved by utilizing a different area of small intestine and this should be addressed specifically if the model is to be developed.

Also worthy of discussion is the appropriate age of animals for optimum yield. Evans et al. utilised suckling rats for OU culture and Sala et al. sacrificed 6 week old piglets. As with all cell culture work, younger animals have a greater proportion of stem progenitors and lead to a typically more productive culture with greater yield. Pigs of significantly differing ages were not used for OU culture in these experiments (all pigs were aged between 4-10 months). It is likely that the optimum age for OU isolation and expansion would be species specific, as growth rates vary between large animals significantly.

The specific process of OU release from crude tissue was varied within experiments. Mechanical tissue chopping tended to pulp tissue and made for difficult handling, with no clear improvement in yield and was therefore avoided. OU were found to be fragile at every step of the isolation process and it was postulated that excessive chopping or agitation at any phase was more likely to reduce yield by disassociating OU. This was supported by data revealing worse or no yield of OU when agitator speeds were increased above 100rpm. Previous protocols have used even lower agitation speeds (80rpm) but these were not trialled (72).

Evans et al. originally described a protocol with a collagenase concentration of 300U/ml to release OU from tissue and most protocols within the literature have typically used 300 or 800U/ml. 300U/ml collagenase was used in pig experiments described above but this produced poor yields and in 2 cases, none at all. This was likely due to insufficient enzymatic activity at concentrations of 300U/ml to release OU from tissue. There may be a species specific importance of this, as collagenase concentration of 300U/ml has been reported previously in use with small animal models only and more recent studies in pigs and humans have used concentrations of 800U/ml exclusively (140-142). The dense muscle layers in porcine and human intestine may be responsible for this increased collagenase requirement. It is noteworthy however that all these latter studies have originated from the same research group, thus suggesting that this is an established protocol within the group. As a result, a collagenase concentration of 800U/ml was

favoured in these studies but concentrations above this were not tested and definitive use of these are not documented in the literature. This would definitely be necessary to develop the model as there may be room for optimization of the use of enzymes to increase OU yield.

The duration of enzyme digestion to yield OU differed significantly from that documented in the literature. Sala et al. utilized a period of 30 minutes to permit release of OU and in more recent human studies, a period of 20 minutes was sufficient (79, 141). In addition, OU have been released from rat tissue successively using collagenase digestion periods ranging from 20 – 40 minutes but often repeated several times over (72, 137, 139). These works were typically reported from TEI studies in which a short period of digestion was favoured to permit rapid implantation and maintain viability of OU. Whilst experimental work in this chapter did not assess OU viability, a short digestion period was sought for similar reasons. This was not effective however and yield of OU was demonstrated to be less from the same tissue after 30 minutes than after 60 in every sample. In conjunction with this, reported yields of OU were considerably less than those documented in previous studies. Genuine reporting of OU yields per weight or length of tissue is scarce as studies have tended to report the protocol and then seeding densities in scaffolds without commenting on effectiveness of the process. Typical seeding densities have been reported as approximately 100,000 OU per 1cm scaffold (122, 138) and as low as 60,000 OU per scaffold in rats (137). Work from this chapter suggested that at 800U/ml collagenase concentration, 6,000 OU per centimetre of pig ileum could be procured. This suggests that a simple 10cm sample of intestinal tissue would provide enough OU to parallel experiments above but this amount of OU was only used to populate scaffolds of 1cm length which typically had a lumen of a few millimetres. The scale up would therefore require at least 10 times as much intestinal tissue to populate even a small scaffold in an equivalent large animal size. Even for allowing optimization in protocol, improvement in yield and use of an optimum aged animal, the use of crude OU for seeding large animal scaffolds currently seems far from reach.

OU from porcine intestine were classified as OU when 100- 300µM in size and displaying the characteristic appearance of an epithelial rim and mesenchymal core. In reality, considerable variation in the shape and size of cell clusters was noted at microscopy and this may be the reason for differing reported counts of OU within the literature. Certain cell clusters may be larger, yet display the appropriate structure and the same may be true of smaller clusters. It is not clear whether this has an effect on regenerative properties and the issue has not been addressed nor would it be easy to do so given the difficulty in separating these cell clusters. With this variation comes the added variation in composition. With the exception of the work of Clevers et al., OU essentially remain a poorly defined cell population that consist of a mixture of epithelial and mesenchymal cells when in solution and characterization remains difficult. As a result of this, where OU have been used for scaffold seeding successfully, it is difficult to know what has been responsible for intestinal regeneration.

The term 'intestinal regeneration' has been used frequently but specific consideration of what this constitutes is necessary. Choi et al. demonstrated the formation of cysts lined by mucosa of columnar cells, goblet cells and Paneth cells in 1997. This finding was reproduced in TEI studies using similar scaffolds within the group. In 2002, Grikscheit et al. demonstrated the presence of 'function' of tissue engineered colon in similar scaffolds by assessing transit times and serum electrolytes. TEI has also been shown to demonstrate clinical benefit in an animal study where it was used to promote recovery after bowel resection (139). Given the heterogeneous nature of OU in suspension described above, it is impossible to know specifically from where these benefits arose – the regenerative properties of the epithelium, the mesenchyme or a combination of both.

Minimal characterisation of OU was undertaken for specific reasons related to the above. Demonstration of composition of a single OU is unlikely to correspond closely to that of another based on microscopy from this work. The presence of intestinal crypt base columnar stem cells (which may stain positively for Lgr5) in a single OU does not guarantee the presence or absence in

another. Presuming some cell viability within a single OU, the presence of dead cells within the OU does not indicate that the important population is dead. Finally, details of specific composition of OU in suspension does not account for what mechanisms have led to intestinal regeneration should seeding experiments be successful.

Since beginning this experimental work, the science of OU has developed towards in vitro expansion which was previously not deemed feasible. Sato and Clevers et al. have postulated that using current technological cell culture methods, a single tissue biopsy (even obtained endoscopically) could be used to culture and expand intestinal epithelial cells, before stimulation to form self-organising 'mini-gut structures', which could be maintained in culture in- vitro for long periods (75). The authors then propose that such structures could be used for experimental, diagnostic or therapeutic purposes. The evidence to support this is clear, as cell sorting of Lgr5 positive cells, followed by expansion and development of mini gut structures has been demonstrated. As the in- vitro approach appears to have potential for better isolation, characterization and expansion before any use in TEI, it seems likely that this method will supersede those previously used involving suspensions directly from in vivo to seeding.

Mesenchymal stem cells (MSC) were successfully isolated and cultured as according to aims. The use of MSCs in research and clinical practice has rapidly increased and, together with rationale for use in TEI, this has been discussed in section 4.1.4. MSCs have been demonstrated to be: successfully isolated from an autologous model, expanded easily in vitro to large populations, involved in intestinal regeneration and contributory to TEI in a human model (47). The ability to use MSCs in any TEI studies is therefore of potentially great utility.

Bone marrow aspiration is a well-described clinical procedure that has been used successfully for years in diagnostics and bone marrow transplantation in haematology and other fields. Transferring this for use in a porcine model proved more challenging than anticipated. The standard site of aspiration in humans is the iliac crest and is described in the literature as a

standard approach for use in pigs (143). The iliac crest proved difficult to access and aspirate from successfully in pigs, perhaps due its documented use only in mini-pigs which are smaller and likely with softer bone cortices. The femoral approach has been described and was found to be successful (144). Aspirate volumes gained in experiments in this work were in keeping with those previously described (15ml in mini-pigs), although large volumes of heparin anticoagulant were essential at a concentration of 5000U heparin/ml in a 1:3 (heparin: aspirate) to prevent clots. This was not in keeping with published protocols indicating lower volumes of less concentrated heparin would be sufficient. Once optimized, bone marrow aspiration models were successfully used in recovery models with no reported morbidity.

Ficoll-Paque separation of bone marrow aspirates required considerable optimisation. The fat content of bone marrow led to a much less clear defining of layers on gradient separation when compared to whole peripheral blood and adequate completion of this stage was essential to obtain a definitive mononuclear cell population. Most protocols described involved centrifugation of Ficoll-Paque layered bone marrow aspirates immediately following layering (144) but using this method, layers were not distinct and a period of 30 minutes standing before centrifugation much improved results, presumably by allowing red cells to gradient separate naturally rather than by centripetal force.

Strategy to initial plating was sufficient but review of methods suggests further optimization of the process is required. Once initial values of mixed mononuclear cell populations were obtained, early aspirates were plated at high density to permit maximum chance of adherence and successful culture of cells. The crude strategy of plating mononuclear cell population from a 15ml bone marrow aspirate to a corresponding single T75 flask was successful. Retrospective cell counts demonstrated an approximate mononuclear cell population of 32.9 x 10^6/ml and plating densities typically equated to 2.2 x 10^6/cm^2. This value is significantly higher than that reported and a review of plating densities of MSC suggests that 1 x 10^6 is at the upper end of reported

plating densities (145). It should be noted however, that this paper by Ikebe et al. reviews the use of human MSCs in clinical trials and may not be applicable to pigs. Additionally, it is worth noting that the same review reports passage seeding densities within the literature and the approximate seeding density used in expansion of cultures (6,666 cells/cm^2), was in keeping with values similar to those in human studies. Details of porcine mononuclear population plating for MSC growth is scarce, even in studies specifically reporting the culture of MSCs (144). Typically, a balance must be struck between optimum culture seeding density for expansion and any practical issues that may be present in expanding large numbers of flasks. Despite this, there is significant evidence in general cell culture work to suggest that a plating density that is too great may have as detrimental an effect on cell expansion as one that is too low (145). More robust data for cell culture plating and seeding densities will enable possible improvements to the protocol that may produce a valuable increase in cell culture yields.

Plastic adherence of MSCs is easily demonstrated during the process of culture as non-adherent cells perish. Other important characteristics of MSCs were demonstrated within limits such as differentiation into osteoblasts, adipocytes and chondrocytes. This remains essential as fibroblasts share many of the same properties as MSC and are equally plastic adherent and likely to be found in crude bone marrow aspirate mononuclear cell populations. The most important difference between fibroblasts and MSCs is that fibroblasts are considered terminally differentiated cells and therefore do not demonstrate the same differentiation potential (146). Passage 2 cells were favoured for differentiation experiments as with progressive passages, phenotype and differentiation potential are altered. The evidence to support when this occurs is not conclusive but studies have demonstrated no change in ability to differentiate into all cell types by MSC in passages well past P5 (136, 144) therefore if MSCs could not differentiate adequately at P2 it is unlikely they had capacity to at all.

Adipocyte differentiation was demonstrated with lipid vesicles staining clearly on Oil Red O. Notably, demonstration of differentiation was not blanketed across the entire cell population in view, and some but not all cells stained positive. Primary culture of adipocytes was successful and demonstrated clear positive control staining. Micro-mass (MM) MSC cultures successfully demonstrated increased Alcian Blue staining and when supplemented with TGFβ this level was increased both visually and on quantitative analysis. Primary chondrocyte MM cultures served as adequate controls and cultures were successful. Differentiation of MSCs into osteoblasts was demonstrated by alkaline phosphatase staining successfully but Alizarin Red staining failed both in MSCs and control human OB samples purchased commercially. The failure to stain even positive controls in this case suggests a failure in experimental protocol and little can be gained from this limited study alone. In all cases of differentiation experiments, staining results demonstrated a population of cells that differentiated amongst those that did not. True MSCs possess 'tri-potent' ability but studies have shown that there is no discrete population of tri-potent cells existing alone and they reside with much less frequency among bi-potent and uni-potent cells (147, 148). Despite this, the scarce staining of cells for differentiation and failure of Alizarin Red staining protocol, in conjunction with the difficulty in reproducing results suggests further optimization of protocols may be beneficial.

