

Scaffold Development to Engineer Human Intestinal Epithelial Tissue Grafts

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Abstract

Epithelial crypts isolated from normal colon mucosa have been cultured *in vitro* in the form of organoids. These organoids have been shown to have regenerative potential when implanted in mouse models of denuded colon mucosa. However, a major limitation in the translation of these studies to a clinical setting is the use of Matrigel, which is clinically unsuitable due to its undefined composition and potential for tumourigenicity. Additionally, enclosed organoids do not fully reproduce the structure and function of native tissue, and do not lead to full integration into the host. Therefore, the overall aim of this thesis was to investigate different scaffolds for their ability to support growth of primary colon epithelial cells in order to form an open monolayer structure that resembles native epithelium.

It was anticipated that *in vitro* culture of primary colon cells would be more efficient with isolated stem cells than with an entire heterogeneous epithelial cell population. Therefore, the side population technique was explored using established cell lines as a method of isolating a population enriched in stem cells, based on their ability to efflux the Hoechst 33342 dye. This was found to be irreproducible and unreliable so was not pursued further.

Various scaffolds were manufactured, characterised and seeded with colon epithelial cell lines to investigate how their morphological and biochemical characteristics influenced cell proliferation and differentiation. Electrospun nanofibre and microfibre scaffolds made from the synthetic material PET, and the natural polymer gelatin were fabricated. All electrospun scaffolds supported proliferation, and cells on gelatin nanofibre scaffolds differentiated to form tight

junctions that are typical of epithelial cells, while also expressing increased levels of the stem cell marker *ABCG2*. Thereby, gelatin nanofibres demonstrated the ability to support appropriate cell differentiation while maintaining a pool of stem cells.

Gelatin in the form of hydrogel scaffolds was also investigated for its ability to support cell proliferation and influence cell differentiation. Moreover, a novel method of rapidly 3D printing scaffolds using unmodified gelatin was presented. Both planar and 3D gelatin scaffolds were able to support proliferation and tight junction formation in Caco2 cells. Thus, it was concluded that gelatin hydrogels are a suitable substrate for proliferation and differentiation of colon carcinoma cell lines, and that the addition of the printed topography did not have any negative effects on cell growth and behaviour.

Epithelial crypts were isolated from human colon mucosa and expanded *in vitro* in the form of organoids, as previously published. It was found that the precise nature and source of organoid media components are critical for successful primary cell culture. A method in which the Wnt signalling cascade is indirectly activated by GSK3 β inhibition was found to be partially successful for growing organoids and may be a potential alternative culture method. Primary organoid cells were seeded onto gelatin nanofibre and hydrogel scaffolds, however the cells did not proliferate in these preliminary experiments. Overall, it was concluded that although the gelatin scaffolds demonstrated promising results with colon carcinoma cell lines, more research would be required in order to produce scaffolds that can support growth of primary colon epithelial cells.

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Abbreviations

ABCG2	ATP-binding cassette sub-family G member 2
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BMP	Bone morphogenetic protein
CBC	Crypt base columnar cell
COOH	Carboxylic acid
DCM	Dichloromethane
DCV	DyeCycle Violet
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECM	Extracellular matrix
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FTC	Fumitremorgin C
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GSK3β	Glycogen synthase kinase 3 beta

HFIP	Hexafluoroisopropanol
HPRT	Hypoxanthine phosphoribosyltransferase
IMS	Industrial Methylated Spirits
KGMG	Keratinocyte Growth Medium Gold
Lgr5	Leucine-rich repeat-containing G-protein coupled receptor 5
mRNA	Messenger Ribonucleic acid
NHS	N-Hydroxysuccinimide
PBS	Phosphate buffered saline
PDMS	Polydimethylsiloxane
PET	Poly(ethylene terephthalate)
PLGA	Poly(lactic-co-glycolic acid)
qPCR	Quantitative real-time polymerase chain reaction
RPM	Revolutions per minute
SD	Standard deviation
SEM	Scanning electron microscopy
SIS	Small intestinal submucosa
SP	Side population
TFA	Trifluoroacetic acid
TNBS	2,4,6-Trinitrobenzene sulfonic acid
UV	Ultraviolet
Zo-1	Zonula occludens-1

Chapter 1: General Introduction

1.1 The gastro-intestinal tract

The human gastrointestinal (GI) tract extends from the mouth to the anus and comprises the oral cavity, oesophagus, stomach, small and large intestines. Its primary functions are to digest food, extracting nutrients in forms that can be utilised by the body, and to remove unusable waste products. It is essentially a hollow tube with a lumen which varies in diameter, surrounded by a wall made up of four main layers: the serosa, muscularis externa, submucosa and mucosa [2].

(Figure 1.1.1)

The serosa is the outermost layer and consists of loose connective tissue covered by a layer of mesothelial cells. The muscularis externa is made up of the inner circular and outer longitudinal muscle layers. The two layers of smooth muscle contract in coordination to produce the peristaltic wave that moves food along the tract. The submucosa is an irregular layer of dense connective tissue with blood vessels, lymphatics and nerves. It includes Meissner's nerve plexus and the enteric nervous system, which regulate smooth muscle contractions.

The mucosa is the inner lining of the GI tract and is composed of the muscularis mucosae, lamina propria and a layer of epithelial cells. The muscularis mucosae layer separates the mucosa from the underlying submucosa and is made up of smooth muscle and elastic fibres. The lamina propria contains connective tissue, myofibroblasts which secrete extracellular matrix and growth factors, blood vessels,

lymphatics and nerves. The epithelium is a selectively permeable barrier that is highly specialised and varies throughout the gastrointestinal tract.

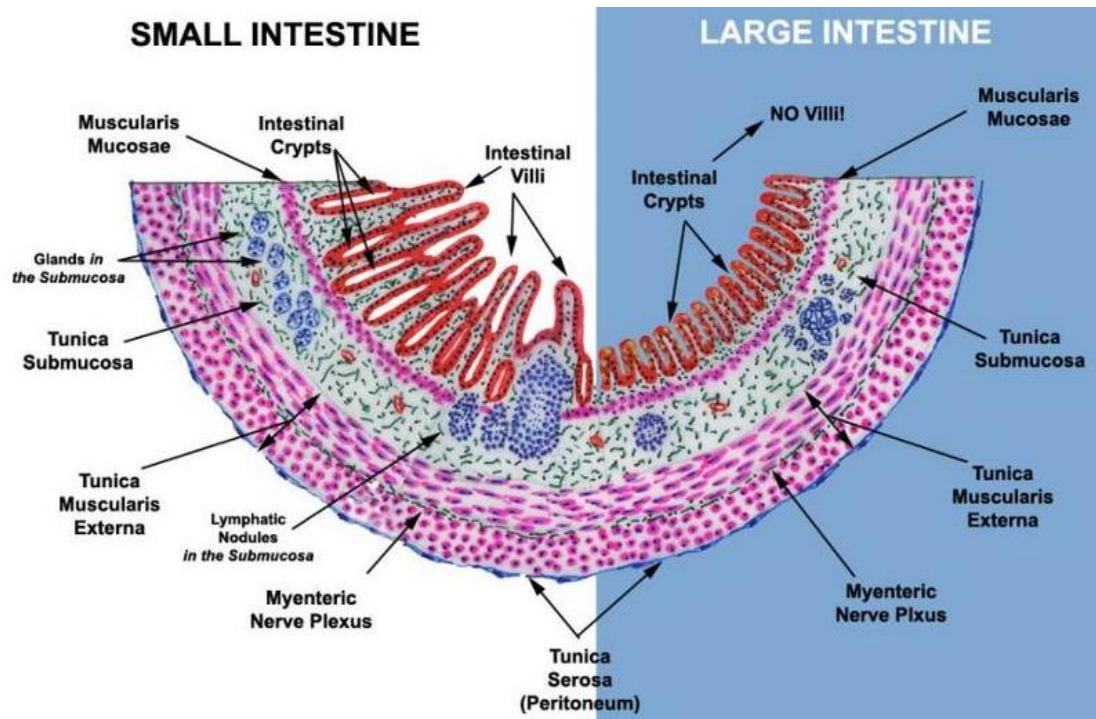


Figure 1.1.1: The structure of the wall in the small and large intestine

Taken from [3]

1.1.1 Epithelium in the small and large intestine

The intestinal epithelium is composed of specialised cells whose primary functions are the absorption of nutrients and to provide a barrier against external microorganisms and toxins. The absorption of most nutrients occurs in the small intestine, whereas the large intestine is responsible for the absorption of electrolytes and water. In order to maximise surface area for absorption in the small intestine, the epithelium forms intraluminal finger-like projections known as villi. Between the villi, there are invaginations in the epithelium called crypts of

Lieberkühn [4]. In the large intestine the epithelium is organised into crypts but villi are not formed.

There are four main cell types in the intestinal epithelium [4]:

Absorptive cells: These are tall, columnar cells with basally situated nuclei and are the most abundant cell type. In the small intestine, microvilli are present on their apical membranes forming the brush border. This further increases surface area and provides the site for small molecule transport.

Goblet cells: These are scattered throughout the epithelium and provide a protective mucus layer into the lumen. The cells secrete mucins and antimicrobial proteins that provide the first line of defence against microbial infection [5].

Enteroendocrine cells: These cells are responsible for controlling physiological and homeostatic functions in the digestive tract by secreting numerous peptide hormones [6].

Paneth cells: These are pyramidal shaped cells normally found only at the bottom of small intestinal crypts. They contain cytoplasmic granules containing enzymes such as lysozyme, defensins and antimicrobial polypeptides, which are released in response to stimuli such as exposure to bacterial products. They have also been shown to play a key role in the intestinal stem cell niche [7].

Due to its key barrier function, the epithelium is subjected to many potentially lethal microorganisms and toxins. This leads to a high level of cell death, with up to 10^{11} cells being lost each day [8]. This results in constant cell-renewal which is driven by intestinal epithelial stem cells.

1.1.2 Stem cells in the intestinal epithelium

Stem cells are self-renewing, undifferentiated cells that have the potential to produce cells of different types. There are two main types of stem cell [9]: pluripotent and adult stem cells. Pluripotent stem cells can self-renew indefinitely and have the potential to differentiate into any cell type. Adult stem cells are isolated from a specific adult tissue or organ. They have a restricted differentiation potential, and can usually only generate cells of the tissue from which they were isolated.

Adult stem cells in the small and large intestines are found at the base of crypts of Lieberkühn. They replicate and differentiate to generate cells of different types. As they differentiate, absorptive, goblet and enteroendocrine cells migrate upwards towards the lumen where they are eventually shed. Paneth cells, present only in the small intestine, migrate downwards to the crypt base. This process of cell renewal in the intestinal epithelium occurs continuously and takes approximately 5 days [4]

The precise stem cell location within the crypt has led to differing reports in the literature. Crypt base columnar (CBC) cells were first described in 1974 [10] as undifferentiated cells found between positions 1-9 at the bottom of the crypt between Paneth cells in mouse small intestine. Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) has since been found to be a biomarker for these cells, which represent long-lived, rapidly cycling, multipotent stem cells that generate all the differentiated cells of the intestinal epithelium [11]. Another niche consisting of quiescent stem cells located above the base of a crypt, at the '+4' position in small intestinal crypts has been proposed. These cells were originally

identified as DNA label retaining cells, which selectively segregate their DNA after replication in order to protect against genetic damage [12]. A schematic representation of cell positions is shown in Figure 1.1.2.

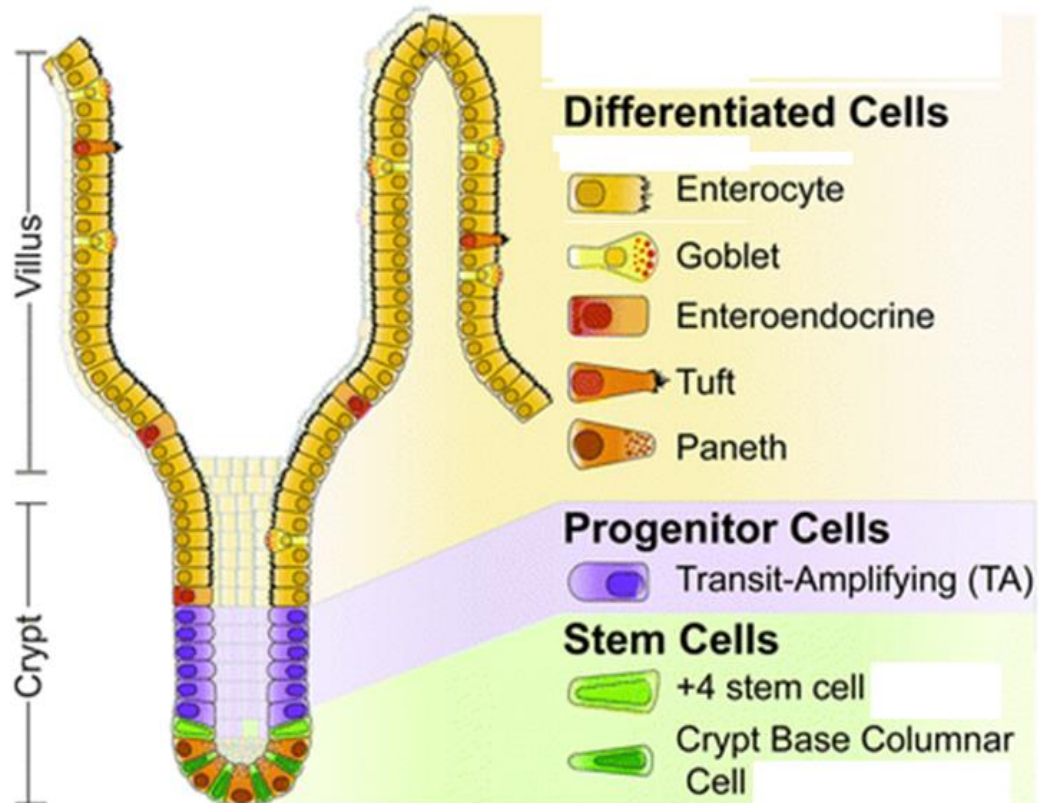


Figure 1.1.2: Schematic showing cell organisation in intestinal epithelium

CBC and '+4' stem cells are found at the base of crypts. Transit-amplifying progenitors arise from the stem cell compartment, and differentiate into all other epithelial cell types. Taken from [13]

1.1.3 Intestinal stem cell niche

The stem cell niche is a stable, custom microenvironment that nurtures stem cells and allows them to maintain tissue homeostasis. It is composed of a subset of mesenchymal cells and extracellular substrates which protect epithelial cells from excessive stem cell production that could potentially lead to organ disruption and cancer, and from differentiation or apoptosis that could deplete stem cell reserves [14].

Subepithelial myofibroblasts are specialised mesenchymal cells located below the basement membrane that are part of the intestinal stem cell niche. Together with extracellular matrix and mesenchymal cells such as enteric neurons and intraepithelial lymphocytes, they play a role in regulating epithelial proliferation and differentiation [15]. This is done through epithelial-mesenchymal crosstalk and the secretion of Wnt ligands.

There are four main signalling pathways controlling stem cell proliferation and differentiation: Wnt, EGF, Notch and BMP [16] (figure 1.3). The canonical Wnt pathway is the primary signalling pathway regulating 'stemness' in the intestinal epithelium. Wnt has 19 family members in humans, which bind to receptors composed of Frizzled and Lrp, and activate the cytoplasmic phosphoprotein 'dishevelled'. This causes inhibition of GSK3 β and accumulation of β -catenin which can migrate to the nucleus to form nuclear β -catenin/Tcf complexes. This activates expression of Wnt target genes, including *c-myc*, *tcf-1*, *cyclin D1*, *c-Jun*, *Fra-1*, *fibronectin*, *CD44* and *MMP* [17]. R-spondin is a Wnt agonist that binds to Lgr5, which is found in complex with LRP and Frizzled [18]. APC is a tumour suppressor

protein and a negative regulator of Wnt signalling. In the absence of Wnt signalling, APC binds β -catenin and targets it for degradation in the proteasome.

The Notch and EGF signalling pathways are also important for maintaining stem cells in an undifferentiated state. When the Notch transcription factor RBP-J was removed or a γ -secretase inhibitor was used to block the Notch pathway, proliferative stem and TA cells differentiated into goblet cells [19]. Additionally, epidermal growth factor (EGF) promotes stem cell proliferation by activation of the Ras/Raf/MEK/ERK signalling pathway [16]. Bone morphogenetic protein (BMP) negatively regulates stem cell proliferation, by repressing nuclear β -catenin accumulation. The BMP pathway plays an important role in controlling stem cell self-renewal thereby maintaining normal crypt size [20]. A diagram summarising the signalling pathways that regulate stem cell proliferation and differentiation is shown in Figure 1.1.3.

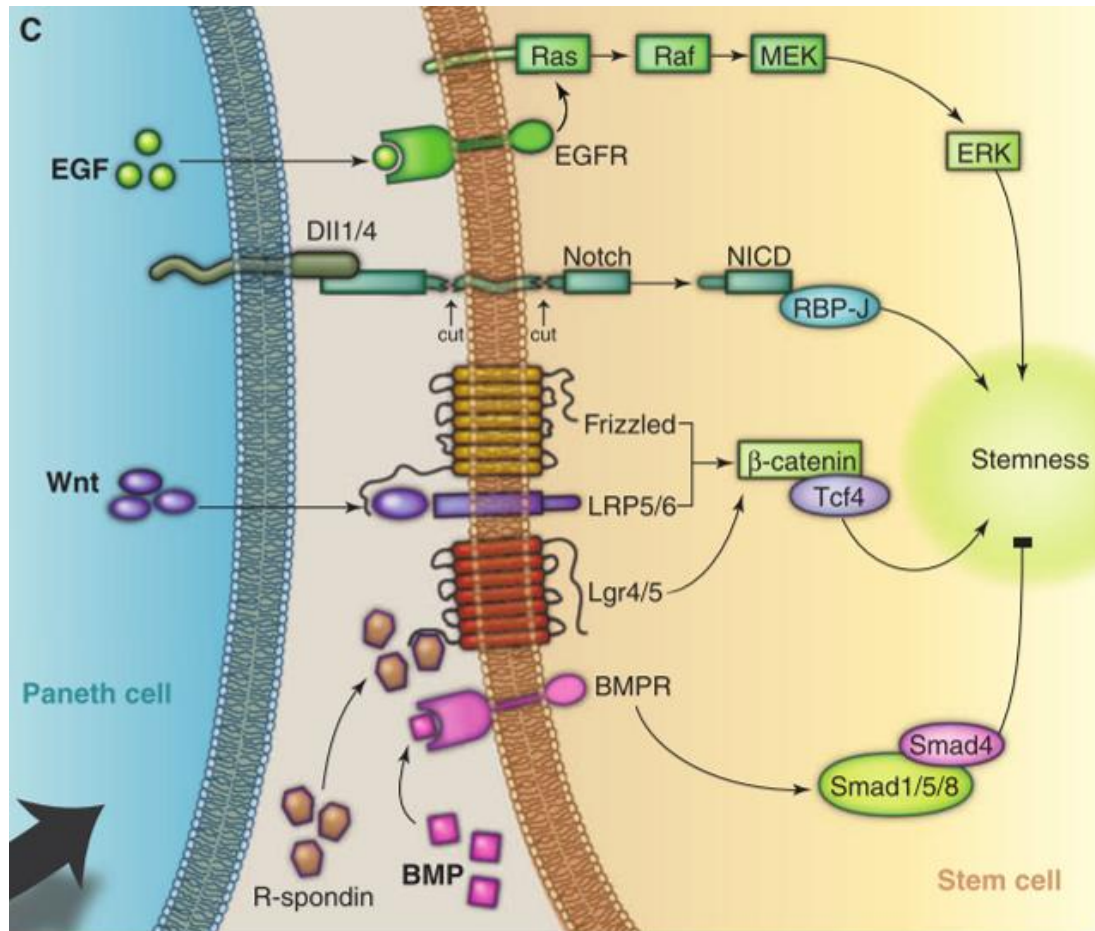


Figure 1.1.3: Signalling pathways involved in controlling stem cell proliferation and differentiation in the intestinal epithelium

Taken from [16]

1.1.4 Intestinal stem cell markers

Identifying stem cells by the expression of a unique combination of markers has been a challenging area of intestinal stem cell research, though a number of intestinal stem cell markers have recently been described. One of the first was Musashi-1 (Msi-1), which has been reported as a stem cell marker in murine small and large intestine [21].

B lymphoma Mo-MLV insertion region 1 homolog (Bmi1) and Doublecortin and CaM kinaselike-1 (DCAMKL-1) mark the '+4' stem cell population, though Bmi1 is limited to the proximal small intestine and DCAMKL-1 may be a marker of stem cell response to injury [22]. Conversely, a number of other markers have been described which label CBC cells specifically, such as: leucine-rich-repeat-containing G-protein-coupled receptor 5 (Lgr5), CD133 (prominin-1), β -catenin, Ascl2 and SRY-box containing gene 9 (Sox9) [23].

The use of genetically engineered mice in which green fluorescent protein (GFP) was integrated into the Lgr5 locus made it possible to isolate cells from murine small intestine based on expression of the CBC marker Lgr5 [24]. Single Lgr5-GFP+ cells were isolated and cultured to form organoids with all differentiated cell types and a crypt-villus structure, thereby providing functional evidence that cycling CBCs are stem cells. GFP in a transgenic mouse has also been used to isolate cells from murine small intestine based on Sox9 expression. Two cell populations were identified based on Sox9 mRNA and protein levels. Sox9-lo cells were found to be CBC cells that were enriched for Lgr5, whereas Sox9-hi cells were found to be distributed at higher crypt positions and in the villi and were identified as differentiated enteroendocrine cells [25]. Further work by the same group showed that a single Sox9-GFP-lo cell could be cultured to generate organoids with all differentiated cell types [26], supporting the previous findings that an Lgr5-GFP+ cell can generate *in vitro* organoids.