Flow cytometry testing of bone marrow MSCs was successful in identifying cell populations, confirming phenotype and demonstrating presence/ absence of anticipated surface markers. Results demonstrated a clear positive signal for CD90, CD29 and CD44 but the absence of the haematopoietic markers CD34 and CD14. These results support the phenotype of a MSC population. Various studies have characterised porcine MSCs but there is no accepted convention as to demonstration of surface markers and this should be borne in mind when discussing results. Sato et al. used purified or directly conjugated antibodies to demonstrate the presence of surface CD44, SLA-class I, CD90 and CD29 but absence of CD31 (133). Comite et al. demonstrated

positivity for CD90, CD105 and CD29 but <5% positivity for CD45 (134). Noort et al. demonstrated similar results with positive staining for CD90, CD44 and CD29 but negative staining for CD34 and CD14. These studies were used as a basis to form the antibody panel but further limitations should be noted in the available antibodies for testing, as antibodies were not all species specific and the potential for cross reactivity exists which was not assessed for. Despite this, the results are limited only by the limited number of cell cultures used and form the basis of a protocol. More rigorous testing with multiple cultures is necessary for further development of the protocol.

Adipose derived MSCs were sought to supplement the quantity of available MSCs for seeding. Protocols for fat retrieval and extraction of mononuclear population were effective and cell plating strategies enabled successful expansion of cultures. Differentiation studies were not undertaken and are required for full characterisation of the cell population as MSCs. Despite this absence, comprehensive flow cytometry experiments were undertaken and the main difference in protocol was the use of CD31-FITC in the FL1 channel (in place of CD29 used in bone marrow MSC), as this is a recognised marker of multipotent stromal cells from fat with MSC like properties (149). Although the scale of experiment was greater (n=27), similar cautions regarding cross reactivity of antibodies should be considered when reviewing results. Notably, in the volume of cells tested, considerably less were identified in the mononuclear population area of interest and substantial debris was noted, especially when compared to BM MSC. This suggests that the AD MSC culture protocol produces a less 'refined' MSC population with approximately one fifth of the cells forming a distinct population with MSC like properties. This is worth considering in future seeding experiments and if very specific outcomes in function are sought, cells used for

seeding should potentially be sorted by flow cytometry before seeding to enable conclusions to accurately be drawn.

In summary, the work described in this chapter achieved the aims of isolating and, where appropriate, characterising cell populations for use in seeding and tissue engineering. In isolating and extracting OU, difficulties in progressing with the project were noted due to the low yield, fragility and need for immediate use for seeding experiments, thus restricting applications. By contrast, MSC cultures were expansive, reproducible and produced high yield. As a result of this, seeding experiments detailed in chapter 5 used MSCs as an initial population and not OU as originally planned.

Chapter 5 Cell Culture in Decellularized Scaffolds

5.1 Background

The process of developing a large animal intestinal scaffold has been described, in addition to the isolation and characterisation of cells for seeding. Despite landmark studies in the development of decellularized organs, the challenge of combining cells and scaffolds to regenerate functional tissue remains the most pressing in TE. Important aspects are reviewed briefly below before describing the approach taken to combine cells and scaffold within this project.

5.1.1 Approaches to Recellularization

Recellularization refers to the repopulation of an organ or tissue by cells. The precise method by which this is achieved can vary and selection of method(s) is likely to be influenced by the target function of the tissue engineered product. Studies are typically embarked on with a specific 'goal' or 'endpoint' and this will be highly influenced by the tissue being engineered and the specific challenges within the model being used. TEI studies provide a good example of this - when considering the previous studies using polymer scaffolds by Vacanti et al., the primary aim was to assess if scaffolds, when combined with organoid units, could generate a neointestinal type tissue (78, 137, 138). Scaffolds were seeded with cells and implanted in vivo shortly after, using the host's physiology to provide conditions for cell proliferation and recellularization. Such an approach however, does not aim to create a fully functional intestinal graft and this is a more complex task which may require a more step-wise approach.

The challenges in TE of organs varies in relation to the function of the organs in question and therefore, the specific goals of studies and methods to achieve them may also vary. In the work of Olausson et al., an allogeneic vein was implanted into a human and seeded by an in vitro process lasting over 2 weeks before implantation (49). In this case, the vein was required to function immediately on implantation, as otherwise, thrombosis would have occurred rapidly and led to

failure. However, the implantation of other tissues may not require immediate total function. An example organ in this case would be the kidney. Although no clinical study has successfully implanted a tissue engineered functioning kidney into a human recipient, if the occasion arose, immediate function would not be necessary in the same fashion that an allogeneic renal graft may endure delayed graft function (DGF). The intestine resembles the kidney in the fact that the range of functions (e.g. secretory, absorptive, peristaltic), may not be immediately necessary on implantation in the same manner as a heart is required to pump and therefore, it is important to determine which functions TEI would be intended to serve and at what stage in any given project.

The decision of whether to utilise in vitro recellularization before implantation, for what time period and under what conditions poses a challenge. Tissue cannot be maintained in vitro indefinitely, even if fully functioning and clinical work in organ preservation strategies has demonstrated this (150). As a result, depending on the study goal, implantation of partially recellularized tissue may have a better long term outcome than that of prolonged in vitro support prior to implantation. One organ in which this may prove beneficial is the liver, due to its excellent capacity for regeneration and promotion of clinical recovery with a limited number of cells (151).

The approach chosen will also depend on the cells of choice for recellularization, as some progenitors may not survive in vitro for prolonged periods and demonstrate a tendency towards dedifferentiation. Cells such as MSCs and fibroblasts survive well in vitro but hepatocytes are not as robust (152). In addition, the mixture and ratios of required cells poses a complex issue as discussed in chapter 4. Fully differentiated cells such as cardiac myocytes may possess the ability to perform desired function but have reduced ability for proliferation and regeneration, both in vitro and in vivo when compared to progenitors. Finally, cell viability is crucial to a successful TE model and therefore any approach must take account of this.

5.1.2 Cell Seeding

Cells may be delivered to scaffolds using different methods. The method of delivery may be a simple, static process with placement of cells on or in scaffold tissue, followed by promotion of cell adherence. The OU and polymer scaffolds discussed previously provide an example of such methods, as OU were placed within scaffolds followed by in vivo implantation. Another such method might involve the injection of cells into structures (57) or the layering of cells on the surface of structures (105). Dynamic seeding may refer to active delivery of cells by pump, or perfusion type mechanisms. In practice, a combination of techniques may be used to administer cells into a scaffold, followed by provision of conditions to facilitate cell uptake and proliferation. For example, in a study by Uygun et al., hepatocytes were injected into a decellularized rat liver scaffold at a series of 10-minute intervals, before placing the scaffold in a perfusion chamber (108). Of note, the injection method used in this study was compared with a continuous infusion of the same number of total cells (50 million) over 40 minutes and found to be superior. The initial seeding of cells appears to be influenced by a number of factors including cell metabolic requirements, scaffold environment and shear stresses (35).

There exists no formula for determining even an approximate number of cells required for regeneration. In the process of seeding, a small proportion of cells may not be viable to begin with, a proportion will die during seeding, some will die later during the culture phase and many may never reach the desired target. The net result cell loss may be large and therefore, required initial counts of cell suspensions may be high. In the study described above, success of hepatocyte seeding into a rat liver scaffold was assessed histologically to compare protocols but this method has limited value, indicating only that cells have reached their target destination. It seems a tempting strategy to use as many cells as possible but aside from the challenges of producing such high numbers in culture, it may also be detrimental if high cell count suspensions occlude delivery lumens, form aggregates and disrupt phenotypes. Experimental model-specific studies

are required to ascertain and determine the correct cell seeding strategies for particular endpoints.

5.1.3 Culture Conditions

Once cells are combined with scaffolds, the appropriate conditions for regeneration must be ensured. Such conditions typically mimic those of the normal physiology of the cell and the regulation of; temperature, pH, oxygen supply and nutrient provision are important considerations. In vivo implantation frequently provides many of these conditions and the work of the Vacanti group described above in TEI studies utilised the recipient's own physiology within rat omentum to provide these conditions. It is well established that physiological conditions are difficult to replicate ex vivo but there are obvious drawbacks to using the in vivo regeneration approach. First, the recipient environment poses substantial immunological risk. As a result, immature scaffold-cell complexes may be placed in a hostile environment and exposed to cells (e.g. macrophages) limiting their proliferation. Second, the precise conditions required to support immature progenitors and promote their proliferation cannot be controlled externally and cells are at the mercy of the recipient's physiological processes.

The term 'bioreactor' is frequently used when discussing TE studies. The term describes a device that supports a biologically active environment and bioreactors have been used for years in industries such as food technology, performing a range of functions. In TE specifically, they may be used to create specific physiological conditions in vitro, to provide an ideal environment for tissue maturation (153). Important considerations in designing appropriate conditions are multiple. The use of standard culture media may not be appropriate, as classical in vitro culture does not utilise a scaffold and is not delivered in a perfusion-type system. As a result, adjustments to media supplementation and viscosity may be required. Oxygen delivery remains an important consideration as without viable supply, cells will perish and this poses a particular problem in thick tissues which may be seeded with cells (154). Perhaps the most important feature of a

bioreactor for TE is the ability to provide biophysical stimuli, which is not possible using standard cell culture techniques. Studies have indicated that provision of biophysical stimuli may promote cell orientation and function within a scaffold in a desired configuration. The work of Ott et al. demonstrated this by subjecting a decellularized rat heart to synchronised electrical stimulation to promote myocardial contractility with success (57). Such studies demonstrate that the careful coordination of biophysical stimuli in conjunction with culture conditions is necessary for optimum tissue regeneration.

5.2 Aims

Experiments aimed to combine cultured cells (bone marrow and adipose derived MSC) with intestinal biological scaffolds to test preliminary properties of scaffolds for recellularization. Specific aims involved; (i) developing a scaled-down, seeded scaffold in-vitro culture model, (ii) comparing utility of AD and BM MSC in seeded scaffold cultures and (iii) developing a method of assessment of cell seeding efficacy in seeded scaffolds.

5.3 Methods

5.3.1 Intestinal Scaffold Preparation for Seeded Scaffold In-Vitro Cultures

5.3.1.1 Scaffold Mounting and Securing on Biopsy Pads

Experiments were scaled down to assess the ability of scaffold tissue to support cells. Scaffold segments were therefore used in combination with cells. Once intestinal decellularization was complete, handling of specimens was often difficult as later described. In order to aid orientation and permit storage before in vitro experiments, full thickness scaffold pieces were mounted and secured on foam biopsy pads as described below. This method of securing specimens was initially attempted with small intestinal scaffolds to assess efficacy as these were more gelatinous and difficult to handle than those from large intestine. Whole small intestinal decellularized scaffolds were transferred to a working tray and cannulas removed from the intestinal lumen. Standard 1x

1.25inch foam biopsy pads (*Surgipath, UK*) were placed within the lumen at the opening using forceps and pressed against the mucosal surface such that the scaffold tissue formed a layer on the surface of the pad. The scaffold was then cut using surgical scissors around the pad edges resulting in a scaffold sheet layer on the pad surface with mucosal surface facing down. The position was then fixed using a 5.0 Prolene suture 'through and through' with a loose knot tied on the scaffold side. This prevented rotation of the scaffold and permitted permanent orientation. The entire structure of intestinal scaffold, foam biopsy pad and suture will be referred to as SPS-complex for simplicity. SPS-complexes were then returned to PBS (1% antibiotic) for further storage.

5.3.1.2 Peracetic Acid Sterilisation Trials

Large intestine was decellularized according to described protocols and scaffolds divided into 1cm^2 pieces. Scaffold pieces were either; mounted on foam biopsy pads, on cassette strainer lids (fixed with a Prolene 5.0 suture) or unmounted. Samples were then placed individually in 50ml centrifuge tubes and 25ml of 0.1% peracetic acid added. Tubes were then sealed and placed on a roller at 60rpm for 1 hour. Following this, tubes were transferred to the cell culture hood and samples removed and transferred to a new 50ml centrifuge tube. 25ml PBS containing 1% antibiotic was then added to the tube and the samples washed for 1 hour under the same conditions. Resultant scaffolds (+/- mounts) were then placed in individual wells in a 6-well plate. MEMα was added and plates transferred to the cell culture incubator. Plates were reviewed daily for 7 days to assess for any obvious signs of contamination and thus provide an assessment of the sterilisation method.

5.3.1.3 Gamma Irradiation Sterilisation of Scaffolds

In order to sterilise small intestinal scaffold pieces for further experiments, SPS-complexes were treated by gamma (y) irradiation. To permit transport of SPS complexes, complexes were sealed by plastic packing using a commercially available vacuum sealing device (*Freshield Elite II,*

Freshield Pty Ltd., Aus). Sheets of 10cm height packing plastic were folded over forming a double layered 5cm high strip. The folded edge was then sealed using the machine, followed by the lateral sides forming a large, 4cm high plastic 'envelope' structure with a single open side. Individual SPS-complexes were then placed within the envelope, sealed at the remaining 2 borders and the sealed complex cut from the main sheet. The process was repeated until >25 SPS-complexes were sealed. SPS-complexes were gamma irradiated using a 60Co gamma-ray source (*Isotron, Berkshire, UK*) according to departmental protocols. Sterilised SPS-complexes were then maintained within sealed plastic pockets and stored at 4^{o}C until further use, when they were transferred to culture hood and opened under sterile conditions.

5.3.2 Development of Seeded Scaffold In-Vitro Culture Model

5.3.2.1 Seeded Small Intestinal Scaffold and AD MSC In-Vitro Culture Pilot Study

An initial pilot study was performed to determine the best method for in-vitro seeded scaffold culture, focussing on developing an optimum cell seeding mechanism and density and anticipating any future experimental challenges. Small intestinal scaffold was selected for this purpose as the scaffold was more fragile, gelatinous and difficult to handle. It was predicted therefore, that suitable culture methods for small bowel scaffolds could be transferred for use in large bowel scaffolds. A 12-well non adherent tissue culture plate (*Sarstedt, UK*) was selected to prevent cells preferentially adhering to well base rather than the scaffold. The 12-well size permitted lower cell concentrations in suspension but allowed for reasonable sized scaffold segments to be used for culture platforms. 1cm^2 small intestinal scaffold pieces were transferred to wells with the aim of laying scaffolds flat on well surface - this was not technically possible due to the lack of rigidity and tendency to curl. It was also not possible to maintain orientation of scaffold surface following cutting 1cm^2 segments and orientation was therefore lost. 9 scaffold pieces were therefore placed as centrally as possible within 9 individual wells and each well filled with 2ml of sterile PBS containing supplemental calcium and magnesium to promote cell

adhesion to scaffolds. The culture plate was then placed in the incubator for 3 hours. Following this period of 'priming', culture plates were returned to the hood and PBS carefully aspirated avoiding contact with the scaffolds. MEMα was then added to each well in a 2ml volume and the culture dish returned to the incubator to remain overnight and permit scaffolds to acclimatise to incubator conditions and immersion in medium.

The following day, scaffolds cells were seeded with cells and cultured. A single negative control scaffold well had no cells added and contained MEMα only. The remaining 8 wells containing scaffolds were then loaded with cell suspensions. AD MSC cells for seeding were taken from already plated cultures of P2. Following standard trypsinization protocols, cells were dissociated and resuspended in 20ml of MEMα. 2ml of cell suspension was then added to each well containing a scaffold and the single well with no scaffold as a control well. The remaining 2ml of cell suspension was cryogenically stored for future DNA analysis (detailed below). The total cell count per well was 17,777 cells and this was used as a preliminary seeding trial count based on available cells in culture. The tissue culture plate was reviewed daily under an inverted microscope. After 5 days, scaffolds were removed from 4 wells and the plate returned to the incubator. Of these removed scaffolds, 2 were snap frozen in liquid nitrogen for future DNA analysis and the remaining 2 were transferred for embedding in agar (described below) before fixation. After 7 days, the remaining 4 scaffolds were removed and treated with the same protocol.

5.3.2.1.1 In-Vitro Culture Pilot Study DNA Quantification

DNA was quantified in control scaffolds and test samples at day 5 and day 7. In addition, DNA quantification in the cryogenically stored aliquot (containing 17,777 cells in 2ml MEMα) was performed in an aim to assess cellular proliferation within co-culture wells. Quantification methods were identical to those previously described in Chapter 3 using the *GenElute mini prep kit* (*Sigma- Aldrich, UK*).

5.3.2.1.2 In-Vitro Culture Pilot Study Histological Processing

Seeded cultured scaffolds due for histological analysis were embedded in 3% agar before fixation to ensure adherent cells were not removed during tissue processing. A 10ml solution of 3% agar in distilled water was prepared by steady warming of agar powder (*Sigma Aldrich, UK*) in water on a hotplate. Once appropriate consistency was achieved, the solution was poured into polyethylene moulding cups to half full, before laying scaffolds in wells as flat as possible and filling the mould to the brim with agar, thus immersing the specimen. Moulds were then placed in a refrigerator at 4^{o}C to set for 1 hour before removal. Moulds were then placed in histological cassettes and fixed in NBF for 48 hours before being processed according to protocols previously described. Samples were then stained for H&E with slight modification of the protocol in appendix 2.4 by counterstaining with Eosin for 10 minutes, instead of 5.

5.3.3 Seeded Small Intestinal Scaffold and MSC In-Vitro Cultures - Biopsy Pad Model

Following the pilot study, significant alterations were made in further experiments. Given the difficulty in handling the scaffold segments and easy loss of orientation due to the lack of scaffold rigidity, culturing using a mount was attempted. The obvious choice for this purpose was the foam biopsy pads on which scaffold pieces were stored. Gamma irradiation protocols were initiated in view of this to permit sterilization of the entire SPS complex. In addition, culture using free cells in suspension was not believed to be an effective method of promoting engraftment of cells within scaffolds and direct seeding was performed as described below.

SPS-complexes were transferred to the hood, plastic envelopes opened and fixing sutures cut. Scaffolds were then inverted such that mucosal side of the intestine was facing upwards and secured again to foam biopsy pads using a suture. SPS-complexes were then placed into wells of 6-well plates, to allow for a greater working volume and size of SPS-complexes. 4 plates were used and 3x SPS-complexes were placed with biopsy pad in contact with well surface and the

wells filled with 4ml PBS supplemented with calcium and magnesium. Tissue culture plates were then placed in the incubator overnight.

The following day, cells were seeded. Two tissue culture plates were removed from the incubator and PBS aspirated. BM MSC were thawed (P3) from cryogenic storage using methods previously described. 5 million cells were resuspended in a volume of 500µL of MEMα. Each tissue culture plate was planned to contain a single well of cell suspension only, a well containing SPS-complex and medium without seeded cells (negative control) and 2 wells with SPS-complexes seeded with cells.

500,000 cells in a volume of 50µL were pipetted directly onto the surface of each scaffold (apart from the negative control), taking care to ensure the entire liquid volume covered the scaffold surface and did not flow off. In the positive control well, cells were pipetted directly into the centre of the well. The 2 plates were then returned to the culture incubator for 3 hours to permit cell attachment. Following this, 4ml of MEMα was added to each well including both positive and negative control wells. The plates were then returned to the culture hood. The use of two separate plates allowed co-cultures to be assessed at 2 separate time points (day 3 and day 7) by removing a single plate for processing. An identical protocol for seeding was performed using AD MSC (P3) with the remaining 2 plates. In total, 4 culture plates were assessed; a pair at 3 days seeded with either BM MSC or AD MSC and a pair at 7 days again seeded with either BM MSC or AD MSC. Culture plates were reviewed daily.

5.3.4 Seeded Small Intestinal Scaffold and MSC In-Vitro Cultures - Mesh Insert Model

Following use of the biopsy pad model, an alternative method was sought due to difficulties encountered (described below). Use of foam biopsy pads was not favoured and therefore an alternative mount was sought. 200µm cell strainers were postulated to be a viable alternative. Though not designed for this purpose, the mesh properties provided sufficient porosity and rigidity to allow passage of cells and form a base on which to lay the intestinal scaffolds. The

surface area was large enough to lay the entire 1cm^2 scaffold segment and the strainers were available in sterile packing for single use. With a minor modification (removing tabs), it was postulated that strainers could be placed within wells with scaffolds inside to form a transwell-like culture system containing scaffolds and allowing engraftment from suspension via the porous mesh insert base.

However, when initiating experiments, it was not possible to lay scaffolds within strainers and maintain orientation and an alternative was therefore sought. The base of the strainers was therefore removed intact from the strainer and used as a sterile mount. Sutures in SPS-complexes were removed and the mesh mount placed on the surface of the scaffold. The sandwiched scaffold was then inverted and the foam pad gently 'peeled' off to demonstrate the scaffold layered on the mesh disc surface, with mucosal surface facing upwards. The entire process was performed under sterile conditions within the culture hood. The mesh/disc/scaffold complex (MDS-complex) was then used for seeded culture experiments in a similar manner to SPS-complexes.

A seeded MDS-complex culture experiment similar to that previously described was then performed using BM MSC only. Full surgical scrub was performed prior to working in the cell culture hood. Meticulous attention to sterile technique was applied. This required an assistant to provide sterile items where necessary. 2x 6 well plates were obtained and MDS-complexes placed in 4 wells in each plate under sterile conditions within the culture hood. PBS supplemented with calcium and magnesium was then added to each well in a 4ml volume and plates covered and placed in the incubator overnight. The following day, 5 million BM MSC (P3) were resuspended in 500µL MEMα. Wells were allocated for testing as described in section 5.3.3. 50µL of cell suspension was then layered gently onto appropriate scaffolds equating to a seeding density of 500,000 cells/cm^2. As previously described, a single scaffold in each plate served as a negative control and had no cells seeded on the surface. A single well within each plate was seeded with

cells alone and served as a positive control. Both plates were returned to the cell culture incubator and removed at either 3 or 7 days before analysis by methods described above.

5.4 Results

5.4.1 Scaffold Mounting and Sterilization

Intestinal scaffold tissue remained difficult to handle and manipulate with small intestinal segments being thinner and more challenging than large. Initial attempts to place scaffold tissue within wells without support was unsuccessful as previously described and orientation could not be maintained. Mounting of scaffolds on foam biopsy pads was successful but tissue had to be 'layered' onto foam as if lifted, sheets were not rigid and lost any sheet-like shape. Altering scaffolds once layered was difficult and if scaffolds were lifted off foam pads, it was not possible to maintain orientation and replace them. As a result, sandwiching scaffolds between 2 foam pads and then manipulating the pads became the preferred method of choice for changing orientation of scaffolds.

Sterilisation methods using peracetic acid were assessed by the ability to maintain scaffolds in media without change of phenol red colour. Change would have corresponded to pH alteration and in the absence of known cells, likely represent contamination. In the testing described above using different methods of scaffold mounting and position, at day of testing no significant change in phenol red colour of medium was noted. This was consistent with an absence of contamination, supporting effective sterilization using peracetic acid methods. Success of gamma irradiated sterilization methods was not assessed.