Despite these advances in intestinal stem cell marker identification, almost all of these markers represent intracellular proteins and are identified in genetically

modified mice or by immunostaining fixed histological tissue sections. An ideal combination of surface markers allowing positive cytological sorting of an enriched ISC population remains elusive.

CD24 was identified as a surface marker that could potentially be used to sort cells without the need for transgenic mice. CD24-lo and CD24-med populations isolated by FACS correlated with Sox9-lo cells and could also generate *in vitro* organoids, although with reduced efficiency compared with Sox9-lo cells. This was followed by investigation of colonic epithelial cells. CD24+ cells from murine colon were enriched for Lgr5 and CD166 indicating that this cell population represents colonic cycling stem cells [27]. Additionally, an antibody against the extracellular domain of ephrin type-B receptor 2 (ephrinB2) has been used to isolate stem cells from human colon mucosa [28]. This report suggests that ephrinB2-high cells identified by flow cytometry are enriched with stem cells and can be cultured and expanded *in vitro*.

An alternative technique for isolating ISC has been described and uses the efflux properties on stem cells for the DNA-binding bis-benzimide blue fluorescent dye Hoechst 33342. In 1996 Goodell *et al.* described a sub-population of murine bone marrow-derived cells which efflux Hoechst 33342 [29]. Hoechst 33342 is a cell permeable, DNA binding dye which enters cells through passive diffusion and is actively effluxed by ATP-binding cassette (ABC) transporters. Cells are analysed by a flow cytometer which includes a UV laser that excites the Hoechst dye. Stem cells possess a greater number of ABC transporters, and therefore do not retain the dye. Consequently, when Hoechst red and blue fluorescence emissions are plotted against each other, a low fluorescence tail of cells can be seen, and this is referred

to as the side population (SP). This technique has been used to sort stem cells from a number of different tissues, including kidney [30], lung [31] and mammary gland [32].

The side population technique was used to sort cells isolated from mouse small intestine, and although the side population was found to be enriched for the stem cell markers musashi-1 and β 1-integrin, it was heterogeneous rather than a pure stem cell population [33]. The side population method was also carried out with human colonic epithelial cells [34]. This study showed the side population cells expressed musashi-1, BerEP4, β 1-integrin and CD133.

1.1.5 *In vitro* culture of intestinal epithelium

To date, several different approaches have been used to culture intestinal epithelium. Clusters of mesenchymal cells surrounded by epithelium, known as 'organoid units' were isolated from rat small intestine [35]. Additionally, pure populations of intestinal epithelial cells in the absence of mesenchymal cells were cultured on a collagen gel with media containing a cocktail of growth factors and small molecules [36]. In both of these early efforts, proliferation could only be maintained for 1-2 weeks. Later on, single cells isolated from colonic crypts were cultured on agar with the addition of conditioned medium from colon carcinoma cell line LIM1863 [37]. With this method, *in vitro* cultures were maintained for 4 weeks. However the unknown nature of the conditioned media and the lack of cell differentiation meant that epithelial architecture was not preserved, limiting the use of this culture method.

More recently, intestinal fragments containing mesenchymal cells, as well as epithelial cells, were grown in a collagen gel with an air-liquid interface and media containing a fusion protein of the Wnt agonist R-spondin-1 and immunoglobulin [38]. With this method, spheroid shaped structures with multi-lineage differentiation and proliferation were sustained for up to 4 weeks. However, the limitation of this method was the lack of a epithelial crypt-villus morphology.

1.1.6 Intestinal organoids

The breakthrough in intestinal epithelial isolation and *in vitro* culture occurred in 2009. Sato and colleagues isolated Lgr5+ stem cells from intestinal crypts and cultured them embedded in Matrigel with a well-defined medium which was based on meeting certain growth requirements of intestinal epithelium [24]. When intestinal epithelial cells are detached from their surrounding extracellular matrix, they have been found to undergo a programmed cell death, known as anoikis [39]. By embedding cells within the laminin-rich Matrigel, this cell death was prevented. The Wnt and EGF signalling pathways are required for crypt proliferation, therefore the Wnt agonist R-Spondin and EGF were included in the culture media. BMP signalling negatively regulates stem cell proliferation; therefore the BMP antagonist Noggin was also included. With this media combination, self-renewing organoids with crypt-villus architecture were formed, without the need for mesenchymal cells. The system was initially developed with murine small intestine. When it was tried with mouse colonic epithelium, it was found that the colon organoids did not produce enough Wnt to maintain the stem cells so Wnt3a was added to the culture medium [40]. When this culture system was applied to human colon, crypts could

only be maintained for up to 7 days. Various compounds were screened to increase plating efficiency and prolong culture periods. Following this, gastrin, nicotinamide and small molecule inhibitors of Alk 4/5/7 and p38 were added to the culture medium. This culture system was able to support human colon organoid expansion for at least 6 months.

This culture system has been shown to have a multitude of applications. For example, intestinal organoids have been modified by genome editing [41] or cultured from cells isolated from gene-deficient mice [42] to create *in vitro* models for investigating the roles of specific genes in disease development. Additionally, they have been used to test therapies for conditions such as inflammatory bowel disease [43] and intestinal fibrosis [44]. Organoids have also been cultured from cells obtained from cystic fibrosis patients and CRISPR/Cas9 genome editing used for gene correction [45]. In addition to these applications, intestinal organoids have also been investigated for use in mucosal regeneration.

1.1.7 Injuries and disease of colonic mucosa

Inflammatory bowel disease (IBD), comprising Crohn's disease and ulcerative colitis, affects more than 5 million people worldwide. This includes approximately 1.5 million Americans and 3 million in Europe [46,47]. In Europe, this translates to a direct healthcare cost of 4.6–5.6 billion Euros/year [48]. Furthermore, the incidence of IBD is increasing worldwide, particularly in traditionally low-incidence regions such as Asia, South America and southern and eastern Europe [49]. Crohn's disease and ulcerative colitis are chronic conditions characterised by mucosal inflammation caused by immunological and genetic abnormalities [17,50]. Current therapeutic

strategies focus on modulation of the immune system using immunosuppressant drugs such as 5-aminosalicylates, corticosteroids, and immunomodulating drugs such as azathioprine and mercaptopurine [51]. However, key features of these diseases include continued epithelial damage and impaired epithelial healing [52,53]. Furthermore, some reports suggest that treating the persistent inflammation does not necessarily lead to complete mucosal healing [54,55]. Therefore, strategies for aiding tissue regeneration are required in addition to conventional immune treatments. Other conditions that can benefit from intestinal epithelial regenerative therapies include necrotising enterocolitis [1], short bowel syndrome [56], radiation injury and intestinal infection [57].

The potential of intestinal organoids for mucosal regeneration has been demonstrated. Yui et al [58] isolated stem cells from colon crypts, expanded them *in vitro* in Matrigel, forming organoids. They then transplanted these organoids into a mouse model with damaged colon epithelium and found that firstly, the cells did engraft despite the inflammation. Secondly, the engrafted cells covered the damaged area, facilitating healing. A further similar study was conducted using colon organoids generated from foetal intestine [59]. Both of these studies showed that organoid transplantation can contribute to the regeneration of an injured epithelium. The limitations of this work include the requirement of Matrigel which is clinically unsuitable due to its undefined composition and patient incompatibility. In order to overcome this limitation, a more recent report demonstrated that the organoids could be expanded in collagen gel, then transferred to a collagen foam for delivery in the form of an enema [57].

While organoids may promote wound healing to some extent, this method does not fully reproduce the structure and function of native tissue, and may not lead to full integration into the host [1]. In order to achieve this, tissue engineering principles will have to be employed.

1.2 Tissue engineering and regenerative medicine

Tissue engineering is an interdisciplinary field in which scaffolds are combined with cells and/or biologically active molecules to form functional tissues. As described by Langer and Vacanti in 1993 in a quintessential definition, tissue engineering ‘applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain or improve tissue function’ [60]. This refers to regenerative medicine which is the field’s most common application. Another major application of tissue engineering is the development of tissues that mimic biological processes outside the body which can be used to model disease progression and test activity and toxicity of pharmaceutical compounds.

The traditional paradigm of tissue engineering in relation to regenerative medicine involves three main elements: 1) a biomaterial or scaffold that can sustain new tissue growth and regeneration, 2) a suitable cell source that can maintain the appropriate phenotype and perform the required biological functions, and 3) a growth factor and other components that provide biochemical signals that enhance tissue regeneration [61,62]. The conventional process involves harvesting cells from a patient, expanding them *in vitro*, seeding them onto scaffolds with the addition of growth factors to promote tissue development, then implanting into the patient [63]. More recently other approaches are being developed, such as the

implantation of acellular scaffolds [64], including those used to deliver drugs, growth factors and oligonucleotides [65], in order to promote *in situ* regeneration.

1.2.1 Scaffolds – functions and design considerations

Generally speaking, the role of scaffolds in tissue engineering is to provide a structure for cell attachment and tissue development and to mimic the functions of extracellular matrix (ECM) [66]. ECM has many functions. Firstly, it is important for providing cells with mechanical support and a physical environment for cell attachment and growth. Additionally, the 3D micro-environment provides bioactive cues that regulate cell signalling cascades and control cell structure, function and differentiation [67]. ECM also provides a degradable and flexible environment to allow neovascularisation, tissue remodelling and wound healing. Therefore, one of the primary approaches in scaffold design is to mimic these structural and biological functions of natural ECM.

A further function of scaffolds is to efficiently deliver cells to the area required. When free cells in suspension have been injected, cell engraftment is poor, as low as 1-5% in some cases [68]. Following cell delivery, control over cell fate is lost and the lack of a template to guide restructuring and tissue formation leads to death of the majority of the transplanted cells [69].

There are many design considerations with regards to scaffold properties. In particular, the choice of biomaterial influences mechanical properties, biodegradability and surface chemistry; all of these can affect cell properties and

thereby have an impact on cell behaviour. Additionally, scaffold shape and topography are important factors to consider.

Biomaterials can generally be categorised as either naturally derived or synthetic. One of the main advantages of using naturally occurring ECM proteins, such as collagen, elastin and laminin, is the ability of their cell-binding sites to interact with cell-surface receptors [70]. Moreover, they can undergo cell-triggered proteolytic degradation and remodelling [71]. However, limitations of these materials include poor mechanical strength, high batch-to-batch variability, difficulties in sourcing and purification and potential for immune reactions due to xenogenic components [72]. By contrast, synthetic materials are used for their improved control and adaptable chemistries, reproducibility, mechanical strength and safety [72]. The poly (α -hydroxy acid) polymers are commonly used for tissue engineering applications due to their biodegradability, biocompatibility and low toxicity [73]. Popular examples include poly (lactic acid) (PLA), poly (glycolic acid) (PGA) and their copolymer poly (lactic-co-glycolic acid) (PLGA). However, the use of synthetic materials is limited by their hydrophobicity and lack of cell recognition sites, leading to poor cell adhesion to the scaffolds. To counteract this, surface modification by the addition of functional groups or molecules has been employed [74,75]. This has led to improvements in cell attachment as well as enhanced cell spreading and growth.

Mechanical properties of scaffolds have been shown to play a significant role in cell behaviour. Many reports have demonstrated that varying substrate elasticity and stiffness can influence adhesion, proliferation, migration and differentiation in a number of different cell types [76–79]. Additionally, scaffold geometry and

topography also play an important part in cell behaviour. Changes in cell shape due to substrate micropatterning can alter cellular phenotype. For example, mesenchymal stem cells grown on micropatterned topographies adopted a rounded morphology in contrast to a flattened morphology when cultured on 2D substrates. This difference in cell shape has been shown to control lineage commitment to either adipogenic or osteoblastic phenotypes [80,81].

1.2.2 Methods for scaffold manufacture

Researchers working in the field of tissue engineering have developed many varied scaffolding strategies for cell growth and delivery, with varying materials and manufacturing methods. Different approaches may be more suitable for different organs, tissues and applications with each having their own advantages and disadvantages.

Scaffold fabrication methods have been broadly split into four categories [82]:

- 1) Solvent casting with particulate leaching, in which a polymer solution is cast in a mould with water soluble porogens. A relatively simple example of this involves freeze drying of an aqueous hydrogel causing the formation of ice porogens which are leached away during rehydration [83].
- 2) Phase separation, which involves separation of a multicomponent system. An example of this method used in scaffold fabrication is thermally induced phase separation (TIPS). At elevated temperatures, a homogenous polymer solution is separated to form two phases: a polymer-rich phase and a polymer-lean phase. When the solvent is removed, a microporous structure is formed [84]. This

technique has been used to produce porous microspheres in which porosity and pore morphology can be controlled by altering phase separation conditions [85].

3) Fibre networking, including textile methods such as braiding yarns [86] and electrospinning, which can be used to produce polymer fibres with diameters ranging from a few nanometres to greater than 5 micrometres [87]. Electrospinning involves the use of an electrostatic field to create a charged polymer jet, which is elongated as it travels to the grounded collector. The solvent evaporates, and a solidified fibre is produced. The technique can be used with a wide range of natural and synthetic polymers and fibre diameter, pore size and porosity can be controlled by altering electrospinning parameters [88].

Solid freeform fabrication, also known as rapid prototyping, in which a 3-dimensional structure is made by deposition or processing of material layers, using computer controlled technology [89]. Methods include stereolithography, selective laser sintering and 3D printing, which can include ink jet printing of a binder onto a powder polymer surface or direct deposition of a polymer solution [90]. The main advantages of these methods are the ability to fabricate complex architectures in a rapid and reproducible manner.

1.2.3 Tissue engineering of the intestinal epithelium – progress to date

Research into tissue engineering of intestinal epithelium focuses both on development of *in vitro* models for drug testing and studying disease as well as grafts for regenerative medicine.

Early studies used isolated organoid units (a cluster of mesenchymal cells surrounded by epithelium) seeded onto scaffolds and implanted back into the animal, using them as bioreactors to generate neomucosa grafts *in vivo* [91–93]. Tissue engineered colon was also generated in a similar way using colonic organoid units [94]. The problem with this approach is the number of organoid units that need to be isolated and implanted is very high as expansion of organoid units *in vitro* is not possible. In order to avoid the need for long term immunosuppressive therapies, autologous transplants are preferable. However, it is difficult to take large numbers of cells from patients with diseased or damaged intestine.

An alternative early approach was the use of acellular polymer matrices. There have been several *in vivo* studies in which scaffold patches were surgically applied to the intestine [95–97]. Although there was some regeneration observed in the animals in this study, none were ever tested in a clinical setting. The approach relies on scaffolds being infiltrated by native cells from nearby tissue, which may not occur in patients with diseased intestine. The most promising acellular approach is the use of decellularised small intestinal submucosa (SIS). Porcine SIS is an FDA-approved product [98] that has been used surgically in a range of regenerative surgeries. The applications include dermal wound repair [99,100], rectal surgery [101], treatment of urinary incontinence [102] and musculoskeletal reconstruction [103]. SIS has also been used in animal studies as an acellular patch for regeneration of colon wall [104,105]. Despite this, there have also been many reports of adverse reactions and/or no clinical benefit after implantation of porcine SIS. These include a study in which inflammatory complications were observed in 31% of patients [106], a

rotator cuff study in which the SIS group had a healing rate of just 26% compared with 60% in the control group [107] and a case of graft-versus-host disease [108].

Carcinoma cell lines such as Caco2 have been employed as an alternative strategy for investigating scaffolds. Cell lines are useful as a model for testing cell response to scaffolds prior to use with primary cells, when obtaining large numbers of primary cells is a challenge. Gupta and colleagues [109] used a rat intestine epithelial cell line in order to study the cytocompatibility of their scaffold, rather than using the large number of rats they would have needed to obtain enough primary cells. They developed a porous scaffold fabricated by particulate leaching and solvent casting made from the nanocomposite polymer POSS-PCL, and found it was able to support proliferation of intestine epithelial cells.

Some studies have evaluated scaffolds intended for acellular delivery with the use of colon cancer cell lines. Moosvi and Day developed bioactive glass particles that were found to stimulate wound healing in intestinal mucosa [110]. They were seeded with epithelial Caco2 cells and CCD-18Co myofibroblast cells. The bioactive glass led to increased secretion of peptide growth factors by the myofibroblasts and enhanced epithelial restitution. In a similar approach, Franck and colleagues used the colon adenocarcinoma cell line T84 to assess the ability of their bilayered silk fibroin scaffold to support cell attachment, proliferation and differentiation [111]. In another study, a porous hyaluronic acid-gelatin-collagen scaffold was produced by solvent casting and freeze drying [112]. In addition to characterisation of the scaffolds' physical characteristics, cytocompatibility was assessed using Caco2 cells. In all of these studies, the scaffolds appeared to support cellular processes of

carcinoma cells *in vitro*. However, they were not tested with primary intestinal cells *in vitro*, and cell infiltration *in vivo* was not evaluated.

A more recent study used intestinal organoids as developed by Sato et al [24] to test their scaffold [113]. They used a serial mould fabrication technique to manufacture a PLGA scaffold with the crypt-villus topography that is typical of intestinal epithelium. The scaffolds were coated with Matrigel and seeded with murine intestinal progenitor cells. The cells were found to proliferate and differentiate on the scaffold, covering the entire villus structures. However it appears that the cells grew in enteroid structures along the villi, rather than a monolayer that is open to the lumen. This is likely to be due to the addition of Matrigel, which also limits the clinical relevance of this work.

In addition to tissue engineering of intestinal epithelium for regenerative applications, there has also been a great deal of research in this area for the development of *in vitro* models. These models are useful for studying disease progression and to test absorption of drugs. Much of this research is looking to replicate crypt-villus structure of the intestinal epithelium. The main difference with this application is that the biomaterial does not need to be biodegradable or biocompatible *in vivo*.

Wang et al [114] fabricated a micropatterned scaffold made from PDMS to determine whether a biomimetic topography plays a role in regulation of intestinal epithelial cells. This was done by first using photolithography to make a mould with the photoresist SU-8. Liquid PDMS was poured into the mould and solidified to create a crypt-like pattern. The ECM protein fibronectin was used to coat the

surface of the PDMS substrate to enhance cell adhesion. Caco2 cells seeded on these scaffolds were able to migrate up the side walls from the bottom of the 'crypts'. Additionally, it was found that the micro-well patterned substrate influenced Caco2 morphology, metabolism and differentiation.

Sung et al created a villi-shaped scaffold made of the natural hydrogel collagen or the synthetic hydrogel PEG-DA [115]. Their fabrication method involved making several moulds of various materials and techniques. The initial plastic mould was made by laser ablation, from which a reverse-mould made of PDMS was created. This was used to make a second sacrificial mould from alginate which was dissolved after being used to create the final hydrogel structure. The scaffolds were seeded with Caco2 cells, which were shown to cover the villi and demonstrate morphology that is similar to native intestine.

Wang et al developed an *in vitro* model of colon epithelium for the purposes of studying colon diseases [116]. They made a micromesh array by UV curing SU-8 photoresist through a photomask. They then loaded the substrate with crypts isolated from mouse colon so that they could be captured by the crypt-shaped well of the scaffold. This initial study was further developed to create a 'colon-on-a-chip'. A PDMS microwell substrate was manufactured with several moulding steps [117]. Primary murine colon crypts were embedded in Matrigel and placed inside the microwells. A combination of 3D and 2D culture resulted in the formation of organoids containing stem cells within the Matrigel at the bottom of the microwells, and a differentiated monolayer above the microwells in the absence of Matrigel.

With this method, they were able to replicate the polarised nature of the colonic epithelium.

1.3 Aims and objectives

To date, there have been no reports of tissue engineered colon epithelium in which primary cells were used to form an open monolayer that resembles native epithelium. The overarching aim of this PhD was to investigate different scaffolds for their ability to support growth of primary colon epithelial cells in order to form an open monolayer structure that resembles native epithelium.

It was hypothesised that epithelial crypts could be isolated from human colonic mucosa and from these, a population enriched with stem cells could be obtained which could then be cultured *in vitro* to achieve cell expansion. It was further hypothesised that scaffolds resembling a 3D ECM topography or specific small and large intestinal epithelial cell microenvironment could be manufactured and used to support proliferation of primary colon crypt cells. Therefore, the initial aim was to isolate, enrich and expand stem cell populations from human colon mucosa. The second aim was to manufacture and characterise scaffolds with different biomaterials and varying structures and investigate their effects on colon epithelial stem cells.

The aims of this project were achieved through the following objectives:

- Investigate the side population method for identifying and isolating stem cells based on their ability to efflux the Hoechst 33342 dye.

- Manufacture and characterise electrospun nanofibre and microfibre scaffolds made from the naturally derived material gelatin and the synthetic material PET which is used as a substitute for PLGA in initial experiments.
- Use gelatin to fabricate planar hydrogel scaffolds and printed scaffolds with small and large intestinal biomimetic topography.
- Investigate colon epithelial cell proliferation and differentiation on all manufactured scaffolds using colon carcinoma cell lines as a model.
- Isolate and culture primary epithelial crypt cells from human colon. Seed primary colon cells on chosen scaffolds to investigate cell proliferation.

From the above objectives, the results were broken down into four distinct parts. In chapter 3, the side population method was investigated for its ability to sort a population of colon epithelial cells enriched with stem cells. Colon carcinoma cells were used as a model with which to validate the method, with the intention of subsequently using the protocol with primary colon epithelial crypt cells. Electrospun gelatin and PET microfibre and nanofibre scaffolds were manufactured, characterised and their influence on colon epithelial cell proliferation and phenotype were assessed in chapter 4. Gelatin hydrogel was investigated as a scaffold material and ink for printing biomimetic scaffolds in chapter 5. In chapter 6, primary epithelial cells from human colon were isolated and cultured. They were seeded onto the chosen electrospun scaffold assessed in chapter 4 and the gelatin hydrogel scaffold from chapter 5 to investigate primary cell proliferation on the scaffolds.