5.4.2 Seeded Scaffold In-Vitro Culture Pilot Study

The scaffold placement within the pilot study was conducted as a result of the unanticipated difficulty in positioning scaffolds as described above. Despite this, the experiment was adapted as described above and continued till completion with no evidence of contamination of cells or

scaffolds. Fig 5.1 demonstrates the quantity of DNA in a single aliquot of 17,777 cells and quantities in 2 seeded scaffolds removed from in-vitro at 5 days and 2 removed at 7 days. Results demonstrate a reduction in DNA after 5 days of approximately 50% and therefore a proposed reduction in cell numbers. By 7 days however, seeded scaffolds displayed an increased quantity of cells compared to the original aliquot suggesting that proliferation had occurred. It is noteworthy however that the increase in cell numbers was not significantly greater than that of the control well of cells alone without scaffolds.

Histological analysis of agar embedded scaffolds posed difficulties with orientation due to fragility and lack of rigidity of scaffolds as previously discussed. As cells were seeded into suspension and not onto scaffolds, any integration of cells and scaffolds was noted to be a significant finding. Images did not demonstrate a representation of scaffold morphology and a few scant cells were noted on scaffold surfaces in test wells.

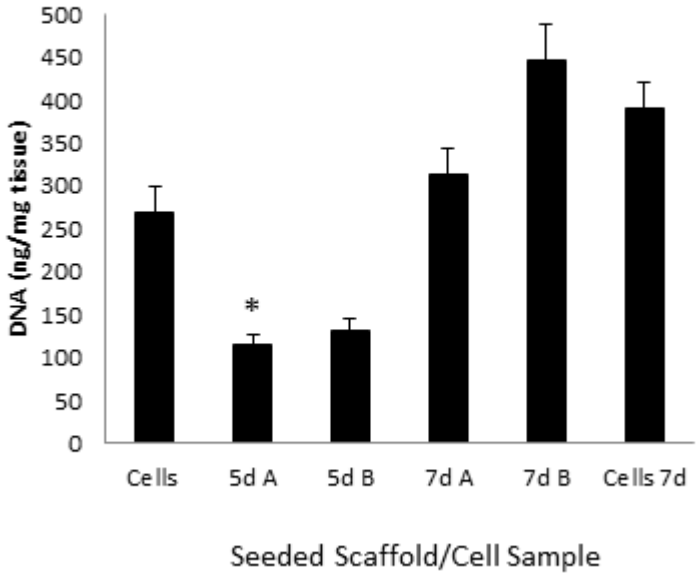

Figure 5-1 – DNA Quantification in Seeded Scaffold In-Vitro Culture Pilot Study of AD MSC

Bars correspond to specific samples and error bars denote standard error. ***'Cells'*** *demonstrates the quantity of DNA in the frozen aliquot of 17,777 cells. 2 scaffold samples* ***(5dA and B)*** *were assessed after 5 days indicating an approximate 50% reduction in DNA quantity when cells were seeded in wells with scaffolds. Quantification in '5dA' demonstrated the only statistically significant difference ($p \leq 0.05$) when compared to 'cells'. After scaffold-cell culture for 7 days* ***(7d A and B)*** *an increase in DNA quantity was noted however.* ***'Cells 7d'*** *corresponds to the DNA quantity in the control well without a scaffold after 7 days indicating an increase and no significant difference when compared to scaffolds at 7 days. All statistical analyses were performed using one-way ANOVA testing.*

5.4.3 Seeded Small Intestinal Scaffold and MSC In-Vitro Cultures

Use of the foam biopsy pad model of in-vitro scaffold-cell culture was complicated by the need to reverse the orientation of scaffolds such that they were exposed with mucosal surface facing up. Following successful orientation, placement in wells and priming with calcium and magnesium, cells were seeded onto scaffolds as described. Unfortunately, within 24 hours, contamination had affected all wells with change of phenol red medium colouring to yellow indicating a change to acidic pH. Seeded scaffold cultures were continued but clear contamination became visible in all wells with cloudy medium appearance and the experiment was therefore abandoned.

The use of the mesh insert seeded scaffold in-vitro culture model was performed in view of the failure of the SPS-complex culture method. Adaptations to the experimental method were performed as a direct result of failure of SPS-complex method. Full surgical scrub techniques were applied to increase sterility and the mesh insert was used in place of the foam biopsy pad as described. However, following priming of scaffolds, seeding again resulted in change of phenol red medium colour and evidence of contamination after 24 hours resulting in abandoning of experiment. As a consequence, minimal data could be gained from both attempts at in vitro culture model following the pilot study.

5.5 Discussion

Experiments conducted within this chapter aimed to combine scaffold tissue with cell progenitors to demonstrate the potential of the described biological scaffold for cell engraftment, adhesion and proliferation but this was not achieved. The proposed use of scaffold pieces measuring 1-2cm^2 was logical to enable assessment of the scaffold tissue in isolation without the necessary use of an entire scaffold in a single experiment. The theoretical advantages of this approach included the ability to conduct a number of experiments utilizing different cell seeding densities, culture conditions and seeding methods to assess optimum conditions for transferring to a larger scale model. Such an approach is in contrast to methods used previously in a number of intestinal tissue engineering studies, which proceeded to use segments of tubular scaffolds, seeded with cells and implanted in-vivo to permit cell proliferation (77, 79, 121). The methods utilized in this study also provide a contrast to in-vitro whole organ engineering seeding strategies previously discussed (58, 112). Thus the attempted use of a scaled-down, in vitro scaffold segment seeding model represents a method with a view to assessing the regenerative properties of the specific scaffold ‘tissue’ in isolation.

The use of single scaffold pieces posed a number of difficulties. Whilst fully intact scaffolds posed no challenges in handling and manipulation, when divided into small sections, the gelatinous and

highly flexible nature of the scaffold tissue proved difficult to work with without a supporting structure. It was not possible to utilise scaffold segments without supports and maintain orientation for experimentation. This meant that direct seeding methods (e.g. injection onto mucosal surface) could not properly be compared with alternative methods without use of supporting structures. The initial pilot studies demonstrated this by the failure to orientate specimens and the use of the resultant non-specific seeding method of combining cells and scaffold in suspension.

The DNA quantification data demonstrated an increase in DNA present after 7 days when compared to that in the original seeded cells at the point of in-vitro seeded scaffold culture. The number of seeded scaffolds cultured for 7 days (n=2) and the small difference makes commentary on the precise quantitative relationship difficult but there was a clear increase in DNA quantity when compared to scaffolds at 5 days which demonstrated a definitive loss of DNA quantity when compared to original seeded quantity. Of note, the positive control well of cells alone demonstrated an increase in cell numbers similar to that of seeded scaffolds cultures at day 7. A possible explanation for these findings is that a proportion of seeded cells died within 5 days but the remaining cells had not proliferated significantly until day 7. A number of cells may have been non-viable at the time of seeding or may have perished following exposure to culture conditions. This would explain the findings to some extent and if correct, poses the question as to whether seeded scaffold in-vitro cultures provided any benefit to cell proliferation. Data gained does not support this, with a small increase and a decrease seen in DNA quantity when compared to cells cultured independently without scaffolds after 7 days. These experiments presume that DNA quantity within small intestinal scaffolds was negligible which is supported by previously published DNA quantification following the decellularization method (84). These findings proved interesting but clearly further, more robust data is required to quantify cell population increase of MSC in given culture conditions after 5 and 7 days independently, before assessing carefully the possible positive effect of culture with scaffold tissue. In addition, experiments would need to be

continued for a greater duration to assess effect of more prolonged seeded scaffold in-vitro culture.

The histological analysis from the pilot study again demonstrated the physical challenges of combining cells and scaffold without structural support. Orientation of scaffolds proved difficult prior to sectioning. Demonstration of cells on the scaffold surfaces was a significant result as this suggests that cells favoured attachment to scaffold over suspension in media. Given the lack of provided stimulus to initiate this cell migration and the lack of direct seeding, the reasons for this are not clear. If the scaffold promoted cell adhesion, it may have been due to retained micro-molecules as postulated in the original experimental hypothesis. However, in the absence of robust data to support cell migration and lack of assessment of any specific factors, such conclusions cannot be drawn. In addition, the DNA and histological data is limited by assessment of cell/ nuclear material presence only and provides no assessment of functionality or cell viability. It remains possible that cells adherent to scaffolds were not viable and if so, the importance of the result is reduced. Further work is necessary to assess cells that do adhere to scaffolds in such a culture system.

Following the pilot study, the importance of the method of culture system was identified. The need for structural support was the initial aim in culture system design and sought to permit scaffold orientation and enable direct seeding onto mucosal surfaces. This led to the development of the SPS-complex model. Foam biopsy pads were identified as ideal to permit scaffold storage and allow flexible mounting. Initial concerns regarding the sterility of biopsy pads were dealt with by gamma irradiation but the initial method of mounting proved to be a clear error in retrospect. Whilst the process of mounting scaffold segments with mucosal surface in apposition to foam pads was technically easier initially, this meant that scaffolds later had to be inverted and re-mounted following sterilisation. This provided a potential source of contamination. The contamination experienced in this experiment confirmed this risk. Another

potential issue was the use of the foam for this purpose. A likely polyurethane composite, the sponge foam's original design use is for tissue processing and not cell culture. Despite the inert chemical properties, the effect of culture conditions on foams is unknown and not validated. Both of these facts played a key role in transfer to an alternative culture system model.

The intended culture system for use following the use of SPS-complexes could be technically described as a 'modified transwell system', attempting to utilize similar principles to that of a traditional Boyden chemotaxis type assay. If experiments had proceeded as planned, scaffolds would have been placed in sterile chambers with mucosal surface orientated up, with a porous membrane beneath and separated from the well. Although direct cell seeding onto mucosal surfaces was originally planned, the model would have permitted genuine 'co-cultures' with potential direct seeding of a single cell type (e.g. MSC) and suspension of alternatives (e.g. OU). Commercially available tissue culture well inserts did not unfortunately fulfil the necessary physical requirements for this purpose and the attempted adaptation of alternative methods failed due to the difficulty in handling the scaffolds. The resultant use of the culture insert mesh membrane as a supporting structure merely provided a similar model to that used in the SPS-complexes but with a supporting material validated for the purposes of cell culture. In practice therefore, as it was not technically possible to suspend scaffold tissue in a chamber with a porous membrane beneath and true cell migration was not assessed in any culture system model used. Any further experiments performed of this type should pay clear attention to a sustainable, reproducible culture system model that enables adequate assessment of desired cell properties.

Maintenance of sterility posed a major issue in all seeded scaffold in-vitro culture systems and this proved one of the most significant findings. In initial SPS-complex experiments, possible reasons for contamination have been discussed above, including scaffold re-orientation, handling and use of non-purpose-specific components within the cell culture system. In the mesh insert culture system, issues surrounding scaffold handling and re-orientation were still present.

Numerous other potential sources for contamination existed despite use of best practice including culture hood, instruments, cell vials, consumables and media. The use of clinical level sterility and full surgical scrub was used to mitigate these risks without success, suggesting that likely sources were from cells, or scaffolds themselves. The use of gamma irradiation to minimize risk of contamination in scaffolds attempted to avert this risk but the increased handling of SPS complexes following this may have provided a source of further contamination. Indeed, gamma irradiation is not without its disadvantages as one of the proposed benefits of a biological scaffold is the retention of micro-molecules such as growth factors and effect of gamma irradiation on growth factors has been demonstrated as deleterious in alternative models (155). Contamination of cells is plausible following cryogenic storage but this problem was never documented in cultures of MSC in vitro before described experiments and therefore seems less likely.

The issue of scaffold sterility is therefore both challenging and complex. Decellularization of animal tissue begins at a stage when numerous sources of contamination are present and these are even greater where definitive contaminated tissue such as bowel are used. Early decellularization procedures utilized antibiotics in washes but the absence of sterile chambers for decellularization and concern regarding prolonged antibiotic use and drug resistance limited application of continuous use. Clearly, the issue of sterility was not addressed adequately in models described above but the cost of escalation of methods (e.g. introduction of sterile chamber) is considerable and further trials of optimisation of current models is probably a more appropriate initial step. In addition, sterility tests should ideally be performed before proceeding with experiments, as otherwise considerable cell numbers may be lost and little data gained as described above.

Experiments described within this chapter were focussed on developing novel methods to assess biological scaffold tissue for ability to provide a platform for cell proliferation and regeneration. The development of a suitable experimental model proved challenging and if the proposed model

of in-vitro assessment of scaffold tissue for regenerative properties is to be pursued, then numerous adaptations are necessary. Many of these have been discussed above and include adequate provision of sterility of scaffolds, cells and conditions for culture. In addition, a robust culture system method must be developed that involves minimal tissue handling, maintenance of sterility, reproducibility and rigorous assessment of cell behaviour. Alternative options include use of scaffolds as a drum skin-like covering over a vessel such as a ring shaped reservoir. The stretched scaffold material then has no requirement for rigidity as the securing the tissue over the ring in a drum skin-like fashion maintains the mucosal surface as desired. Following this, cell suspensions could be poured into the inverted ring structure, permitting direct seeding by contact. Such a structure could be placed in different ways to permit engraftment of cells onto the opposite surface, for example by immersion of the ring in a cell suspension. Alternative techniques could involve seeding short segments of intestine by filling the lumen with a cell suspension and occluding both openings to permit a period of cell engraftment. Such techniques could be adapted or optimised to create a purpose designed 3D scaffold-cell culture system.

The question should be posed however, as to whether this method of in vitro scaffold tissue co-culture is a suitable one. Obvious limitations concerning the applicability of in vitro cell behaviour to in-vivo are always present and there is no guarantee that demonstration of cell-scaffold behaviour in such conditions is transferrable to the in-vivo setting. Clearly, the challenges experienced whilst using these small intestinal tissue segments provides a case for avoiding such challenges entirely and proceeding to a much larger scale model, perhaps where small segments of continuous intestinal scaffold were used for seeding in a mini-bioreactor setup. This obviously represents huge up-scaling from described models and the inability to control conditions on a scale that was described as a benefit to the originally proposed models. These challenges are significant and the path to further progression is not demonstrated clearly as a result of experiments from this chapter. The implications of this and future experiments will be discussed further in chapter 6.

Chapter 6 General Discussion

6.1 Scaffold Production in Tissue Engineered Intestine

Experimental work from chapter 3 detailed the successful production of a large intestinal biological scaffold with capacity for perfusion. Surgical techniques were used effectively to enable removal of large animal tissue after death, with retention of en-bloc vascular perfusion circuit. This indicated that there is no requirement for intestinal retrieval under anaesthesia (IRUA), provided the appropriate surgical experience is available. This is significant as, if the model were to be adapted for human use, the ability to utilise donors after cardiac death greatly expands the pool of available organs. In addition, if the model were to be used in a semi-xenogeneic application, use of pigs after death would reduce costs and permit a more widely applicable protocol not requiring anaesthetic facilities.

The time after death at which intestine should be retrieved for scaffold production may be important, as it is likely that as increasing time elapses, micro-thrombi may form within vessels and potential for perfusion is lost. Alteration in anticoagulation protocols within this work permitted retrieval and decellularization of tissue later than had previously been attempted after death. Practical experience suggested that as increased time elapsed before perfusion decellularization, the quality of perfusion and ability to complete the process was reduced. No assessment was made of the maximum time that could be elapsed before successful retrieval and perfusion decellularization of tissue but such an assessment may provide useful information, as if the model were ever clinically successful, it would potentially expand the donor pool.

The retrieval of specimens, followed by bench preparation and freezing before successful decellularization represents a method of scaffold production more commonly seen in hard tissues than soft. The successful completion of this process in the large animal intestine using vascular perfusion is significant as it might permit a genuine ‘off-the-shelf’ approach to creating complex

soft tissue scaffolds for storage and use when required. Literature suggests that the act of freezing has a detrimental effect on ECM structure however (45) but scaffold specific experiments are required to determine if such effects would be so detrimental as to justify preventing the development of a 'tissue bank' from which to create scaffolds and what the appropriate storage time and temperature would be. In practice, such experiments would be complex, requiring a clear definition of what outcomes demonstrate an improved scaffold and how to compare these between samples. The level of detail required by such experiments in the absence of a genuine method of assessing scaffold utility indicates such a study would be of questionable value until methods of production have been highly refined.

Perfusion decellularization has been used historically in different studies but has typically been by retrograde or by other non-physiological methods (57, 115), with genuine arterio-venous physiological perfusion rarely documented (83). One could postulate that physiological perfusion is more likely to retain ECM and be less traumatic to tissues due to distribution of shear stresses in vessels designed for purpose but there is no evidence that physiological type perfusion decellularization has benefit over any other method. The value of assessing any difference in scaffold quality using different perfusion methods of vasculature is questionable, but where organs contain additional networks such as the intestinal lumen or hepatic ducts, the ideal pattern of perfusion remains uncertain. Multiple approaches to decellularization were attempted within this work, with successful results but the potential for changes to the decellularization process by alteration in the balance between intestinal and vascular perfusion circuits provide possibilities for potentially simplifying the decellularization process. Further studies with more control in decellularization variables may permit development of protocols in relation to this.

The process of decellularization requires further discussion. The effects of timing of IRAD, duration of freezing before decellularization and method of perfusion decellularization all exert unpredictable effects on the decellularization process that has not been formally assessed within

this body of work. The steps involved in decellularization (e.g. reagents used, rate of infusion, duration of washes etc.) similarly have not been explored with respect to the precise effects of altering each of these variables. Experiments within chapter 3 demonstrated relationships of importance such as, the fact an increased rate of pump perfusion corresponded to a more decellularized specimen, but with less retention of ECM. Similar inferences were made regarding other variables during the process (e.g. duration of washes, concentration of reagents) but there is no definitive quantifiable relationship that can be described. This situation is exceptional within the of published literature: in papers in which decellularization protocols are described, the method by which the 'optimum protocol' has been reached is frequently unclear (115, 156, 157). One difficulty encountered within these studies in attempting to define an optimum protocol was the natural variability in animal tissues. This is obvious in large animals, as different segments of retrieved porcine intestine varied in size, weight and age. In addition, en-bloc perfusion vessels had normal anatomical variations in size and shape. These factors may have led to altered delivery of reagents into tissues of different cellular densities. Such differences may have accounted for some of the variation in DNA quantity seen in different specimens and supports the theory that a single complex decellularization protocol may affect 2 anatomically identical animal tissues differently leading to results which cannot be accurately predicted.

Perhaps the most important discussion point with regard to scaffold production is the process of characterisation for tissue engineering. Studies in TEI mirror those of other tissues where a similar set of tests are used to ascertain if scaffolds are decellularized and with retention of appropriate properties. Such tests typically focus on removal of cells/cellular material (e.g. H&E staining, DNA quantification) and retention of ECM components (e.g. histological stains, assays). A myriad of methods have been used for characterisation including; mechanical testing, immunohistochemical staining, electron microscopy and proteomics (57, 58, 64, 115, 158). It is often not apparent however, precisely what affect retention of such characteristics have on future potential for tissue engineering in most cases. The quantification of DNA provides a good

example of such a test as discussed in section 3.5. Literature suggests a biological scaffold should retain <50ng/mg DNA (45) but there is no tissue engineering related evidence to support this claim. As discussed, the effect of residual DNA within a scaffold is likely to vary depending on cell types seeded, recipient host response and use of allogeneic/xenogeneic models. Further studies are needed to ascertain the effects of different levels of retained DNA quantity with respect to the application of tissue engineering.

Similarly, other variables require further discussion. Mechanical properties are frequently assessed in scaffolds and results compared with control tissue before decellularization (47, 64) but a decellularized scaffold is often not intended to serve the exact same mechanical function as native tissue as typically, scaffolds undergo a period of recellularization using ex/in vivo methods before implantation. The demonstration of mechanical properties identical to control tissue could, in theory indicate an increased tissue density and properties that might potentially reduce successful cell seeding. Given the above therefore, caution should be exercised when reviewing characterisation tests of scaffolds as surrogate markers for successful tissue engineering as there is often minimal evidence to support this approach.

Biocompatibility testing described within chapter 3 demonstrated survival of rats in all experiments, without significant adverse effects. This is encouraging but it should be noted that genuine assessment of biocompatibility requires implantation of tissue/device as intended for final use (159). As intestinal scaffolds were intended for seeding, ideal further biocompatibility testing requires seeding of scaffolds before implantation. In the absence of such seeding, conclusions can only be made regarding scaffold degradation and the presence/ absence of any severe immunological reaction triggered by the scaffold alone. In addition, proposed seeding of scaffolds involved use of cells with immunomodulatory properties (MSC) and this may have had a marked effect on improving scaffold survival and tissue regeneration.

There are significant challenges in assessing scaffolds for tissue engineering purposes. The effect of the decellularization process is difficult to predict accurately in complex tissues and whilst decellularization experiments were successful within this work, determining the benefit of one protocol over another was difficult. Characterisation tests, are seen as surrogate markers of 'successful' decellularization, but may not truly reflect the potential of a scaffold to engraft cells, promote tissue regeneration and survive within a host. As definitive success of scaffolds can only be confirmed by demonstration of tissue regeneration, it is argued that characterisation tests should be minimised and the transfer to cell seeding be made more rapidly with assessment of tissue regeneration viewed as a genuine, effective outcome of success/failure of scaffolds.

6.2 Cells for Seeding in Tissue Engineered Intestine

The successful isolation of organoid units (OU) was demonstrated in chapter 4 using adaptation of previously published protocols (74, 78). Despite multiple isolations (n=12), considerable optimisation of the protocol could be performed to further increase yields based on data gained. Increasing the duration of enzymatic digestion or altering the concentration may prove beneficial and the use of younger animals would almost certainly increase yields. Finally, isolation could be attempted at alternative sites (e.g. jejunum) or on a larger scale. Expansion of OU in cultures was not attempted and attempts to identify the presence of intestinal stem cells (e.g. Lgr5 positive) were not made. If further work in porcine OU were to be performed, the above additions to experiments performed would provide a logical base from which to progress.

OU were not used for the initial seeding experiments as a highly expandable, more robust, easily isolated and cultured cell type was favoured to assess scaffold potential for supporting cells. In addition to the technical challenges in culturing OU, the heterogeneous nature of OU as clusters of groups of cells meant that, if regenerative studies had been successful, it would have been difficult to attribute advantageous/ detrimental events to a specific cell population. Advances in OU culture have been reported and there is great promise for the future given continuing

advances in the; yield of OU from culture, the rate of expansion in scaffolds and use of new techniques in stem cell biology (89). This study sought to utilise cells to demonstrate efficacy of the scaffold to support cellular engraftment and experiments demonstrated that, despite initial aims, MSC were more suitable for this purpose.

Experiments described within chapter 4 permitted the successful isolation and culture of MSC from porcine bone marrow using a reproducible protocol. In addition, culture and expansion of MSC from adipose tissue was achieved. Both populations demonstrated criteria corresponding to MSC according to guidelines issued by the International Society for Cytotherapy but of note, these guidelines are applicable to human MSC specifically. Full characterisation with demonstration of differentiation into 3 cell types was not achieved in BM MSC and not attempted in AD MSC. A substantially more robust characterisation of surface markers by flow cytometry was performed in AD MSC when compared to BM MSC. Formal cell characterisation would involve further experiments to complete the above.