Chapter 2: Materials and Methods

2.1 Quantifying gene expression using quantitative real-time polymerase chain reaction (qPCR)

2.1.1 Introduction

Polymerase chain reaction (PCR) is a molecular biology technique that exponentially amplifies a small amount of DNA, generating several million copies of the DNA sequence of interest. The method uses thermal cycling in which cyclic temperature changes occur which allows each step of the DNA replication process to take place repeatedly. The technique requires short strands of DNA that are complementary to the target DNA, known as primers. It is also reliant on a heat-stable Taq polymerase enzyme which synthesises new DNA that is complementary to the DNA template formed by the primers [118].

Reverse-transcription PCR (RT-PCR) is a useful method for analysis of gene expression. It includes an additional step in which RNA is reverse-transcribed to generate complementary DNA (cDNA) which then undergoes PCR amplification. For quantification of gene expression, real-time quantitative PCR (qPCR) is used. During qPCR, primers bind to the single stranded cDNA template and double stranded DNA (dsDNA) is synthesised. A fluorescent probe is used which binds to the newly synthesised DNA. The amount of fluorescence generated is therefore proportional to the amount of DNA. A fluorescent probe that contains a sequence of bases which is specifically complementary to the gene of interest may be used. This ensures that the results are not affected by the presence of other double stranded DNA.

However, this is not generally required as long as sufficient checks have been done to ensure the primers do not bind to each other, forming primer-dimers. Therefore an alternative method of detection uses a fluorescent dye such as Sybr Green which binds to all double stranded DNA [119].

As the PCR reaction occurs, the amount of fluorescence increases and is directly proportional to the amount of newly synthesised dsDNA. The number of cycles required for the amount of fluorescence to reach a given threshold is known as the Ct value. If there is more target DNA present in the starting material the threshold fluorescence is reached earlier and therefore the sample has a lower Ct value. The threshold fluorescence is set at 10 standard deviations above the baseline fluorescence. It must be within the linear phase of exponential amplification before the efficiency drops due to reaction components being used up [120].

2.1.2 RNA isolation

Total RNA was isolated from cellular samples using a spin column based system with the GenElute mammalian total RNA miniprep kit (Sigma Aldrich), according to the manufacturer's instructions. Cells were lysed by addition of the lysis solution and 2-mercaptoethanol, provided in the kit, directly to the cells either on tissue culture plastic or scaffolds. After lysis, samples were transferred to 1.5 mL centrifuge tubes and stored at -80°C until further use. Cells were thawed at a later date. Following addition of 70% (v/v) ethanol (BioUltra, for molecular biology, Sigma-Aldrich), samples were vortexed and added to silica membrane filters in microcentrifuge tubes, in order for RNA to bind to the filters. DNase treatment was conducted using the On-Column DNase I Digestion Set (Sigma-Aldrich), according to the

manufacturer's instructions. Following a series of washes and the DNase treatment, RNA was eluted in 50 μ L Elution Solution. The quantity and quality of RNA was assessed using a Nanodrop 2000 spectrophotometer (Thermo Scientific) and samples were stored at -80°C.

2.1.3 Reverse Transcription

Reverse Transcription was conducted to form single stranded complementary DNA (cDNA) from the extracted RNA. The quantity of RNA reverse transcribed in each experiment was dependent on the RNA concentrations as assessed with the Nanodrop spectrophotometer. In each experiment, equal quantities of RNA were made up to 12.5 μ L with the addition of nuclease-free water (Promega) in 0.2 mL PCR tubes. For each sample, a negative control tube in which no reverse transcription enzyme was added was included. To each tube, 2.5 μ L of random hexamer primer (100 μ M, Fermentas, Thermo Fisher) was added. Tubes were placed in a thermocycler set to 70°C for 5 minutes to melt the secondary structure, and then placed immediately on ice to prevent the secondary structure reforming.

RT positive and RT negative master mixes were made. Table 2.1 shows the quantities for a single sample. These were multiplied by the number of samples in each experiment to make bulk master mixes. For each RNA sample, 10 μ L master mix was added to give final reaction volumes of 25 μ L. Samples were placed in the thermocycler set to 37°C for 1 hour for strand synthesis, followed by 10 minutes at 95°C to denature enzymes. The resulting cDNA was stored at -20°C until use.

Table 2.1: Reagents for a single reverse transcription reaction.

All quantities were multiplied by the number of samples to obtain bulk master mixes.

Reagent	RT+ (μL)	RT- (μL)
M-MLV RT buffer 5x (Promega)	5	5
dNTP (Thermo Scientific, 10mM)	1.25	1.25
M-MLV reverse transcriptase (Promega, 200U/μL)	1	---
Nuclease-free water (Promega)	2.75	3.75

2.1.4 Real-time PCR

Each sample was run in triplicate, and included RT+ and RT- reactions. A no template control (in triplicate) was also run for each gene. Alongside genes of interest, the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) was included as an internal control. Bulk master mixes were made for each gene by multiplying volumes shown in Table 2.2 by the total number of reactions. In a MicroAmp optical 96-well reaction plate (Applied Biosystems), 18 μL master mix and 2 μL cDNA was added to each well. RT-PCR was carried out on the ABI 7500 Fast Real-time PCR machine (Applied Biosystems) and analysed with the ABI 7500 v2.3 software. The run method is detailed in Table 2.3 and primer sequences shown in Table 2.4.

Table 2.2: Reagents for a single qPCR reaction.

All quantities were multiplied by the number of samples to obtain bulk master mixes.

Reagent	Volume per reaction (μL)
GoTaq qPCR master mix 2x (Promega)	10
Forward primer (Eurofins, 6.25 μM)	0.8
Reverse primer (Eurofins, 6.25 μM)	0.8
Nuclease-free water (Promega)	6.4

Table 2.3: Run method for qPCR

	Temperature (°C)	Time (seconds)
Holding stage	95	20
Cycling stage (40 cycles)	95	3
	59.5	30

Table 2.4: Primer sequences for qPCR

Gene	Forward primer	Reverse Primer
HPRT	AAATTCTTTGCTGACCTGCT	TCCCCTGTTGACTGGTCATT
ABCG2	TGGCTTAGACTCAAGCACAGC	TCGTCCCTGCTTAGACATCC
Lgr5	GAGGATCTGGTGAGCCTGAGAA	CATAAGTGATGCTGGAGCTGGTAA

2.2 Scaffold manufacture

2.2.1 Electrospun scaffolds

Electrospinning is a simple and flexible method that utilises electrostatic forces to produce fibrous scaffolds. The process involves a polymer solution being pumped at a constant rate out of a needle tip. A voltage is applied which causes droplets at the needle tip to become cone-shaped due to mutual repulsion forces, creating what is known as a Taylor cone. The solution is then elongated and the solvent evaporated as it travels to the grounded metallic collector where polymer fibres are deposited. This continues until a mat of randomly deposited non-woven fibres is formed. Electrospinning parameters can be altered to produce fibres with diameters from 3 nm to greater 5 μm [121]. The fibrous scaffolds can be designed to mimic the structure of the extracellular matrix, and therefore provide a surrounding for cells that is similar to their *in vivo* environment.

Electrospun scaffolds were made using Climate-Controlled Electrospinning Apparatus (IME Technologies, Netherlands). The electrospinning apparatus were placed in a confined chamber with a climate-control system allowing regulation of temperature and relative humidity within the chamber. A diagram of the apparatus is shown in Figure 2.2.1.

Polymer solutions were prepared by dissolving polymer in solvent overnight with a magnetic stirrer. Gelatin (Type A, 300 bloom, Sigma-Aldrich) was dissolved at 37°C in hexafluoroisopropanol (HFIP), whereas PET (recycled from drinks bottles) was dissolved at room temperature in a mixture of 50% (v/v) trifluoroacetic acid (TFA)

and 50% (v/v) dichloromethane (DCM). The polymer solution was put in a 5 mL syringe, and was delivered at a constant flow rate with a syringe pump. The solution was delivered through an 18 mm needle and fibres were collected on the Rotating Drum Collector Module (IME Technologies), set to rotate at 20 RPM. The temperature was set to 20°C and the relative humidity to 44% to provide ambient conditions. Electrospinning parameters for the gelatin and PET microfibre and nanofibres scaffolds are described in Table 2.5.

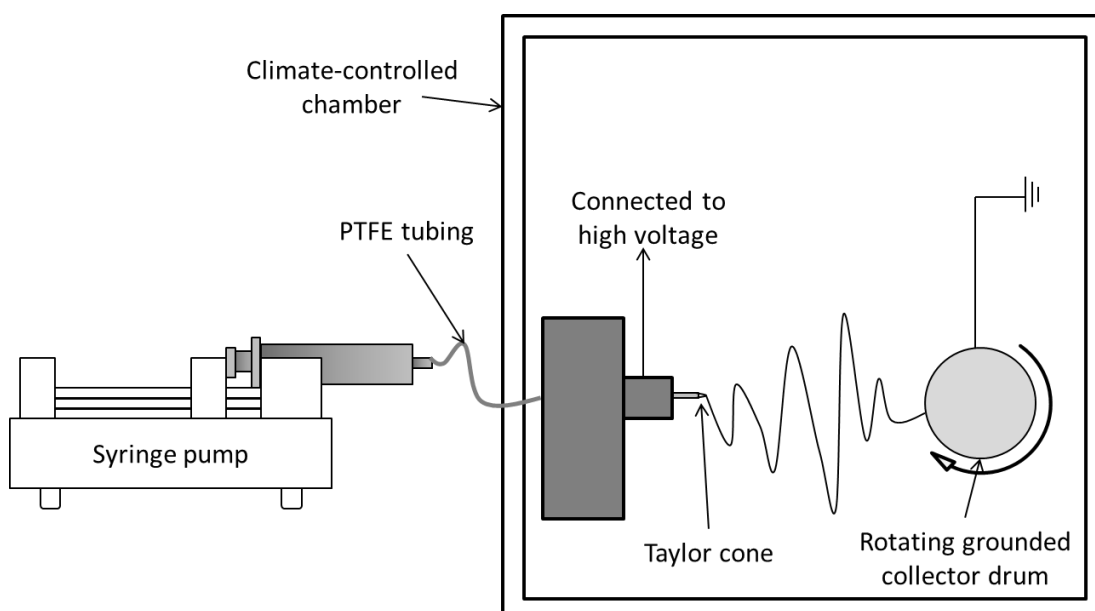


Figure 2.2.1: Diagram showing electrospinning apparatus in a climate-controlled chamber

Table 2.5: Electrospinning parameters

	Concentration (%w/v)	Solvent	Flow rate (mL/hr)	Voltage (kV)	Distance between spinneret and collector (cm)
PET Nanofibres	10	50% (v/v) TFA + 50% (v/v) DCM	0.75	14	15
PET Microfibres	25	50% (v/v) TFA + 50% (v/v) DCM	4	19	11
Gelatin Nanofibres	8	HFIP	1.2	15	17
Gelatin Microfibres	10	HFIP	1.4	15	17

2.2.2 Gelatin hydrogel

A 10 % (w/v) gelatin solution was made by dissolving gelatin (Type A, 300 bloom, Sigma-Aldrich) in distilled water at 37°C with stirring for approximately one hour until fully dissolved. After complete dissolution, 10 mL solution was poured into a petri dish with a diameter of 85 mm and allowed to set at room temperature.

2.2.3 Printed gelatin scaffolds

Printing of gelatin scaffolds was carried out by Hagit Gilon in the Additive Manufacturing and 3D Printing group, Faculty of Engineering, at the University of Nottingham. The printing was done on a substrate that was created by casting a 10% (w/v) gelatin solution in a mould with dimensions of 10mm x 10mm x 2mm, and cooling at room temperature to allow the gel to set. An embedded print head in a three-axis motion system as shown in Figure 2.2.2 was used. The print head is a drop-on-demand (DOD) jetting valve (Pico Dot LV, Nordson EFD) driven by a driver

and controller (DCON, Nordson EFD). The jetting process of the print head is synchronised with a three-axis custom made high performance linear stage connected to a computer with an overall accuracy of 0.5 μm . LabVIEW (National Instruments, USA) software code was written to synchronize between the three-axis motion and print head jetting. The ink is jetted through a 50 μm nozzle by an applied air pressure of 1 Bar and a rod movement driven by two piezoelectric actuators.

To create a villus pattern, the gap between the substrate and the nozzle was set to 3 mm and a 10x10 array of pillars were printed with a drop spacing of 400 μm . The ink was made by dissolving gelatin in distilled water at 37°C to create a 10% (w/v) solution. It was kept warm throughout the printing process in a heated reservoir. The substrate was placed on a custom made Peltier cold stage that cooled to $1 \pm 0.5^\circ\text{C}$. This allowed printed drops to solidify immediately, reducing drop size by preventing spreading and resulting in greater printing resolution.

2.2.4 Crosslinking of gelatin scaffolds

As gelatin is extremely soluble in water, it needs to be crosslinked prior to use for cell culture. Chemical crosslinking using 1-ethyl-3-(dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxyl succinimide (NHS) was chosen as it is a simple method that is non-toxic to cells [122]. It is a zero-length method, meaning that covalent bonds are formed between carboxylic acid and amine residues without addition of reagents into the material structure. The reaction scheme is shown in Figure 2.2.3.

Electrospun gelatin scaffolds were held in place either in cell culture inserts or glued to acetate to prevent scaffolds shrinking during crosslinking. A 9:1 solution of IMS:dH₂O containing 25 mM EDC and 10 mM NHS was made. Gelatin was crosslinked by immersion in this solution for 24 hours at 4°C. After crosslinking, scaffolds were rinsed once with PBS and washed several times with dH₂O.

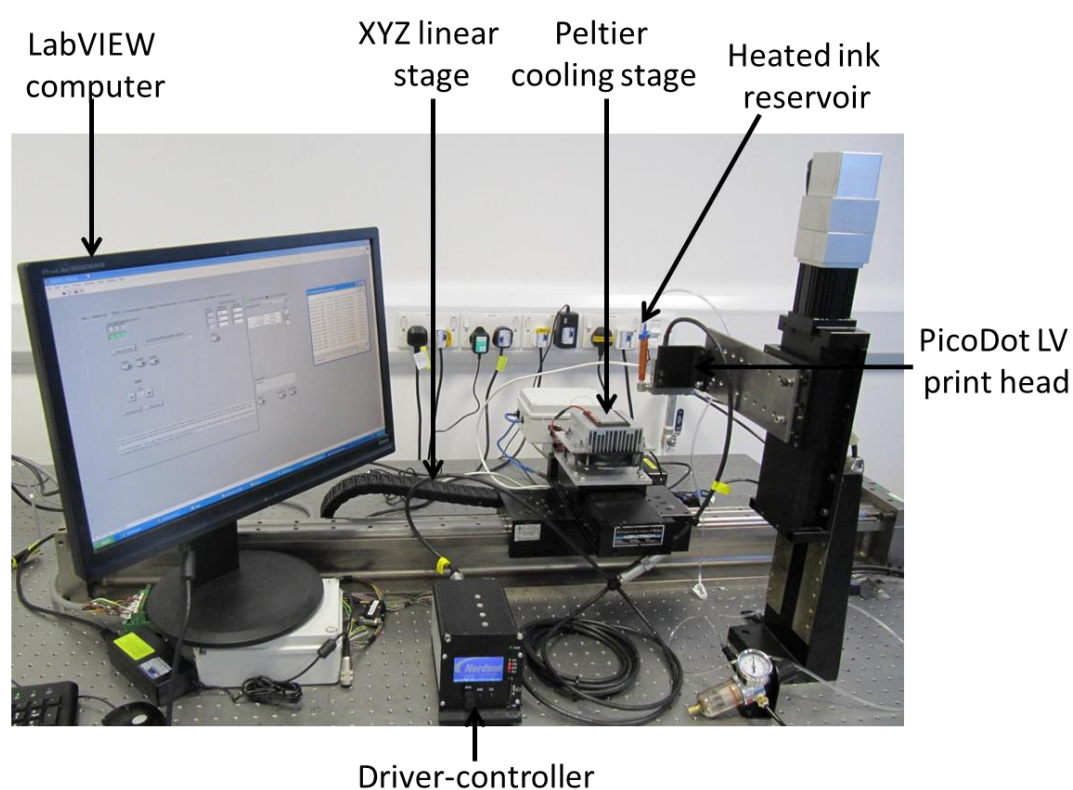


Figure 2.2.2: Diagram of 3D printing apparatus

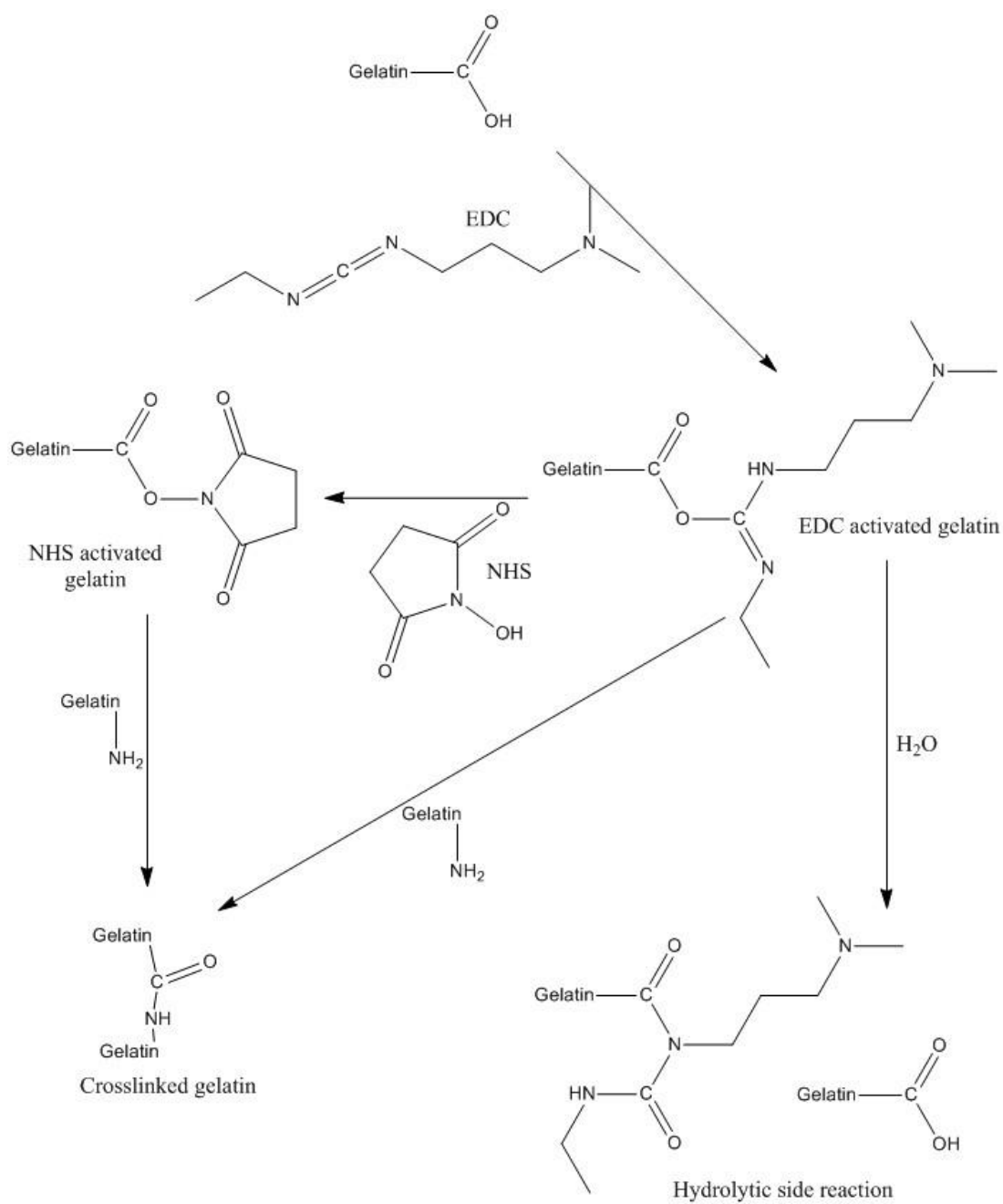


Figure 2.2.3: Reaction scheme for crosslinking gelatin with EDC and NHS

2.3 Scaffold characterisation

2.3.1 Scanning electron microscopy

Scanning electron microscopy (SEM) is a technique for high magnification, high resolution imaging. A focussed beam of electrons is scanned across a sample, where they interact with atoms in the sample. Secondary electrons are transmitted from the surface of the sample, providing information about the surface topography [123].

2.3.1.i Sample preparation

Crosslinked acellular gelatin scaffolds were frozen at -80°C for an hour and then freeze-dried overnight prior to SEM imaging. PET and non-crosslinked gelatin scaffolds were not treated before SEM imaging.

Scaffolds with cells and blanks were fixed in 3% (w/v) glutaraldehyde then dehydrated in increasing concentrations of denatured ethanol in distilled water (25, 50, 70, 90, 100 % (v/v)). The scaffolds were then allowed to air dry at room temperature overnight before SEM imaging.

2.3.1.ii SEM imaging

Prepared samples were mounted on aluminium stubs using double-sided carbon tape, and sputter coated using a Leica EM SCD005 Sputter Coater with gold under an argon atmosphere for 5 minutes. The coated samples were visualised with a JOEL JSM-6060LV scanning electron microscope (SEM) at accelerating voltages of 10-12kV at varying magnifications as shown on individual images.

2.3.2 Porosity measurements

Scaffold porosity was estimated using the gravimetric method as previously reported [124]. Gelatin scaffolds were cut into rectangles, glued into acetate 'windows' and crosslinked as previously described in section 2.2.4, then freeze dried overnight. PET and crosslinked gelatin scaffolds were cut into rectangles. Width and length were measured to the nearest millimetre with a ruler and recorded, and the thickness was estimated using callipers. The dimensions were used to calculate scaffold volume, using the following equation:

$$volume = length \times width \times thickness$$

The mass of the scaffolds was measured using a balance (Mettler Toledo XS105 Dual Range; max=41g/120g, d=0.01mg/0.1mg), and used to calculate apparent density ρ () of the scaffolds:

$$\rho_{scaffold} = \frac{mass}{volume}$$

Total porosity was calculated using the following equation:

$$Total\ porosity\ (\%) = 100 - \frac{\rho_{scaffold}}{\rho_{material}}$$

Where $\rho_{material}$ of PET is 1.38 g/cm³ and $\rho_{material}$ of gelatin is 0.68 g/cm³ [125]

2.3.3 Tensile testing

Tensile testing is a common method of determining mechanical properties of materials. Using a Stable Micro Systems TA.HD.plus texture analyser, the material is held between two grips and elongated at a constant rate. The force applied to the material (stress) and the amount of deformation (strain) are recorded and plotted

on a graph. From this graph it is possible to ascertain the Young's modulus from the gradient of the initial section of the stress-strain curve and the ultimate tensile strength, which is the maximum stress before the material breaks.

As the scaffolds were too thin to be held firmly in the texture analyser grips, rectangular scaffolds were cut, and pieces of acetate (Fingerprint lifting sheet 175 μm thick acetate, Cobex) were glued with silicone adhesive (King British) onto each end to form a dog bone shape (Figure 2.3.1A). The glue was allowed to air dry for at least 48 hours, and then the gelatin scaffolds were crosslinked as described. Dimensions were measured using callipers and entered into the software prior to each measurement, along with settings as shown in Figure 2.3.1B. For each scaffold type, 3 samples were tested from 3 different batches.

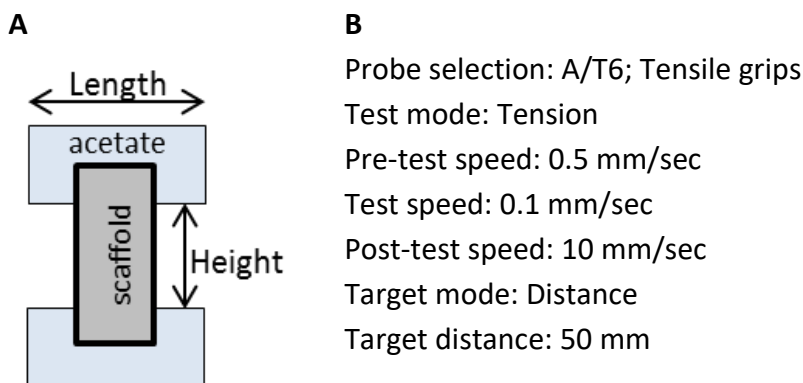


Figure 2.3.1: Sample preparation and texture analyser settings for tensile tests

Scaffolds were glued to acetate prior to tensile strength testing (A). Width = scaffold thickness; Contact area = width x length. Settings inputted into texture analyser are listed in B.

2.3.4 Compressing testing

A Stable Micro Systems TA.HD.plus texture analyser was used to measure mechanical properties of the hydrogels. The system works by using a probe to compress the material at a constant rate, then recording the force applied to the material (stress) and the amount of material deformation (strain) on a graph. Young's modulus can be calculated from the gradient of the initial part of the stress-strain graph. Softer gels will deform more easily with lower amounts of force, therefore the stress-strain graphs have a steeper gradient and a higher elastic modulus is calculated [126].

Crosslinked gels were cut using a biopsy cutter to obtain circles with a diameter of 8 mm, and kept in PBS so that they were fully hydrated to mimic culture conditions. Non-crosslinked gels were carefully pipetted onto a glass slide to form a droplet approximately 8 mm in diameter, and 1 mm in height. Precise measurements were taken using callipers and inputted into the software for each sample. All experiments were performed in triplicate, and carried out in a Peltier hood set to 37°C. The texture analyser settings used for all compression tests are in Table 2.6.

Table 2.6: Texture analyser settings for compression tests

Probe selection	P/10 10 mm DIA Cylinder Delrin
Test mode	Compression
Pre-test speed	1 mm/sec
Test speed	0.1 mm/sec
Post-test speed	10 mm/sec
Target mode	Strain
Target strain	20 %

2.3.5 TNBS assay for determining crosslinking efficiency

Determination of free, un-crosslinked amino groups using a TNBS assay was used to assess degree of crosslinking. 2,4,6-Trinitrobenzene Sulfonic Acid (TNBS) reacts with primary amines to form a coloured product [127], the absorbance of which can be measured at 345 nm.

Crosslinked scaffolds were dehydrated, weighed and put into glass scintillation vials. Similar masses of non-crosslinked gelatin powder were also included, as well as a blank control with no gelatin. A 0.01 M TNBS solution was made by adding 1.65 mL TNBS (5% (w/w), Sigma-Aldrich) to 31.35 mL 4% (w/v) sodium hydrogen carbonate solution. The vials were incubated at 37°C for 3 hours to allow the reaction to occur. To each vial, 3 mL 6.0N hydrochloric acid (Alfa Aesar) was then added, and incubated at 80°C for 1 hour to hydrolyse and dissolve any insoluble material. The vials were cooled to room temperature, and the solution was diluted with the addition of 4 mL distilled water. Triplicate 200 µL aliquots were transferred from each vial to wells in a 96-well plate. Absorbance was measured at 345 nm with a Tecan Infinite M200 plate reader. The amount of free amine groups was calculated from absorbance value, using the equation below, as previously reported [127]:

$$\frac{\text{moles primary amine groups}}{\text{g gelatin}} = \frac{2(\text{absorbance})(0.020\text{L})}{(1.46 \times 10^4 \text{L/mole} \cdot \text{cm}) \times b \times x}$$

$1.46 \times 10^4 \text{ L/mole} \cdot \text{cm}$ = molar absorptivity of TNP-lys

b = cell path length in cm (1cm is the normal cell path length for absorbance)

x = sample weight in g

Degree of crosslinking was then calculated using the following equation:

$$\text{Degree crosslinking (\%)} = \frac{n - s}{n} \times 100$$

n = moles free amine groups/g non-crosslinked gelatin

s = moles free amine groups/g crosslinked gelatin sample

2.3.6 Buffer uptake capacity (swelling)

Swelling ratio of crosslinked gels was calculated using the following equation:

$$\text{Swelling (S)} = \frac{(W_{\text{wet}} - W_{\text{dry}})}{W_{\text{dry}}}$$

W_{wet} = Wet weight after 6 hours

W_{dry} = Dry weight

Scaffolds were dehydrated by freezing at -80°C for at least one hour, then freeze dried overnight. They were weighed to obtain dry weights (W_{dry}). Each dry scaffold was placed in a well of a 24-well plate to which 500 μL PBS was added. The plate was incubated at 37°C until pre-determined timepoints (when equilibrium was reached), scaffolds were removed from PBS, excess moisture removed with filter paper and wet weights (W_{wet}) were taken. Experiments were conducted in triplicate and mean swelling ratios were plotted for each sample type.

2.4 Mammalian Cell Culture

All mammalian cell culture was conducted in Class II Microbiological Cabinets (MSC), fitted with high efficiency particulate air (HEPA) filters, using aseptic

technique. Cultures were maintained in incubators at 37°C in a humidified atmosphere containing 5% (v/v) CO₂ in air.

2.4.1 Culture of cell lines

Immortalised cell lines are a population of cells that can undergo an indefinite number of cell divisions. In this work, a number of different cell lines were used. SW620, SW480, DLD1, Caco2 and HT29 (kind gifts from Prof Ian Tomlinson, University of Oxford) are all epithelial cell lines derived from human colon carcinomas. MCF7 cells (a kind gift from Dr Luisa Martinez-Pomeres, University of Nottingham, UK) are obtained from human breast adenocarcinoma. All cell lines were maintained in Dulbecco's modified eagle medium (DMEM, Life Technologies) supplemented with 10% (v/v) foetal bovine serum (FBS, Fischer Scientific) and 1x Antibiotic Antimycotic solution (Sigma Aldrich; final concentrations 10 µg/mL streptomycin and 25 ng/mL amphotericin B).

Cells were passaged using a standard procedure after reaching approximately 90% confluence. Cells were rinsed in PBS, then a solution of 0.25% (w/v) trypsin / 0.02% (w/v) ethyl amine tetra acetic acid (EDTA) (Sigma Aldrich) in PBS was added and the flask was incubated at 37°C for 2-5 minutes. After gentle tapping to dislodge the cells, cell culture medium supplemented with FBS was added to the flask to neutralise the trypsin. The cell suspension was transferred to a 15 mL centrifuge tube and centrifuged at 200 xg for 5 minutes. The supernatant was removed and the cell pellet resuspended in 2 mL cell culture medium. The appropriate volume (150 µL for a 1:13 passage in Caco2 cells; 100 µL for a 1:20 passage in the other cell lines) was then transferred to a fresh tissue culture flask prepared with warmed cell

culture medium. All experiments reported throughout the thesis were conducted within 10 passage numbers.

2.4.2 Isolation and culture of human colon crypts

Ethical approval and access to normal colonic mucosa (supplementary to diagnosis) from surgical resection specimens was granted by the Nottingham Health Science Biobank (Reference no.: ACP00007). Normal mucosa was dissected off surgical resection specimens and cut into fragments measuring approximately 5 mm². The isolation and culture of colonic crypts was adapted from previously published work [37,128]. The tissue was sterilised with 0.04% (v/v) sodium hypochlorite for 15 minutes at room temperature, washed with PBS and incubated in EDTA/DTT solution (3 mM EDTA + 0.05 mM DTT in PBS, filter sterilised) for 90 minutes at room temperature. The EDTA/DTT solution was removed and replaced with 15 mL PBS. Crypts were released by gentle shaking for 30 seconds, and crypt-containing PBS was transferred to a 15 mL centrifuge tube. This process was repeated a further 2 times. The crypts were centrifuged at 40 *xg* for 2 minutes at 4°C, the supernatant was removed and the crypts were resuspended in medium (Advanced DMEM/F12 + 2mM L-glutamine + 10 mM HEPES). The crypts were counted, centrifuged again and resuspended in chilled Growth Factor Reduced Matrigel (Corning) at a concentration of 2000 crypts per mL. Matrigel (25 µL) containing 50 crypts was added to the centre of each well of a pre-warmed 48-well plate, and incubated at 37°C for 15 minutes to allow the Matrigel to solidify. The crypts were overlaid with 250µL culture media, which was changed every 2 days.

2.4.2.i Crypt dissociation

In some experiments, crypts were dissociated into single cells prior to plating. Isolated crypts were centrifuged at 40 *xg* for 2 minutes, then resuspended in 2 mL TrypLE Express (Gibco) and incubated at 37°C for 30 minutes in order to produce a suspension of single cells. Cells were then centrifuged at 100 *xg* for 5 minutes and resuspended in advanced DMEM/F12 + 2mM L-glutamine + 10 mM HEPES. The cell suspension was filtered using a 40 µm cell strainer in order to remove any cell aggregates.

2.4.2.ii Organoid passage

Organoids were mechanically disrupted with a P1000 pipette tip and transferred to a 15 mL centrifuge tube. The cells were diluted with the addition of advanced DMEM/F12 + L-glutamine + HEPES up to 15 mL and centrifuged at 100 *xg* for 5 minutes. The supernatant was removed, cells were resuspended in 2 mL TrypLE Express and incubated at 37°C for 20 minutes. Cells were centrifuged at 100 *xg* for 5 minutes, supernatant was removed and cells were resuspended in chilled Matrigel. Cell-containing Matrigel was plated as described previously (25 µL / well of a 48-well plate), allowed to solidify for 15 minutes at 37°C and overlaid with culture media.

2.5 Methods to assess cell viability

2.5.1 Presto blue assay

The Presto Blue assay was used to measure the metabolic activity of cells, which can be related to cell number. The assay uses the non-fluorescent dye resazurin, which is taken up by live cells and metabolised to form the pink fluorescent product resorufin [129]. The amount of fluorescence emitted by resorufin is measured by a plate reader. Cell number can be determined through conducting the experiment with a calibration curve of known cell number.

At each time point, medium was removed from samples, and replaced with 10% (v/v) Presto Blue reagent in complete DMEM. After incubation at 37°C for 40 minutes, 3 x 100 µL Presto Blue solution was transferred from each sample to the wells of a black, flat-bottomed 96-well plate. Fluorescence was measured at Ex/Em 560/590nm using Tecan Infinite M200 plate reader. For each substrate, the assays were conducted in triplicate and a blank containing no cells was included. The blank fluorescence was deducted from each sample's fluorescence to account for background fluorescence. Following the assay, Presto Blue solution was removed from the cells, replaced with fresh medium and plates were returned to the incubator.

2.5.2 Live/Dead staining

LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Thermo Scientific) was used to obtain a visual representation of cell viability. The kit contains 2 dyes: calcein-AM which emits green fluorescence when metabolised by viable cells and

ethidium homodimer-1 (EthD-1) which fluoresces red when taken up by dead cells due to their loss of membrane integrity [130]. With these dyes, it is therefore possible to use a fluorescence microscope to visualise both live and dead cells simultaneously.

To each 1 mL of cell culture media, 1 μ L EthD-1 (stock concentration = 2 mM) and 0.25 μ L calcein-AM (stock concentration = 4 mM) was added to give final concentrations of 2 μ M and 1 μ M respectively. This solution was added to the cells and incubated at 37°C for 30 minutes. Cells were imaged on a Leica DM IRB fluorescence microscope. EthD-1 was detected with an excitation filter of 515-560 nm and emission at 590 nm. Calcein-AM was detected using an excitation filter of 450-490 nm and emission of 515 nm.

2.5.3 CellTox Green Cytotoxicity Assay

This assay uses a DNA-binding dye that stains dead cells due to loss of cell membrane integrity. When the dye enters cells and binds DNA, fluorescence is enhanced, and this can be measured in order to assess the levels of dead cells present in a sample. As it is non-toxic it can be used in combination with the CellTiter Glo 3D assay to obtain further information regarding cell viability from the same samples [131].

CellTox Green Cytotoxicity Assay (Promega) was conducted according to the manufacturer's instructions for multiplexing applications. A 5x solution of the reagent was made by addition of 20 μ L CellTox Green dye to 2200 μ L assay buffer (all reagents supplied in the kit). In each well of a 24-well plate, 500 μ L media was

present. From this volume, 100 μL media was removed and replaced with 100 μL prepared 5x reagent. The Tecan Infinite M200 plate reader was set to orbital shaking for 1 minute at 432 RPM, waiting for 15 minutes before reading fluorescence at Em/Ex 490 nm/525 nm.

2.5.4 CellTiter Glo 3D Cell Viability assay

This assay assesses cell viability based on the quantity of adenosine triphosphate (ATP) present, a marker of metabolically active cells. The method uses a luciferase which catalyses luciferin in the presence of ATP to generate a luminescent signal that is proportional to the amount of ATP present [132]. This is an endpoint assay therefore different samples are required to assess cell viability at different time-points.

Immediately after the CellTox Green assay was conducted as described above, 250 μL of the media/CellTox Green solution was removed and replaced with 250 μL CellTiter Glo 3D reagent (Promega). The Tecan Infinite M200 plate reader was set to orbital shaking for 5 minutes at 432 RPM, waiting for 25 minutes before reading luminescence.

2.6 Immunocytochemistry

Immunocytochemistry is a method used to visualise proteins in a cell using an antibody that binds specifically to the protein of interest. The primary antibody may be directly conjugated to a fluorophore that is imaged by a fluorescence microscope. Alternatively, a secondary antibody-fluorophore conjugate that binds to the primary antibody may be used. Additionally, a DNA-binding dye such as Dapi or Hoechst is usually added to visualise the cell nuclei. In this work, the fluorophore was conjugated directly to the primary antibody against the protein zonula occludens-1 (zo-1) [133].

Cells were fixed at room temperature in 3.7% (w/v) paraformaldehyde for 15 minutes, washed three times with PBS, permeabilised with 0.05% (v/v) Triton-X in PBS for 5 minutes at 4°C, and then washed again with PBS. The samples were blocked for 30 minutes at room temperature with 3% (w/v) BSA/1% (w/v) glycine, then incubated for 2 hours in the dark at 4°C in anti zo-1 monoclonal antibody conjugated to Alexa Fluor 488 (Life Technologies, 1:200 dilution in PBS). After rinsing with PBS, the samples were incubated for 5 minutes in Hoechst 33342 for nuclei observation (Life Technologies, 0.5 µg/mL in PBS). Samples were rinsed again with PBS, and then mounted onto microscope slides with Fluorescence Mounting Medium (Dako). In the confocal images, nuclei appear blue due to Hoechst 33342 and Zo-1 is displayed green due to Alexa Fluor 488.

2.7 Statistical analysis

All data was analysed using Graphpad Prism 7 software. Where appropriate, mean $\pm$ standard deviation was calculated, with the exception of qPCR data, in which mean, maximum and minimum was assessed and presented. Statistical differences between two samples were evaluated using student's t-test. Where there were more than two samples, one-way ANOVA was used to assess statistical differences, with the exception of Presto Blue data which was assessed with 2-way ANOVA. Differences were deemed statistically significant if $p \leq 0.05$. Symbols of further levels of statistical significance are shown in Table 2.7.

Table 2.7: Symbols of statistical significance

*	$P \leq 0.05$
**	$P \leq 0.01$
***	$P \leq 0.001$
****	$P \leq 0.0001$

Chapter 3: Side population method for identifying and sorting colon epithelial stem cells

3.1 Introduction

In order to develop a clinically applicable tissue graft, an appropriate cell source for transplantation is required. Various attempts have been made to isolate stem cells from human intestinal epithelium. The preferred method is to use antibody based fluorescence activated cell sorting (FACS), although this requires an appropriate cell surface marker which is selectively expressed by stem cells. Lgr5 is the most convincing and widely accepted marker of colon epithelial stem cells [7,11]. However, the currently available antibodies against the extracellular domain of Lgr5 have been found to perform poorly in experiments. This has lead to the use of genetically engineered mice in which green fluorescent protein (GFP) is integrated into the *Lgr5 locus* to identify and sort Lgr5⁺ cells [58,134]. As this is not feasible with human cells destined for the clinic, an alternative method of sorting stem cells is required.

The side population technique, first described in 1996 [29] is a method of identifying stem cells based on their ability to efflux the vital dye, Hoechst 33342. Hoechst 33342 is a cell permeable, DNA binding dye which enters cells through passive diffusion and is actively effluxed by the family of cell surface ATP-binding cassette (ABC) transporters, in particular, ABCG2. Cells are analysed by a flow cytometer, which includes a UV laser that excites the Hoechst dye, and the emitted fluorescence is measured through a blue 450/50 band pass filter and a red 675/20 long pass filter. Stem cells possess a greater number of ABC transporters than

differentiated cells [135], and are therefore able to pump the dye out of the cytoplasm. When Hoechst red and blue fluorescence emissions are plotted against each other, a tail of cells which has low fluorescence can be seen, and this is referred to as the 'side population'.

Fumitremorgin C (FTC) and Verapamil are compounds that inhibit ABC transporters [136]. They are used as controls in the side population method, as they prevent dye efflux. When an inhibitor is present, the side population is not visible as the ABC-expressing cells retain the dye and remain fluorescent.

DyeCycle Violet (DCV) is a DNA-binding dye with structure and permeability characteristics that are similar to Hoechst 33342. However, it has a longer wavelength excitation maximum of 369 nm, compared with Hoechst 33342 which has an excitation maximum of 350 nm [137]. DCV can be excited with a violet laser diode which is often more easily available than UV lasers. It is therefore useful for side population analysis and sorting when there is no UV laser available on the flow cytometer. When tested with mouse and human haematopoietic cells, the side populations isolated with DCV were found to have identical expression of stem cell lineage surface markers to side population cells isolated with Hoechst 33342 [137]. This indicates that DCV can be used as an adequate substitute for Hoechst 33342 for identifying a side population.

The side population (SP) has been reported in numerous studies to identify both cancer stem cells as well as normal stem cells. Cancer stem cells identified with the presence of specific surface markers have been found to be present in multiple tumour types and have been shown to share characteristics with SP cells sorted by

dye exclusion [138,139]. Moreover, side populations have been shown to possess intrinsic stem cell properties, including the ability to generate non-SP cells and increased expression of stemness genes such as *Notch* and *β -catenin*. Side population cells in breast cancer have been found to exhibit increased drug resistance associated with a survival advantage in the presence of chemotherapeutic agents [140]. Similarly, in bone sarcomas, side population cells demonstrated increased cancer-initiating activity *in vitro* and *in vivo* [141]. In colorectal cancer cell lines SW480, HT29 and HCT15, SP cells have shown increased expression of stem cell marker genes such as *Sox2* and *Lgr5*, and resistance to chemotherapeutic drugs [142]. Similar results have been demonstrated in a range of gastrointestinal cancer cell lines, where SP cells showed self-renewal characteristics and resistance to a range of anti-cancer drugs [143].

Side populations have also been identified in non-cancerous primary cells from a variety of organs. The side population represented less than 0.1% of cells isolated from murine lung and were shown to be enriched for stem cell activity [31]. In another study, side population was isolated from primary rat kidney cells [30]. These cells were then transplanted intravenously into rats and were found to differentiate into a range of cell types including skeletal muscle, liver and blood cells. Side population has also been isolated from primary human mammary gland cells [32]. The SP cells in this study were capable of self-renewal and differentiation into mammary epithelial cell lineages. Furthermore, side population cells have been isolated from colon epithelial crypts [34]. The colon epithelial SP cells demonstrated stem cell characteristics and expressed putative stem cell markers *musashi-1*, *β 1-integrin*, *BerEP4*, and *CD133*.