The porcine models permitted expansion of both AD MSC and BM MSC for use in cell seeding. AD MSC populations analysed by flow cytometry contained considerably more debris and other cell types compared with BM MSC, indicating a less pure population. It is probable that the isolation process from adipose tissue permitted culture of other plastic adherent cell types to co-exist in culture with AD MSC such as fibroblasts. The precise proportion of true MSC with tri-potency in cultures derived from adipose tissue or bone marrow is difficult to determine accurately. Fluorescence activated cell sorting (FACS) would be an appropriate method to isolate different cell types, but there are 2 key issues with this approach. The first is the reliability of the process as, there is a lack of species-specific antibodies and issues regarding cross-reactivity have not been fully assessed. In addition, there is insufficient evidence in the literature with regard to precisely which antibodies' presence or absence reliably demonstrates MSC in pigs, particularly in AD MSC populations. The second issue is as to whether any benefit can be gained from a tissue

engineering perspective in sorting and isolating such cell populations. There has been much discussion regarding the regenerative properties of MSC within this work but, as described in section 4.1.4, the definitive mechanism(s) by which this occurs remains unknown. Given the likely reduction in yield and complexities in FACS of MSC for TE, an argument can be made for avoiding the process in TEI unless a clear benefit can be demonstrated.

Successful studies to date in whole organ engineering from other tissues have typically used cell combinations and have usually involved use of stem cell populations expanded in vitro, in addition to seeding with selected specialised cells of choice. Such an example of this is provided by the study of Uygun el al. in which a decellularized liver matrix was seeded with hepatocyte and endothelial cells which were expanded in vitro in large numbers before use (108). This study is not unique as similar results have been demonstrated using the same principles in different tissues (46, 58). A reliance on specialised cells from primary culture seems to be essential in whole organ regeneration at present and the challenge lies in reducing this reliance. There are two methods by which this can be achieved: improving the yield of expanded primary culture cells in vitro before use, and reducing the need for such a high quantity of these cells. This might be achieved by supplementation of alternative cell types which may produce a 'synergistic' regenerative effect. Patil et al.'s study provides evidence as to the extent of intestinal regeneration that can be achieved using MSCs in the absence of primary culture cells within a scaffold (47). Increased understanding of stem cell niches reiterates the importance of the interaction between groups of cells and therefore cells such as MSCs are likely to play a role in TEI. The work of Sato et al. gives promise to the successful in vitro culture of intestinal stem cells for the first time in a demonstrable, reliable and sustainable way (75). Based on work completed within this study and other published studies it may soon be possible to apply these two principles in TEI. Isolating intestinal stem cells using a small amount of tissue, culturing, expanding and seeding in conjunction with MSCs may prove to be an effective strategy to the challenge of cell seeding of scaffolds.

6.3 Recellularization in Tissue Engineered Intestine

Difficulties in scaffold handling, maintenance of sterility and seeding in chapter 5 within this work exposed some of the challenges in attempting to demonstrate regenerative properties of intestinal scaffolds and although other groups have done this more convincingly, specific applications of such studies have often not been demonstrated (47). Combining cells and scaffold to demonstrate regeneration of intestine represents a significant achievement and is the ultimate test of the efficacy of a scaffold. In the absence of demonstrable tissue regeneration, scaffolds remain unproven. This concept was a driving factor in aiming to develop a model for assessing regenerative properties of scaffolds on a manageable scale before proceeding with further experiments.

Scaffold sterility remained a major issue encountered using methods of decellularization and seeding. The multiple possible sources for this make identification of the primary problem difficult and further attention to sterility, in combination with suitable testing is necessary in future experiments. Gamma- irradiation proves attractive as a method of reducing risk of contamination but as demonstrated, in isolation it may not be successful in preventing contamination. In addition, as discussed in chapter 5, the potential loss of growth factors and the effects of sterilisation on the ECM are unclear and may be deleterious. Cell culture rarely demonstrated contamination when performed in isolation and suggests strongly that sources of contamination were present within the scaffold or in the process of seeding. Future experiments should focus on improving sterility within the decellularization process initially and assessing scaffolds robustly for contaminants prior to undertaking seeding experiments.

An initial goal in chapter 5 was to demonstrate the potential of scaffolds to support cell survival and proliferation. The use of a scaled-down model utilising cut pieces of intestine was not effective however. The precise nature and structure of the scaffold, combined with the need to demonstrate cellular engraftment in relation to morphology necessitated the need for use of

supporting structures for scaffold tissue, due to the lack of rigidity of scaffold pieces. Methods used to overcome this problem demonstrated variable success in studies described in chapter 5, although similar measures have proved successful elsewhere in other groups using different approaches (160). Unfortunately, no method used within this study permitted completion of the experiment with ability to determine accurately the potential for cellular engraftment within scaffolds. Further optimisation of methodology and improvement in sterility could enable this.

The relevance of any scaled down model of cell seeding to the final model is important. The demonstration that isolated scaffold tissue possesses properties for regeneration is desirable but whole organ regeneration strategies require many other considerations. When seeding a full segment of intestinal scaffold with cells, the mode of cell delivery, maintenance in sterility and assessment of engraftment will differ based on the size and nature of the scaffold. The initial aim of scaled down cell seeding models sought to overcome difficulties in using a larger size scaffold which requires a greater number of cells. Given the challenges that were encountered within the scaled down model, an argument can be made for avoiding its development as the model will again need to be altered significantly for final use. The problems encountered in the work described in chapter 5 suggests that development of a model for seeding within a segment of intestinal scaffold, with optimised conditions for sterility and cell delivery before seeding with cells may be a better route to the final goal.

The final issue of how best to assess the success of intestinal scaffold regeneration was not explored in detail within this work. The ability of scaffolds to support and sustain cells was identified as an initial goal but this represents a single aspect of the process. Cellular proliferation, differentiation and activity are all essential to full tissue regeneration and adequate methods of assessment will need to be developed if the model is to be successful. Analysis of the scaffold following seeding using techniques such as immunohistochemistry provides one method of static analysis. Such analysis however would be performed in fixed tissue and would not be suitable for

assessment of function or proliferation. The need for further assessment methods combined with the challenges of supporting cells with oxygenation and nutrients, illustrate the magnitude of the work required in the future development of the model.

6.4 Final Considerations in Tissue Engineered Intestine

6.4.1 Clinical Utility

The clinical rationale for the development of tissue engineered intestine was outlined in chapter 1 and important considerations with respect to this include the incidence of relevant disease, the extent of disease morbidity and the limitations of current treatments. As discussed, studies in TEI have historically predominantly focussed on the small intestine and development of TEI for use as a replacement for absent intestine (47, 76, 79, 121, 122, 139). In theory, such replacement intestine would serve as a treatment for patients with irreversible type III intestinal failure who were dependant on TPN and faced an impaired quality of life as a result. To make a useful impact on clinical practice, TEI strategies will need to be superior to existing therapies – long-term parenteral nutrition and allogeneic intestinal transplantation. At present the number of patients treated for Type III intestinal failure is relatively low, at least compared to the number of patients with renal or hepatic failure that fulfil the criteria for transplantation (24). However, narrowing the application of TEI to patients with small intestinal disease is to underestimate its potential, which should include many more patients with large intestinal pathology, including inflammatory bowel disease and cancer.

This work represents the first documented research into large animal, large intestine tissue engineering. Such an approach permits application of TEI for a different patient group that has previously received less attention, despite being greater in number (albeit whilst experiencing less morbidity and mortality as a result of disease).

6.4.2 Applications of Large and Small Intestinal Scaffolds

Experiments described in chapter 3 detailed the development of the large intestinal biological scaffold. It is noteworthy that the original aims of the project did not include full regeneration of 'large intestinal tissue' and sought instead to create a scaffold which could demonstrate engraftment and proliferation of cells. It is logical that a biological scaffold derived from a given tissue type would be ideal to replace that specific tissue type, due to retention of an identical ECM. However, the differences between the large and small intestine are not substantial when compared to other tissues. This fact, in conjunction with the concept of intestinal adaptation suggests that a large intestinal scaffold could be used to regenerate a small intestinal substitute and vice versa. There is definitively no evidence to suggest contrary to this and it should also be remembered that matrices derived from certain tissues may be used to augment different tissue types (e.g. use of SIS in bladder reconstructive surgery). Results gained to date however, do not provide sufficient level of detail to compare the small and large intestinal scaffolds from this study with respect to either scaffold characteristics or recellularization potential. It is hoped that by continuing this work and thus broadening the research field, better insights into a greater number of potential applications can be obtained and particularly any significant differences between the large and small intestine.

6.4.3 Defining Goals

When work from this project is reviewed in conjunction with previous literature, the case can be made for more defined goals of TEI studies. As discussed previously, no application of TEI has progressed to the stage of a clinical trial in patients with intestinal disease and this clearly demonstrates a challenge. It seems possible that this situation has arisen from over-ambitious projects in pre-clinical models that lack specific uses for seeded scaffolds. The majority of research studies in both small and large animals have sought to generate replacement intestine, or segments of it but a graduated approach to applications of TEI has not been favoured. For

example, the development of a simple patch of intestinal tissue that could be used to directly repair a large perforated duodenal ulcer would offer a tissue engineering solution where no alternative currently exists. The goal of creating such a product is distinctly different to attempting to provide an entire segment of replacement intestine, which has been the focus of numerous previously published studies instead. This study retains similarly ambitious goals but the emphasis placed on retention of vascular arcades permits potential use of the model in humans by in vitro scaffold regeneration or direct transplantation, should ex- vivo regeneration prove only partially successful. In addition, the animal model scaffold has the potential for use in a semi- xenogeneic model of transplantation. Other studies have demonstrated excellent general outcomes such as; Patil et al.'s recellularization of a human intestinal biological scaffold with MSC, or Levin et al.'s development of full thickness human TESI in a mouse scaffold in vitro but both studies lead to production of a recellularized scaffold of which the use remains unclear (47, 140). Such studies, in addition to a number of others, demonstrate proof of principle but in the absence of a proposed use for the scaffold in question. This remained a strongly influencing factor in both the original experimental model and the reluctance to rely on OU for any long term strategies in TEI production.

6.4.4 Ideal Approaches

Consideration of the above in conjunction with experimental work completed provides possible explanations as to the barriers in transferring pre-clinical TEI studies to the bedside. In 2006, Gupta et al. reviewed the 'current status' of TEI and almost every study cited involved the use of OU or small animal models (161). There has been progress within the 10 years since then, with notable studies utilising large animal models and involving human progenitors, but transfer to the clinical phase remains elusive. A failure to adopt a suitable approach to the problem could be a cause, although it is not apparent from this work or previous studies if a 'correct' approach exists. With the need for TEI to perform many functions (e.g. peristalsis, ion transport, secretion etc.) and the differences between species and large and small animal models, many questions remain

unanswered: if the model should be demonstrated in a small animal and scaled up, if regeneration should take place in vivo or ex vivo, and which cell progenitors should be used to repopulate scaffolds? Experiments described in chapters 3-5 provided one approach by utilising a large animal scaffold with allogeneic cells but did not proceed beyond the stage of cell seeding as summarised in chapter 5. Scaffold transplantation undertaken in chapter 3 clearly demonstrated that immediate revascularisation of scaffolds is not a viable regenerative strategy due to thrombosis. Therefore, should the scaffold continue to be used with retention of current properties, at least some aspect of regeneration would need to occur in vitro. To truly ascertain if this model is sustainable, cells will need to be successfully seeded onto scaffolds initially before regeneration is demonstrated and the feasibility of this approach remains unassessed. The use of purpose-built bioreactors in successful whole organ engineering has been demonstrated in other organs (57, 58, 108) and more recently in human intestine and this suggests that the strategy is feasible (47).

6.4.5 A Proposed Future Strategy

Based on completed work and review of the literature, the ideal experiment for clinical utility would likely involve a combination of experimental methods from various studies. The case for use of biological scaffolds over synthetic is supported by the successful whole organ regeneration studies performed previously using other organs (57, 58). Work completed within chapter 3 has demonstrated that perfusion decellularization of large animal colon can be achieved and this work is supported by previously published work in the small intestine (84, 162). In addition, decellularization and recellularization of human cadaveric intestine has been achieved and therefore perfusion decellularization of human cadaveric tissue remains the next logical approach and brings the field closer to clinical application (47). As previously discussed, characterisation of the scaffold should be purpose-specific and brief, as genuine assessment of biocompatibility and regenerative properties can only be made when combined with cells. All evidence supports a tissue engineered approach over one of guided tissue regeneration. Scaffolds must retain the

ability to permit cell adherence, attachment and proliferation and a purpose built bioreactor is likely to be necessary for this, with emphasis on maintenance of sterility and delivery of cells. Adequate technical delivery of cells to scaffold should be demonstrated before appropriate administration of necessary progenitors. A suitable combination of MSCs in combination with in vitro cultured OU or isolated intestinal stem cells would provide an ideal pool of cells to promote regeneration and indicate appropriate ratios and conditions for maximal regeneration. Scaffolds should be seeded at intervals with optimum conditions to permit tissue regeneration. The prospect of ex-vivo normothermic haematogenous perfusion provides an exciting option for assessing the scaffold response to more physiological conditions prior to any implantation. Based on the response to this, plans for in-vivo implantation could be made. In an ideal experimental model, the above could be demonstrated in a large animal before final transplantation and anastomosis of TEI into a recipient in which the scaffold has been seeded with autologous cells. Based on success of this approach, progenitors could be changed to allogeneic in another model and response assessed. If the model proved successful, this may form a theoretical case for transferring at least some aspect of the model to the clinical setting.

The above provides an ideological view of future aspects of TEI and for this to be realised will take time. Each step will require considerable development of methods and specialised techniques utilised by specific groups must be better understood and applied by others (e.g. expansion of in-vitro OU as per Clevers et al.). Finally, should the model prove successful in providing a scaffold that can support and permit cell proliferation, the optimum combination of cells for implantation and transfer from cultured tissue to in vivo implantation and possible future clinical use represents a considerable challenge for many reasons. Thus whilst the future of TEI is bright, the challenges remain significant.

Chapter 7 Conclusions and Future Work

This study hypothesised that a large animal, large intestinal scaffold could be created by perfusion decellularization and subsequently retain its vascular network. Experiments undertaken demonstrated that complex decellularization processes could be undertaken following animal death, with retention of vascular networks even after freezing specimens for 6 months. This important finding suggests that future application of such methods could be applied to a large pool of donor tissue and stored at convenience. A perceived benefit of maintaining vascular networks is the ability to use vessels as a route for recellularization but this was not assessed within this study.

Scaffold characterisation undertaken demonstrated an absence of cell nuclei and retention of important extracellular matrix components such as glycosaminoglycans. Further analysis demonstrated difficulties in reproducing results from molecular analyses and residual quantities of DNA within scaffolds. The high sensitivity of such assays, coupled with the complexity of tissues and variation among animals was thought to account for these variable results and the benefit of extensive scaffold characterisation questioned in its value in tissue engineering studies. Scaffolds were assessed immunologically by biocompatibility testing and displayed translational potential in a xenogeneic model without adverse effect to rat recipients.

Isolation of Organoid Units (OU) was successfully undertaken and a protocol developed with potential for further optimisation. Precise characterisation and culture of OU was not undertaken but recent advances in the field suggests that this may now prove an effective method for expansion of progenitors for intestinal tissue engineering. Mesenchymal stromal cells were cultured successfully from adipose and bone marrow sources and the good accessibility and expansion potential of these cells makes them a valuable source for aiding tissue regeneration. Cell-scaffold combined cultures attempted within this work were unsuccessful but alteration in protocols may provide options for improving systems. The challenges faced in such scaled-down

models was substantial and benefit must first be proven before developing such models instead of scaling up to larger co-culture models.

The findings of this work have been encouraging but considerable challenges still remain in the pre-clinical phase of tissue engineered intestine production. Further studies should ideally adopt a step-wise approach to assessing and characterising scaffolds, in order to determine which properties of scaffolds are beneficial for tissue regeneration. Such properties should not however be viewed in isolation alone and scaffold response to different mechanisms of cell seeding should be assessed simultaneously to ensure that scaffold development has true regenerative utility.

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Appendices

Appendix 2.1 – Tissue Processing Protocol

Step	Processing Reagent	Time	Temperature
1	10% Neutral Buffered Formalin (NBF)	2 hours	Room temperature
2	70% Industrial Methylated Spirit (IMS 99)	1 hour	Room temperature
3	90% IMS 99	1 hour	Room temperature
4	100% IMS 99	1 hour	Room temperature
5	100% IMS 99	1 hour	Room temperature
6	100% IMS 99	1 hour	Room temperature
7	50:50 IMS 99/ Toluene	1 hour	Room temperature
8	Toluene	0.5 hours	Room temperature
9	Toluene	1 hour	Room temperature
10	Toluene	0.5 hours	Room temperature
11	56^{o}C Paraffin Wax	0.5 hours	60^{o}C
12	56^{o}C Paraffin Wax	1.5 hours	60^{o}C
13	56^{o}C Paraffin Wax	1.5 hours	60^{o}C

All steps were performed under pressurized conditions in a vacuum according machine conditions (*Tissue-Tek® VIP 3000, Sakura, Neth*). *Tissue processing was typically carried out overnight.*

Appendix 2.2 – Protocol for De- Waxing and Paraffin Rehydration of Slides

Step	Reagent	Time
1	Xylene 1	5 mins
2	Xylene 2	2 mins
3	100% IMS	2 mins
4	95% IMS	2 mins
5	70% IMS	2 mins
6	Running Water	2 mins

Slides were loaded into standard staining troughs and bathed in reagents as above to remove wax and permit rehydration before staining protocols. Note 'dehydration' protocols essentially involved the reversed process.

Appendix 2.3 – Protocol for 3-(Aminopropyl)triethoxysilane (APTS) Coating of Slides

Step	Reagent	Time
1	2% APTS solution in IMS	2 mins
2	Distilled Water	2 mins
3	Distilled Water	2 mins

For APTS preparation staining, slides were loaded into standard staining troughs and bathed in reagents as above to permit coating.

Appendix 2.4 – Protocol for Haematoxylin and Eosin (H&E) Staining

Step	Reagent	Time
1	Gill's Haematoxylin	90 sec.
2	Running Water	5 mins
3	1% Hydrochloric Acid in 70% IMS	3 sec.
4	Running Water	4 mins
5	0.5% Aqueous Eosin	5 mins
6	Running Water	20 sec.
7	Dehydrate and Mount with Distyrene Plasticizer Xylene	

Appendix 2.5 – Protocol for Picrosirius Miller's Elastin (PME) Staining

Step	Reagent	Time
1	Acid Potassium Permanganate	5 mins
2	Distilled Water	1 minute
3	1% Aqueous Solution of Oxalic Acid	2 mins
4	Distilled Water	5 mins
5	70% IMS	1 minute
6	90% IMS	1 minute
7	Miller's Stain	1 hour
8	95% IMS	1 minute
9	Tap Water	2 mins
10	Weigert's Haematoxylin	10 mins
11	Tap Water	1 minute
12	1% HCl in 70% IMS	5 sec.
13	Tap Water	5 mins
14	Distilled Water	30 sec.
15	Picrosirius Red	45 mins
16	Dehydrate and Mount with DPX	

Appendix 2.6 – Protocol for Masson's Trichrome Staining

Step	Reagent	Time
1	Wiegert's Haematoxylin	10mins
2	Tap water	5 mins (wash)
3	Biebrich Scarlet Acid Fuchsin	10 mins
4	De-ionised water	2 mins
5	Phosphomolybdic Acid- Phosphotungstic Acid	15 mins or until collagen no longer red
6	Aniline blue	10 mins
7	De-ionised water	1 minute rinse
8	1% Acetic acid	3 mins
9	De-ionised water	1 minute wash
10	Dehydrate and Mount with DPX	

Appendix 2.7 – Protocol for Alcian Blue Staining

Step	Reagent	Time
1	De-wax and rehydrate slides	15 mins
2	1% Alcian Blue in 3% Acetic Acid (pH 2.5)	5 mins
3	Tap Water	2 mins
4	0.1% Nuclear Fast Red in 5% Aluminium Sulphate	1 minute
5	Tap Water	20 sec.
6	Dehydrate and Mount with DPX	5 sec. quick rinse

3.1 DNA Quantification Protocol

Step	Process	Time
1	Add 180µL Lysis T solution to samples in 1.5mL microcentrifuge tubes	
2	Add 20µL Proteinase K[1] solution to samples and vortex mix	
3	Place samples in heating block at 55°C, vortex mixing every 30 minutes until tissue dissolved	2 – 4 hours
4	Add 20µL RNAse to samples and incubate at room temperature	2 minutes
5	Add 200µL Lysis Solution C and vortex mix. Incubate at 70°C	10 minutes
6	Add 200µL 95% ethanol to lysate and vortex mix	10 seconds
7	Transfer tube contents to corresponding prepared binding columns and centrifuge at 6,500G	1 minute
8	Discard collection tube and replace column in new binding tube. Add 500µL wash solution to binding column and centrifuge at 6,500G	1 minute
9	Discard collection tube and replace column in new binding tube. Add 500µL wash solution to binding column and centrifuge at 12,000G	3 minutes
10	Discard collection tube and replace column in new binding tube. Pipette 200µL of elution solution directly into the centre of the binding column and incubate at room temperature before centrifuging at 6,500G to elute DNA	1 minute

All reagents supplied by Sigma-Aldrich, UK.
1 – Proteinase K 20mg/ml solution

3.2 Collagen Quantification Protocol

Step	Process	Time
1	1.5mL pepsin in acetic acid[1] added to 50mg of sample tissue in 1.5mL microcentrifuge tubes. Incubated at 4°C	Overnight
2	1mL aspirated from each tube and transferred to new tube. Add 100µL acid neutralising reagent and 200µL cold isolation and concentration reagent. Mix tubes by inversion and place in rack in a container half filled with an ice water mix. Incubate at 4°C	Overnight
3	Centrifuge tubes at 12000 rpm	10 minutes
4	Remove supernatant using micropipette and add 1mL Sircol dye reagent. Mix by inversion and place tubes in mechanical shaker	30 minutes
5	Centrifuge tubes at 12000 rpm	10 mins
6	Pour off supernatant. Gently pipette 750µL ice-cold acid salt wash reagent to the collagen dye pellet. Centrifuge at 12,000rpm	10 minutes
7	Discard wash supernatant. Add 250 µL alkali reagent to samples and vortex mix	
8	Transfer 50µL of sample(s) to microcuvettes for analysis in spectrophotometer	

All reagents supplied in Assay Kit (Sircol, Biocolor,UK). Samples tested against standards made using kit and 'blanks'
1- Pepsin at 0.1mg/ml dissolved in 0.5M acetic acid

3.3 Glycosaminoglycan Quantification Protocol

Step	Process	Time
1	1ml Papain[1] extraction buffer added to 50mg tissue in 1.5mL microcentrifuge tube. Incubate at 65°C in water bath	3 hours
2	Pipette 50µL of digested solution in new tubes and make up to 100µL with distilled water then add 1mL Blyscan dye reagent. Place in mechanical shaker	30 minutes
3	Centrifuge tubes at 12,000rpm	10 minutes
4	Drain supernatant from tubes leaving GAG complex at base	
5	Add 0.5mL dissociation reagent, cap and vortex mix	
6	Transfer 50µL of sample(s) to microcuvettes for analysis in spectrophotometer	

All reagents supplied by in Assay Kit (Blyscan, Biocolor,UK). Samples tested against standards made using kit and 'blanks'
1- Papain extraction buffer: 50mL 0.2M sodium phosphate buffer supplemented with 400mg sodium acetate, 200mg EDTA disodium salt, 40mg cysteine HCl and containing 5mg of papain.