3.1.1 Aims and hypotheses

The ultimate aim of this project was to isolate primary crypt cells, expand them in culture and seed them on scaffolds for the development of a tissue-engineered graft. Primary epithelial crypts contain a heterogeneous population including stem cells as well as differentiated cells of various types. It was anticipated that *in vitro* culture and cell expansion would be more efficient with isolated stem cells than with the entire heterogeneous crypt cell population. It was hypothesised that side population cells are enriched with stem cells and could be used to generate colon organoids more efficiently than the whole population of colon crypt cells. The primary aim of this chapter was to optimise and validate the side population method. Before using the side population technique with primary crypt cells obtained from human colon, colorectal cancer cell lines were used to validate and optimise the technique. A range of cell lines were chosen, each containing a different genetic and mutation profile. Therefore, when the results of different cell lines are taken together they may provide a more complete picture of how primary cells will behave in similar circumstances.

3.2 Experimental

3.2.1 General side population method

Stock solutions were made and stored at 4°C in the dark for up to six months. DyeCycle Violet (Invitrogen, V35003) was available in solution in DMSO at a concentration of 5mM. Fumitremorgin C (EMD Millipore, 344847; 250 µg) was reconstituted in 132 µL DMSO to make a stock solution with a concentration of 5

mM. Hoechst 33342 (Molecular Probes, H-3570) was available as a stock concentration of 10 mg/ml. A 25 mM stock solution of verapamil (Sigma, V4629) was made by dissolving 246 mg in 20 mL methanol.

Colon carcinoma cell lines were removed from their flasks using 0.25% trypsin/0.02% EDTA in PBS, as described in chapter 2, section 2.4.1. The cells were centrifuged at 200 *xg* for 5 minutes and resuspended in fresh media. Cells were counted, and 1×10^6 live cells in 1 mL media were transferred to 1.5 mL microcentrifuge tubes.

To identify the side population, either 50 μ M verapamil or 10 μ M Fumitremorgin C (FTC) was added to some of the tubes, by addition of the appropriate volume of stock solutions. The inhibitors were either added at the same time as the dye or 15 minutes prior to addition of the dye. These tubes were used to demonstrate the role of ABC transporters in the appearance of a side population by showing that the cells in the side population are not present upon ABC transporter inhibition.

Hoechst, at a variety of concentrations (2.5 – 10 μ g/mL) or 10 μ M DyeCycle Violet was added to the cells. The cells were then incubated at 37°C for 30-90 minutes, with or without shaking. Following this incubation step, the cells were immediately placed on ice then washed 3 times by centrifuging at 200 *xg* for 5 minutes at 4°C. The supernatant was removed and the cells were resuspended in chilled Hank's Balanced Salt Solution (HBSS) containing 2% (v/v) foetal bovine serum (FBS). It was crucial to keep the cells cold (on ice) until cytological analysis to prevent passive efflux of the dye.

When DyeCycle Violet was used, cells were analysed on a Beckman Coulter MoFlo XDP cell sorter. The dye was excited with a violet laser at a wavelength of 405 nm. Fluorescent emissions were measured at two wavelengths using a 450/50nm Band Pass (BP) blue optical filter and 620nm Long Pass (LP) red optical filter.

When Hoechst 33342 dye was used, cells were analysed on the BD LSRII flow cytometer. The dye was excited with a UV laser at a wavelength of 325nm and fluorescence was measured using 440/40 nm and 530/30 nm optical filters.

3.2.2 Method optimisation

Three different colon carcinoma cell lines were used: SW620, SW480 and DLD1. In an attempt to optimise the method, several different variations were explored. Schematics describing the experiments conducted with each dye (DyeCycle Violet and Hoechst 33342) are shown in Figure 3.2.1. With the DyeCycle Violet experiments, the ABC transporter inhibitor was initially added at the same time as the dye. However in many published studies, the cells are pre-incubated with the inhibitor prior to dye addition [142,144,145]. The reasoning behind this is that it allows the inhibitor to be more effective so that a clearer negative control can be observed. The other step that was altered when DCV was used was the length of time for dye incubation. The longer incubation time allows a state of equilibrium to be reached with regards to how much dye remains inside the cells. However, it is also possible that a longer incubation may negatively affect cell viability.

Resolution of the side population has been reported to be less sharp when using non-UV lasers [146,147]. Therefore, Hoechst 33342 was used instead of DCV with a

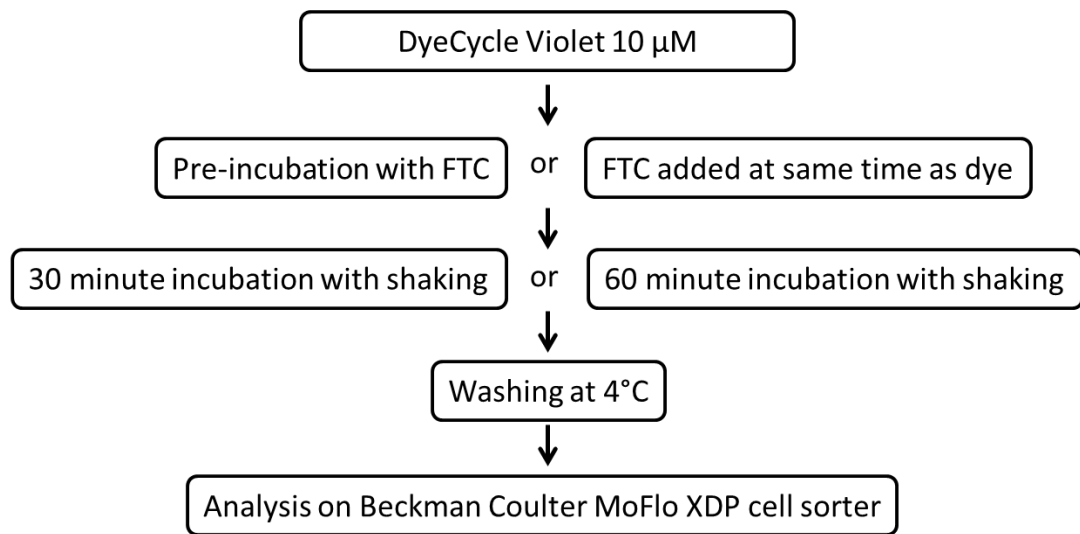
UV laser on a BD LSRII flow cytometer. This flow cytometer could only be used for analysis, not sorting. Hoechst concentrations ranging from 2.5 – 10 µg/mL were used to find an optimal concentration for the cell lines used. Both of the ABCG2 blockers FTC and verapamil were tested, as they have different selectivity. Fumitremorgin C selectively inhibits ABCG2, which is the main transporter used to actively efflux the Hoechst dye [148]. Verapamil is a competitive non-selective ABC inhibitor that also targets other transporters such as ABCB1 and multiple drug resistance protein 1 (MDR1), which also have a role in active efflux of Hoechst dye.

Some antimicrobials, especially antifungals are substrates for ABCG2 [149]. Therefore their presence may interfere with side population experiments by providing competition for the available transporters. Consequently, in all the experiments with Hoechst, antibiotics were omitted during the dye-incubation step. To further ensure that there were no antibiotics present to interfere with the experiments, the medium in the cell culture flasks was changed to omit antibiotics 2 days before the side population experiments were conducted.

3.2.3 Expression of ABCG2 by qPCR

Cell lines were grown to confluence in tissue culture flasks. RNA was extracted, transcribed to cDNA and qPCR carried out to assess gene expression of *ABCG2*. The methods for this are described in detail in chapter 2, section 2.1. The data was analysed using the relative standard curve method. Standard curves for both *ABCG2* and the housekeeping gene *HPRT* were created using serial dilutions of MCF7 cDNA (see Appendix B). This was then used to quantify expression of *ABCG2* relative to *HPRT*.

A



B

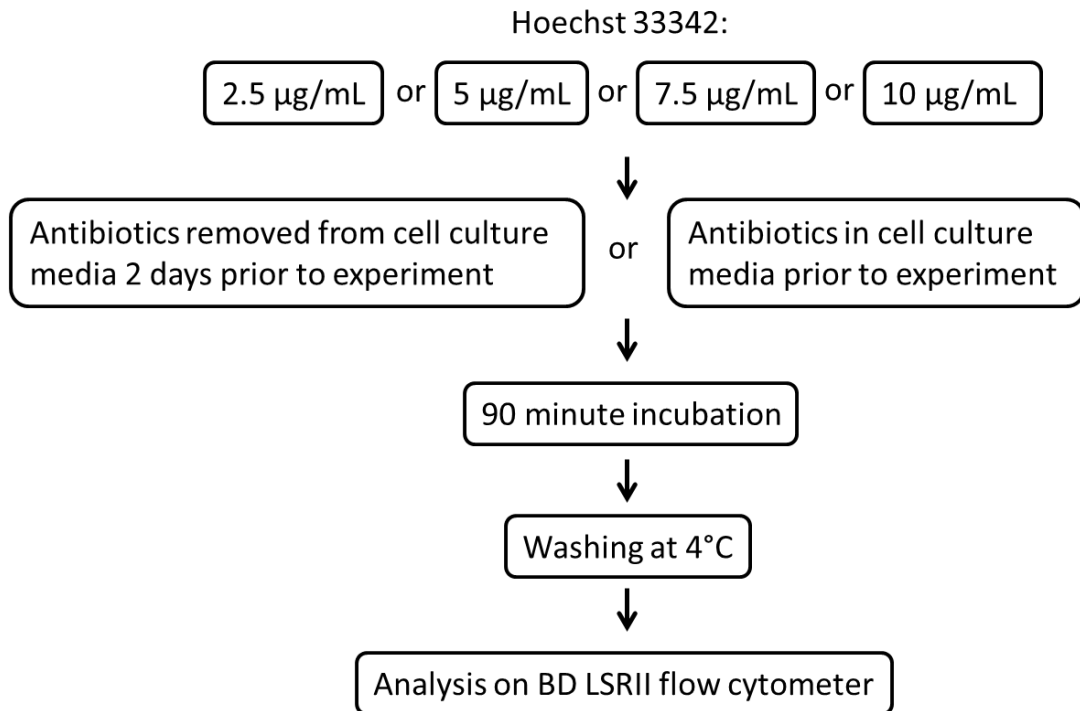


Figure 3.2.1: Schematic summarising the side population experiments

Various experimental modifications were conducted DyeCycle Violet (A) and Hoechst 33342 (B)

3.2.4 Data analysis

All flow cytometry data was analysed using Weasel v3.0.2 software. Each dot plot presented is from a single experiment.

Viable cells were initially identified by forward and side scatter analysis. Non-viable cells found close to the plot origin, and cell clumps with large forward and side scatter deflection were removed from further analysis. The remaining viable cells were analysed for Hoechst red fluorescence and blue fluorescence.

Side population gates were drawn if low-fluorescence cells were present with the dye alone and if these cells were absent in the presence of an ABC transporter inhibitor. The percentage of cells within the side population gates was then calculated.

3.3 Results

3.3.1 Side population differed in replicate experiments using identical protocols

The initial aim of this chapter was to establish and validate the side population method in colon carcinoma cell lines. Therefore replicate experiments were conducted to ensure reproducibility. SW620 cells were incubated with DyeCycle Violet for 30 minutes with shaking, either alone or in the presence of the ABCG2 blocking agent Fumitremorgin C. Cells exhibiting low levels of red and blue fluorescence following excitation of the dye with a violet laser are considered to be

within the side population region. If the number of cells within this region is reduced in the presence of FTC, then they are deemed a true side population.

This protocol was repeated twice with SW620 cells. Each time, there was a side population, in which the proportion of cells was reduced in the presence of FTC. However, there was a 10-fold increase in the percentage of cells within the side population region, from 3.24% (in experiment 1) to 34% (in experiment 2; Figure 3.3.1).

The same protocol was also used in DLD1 cells. In the first experiment, there was a side population of 10.85% that was reduced to 1.8% upon inclusion of FTC. However, when this was repeated, there was a larger proportion of cells with low fluorescence, and the shape of the plot was different from the initial experiment (Figure 3.3.2).

In order to investigate whether the presence of a higher proportion of cells within the side population region was due to the cells not taking up the DCV, the protocol was adjusted to increase the incubation with DCV to 60 minutes with shaking. The first time the adjusted protocol was used, the percentage of low-fluorescent cells was 1.13% which reduced to 0.68% in the presence of FTC. This suggested that the increased incubation time had indeed increased DCV uptake. However, when this method was repeated, a very different dot plot was obtained (Figure 3.3.3). The cells that were positive for red and blue fluorescence were concentrated in the middle of the plot, rather than being spread across the plot as they had been previously. There were also no cells present in the side population region.

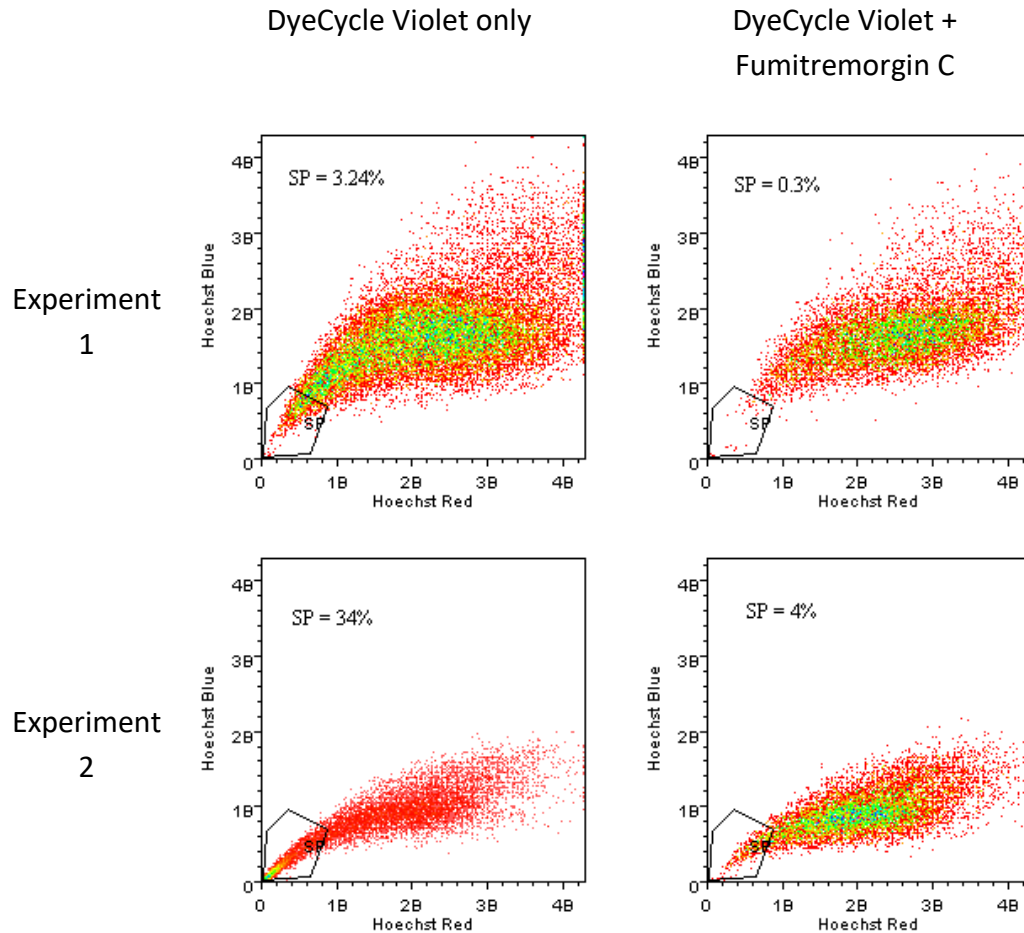


Figure 3.3.1 The side population size in SW620 cells changed after replication with the identical protocol.

SW620 cells were incubated with 10 μ M DyeCycle Violet for 30 minutes with shaking, alone or with 10 μ M Fumitremorgin C (FTC). This experiment was conducted twice and the proportion of cells (SP) with low fluorescence reduced with FTC on both occasions.

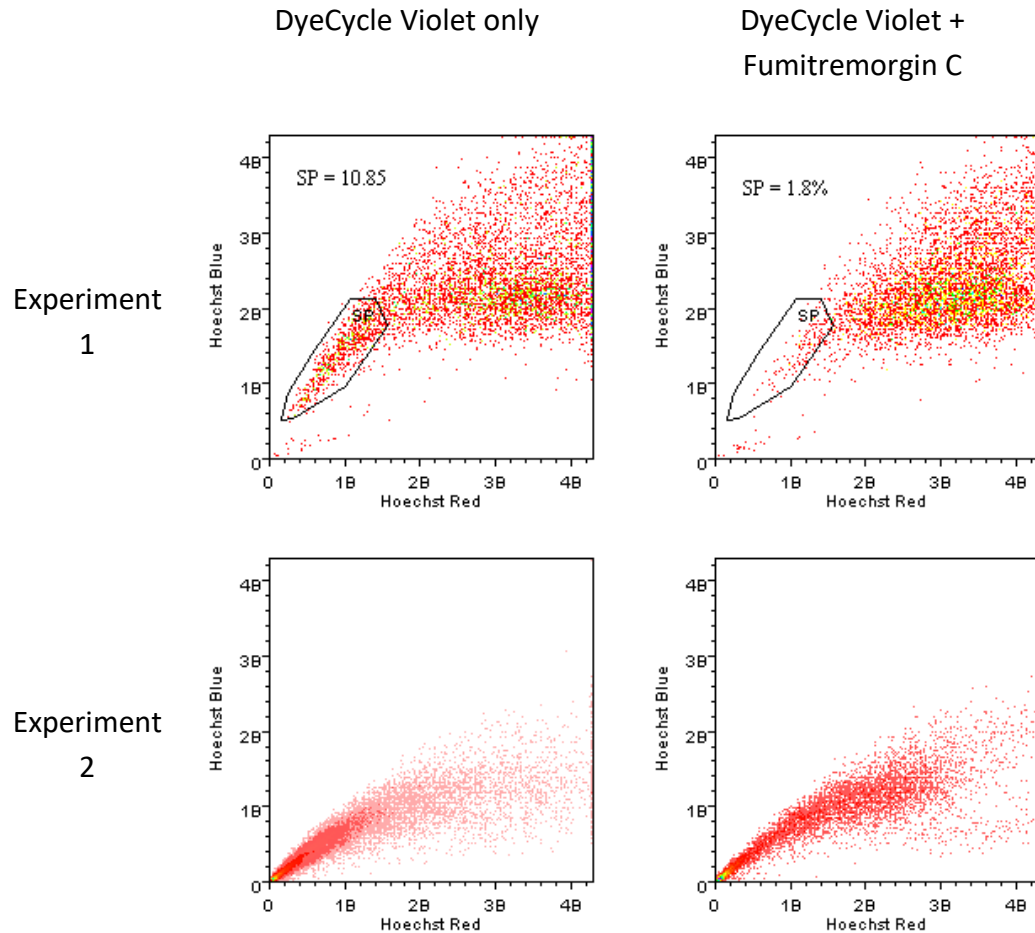


Figure 3.3.2: The side population size in DLD1 cells changed after replication with the identical protocol.

DLD1 cells were incubated with 10 μ M DyeCycle Violet for 30 minutes with shaking, alone or with 10 μ M Fumitremorgin C (FTC). On the first occasion, a side population (SP) of 10.85% was observed which reduced with addition of FTC. However, when repeated there was a much larger proportion of negative cells.

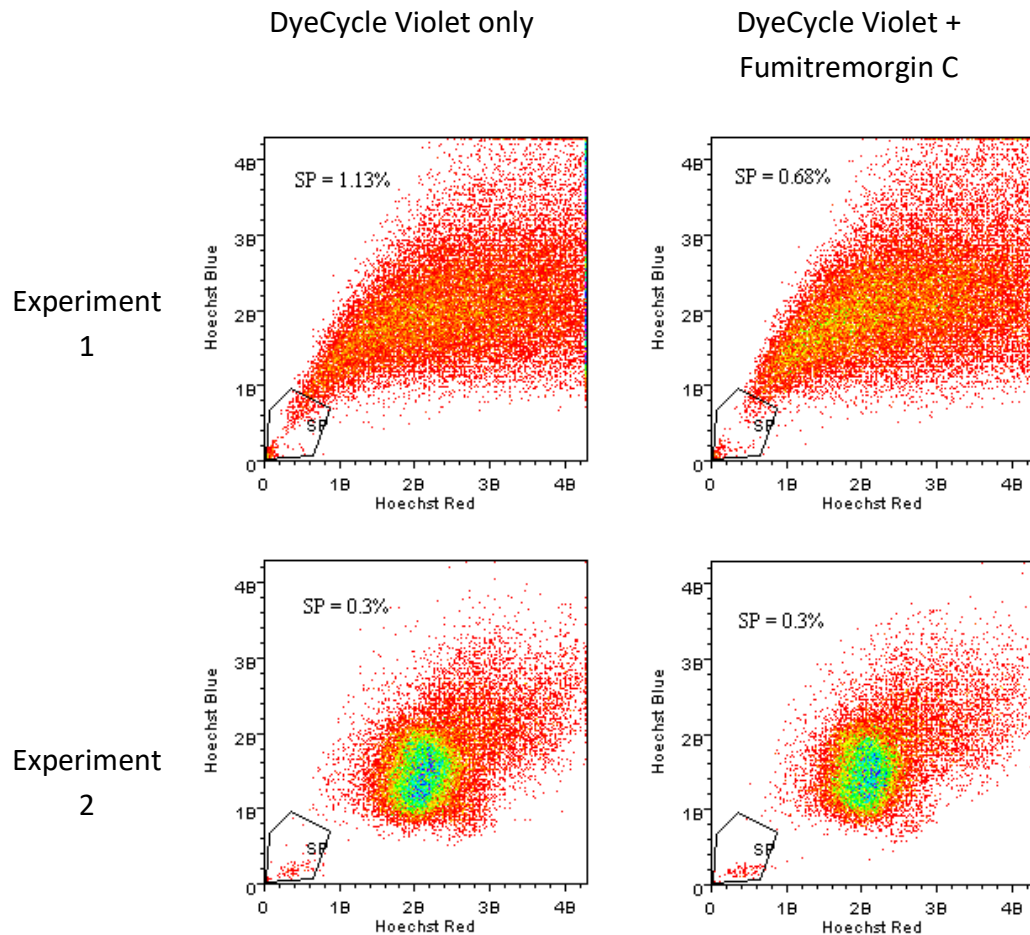


Figure 3.3.3: The fluorescence plot changed greatly after repeating the same staining protocol in SW620 cells.