3.4 – MHC II Immunostaining Protocol

Step	Process	Time
1	Slides de- waxed and rehydrated (see appendix 2.2)	15 minutes
2	Antigen Retrieval with Citrate buffer at 95^{o}C	15 minutes
3	Rinse with PBS x 3	
4	Apply endogenous block with 3% hydrogen peroxide	30 minutes
5	Rinse with PBS x 3	
6	Add normal blocking serum	30 minutes
7	Add Primary antibody 1:100 (*VMRD MHC II*)	2 hours
8	Rinse with PBS x 3	
9	Add biotinylated secondary antibody	30 minutes
10	Rinse with PBS x 3	
11	Add Vectastain ABC reagent	30 minutes
12	Rinse with PBS x 3	
13	Add DAB peroxidase substrate solution	90 seconds
14	Immerse in distilled water	5 minutes
15	Counterstain with Harris haematoxylin	1 minute
16	Wash with tap water	5 minutes
17	Differentiate in Acid Alcohol	3 seconds
18	Wash in tap water	5 minutes
19	Dehydrate and mount with DPX	

3.5 Small Intestinal Decellularization Protocol

Step	Decellularization Reagent	Temperature	Time
1	0.075% Sodium Dodecyl Sulphate (SDS)	Room	90 minutes
2	PBS wash x 3 (15 minutes each)	Room	45 minutes
3	0.05% Trypsin & 0.05% EDTA	37^{o}C	90 minutes
4	PBS wash x 3 (15 minutes each)	Room	45 minutes
5	DNAse (2000U)	37^{o}C	120 minutes
6	PBS wash	Overnight	
7	0.075% Sodium Dodecyl Sulphate (SDS)	Room	90 minutes
8	PBS wash x 3 (15 minutes each)	Room	45 minutes
9	0.05% Trypsin & 0.05% EDTA	37^{o}C	90 minutes
10	PBS wash x 3 (15 minutes each)	Room	45 minutes
Arterial Circuit then perfused with PBS only			
12	0.075% Sodium Dodecyl Sulphate (SDS)	Room	90 minutes
13	PBS wash x 3 (15 minutes each)	Room	45 minutes
14	0.05% Trypsin & 0.05% EDTA	37^{o}C	90 minutes
15	PBS wash x 3 (15 minutes each)	Room	45 minutes

Note following step 10, the vascular circuit was perfused with PBS only until completion of the protocol. Decellularization of the intestinal circuit was continued as per steps 12- 15.

3.6 Phase I Decellularization Experiments – Determining Important Variables

Study No.	Decellularization Reagent and Duration (minutes)					RPM	Reagent Volumes (L)	No. of washes between reagents	Wash Durations	Quality of Perfusion	Decellularization Status (histology)	Comments
	SDS (0.075%)	TRYPSIN + EDTA (0.05% + 0.05%)	DNase I (2000 U)	SDS (0.075%)	TRYPSIN + EDTA (0.05% + 0.05%)							
1	60	60	60	60	60	60	2	1	45	Poor	Cells remaining	Analogue pump
2	60	60	60	60	60	60	2	1	45	Average	Cells remaining	Analogue pump
3	60	60	60	60	60	60	2	1	45	Average	Cells remaining	Digital pump
4	60	60	60	60	60	90	2	3	45	Average	Few cells remaining	Damage to small vessels
5	90	60	120			60	2	3	15	Good	Few cells remaining	Ceased due to fracture
6	90	30	30			60	2	3	15	Good	Few cells remaining	Volumes excessive
7	90					15	1	3	15	Poor	Not assessed	Inadequate perfusion to complete study
8	60	30	60			30	1	3	15	Average	Cellular components present	None
9	60	30	120	60		30	1	3	15	Average	Cellular components present	None
10	60	30	60			30	1	3	15	Average	Nuclear debris only remaining	None
11	90	90	120	90	90	30	1	3	15	Poor	Cellular components present	Vascular circuit only pumped
12	30	30	30	30	30	30	1	3	15	Good	Cells remaining	Macroscopy inconsistent with histology
13	60	30				30	1	6	15	Good	Cells remaining	Ceased due to fracture
14	30	30	30	60		30	1	3	15	Excellent	Few cells remaining	Macroscopically excellent
15	30	30	30	60		30	1	3	15	Good	Few cells remaining	Proximal end increasingly fragile

4.1 Oil Red O Staining Protocol

Step	Process	Time
1	Remove medium and wash cells with Dulbecco's PBS (without Ca^{+} and Mg^{+})	
2	Add neutral buffered formalin (10%) to cover the cell monolayer	30 minutes
3	Remove NBF and wash with distilled water	
4	Remove water and add 60% isopropanol[1] to cover the cell monolayer	5 minutes
5	Remove isopropanol and add enough Oil Red O solution[2] to cover monolayer	15 minutes
6	Remove Oil Red O solution and wash layer with distilled water 3 times	
7	Invert plate and blot on absorbent paper to dry	
8	Counterstain with Harris Haematoxylin[3]	1 minute
9	Aspirate haematoxylin and wash 3 times until monolayer clear	
10	Add PBS before review under inverted microscope	

1- *Isopropanol (Sigma-Aldrich, UK) dissolved in distilled water*
2- *Stock solution of 0.3% Oil Red O in isopropanol diluted 3:2 with distilled water and filtered. Must be used within 30 minutes*
3- *Harris Haematoxylin (Sigma-Aldrich, UK)*

4.2 Alcian Blue Staining of Micro-Masses Protocol

Step	Process	Time
1	Remove medium and wash micro-masses twice with Dulbecco's PBS (without Ca^{+} and Mg^{+})	
2	Add glutaraldehyde (4%) to cover the micro- masses	15 minutes
3	Remove glutaraldehyde and wash with 1M hydrochloric acid	
4	Add Alcian Blue Staining solution[1] to cover micro- masses. Leave in the dark.	Overnight
5	Remove Alcian Blue solution and wash with distilled water 3 times	

1- *Alcian Blue (Carl Roth, Germany)*

4.3 Alkaline Phosphatase Staining of MSC Differentiation Protocol

Step	Process	Time
1	Remove medium and wash with Dulbecco's PBS (without Ca^+ and Mg^+)	
2	Add neutral buffered formalin (10%) to cover the cell monolayer	60 seconds
3	Remove NBF and wash with washing buffer[1]	
4	Add BCIP-NBT substrate solution[2] to cover monolayer. Incubate in the dark	10 minutes
5	Remove solution and wash with washing buffer	
6	Remove washing buffer and add PBS before imaging	

1- *Washing Buffer – 0.05% Tween 20 in Dulbecco's PBS (without Ca^+ and Mg^+)*

2- *BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) solution - SIGMA FAST™ BCIP/NBT single tablet dissolved in 10ml distilled water, stored in the dark and used within 2 hours*

4.4 Alizarin Red S Staining of MSC Differentiation Protocol

Step	Process	Time
1	Remove medium and wash with Dulbecco's PBS (without Ca^{+} and Mg^{+})	
2	Add neutral buffered formalin (10%) to cover the cell monolayer	30 minutes
3	Remove NBF and wash with distilled water	
4	Add Alizarin Red S[1] staining solution to cover monolayer. Incubate in the dark	45 minutes
5	Remove staining solution and wash with PBS before reviewing	

1- *Alizarin Red S staining solution – 2g Alizarin Red S (Sigma-Aldrich, UK) dissolved in 100ml and pH adjusted to 4.1-4.3*

4.5 Porcine Adipocyte Culture Solutions

Krebs-Ringer Bicarbonate (KRBH) Buffer:

containing the following dissolved in solution (pH 7.4):

- 10mM $NaHCO_3$
- 30mM HEPES
- 200nM Adenosine
- 1% BSA

Dulbecco's Modified Eagle's Medium 1 (pH 7.4):

- 25mM Glucose
- 2mM Glutamine
- 200nM RN adenosine
- 100µg/mL gentamycin
- 25mM HEPES

Dulbecco's Modified Eagle's Medium 2 (pH 7.4):

DMEM (1) with 7% Bovine Serum Albumin (BSA) in solution

4.6 Raw Data of Porcine Organoid Unit Isolation Protocol

Animal study number	Sample Number	Mechanical Tissue Chopping	Collagenase conc. (U/ml)	Enzyme incubation time (mins)	Agitation velocity (RPM)	Flask position	OU count (OU/ml)	OU count total (OU/ml)
1	1	Yes	300	30	120	Vertical	1,250	6,250
				60	120	Vertical	5,000	
	2	Yes	800	30	120	Vertical	125,000	126,250
				60	120	Vertical	1,250	
	3	Yes	800	30	120	Vertical	0	0
				60	120	Vertical	0	
2	1	No	300	30	150	Horizontal	0	0
				60	150	Horizontal	0	
	2	No	300	30	150	Horizontal	0	0
				60	150	Horizontal	0	
3	1	No	300	30	100	Horizontal	0	5,000
				60	100	Horizontal	5000	
	2	No	800	30	100	Horizontal	6,250	8,750
				60	100	Horizontal	2,500	
4	1	No	300	30	100	Horizontal	2,500	2,500
				60	100	Horizontal	0	
	2	No	300	30	100	Horizontal	1,250	5,000
				60	100	Horizontal	3,750	
5	1	No	800	30	100	Horizontal	2,500	13,750
				60	100	Horizontal	11,250	
	2	No	800	30	100	Horizontal	3,750	6,250
				60	100	Horizontal	2,500	
	3	No	800	30	100	Horizontal	1,250	6,250
				60	100	Horizontal	5,000	

4.7 Raw Data of Porcine Mesenchymal Stem Cell Culture from Bone Marrow

Animal Study Number	Age of pig	Aspirate volume	Passage 0 (Success/ Failure)	Passage 1 (Success/ Failure)	Passage 2 (Success/ Failure)	Passage 3 (Success/ Failure)	Total number of cultured cells (approx.)
1	4m	20	Success – passaged only	Preserved and Passaged	Preserved and Passaged	Terminated	7×10^5
2	10m	35	Success – passaged only	Preserved	Failed to proceed	Failed to proceed	3×10^5
3	4m	60	Preserved and Passaged	Preserved and Passaged	Preserved and Passaged	Preserved and Passaged	13×10^6
4	9m	25	Failed to proceed	X	X	X	0
5	6m	30	Passaged	Preserved and Passaged	Preserved and Passaged	Preserved and Passaged)	6×10^6
6	4m	45	Preserved and Passaged	Preserved and Passaged	Preserved and Passaged	Preserved and Passaged	8×10^6
7	6m	50	Passaged	Preserved and Passaged	Preserved and Passaged	Preserved and Passaged	Not recorded
8	6 weeks	40	Preserved and Passaged	Preserved and Passaged	Preserved and Passaged	Preserved and Passaged	Not recorded
9	6 weeks	25	Preserved and Passaged	Preserved and Passaged	Preserved and Passaged	Preserved and Passaged	Not recorded
10*	7 weeks	10	Preserved and Passaged	Preserved and Passaged	Preserved and Passaged	Preserved and Passaged	Not recorded

*Note *n10 was used to determine initial plating density (2.2×10^6) following previous successful cultures.*