SW620 cells were incubated with 10 μ M DyeCycle Violet for 60 minutes with shaking, alone or following a 15 minute pre-incubation with 10 μ M Fumitremorgin C (FTC). Initially, a side population (SP) of 1.13% was reduced to 0.68%. The second experiment shows a very different dot plot in which 0.3% of cells have low fluorescence, which doesn't change in the presence of FTC.

3.3.2 Pre-incubation with ABCG2 inhibitor prior to addition of DyeCycle

Violet did not diminish the low-fluorescence cell population.

In order to ensure that the ABCG2 blocker was efficiently blocking dye efflux, the cells were pre-incubated with FTC for 15 minutes before addition of DCV. SW620 cells were stained and tested with this adjusted protocol. Yet contrary to what was expected, the percentage of cells within the side population region increased in the presence of FTC (Figure 3.3.4). The proportion of cells within the low fluorescence region was 12.83%, which was higher than anticipated based on the previous experiments with SW620. When FTC was added, instead of a reduction in low fluorescence cells, this proportion increased to 16%.

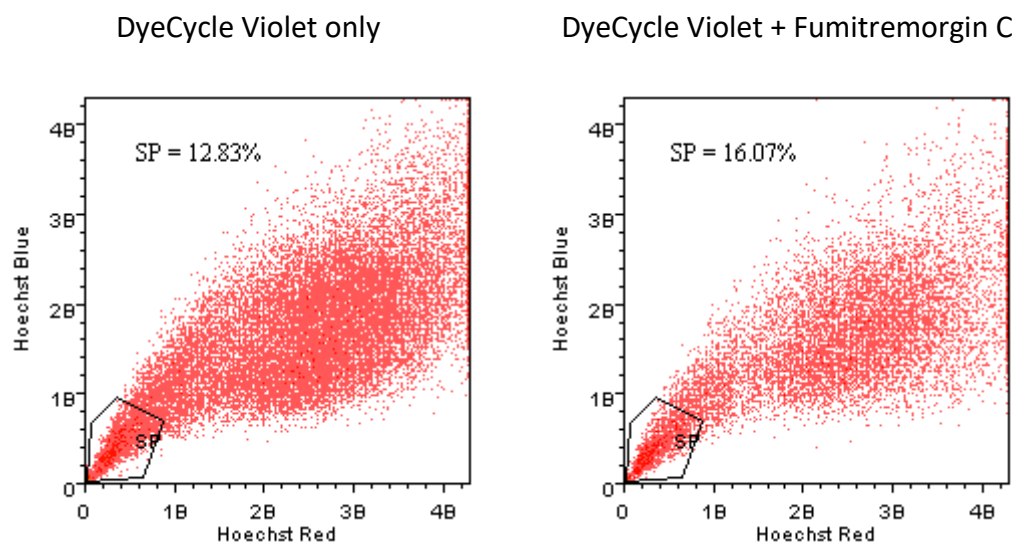


Figure 3.3.4 The side population in SW620 cells did not decrease when pre-incubated with FTC before addition of DCV

SW620 cells were incubated with 10 μ M DyeCycle Violet for 30 minutes with shaking, alone or following a 15 minute pre-incubation with 10 μ M Fumitremorgin C (FTC). The proportion of cells in the side population (SP) increased from 12.83% to 16.07% with FTC.

3.3.3. Increasing dye concentration reduced low-fluorescence population, but this was not reproducible

The side population technique was undertaken with Hoechst 33342 as well as DyeCycle Violet. Two concentrations of Hoechst were initially tested with SW620 cells: 2.5 µg/mL and 5 µg/mL. It was initially apparent that increasing dye concentration resulted in increased dye uptake into the cells and a reduction of the percentage of cells within the low-fluorescence side population region (Figure 3.3.5). On doubling the Hoechst concentration from 2.5 to 5 µg/mL, the percentage of cells within the side population region almost halved from 20.21% to 11.19%. The hypothesis was that increasing dye concentration led to greater dye uptake into the cells and thereby a lower proportion of low-fluorescence cells. Therefore, the experiment was repeated but with higher concentrations of 7.5 µg/mL and 10 µg/mL included. This was done to investigate whether increasing the Hoechst concentration would further reduce the number of cells in the side population region. Apart from the addition of two higher Hoechst concentrations, the protocol was identical to that which was used previously when a side population was observed. Despite this, no side population was observed, even with 5 µg/mL Hoechst (Figure 3.3.6). The shapes of the dot plots were entirely different to what was previously found, and no cells were found in the low fluorescence region.

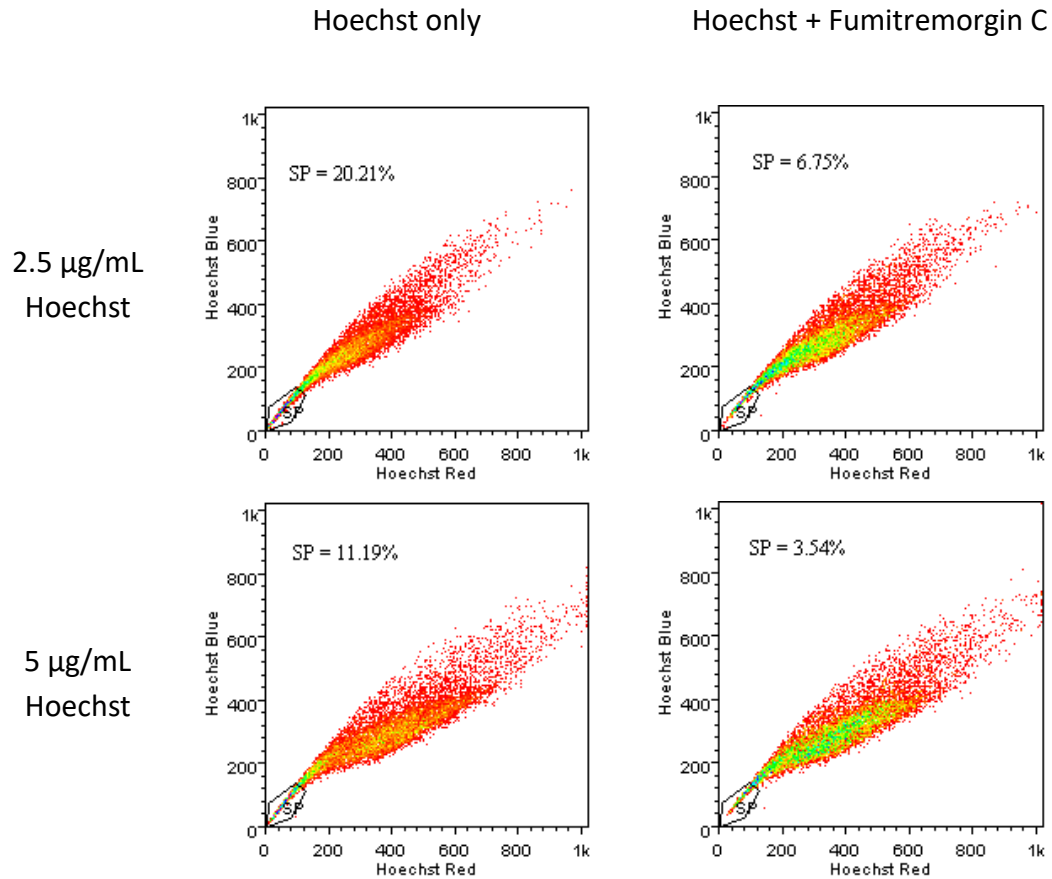


Figure 3.3.5: A side population (SP) was observed in SW620 cells with Hoechst dye although it appears that not all the cells took up the dye at the lower concentration

SW620 cells were incubated with 2.5 $\mu\text{g/mL}$ or 5 $\mu\text{g/mL}$ Hoechst 33342 dye, either alone or with FTC, for 90 minutes with no shaking in media without antibiotics. The proportion of low fluorescence cells decreases with the higher dye concentration and in the presence of FTC.

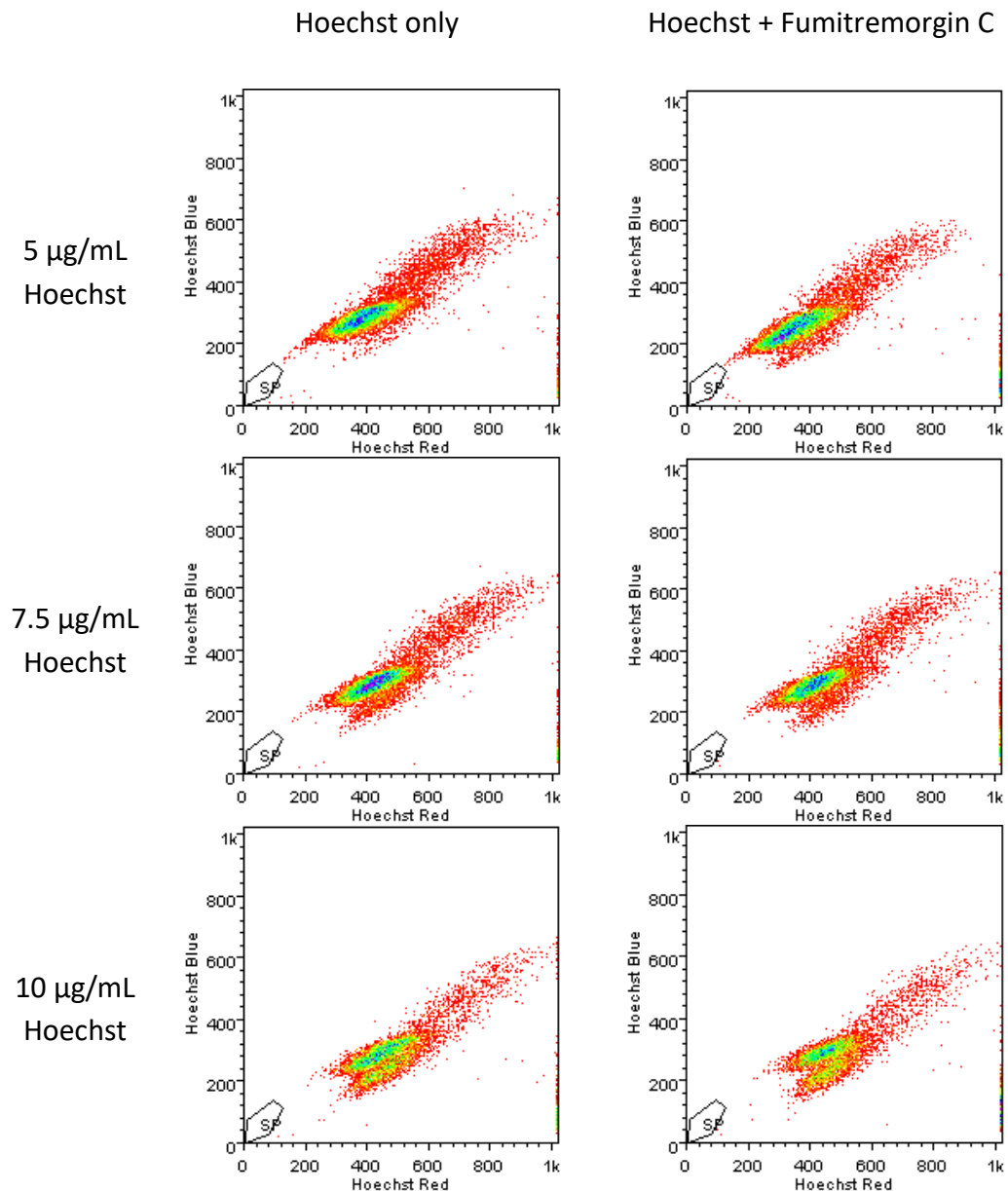


Figure 3.3.6: When higher concentrations of Hoechst were used in the repeat experiment in SW620 cells, no side population (SP) was observed

SW620 cells were incubated with 5, 7.5 or 10 µg/mL Hoechst 33342 dye, either alone or with FTC, for 90 minutes with no shaking in media without antibiotics. No side population was observed.

SW620 cells were incubated with 5, 7.5 or 10 $\mu\text{g/mL}$ Hoechst 33342 dye, either alone or with FTC, for 90 minutes with no shaking in media without antibiotics. No side population was observed in this experiment.

SW480 cells were tested with three different concentrations of Hoechst 33342: 2.5 $\mu\text{g/mL}$, 5 $\mu\text{g/mL}$ and 7.5 $\mu\text{g/mL}$. With the lowest concentration of Hoechst, less than 1% were low-fluorescence cells in the side population region, which reduced further in the presence of FTC. This was eradicated with the higher concentration of dye, where no side population was apparent (Figure 3.3.7). On increasing dye concentration, the entire population appeared to shift to the right as higher levels of red fluorescence were found in all of the cells.

3.3.4. Presence of antibiotics had no effect on the side population

Some antimicrobials, especially antifungals, are substrates for ABCG2, and their presence may interfere with side population experiments by providing competition for the available transporters. In all the experiments with Hoechst, antibiotics were omitted during the dye-incubation step. To further ensure that there were no antibiotics present to interfere with the experiments, the medium in the cell culture flasks was changed 2 days before the side population experiments were conducted, to omit antibiotics. Additionally, both of the ABCG2 blockers FTC and verapamil were tested. SW620 were incubated with 5 $\mu\text{g/mL}$ Hoechst 33342 for 90 minutes with either FTC or verapamil. SW480 cells were incubated with 2.5 $\mu\text{g/mL}$ Hoechst as some cells were apparent in the side population when this concentration was used previously (Figure 3.3.7). A side population was not identified in either SW620 or SW480 experiments when antibiotics were eliminated (Figure 3.3.8 and Figure

3.3.9). The plots appear to be similar in shape with every condition, and no low-fluorescent cells were present.

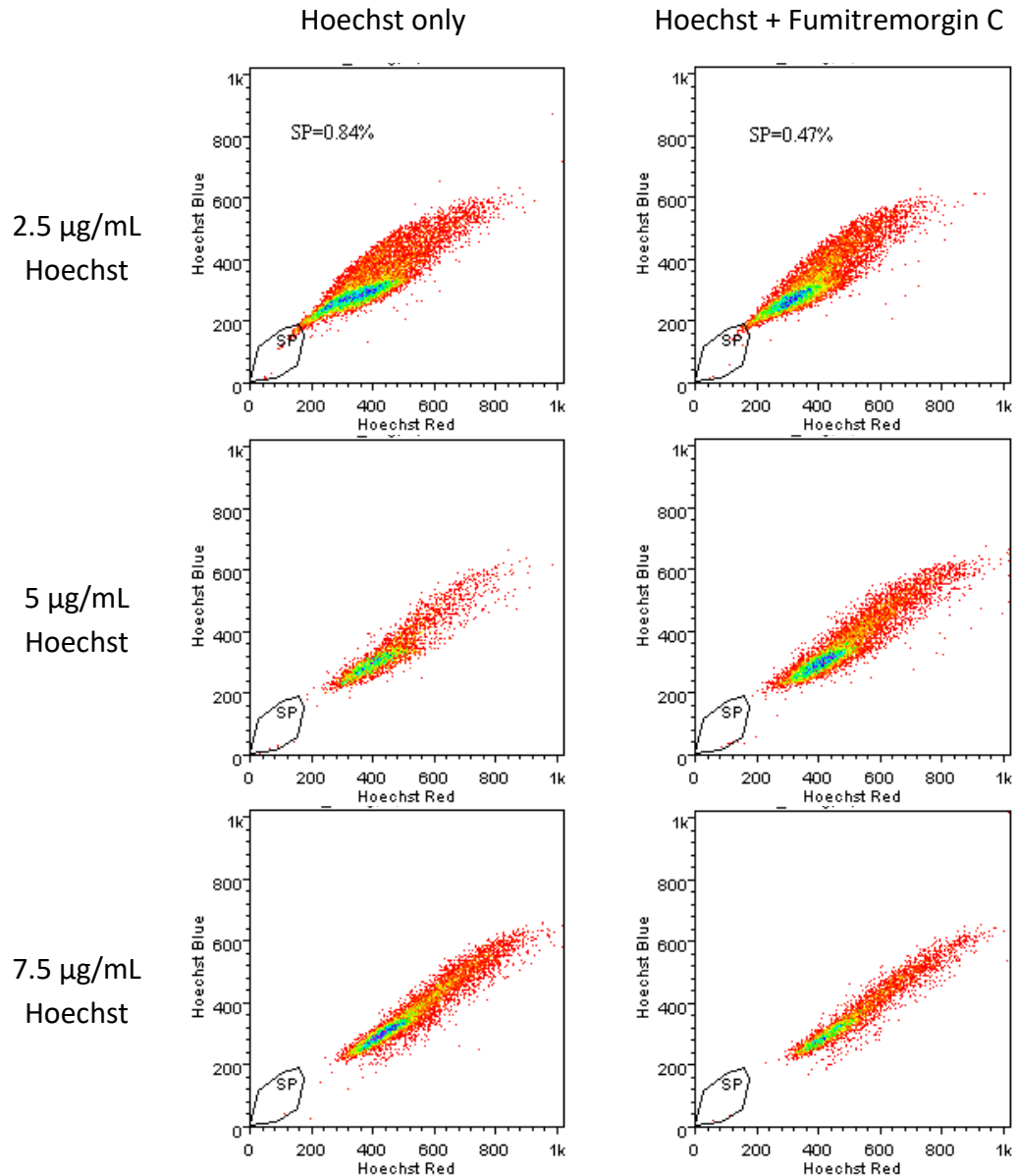
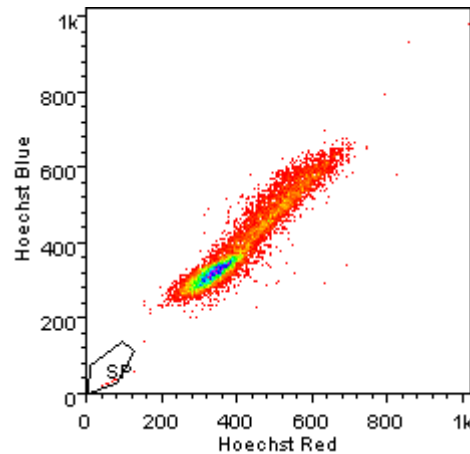


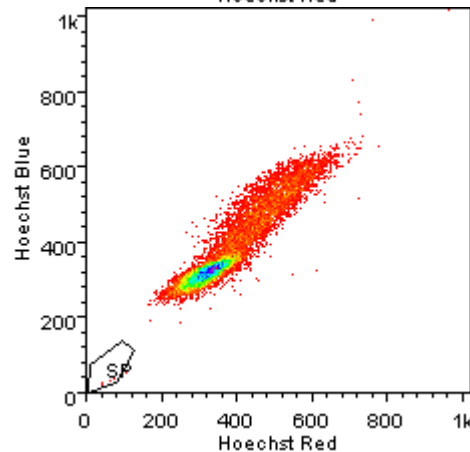
Figure 3.3.7: A side population (SP) of less than 1% was observed in SW480 cells with 2.5 µg/mL Hoechst. No side population was detected with 5 and 7.5 µg/mL Hoechst

SW480 cells were incubated with 2.5, 5 or 7.5 µg/mL Hoechst 33342 dye, either alone or with FTC, for 90 minutes with no shaking in media without antibiotics. No side population was observed in this experiment.

Hoechst alone



Hoechst + FTC



Hoechst +
Verapamil

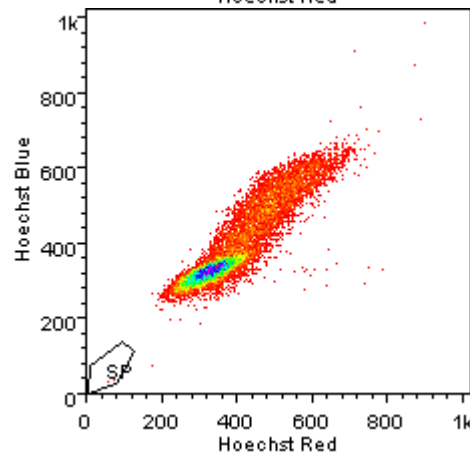


Figure 3.3.8: No side population (SP) was observed with 5 $\mu\text{g}/\text{ml}$ Hoechst in SW620 cells when antibiotics were removed from the media 2 days prior to the experiments

SW620 cells were grown in media without antibiotics for 2 days prior to the experiment. The cells were incubated with 5 $\mu\text{g}/\text{mL}$ Hoechst 33342 dye, either alone, with FTC, or with verapamil, for 90 minutes with no shaking in media without antibiotics. No side population was observed.

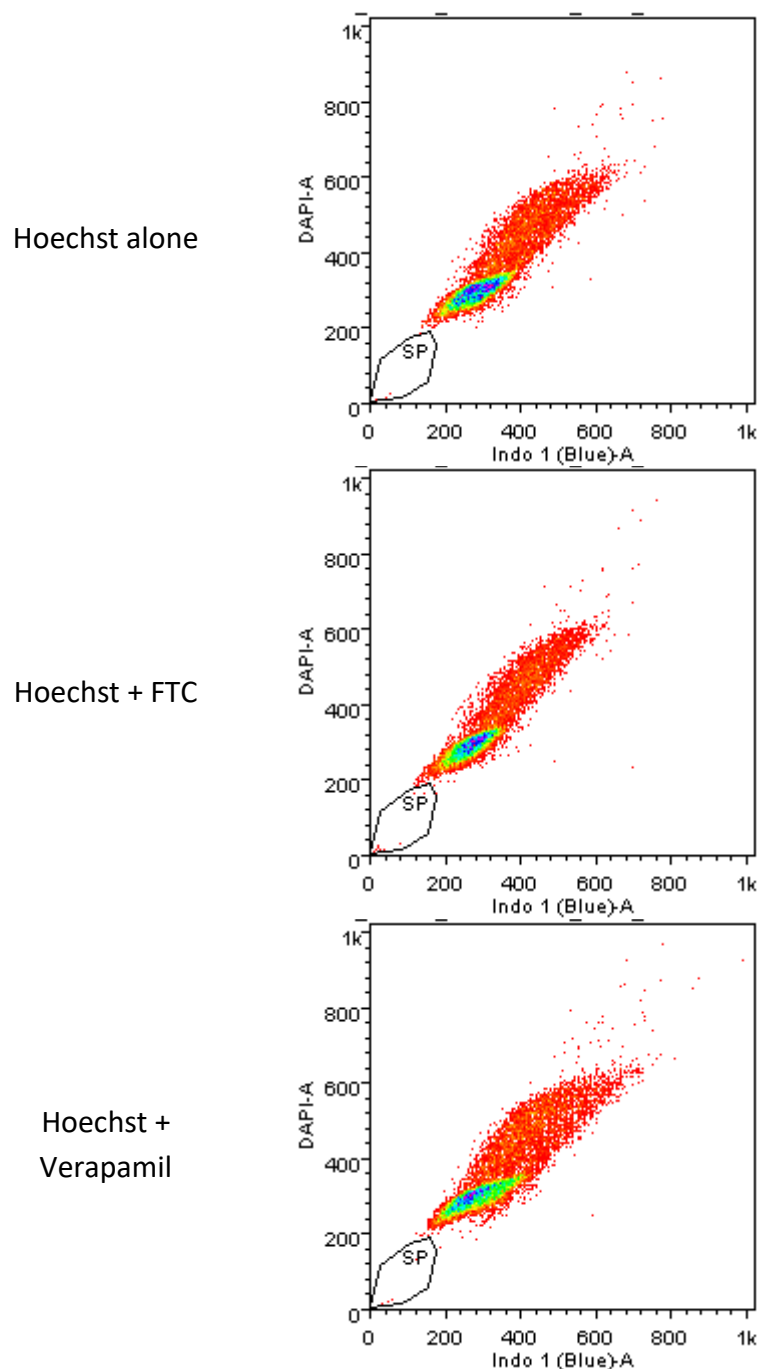


Figure 3.3.9: No side population was observed with 2.5 $\mu\text{g}/\text{ml}$ Hoechst in SW480 cells when antibiotics were removed from the media 2 days prior to the experiments.

SW480 cells were grown in media without antibiotics for 2 days prior to the experiment. The cells were incubated with 5 $\mu\text{g}/\text{mL}$ Hoechst 33342 dye, either alone, with FTC, or with verapamil, for 90 minutes with no shaking in media without antibiotics. No side population was observed.

3.3.5. MCF7 cells express high levels of ABCG2 but a side population in MCF7 could not be found

The MCF7 breast cancer cell line is known to contain a side population as well as highly express the ABCG2 transporter that is responsible for the side population phenotype. The levels of ABCG2 expression were investigated in the colon cancer cell lines compared with MCF7 cells using qPCR. The results show that all of the colorectal cell lines used express lower amounts of ABCG2 than MCF7. SW620 and SW480 express extremely low amounts of ABCG2 (Figure 3.3.10). MCF7 cells express almost as much ABCG2 as they do the housekeeping gene HPRT. Whereas ABCG2 expression in DLD1 is approximately a sixth of HPRT expression and in SW620 and SW480, ABCG2 expression is less than a hundredth of HPRT expression.

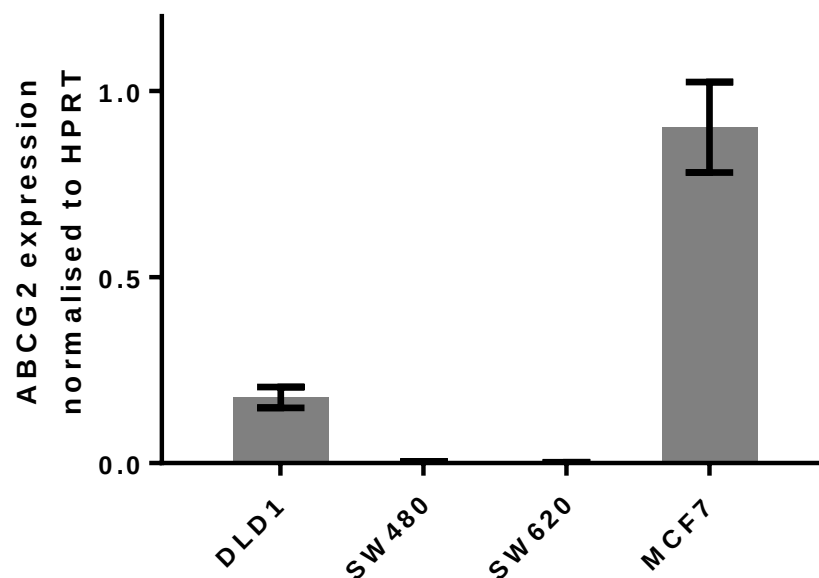


Figure 3.3.10: ABCG2 expression in the human intestinal epithelial cell lines (DLD1, SW480, SW620) and human breast carcinoma cells (MCF7) as determined by q-PCR

The graph shows expression of ABCG2 normalised to the housekeeping gene HPRT. Error bars represent $\pm$ standard deviation for a single experiment with 3 replicates.

As MCF7 was confirmed as a high expresser of *ABCG2*, it was employed as a positive control for the side population protocol. There were a large percentage of cells in the side population region (67%), which was reduced slightly to 61% in the presence of FTC but increased to 80% with verapamil (Figure 3.3.11).

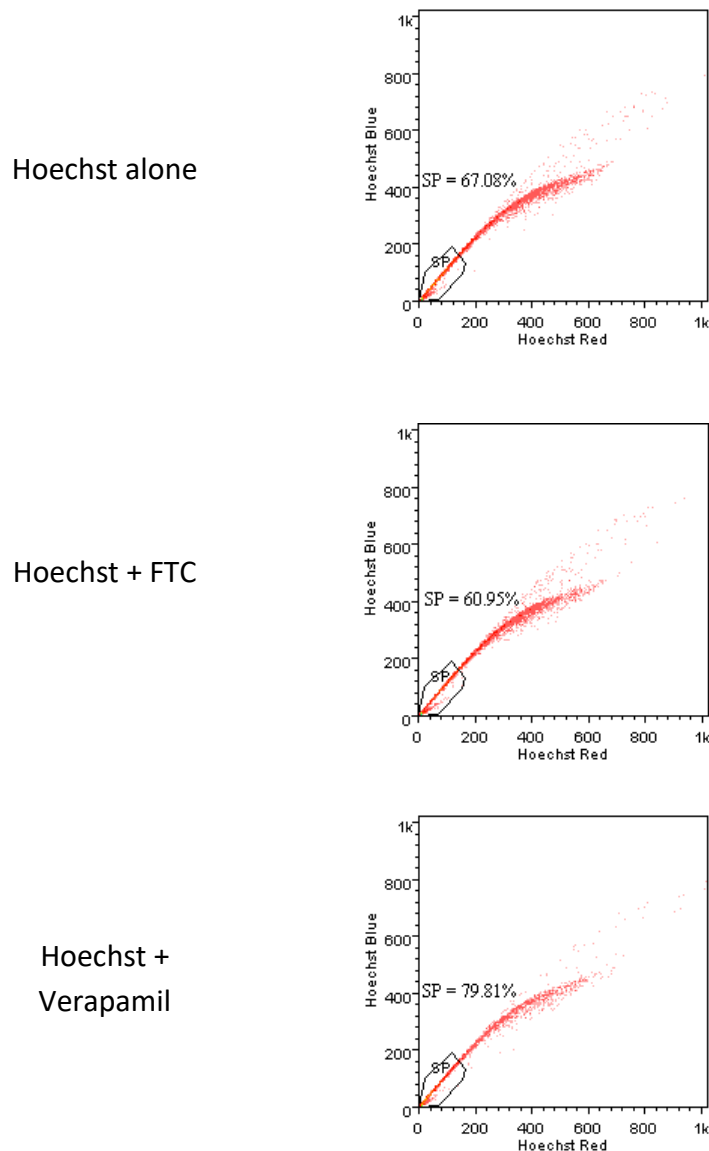


Figure 3.3.11: In MCF7 cells, the percentage of side population (SP) cells did not decrease in presence of ABCG2 blockers.

MCF7 cells were incubated with 5 μ g/mL Hoechst 33342 dye, either alone, with FTC, or with verapamil, for 90 minutes with no shaking in media without antibiotics. A large percentage of low-fluorescence cells were observed which did not reduce in the presence of verapamil or FTC.

3.4 Discussion

The side population method has been described in many publications as a way of isolating a population of cells that is enriched in stem cells. It has been used to sort primary cells from many different organs, including kidney [30], lung [31], mammary gland [32] and colon [34]. It has also been used to sort various types of cell lines, including colon cancer cell lines [142,150,151]. The aim in this work was to establish and optimise the side population method in colon cancer cell lines, with the eventual goal of using it to sort primary cells isolated from normal human colon.

The side population method was not very robust, and results have varied between experiments despite using the same protocols and conditions. Cell passaging can alter the proportion of stem cells within a population [152]. As a result, increasing passage numbers through extended culture would affect side population results. In order to counteract this, fresh aliquots of frozen cells were used for repeat experiments. Additionally, several different variations of the protocol were investigated.

DyeCycle Violet was used initially because the available MoFlo cell sorter only has a violet laser, rather than an ultraviolet laser which is preferred for use with the Hoechst dye. Resolution of the side population has been reported to be less sharp when using non-UV lasers, either with Hoechst or Dye Cycle Violet [146,153]. Therefore, after the method with DyeCycle Violet was found to be unsuccessful, Hoechst 33342 was used with a BD LSR II flow cytometer which includes a UV laser. This instrument can be used only for analysis, not cell sorting.

Fumitremorgin C selectively inhibits ABCG2 (also known as breast cancer resistance protein 1 (BCRP1), which is the main transporter used by stem cells to actively efflux the dyes [148]. Other transporters involved are ABCB1 (also known as P-glycoprotein (P-gp)) and multiple drug resistance protein 1 (MDR1) [139]. Verapamil is a competitive non-selective ABC inhibitor. Therefore, both inhibitors were tested as controls to inhibit dye efflux and prevent appearance of the side population.

It has been reported that some antimicrobials, in particular antifungals, are substrates for ABCG2 [149]. In case their presence was interfering with the experiments, antibiotics were not included in the media during dye incubation. As a further variation to explore whether antibiotics were having an effect, the cell culture media was changed and replaced with antibiotic free media 2 days prior to the experiment. This was found to not have any impact on the results.

The cell lines used in this study were SW480, SW620 and DLD1. Side populations have been identified from these cells by Hart and colleagues [150]. Their staining protocol involved a 15 minute pre-treatment with verapamil followed by 90 minute incubation at 37°C with 5-10 µg/mL Hoechst 33342. With this method, they found side populations in DLD1 of 1.14%, 0.3% in SW480 and 0.19% in SW620. The very low side population percentages appear to be in line with the low expression of ABCG2 found in these cell lines in this study. They also found that all the cell lines express high levels of ABCB1 (p-gp), which is another transporter that contributes to dye efflux in the side population phenotype. Although it would appear from the low side population percentages, despite the high ABCB1 expression that ABCB1 does not play a large part in the presence of the side population.

Side population from SW480 cells have been identified in several previous publications [142–144,151,154]. The size of the side population varies from 0.7%-3.3% in these reports, despite only small variations in methods. The method used by Chikazawa and colleagues [151] involved verapamil being added at the same time as the Hoechst 33342, rather than a pre-incubation step. They used 5 µg/mL Hoechst and incubated for 60 minutes at 37°C with intermittent mixing. With this method, a side population of 0.89% was found in SW480 cells. This is more than double the percentage found by Hart and colleagues [150].

Inoda et al [142] used SW480 among other different cell lines. Their protocol included a 10 minute pre-incubation step with verapamil followed by 90 minute incubation at 37°C with 5 µg/mL Hoechst. They found a side population 3.3% in SW480 cells. This is much larger than the percentages found by Hart et al [150] and Chikazawa et al [151] in the same cell line. It is also worth pointing out that the dot plot in which the side population is presented by Inoda et al seems to have far fewer cells in the main population as well as the side population in the presence of verapamil. The plot from this publication is shown in Figure 3.4.1. This would indicate that there were overall a much lower number of cells in their control samples and therefore the side population they indicate may not be valid.

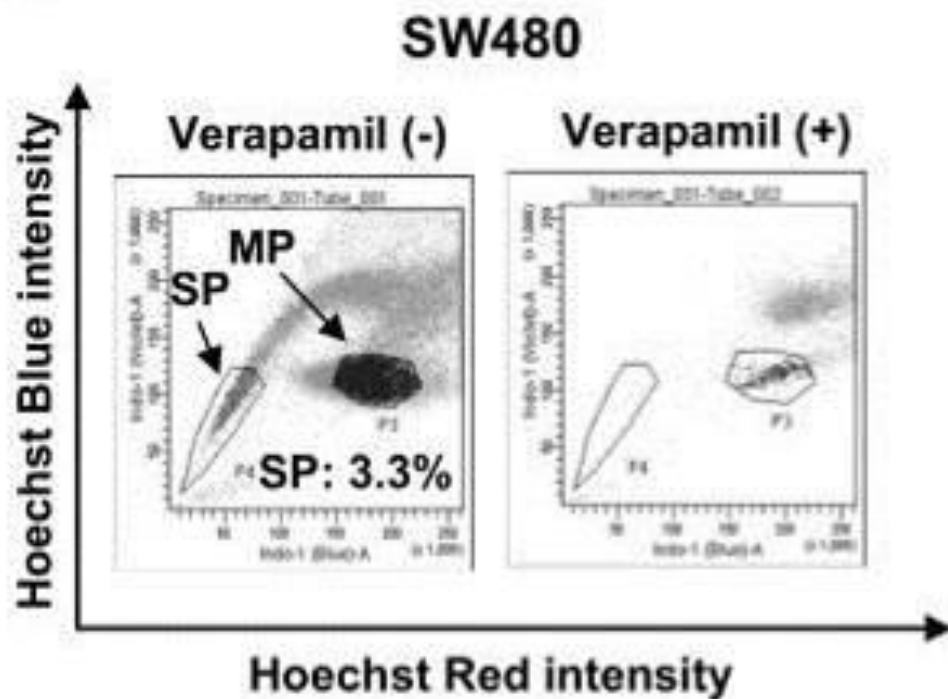


Figure 3.4.1: Side population (SP) in SW480 cells as found by Inoda et al [142].

When verapamil is included, there are fewer cells in the main population (MP) as well as the side population.

The protocol used by Sussman and colleagues [144] involved incubation with 5 $\mu\text{g}/\text{mL}$ Hoechst 33342 for 90-120 minutes, with gentle vortexing of cells every 30 minutes. A pre-incubation of 10 minutes with verapamil was included in control samples. Although they used several colorectal cancer cell lines, they only show data for SW480. They found that 1.89% of SW480 cells were in the side population region. Haraguchi et al [143] identified a side population in SW480 cells of 0.7%. They used 5-10 $\mu\text{g}/\text{mL}$ Hoechst 33342 incubated for 70-90 minutes at 37°C with no pre-incubation.

The breast cancer cell line MCF7 is well established as containing a side population [155,156]. It has also been previously found to highly express the ABCG2

transporter [140]. The qPCR data from this study also found high levels of ABCG2 expression in MCF7 cells. As the colon cancer cell lines expressed much lower levels of ABCG2, it was considered that this was the reason for the lack of consistency in side population. Therefore, the side population protocol was tested in MCF7 cells to ascertain whether the problem was with the method or the cells. It was found that MCF7 express high levels of ABCG2 but a side population was not detected. As the SP method was not successful in MCF7, this indicates that the method itself is the issue rather than the cell lines.

Issues of reliability and consistency of side population results between laboratories have been discussed by Golebiewska and colleagues [147]. They highlight that dye efflux is an active and dynamic process. This makes it a very sensitive procedure in which even very small and unintended changes in protocol can lead to large variability in results. They suggest that reproducibility between laboratories can be improved with more detailed reporting of protocol details in publications. This can allow better comparison of results between different laboratories.

Even within the same laboratory, choice of dye and flow cytometer are further variables that can influence the side population, as seen in results by von Furstenberg et al [145]. They used epithelial cells from mouse jejunum and incubated with either 10 $\mu\text{g/ml}$ Hoechst 33342 or 10 μM DCV for 90 minutes at 37°C, with a 20 minute pre-incubation with verapamil. They also used three different flow cytometers, with ultraviolet and violet lasers. Results from this paper are shown here in Figure 3.4.2. It is apparent from these dot plots that side

population size and shape do change quite considerably when the dye and flow cytometer are varied.

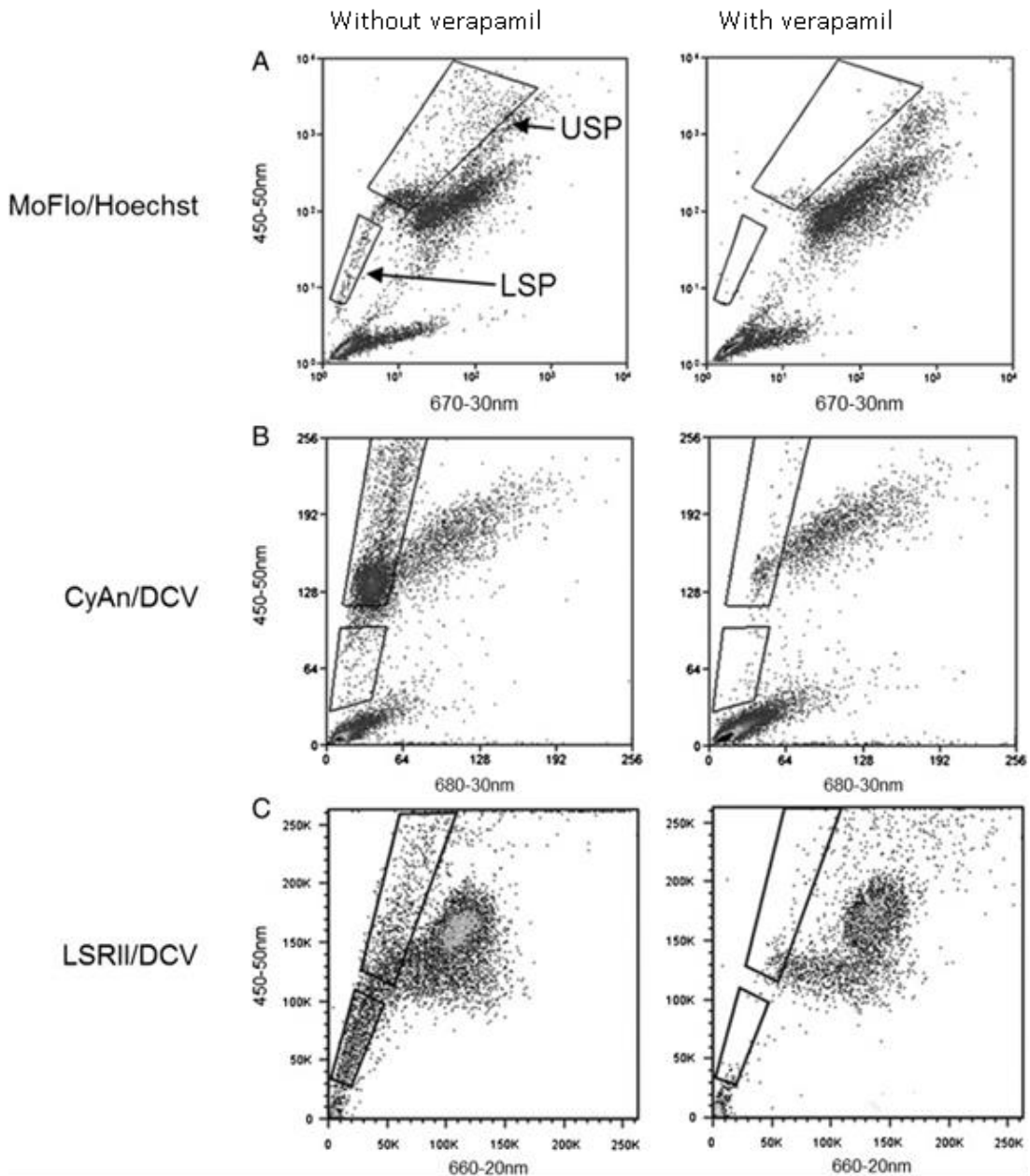


Figure 3.4.2: Data from publication by von Furstenberg et al [145].

Epithelial cells isolated from murine small intestine were stained with either Hoechst 33342 or DyeCycle Violet (DCV). Cells stained with Hoechst were analysed using a UV laser on a MoFlo cell sorter, while DCV samples were excited with a violet laser on a CyAn flow cytometer or an LSRII flow cytometer. For analysis, the side population (SP) was sub-divided into two regions: upper SP (USP), and lower SP (LSP).

It has been acknowledged by Sales-Pardo and colleagues that the side population method is found difficult by most investigators [157]. This is due to several reasons, which include the very low percentage of dye-effluxing cells in most tissue (less than 0.1% of total cell population), the difficulties in establishing correct experimental and acquisition settings, and the extensive expertise required analysing the acquired flow cytometry data to accurately identify the side population. They describe the multitude of seemingly small variables that can have a large impact on the side population results. These factors include power settings and alignment of the laser during flow cytometric data acquisition, the batch of Hoechst 33342 used, sample incubation and sample origin. These reports support the issues described in this chapter in being able to establish a method that achieves consistent results in SP detection.

3.4.1. Conclusions

It was hypothesised that side population cells are enriched with stem cells and could be used to generate colon organoids more efficiently than the whole population of colon crypt cells. The method was initially validated in colon carcinoma cell lines. However, it was not possible to obtain consistent results with the side population in these cell lines. As already described, researchers in different laboratories have found differing percentages of side populations in the same cell lines. Additionally, researchers in the same laboratory have found varying side populations by using a different dye or flow cytometer. It is apparent that this method is too sensitive and variable and cannot be used as a robust method for the identification of stem cells. As a consequence of this, the entire heterogeneous

population of primary colon crypt cells were subsequently cultured rather than an isolated population of stem cells; this work will be presented in Chapter 6. Additionally, in the following two chapters the influence of cell culture surface topography on cell stemness will be investigated by assessing gene expression of *ABCG2* as well as *Lgr5*.

Chapter 4: Evaluation of gelatin and PET electrospun scaffolds for the culture of human colon carcinoma cells

4.1 Introduction

Epithelial cells in the intestine are separated from the underlying layer of mesenchymal cells by a form of extracellular matrix (ECM) known as basement membrane. This membrane is a specialised structure that plays a key role in supporting and modulating epithelial cell growth [158]. It consists of a highly crosslinked network of collagen which provides mechanical stability [159], and a more dynamic, non-covalent network of laminin molecules [160]. These networks are connected and stabilised by the binding protein nidogen [161]. Additional components of the basement membrane include proteoglycans, calcium binding proteins such as fibulin and other structural and adhesive proteins [162]. Epithelial cells grown on stiff substrates such as tissue culture plastic have been found to lose regulatory control of proliferation and differentiation leading to unwanted malignant phenotypes [163]. Therefore, scaffolds that mimic the structure of native basement membrane are increasingly being investigated.

Electrospinning is a simple, versatile method of creating porous, fibrous scaffolds that have been shown to closely resemble native ECM [164]. The process involves a polymer solution being pumped at a constant rate out of a needle tip. A voltage is applied causing droplets at the needle tip to become cone-shaped due to mutual repulsion forces, creating what is known as a Taylor cone. The solution is then elongated and the solvent evaporated as it travels to the grounded metallic

collector where polymer fibres are deposited. This continues until a mat of randomly deposited non-woven fibres is formed. Parameters can be altered to vary fibre diameters from 3 nm to greater than 5 μm [121]. It can also be used with a range of synthetic and naturally-derived polymers so scaffolds can be adapted to the required function.

Electrospun scaffolds have been studied for tissue engineering of a range of tissues and organs. There is particular interest in their use for supporting growth of epithelial cells. Nanofibre scaffolds made of the synthetic polymer poly (l-lactide-co-caprolactone) (PLLC) coated with fibronectin have been investigated with oesophageal epithelial cells [165]. It was found that the electrospun scaffolds supported the epithelial cell phenotype and promoted regeneration. Polyethylene terephthalate (PET) has been used to fabricate electrospun scaffolds for *in vitro* engineering of lung epithelium [166]. The 3D topography created by electrospun fibres of different diameters was shown to influence cell response and enhance epithelial barrier formation. A further application of electrospinning is the generation of aligned fibres that can control cell orientation. Aligned gelatin-grafted poly (caprolactone) (PCL) nanofibres have been shown to promote endothelial cell spreading and control cell shape and orientation so that they form spindle shapes parallel to the aligned fibres [167].

In addition to electrospinning of synthetic polymers, naturally derived materials have also been used. Collagen and elastin have been electrospun in order to replicate the structure of blood vessel walls for vascular tissue engineering [168].

Furthermore, gelatin nanofibres have been used with periodontal ligament cells and were found to support good cell adhesion and proliferation [169]. Another basement membrane protein, laminin, was electrospun to create nanofibre scaffolds [170]. In this study, laminin nanofibres were found to better support attachment, growth and differentiation of adipose stem cells, compared with laminin films, thereby demonstrating the advantage of the fibrous scaffold structure.

By adjusting electrospinning parameters, fibre diameter can be altered [164]. This can influence cell growth and behaviour in a number of ways. Smaller fibres are usually associated with smaller pore size and lower porosity as the fibres are more tightly packed together. It has been found that increasing fibre diameter reduces the adhesion and proliferation of fibroblasts [171]. This is due to the increased surface area with smaller fibres [172]. Conversely, microfibre scaffolds tend to be more highly porous with larger pores that allow for cell infiltration [173,174].

4.1.1. Aims and hypotheses

The eventual goal of this work is to culture primary epithelial cells from human colon on scaffolds, for the development of a regenerative epithelial graft. Due to difficulties in primary cell expansion in Matrigel and the expense of the media (see chapter 6 for further details), this was not possible with all the scaffold types immediately. Therefore, colon carcinoma cell lines were used to model how primary epithelial cells may react to the different substrates. Caco2 and HT29 cells are commonly used in *in vitro* studies to model intestinal function and differentiation

and in pharmacological and toxicological studies. Caco2 cells differentiate to generate enterocyte-like cells [175] which form monolayers with tight junctions between cells. HT29 is a pluripotent cell line that has the capability of generating of different intestinal cell types depending on culture conditions and addition of differentiation inducers [176,177]. Gene expression profiling has been done to compare the cell lines with intestinal mucosa taken from healthy human subjects [178]. This study found that there is a relatively good correlation between these cell lines and intestinal mucosa, with regards to expression of genes encoding efflux transporters and proteins involved in drug metabolism. Unlike Caco2 and HT29, SW480 cells do not differentiate. They are a heterogeneous population of cells, of which the dominant cell type are flat polygonal cells that form epithelial colonies [179]. SW480 and HT29 have been shown to include a population of cells identified by Lgr5 expression that exhibit cancer stem cell-like properties such as increased proliferation, migration and colony forming *in vitro* and tumourigenicity *in vivo* [180]. Cancer stem cells have also been shown to be present in the Caco2 cell line [181,182]. Therefore, these cell lines are suitable for assessing the expression of a stem cell phenotype. By using the three different cell lines, the aim is to get a more complete, rounded picture of how the primary cells might respond.

The naturally derived material gelatin and the synthetic polymer PET were used to synthesise scaffolds in this study. Gelatin is a heterogeneous mixture of polypeptides obtained by the controlled hydrolysis of collagen, a major component of basement membrane. It has advantages over unprocessed collagen such as

increased aqueous solubility and reduced potential for antigenic and immunogenic responses [183], and is already widely used in pharmaceuticals and food products.

Poly(lactic-co-glycolic acid) (PLGA) is an example of a synthetic biomaterial that is commonly used in sutures, implants and prosthetic devices. It has the advantage of reproducibility due to its well-defined chemical composition and can be formulated to have specific mechanical properties and degradation rate [184]. Polyethylene terephthalate (PET) is a synthetic polymer that has been found by previous work within the Tissue Engineering Group at Nottingham to support cells in a similar manner as PLGA [185]. PET is not biodegradable, but is more straightforward to work with and handle during experiments, therefore has been used instead of PLGA in these preliminary experiments as a means to determine scaffold characteristics that would be suitable for further development.

It was hypothesised that the 2D microenvironment provided by electrospun scaffolds would support colonic epithelial cell growth and differentiated state in a way that resembles their behaviour *in vivo*. It was further hypothesised that differences in scaffold biomaterial and fibre diameter would influence this phenomenon. Therefore, the aims of this chapter were to fabricate electrospun scaffolds made from gelatin and PET and with fibres in the nano- and micro-range, and investigate their effect on cell growth using three different colonic carcinoma epithelial cells lines as exemplar cell types.

These aims were achieved with the following objectives:

- Manufacture electrospun microfibre and nanofibre scaffolds from the naturally derived material gelatin and the synthetic material PET.
- Characterise the electrospun scaffolds in terms of fibre diameter, porosity and mechanical strength.
- Evaluate the ability of the electrospun scaffolds to support colon epithelial cell proliferation, differentiation to a typical epithelial morphology and expression of stem cell markers Lgr5 and ABCG2.
- Choose the most appropriate scaffold to test with primary colon crypt cells.

4.2 Experimental

4.2.1 Scaffold fabrication and characterisation

Electrospun gelatin and PET nanofibre and microfibre scaffolds were manufactured, as described in chapter 2, section 2.2.1. To prevent scaffold dissolution in cell culture media, gelatin scaffolds were crosslinked with EDC and NHS, as described in chapter 2, section 2.2.4.

4.2.1.i Fibre diameter measurement

Fibre diameter was measured using ImageJ software from SEM images of the scaffolds. A detailed method for SEM can be found in chapter 2, section 2.3.1. For each scaffold type, 100 diameter measurements were taken from each scaffold batch. Three batches were assessed for inter-batch variation, therefore a total of 300 measurements were taken for each scaffold type.

4.2.1.ii Porosity

Scaffold porosity was estimated using a gravimetric method. Length, width, thickness and mass were measured from a total of 6 samples from 2 different batches of each scaffold type. Together with the material density, these measurements were used to estimate scaffold porosity. A detailed method is described in chapter 2, section 2.3.2.

4.2.1.iii Mechanical properties

Uniaxial tensile testing using a texture analyser was conducted in order to assess mechanical properties of the electrospun scaffolds. Rectangular pieces of scaffold were cut and glued to acetate that could be held securely by the grips of the texture analyser. Scaffolds were pulled apart until breaking and from the resulting stress-strain curves, Young's modulus and ultimate tensile strength were derived. Details of the method can be found in chapter 2, section 2.3.3. For each scaffold type, 3 samples were tested from 3 different batches.

4.2.2. Effects of altering temperature and relative humidity on morphology of gelatin microfibres

The experimental work in this part of the chapter was performed by undergraduate MPharm student Wai Chow under my supervision. Gelatin microfibres were electrospun using identical parameters to those already described in chapter 2, section 2.2.1, with the exception of temperature and relative humidity. Two sets of experiments were conducted. Firstly, the relative humidity was kept constant at

44% and the temperature was varied to 20°C, 30°C and 40°C. Secondly, the temperature was kept constant at 45°C and the relative humidity was changed to 44%, 22% and 11%. For each set of conditions three batches of scaffolds were electrospun. From each batch, 3 scaffolds were cut and imaged by SEM, as already described (see chapter 2, section 2.3.1). The images were used to assess scaffold morphology and fibre diameter measurements were obtained using ImageJ software. For each condition a total of 360 fibre diameter measurements were made across 3 different scaffold batches.

4.2.3. Effects of fibrous topography on the proliferation and differentiation status of colon epithelial cells

Scaffolds were cut into circles, placed in CellCrown inserts, as shown in Figure 4.2.1A. They were sterilised by soaking in 10x antibiotic-antimycotic solution (100 µg/mL streptomycin and 250 ng/mL amphotericin B) for at least 3 hours at 4°C. Antibiotic-antimycotic solution was then removed, complete media (DMEM containing 10% foetal calf serum and 1x antibiotic-antimycotic solution) was added and the scaffolds were incubated at 37°C overnight. The colon carcinoma cell lines SW480, Caco2 and HT29 were cultured at 37°C in complete medium as described in chapter 2, section 2.4.1. They were grown to confluence in T75 flasks, then removed by trypsin digestion and counted. Cells (2×10^4) in 50 µL complete medium were added to each scaffold in triplicate, and incubated at 37°C for 3 hours to allow cells to adhere before 1 mL complete media was added to each well. Cultures on the 4 types of electrospun scaffolds and tissue culture plastic control were

compared by proliferation, SEM, immunocytochemistry and qPCR as shown in the schematic in Figure 4.2.1B.

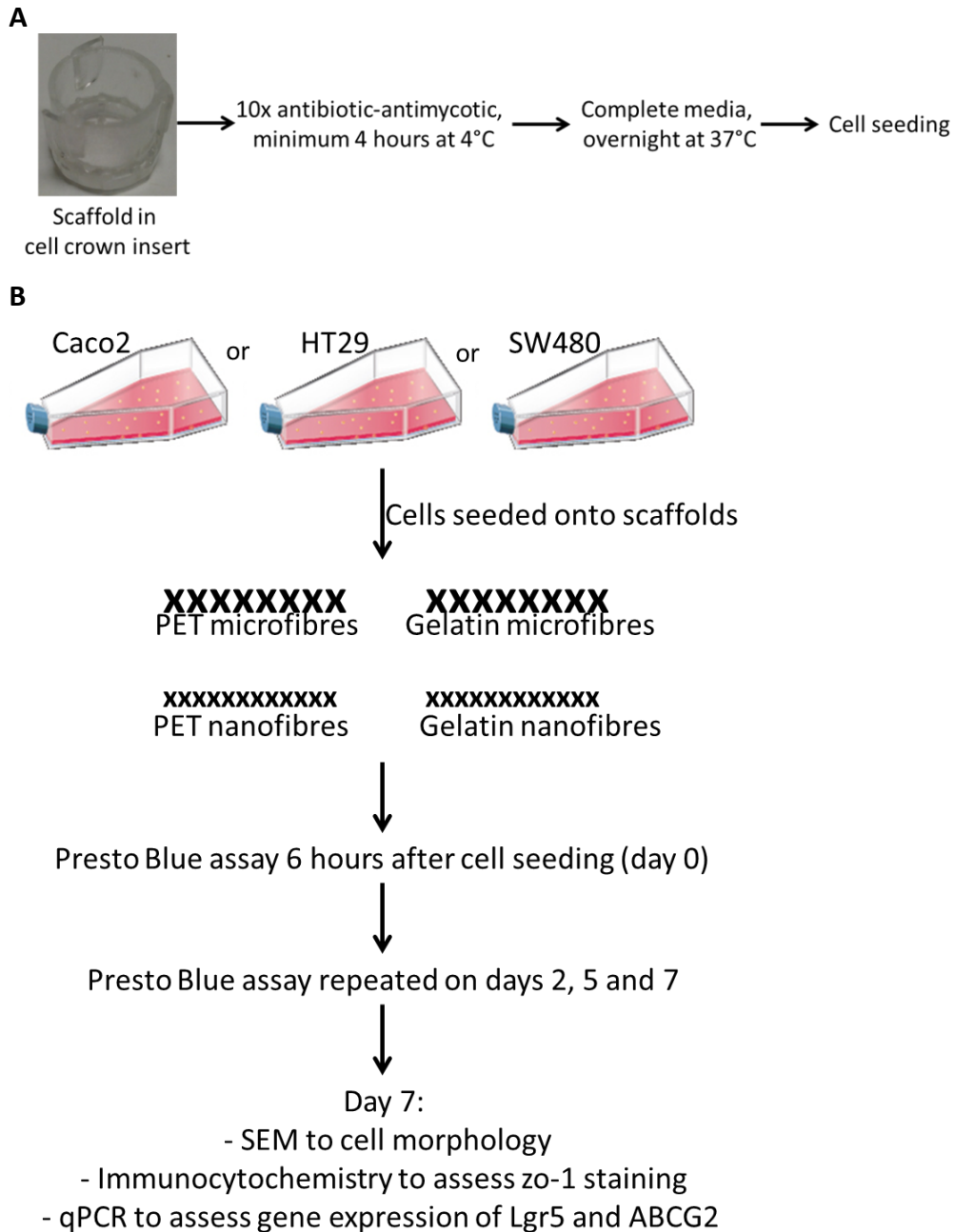


Figure 4.2.1: Schematics showing procedures for scaffold preparation and set of experiments to assess colon epithelial cell response to electrospun scaffolds

Scaffolds were sterilised and soaked in complete media prior to cell seeding (A). A simplified version of the experimental design is presented in B, assessing cell proliferation for 7 days, then investigating cell morphology and expression of stem cell markers.

4.2.3.i Proliferation

Cell adhesion and proliferation was assessed using the Presto Blue assay. This was conducted 6 hours after cell seeding (day 0) and repeated on days 2, 5 and 7. Details of the Presto Blue assay can be found in chapter 2, section 2.5.1.

4.2.3.ii SEM to assess cell morphology

Cellular morphology on the scaffolds was evaluated by SEM imaging. Scaffolds were fixed 7 days after cell seeding with 3% (w/v) glutaraldehyde then dehydrated with in increasing concentrations of IMS. Details of the SEM method can be found in chapter 2, section 2.3.1.

4.2.3.iii Immunocytochemistry to assess tight junction formation

Immunocytochemical staining for the tight junction marker zonula occludens protein-1 (zo1) was used to assess the ability of Caco2 and HT29 cells to differentiate and form an epithelial barrier. Scaffolds were fixed with 3.7% (w/v) paraformaldehyde 7 days after cell seeding. Cells were permeabilised, blocked to prevent non-specific binding and stained with anti-zo-1 antibody conjugated to Alexa Fluor 488. Samples were imaged by confocal microscopy. A detailed method can be found in chapter 2, section 2.6.

4.2.3.iv qPCR to assess gene expression of stem cell markers

Due to the heterogeneous nature of the cell lines used and the suggestion that some of these populations may be stem cells [186], the expression of stem cell markers were assessed. Gene expression of *Lgr5* and *ABCG2* was assessed using qPCR after 7 days culture on the scaffolds. RNA was extracted, transcribed to cDNA and qPCR was conducted. All values were normalised to the housekeeping gene HPRT and gene expression was reported in comparison to tissue culture plastic controls. Detailed methods can be found in chapter 2, section 2.1.

4.2.4. Statistical analysis

Data were expressed as mean $\pm$ standard deviation. GraphPad Prism 7 software was used to perform statistical analysis. One-way ANOVA was used to assess statistical differences, with the exception of Presto Blue data which was analysed with repeated measures ANOVA. Differences were deemed statistically significant if $p \leq 0.05$. Symbols of further levels of statistical significance are detailed in chapter 2, section 2.7.

4.3 Results

4.3.1 Smooth, uniform fibres with clear differences in diameter between nanofibres and microfibres were produced

From observation of SEM images, it is apparent that all of the scaffolds were made of smooth, uniform, bead-less fibres (Figure 4.3.1;). The diameters of the nanofibres were primarily between 300-600 nm and the microfibres were between 1-2 μm (

Figure 4.3.1 C-D; E-F). Three batches of each scaffold type were manufactured and inter-batch consistency in fibre morphology and diameter was observed. Prior to crosslinking, gelatin scaffolds rapidly dissolved in water, however after crosslinking with 25mM EDC and 10 mM NHS, they could be immersed in water and remained intact. The morphology of both the gelatin fibres appeared to change after crosslinking. After crosslinking, the gelatin nanofibres became swollen and the fibre diameters increased slightly. In contrast, crosslinked gelatin microfibres appeared to change shape, becoming less flat, and there was a decrease in fibre diameter. After crosslinking the fibres also seemed to adhere to each other so discrete fibres can be seen less clearly.

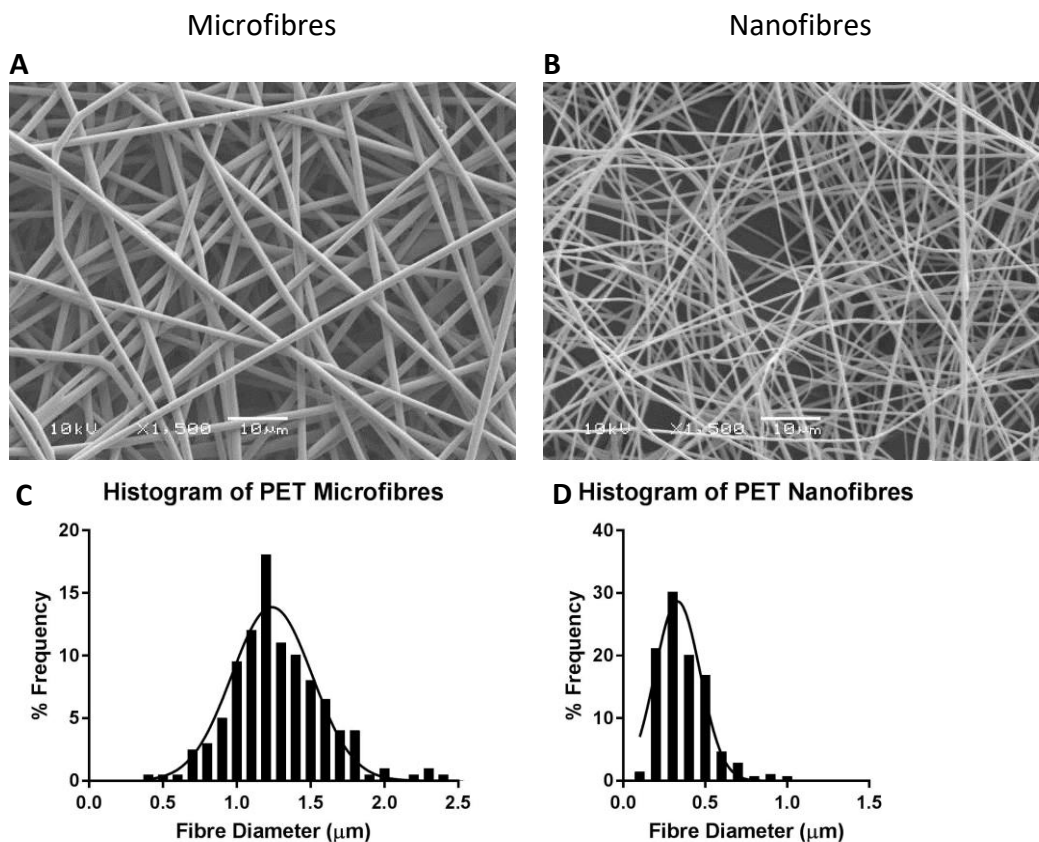


Figure 4.3.1: SEM images and fibre diameter distribution of PET electrospun microfibres and nanofibres.

PET, 25% or 10% (w/v), was dissolved in 1:1 (v/v) DCM: TFA and electrospun to produce scaffolds with fibre diameters either in the micro- or nano-metre range, respectively. Representative SEM images are shown (A; B). Scaffold fibre diameter distributions of PET microfibres (C) and nanofibres (D) were obtained by taking 300 individual fibre measurements from 3 batches of scaffolds. Scale bars = 10 μm.

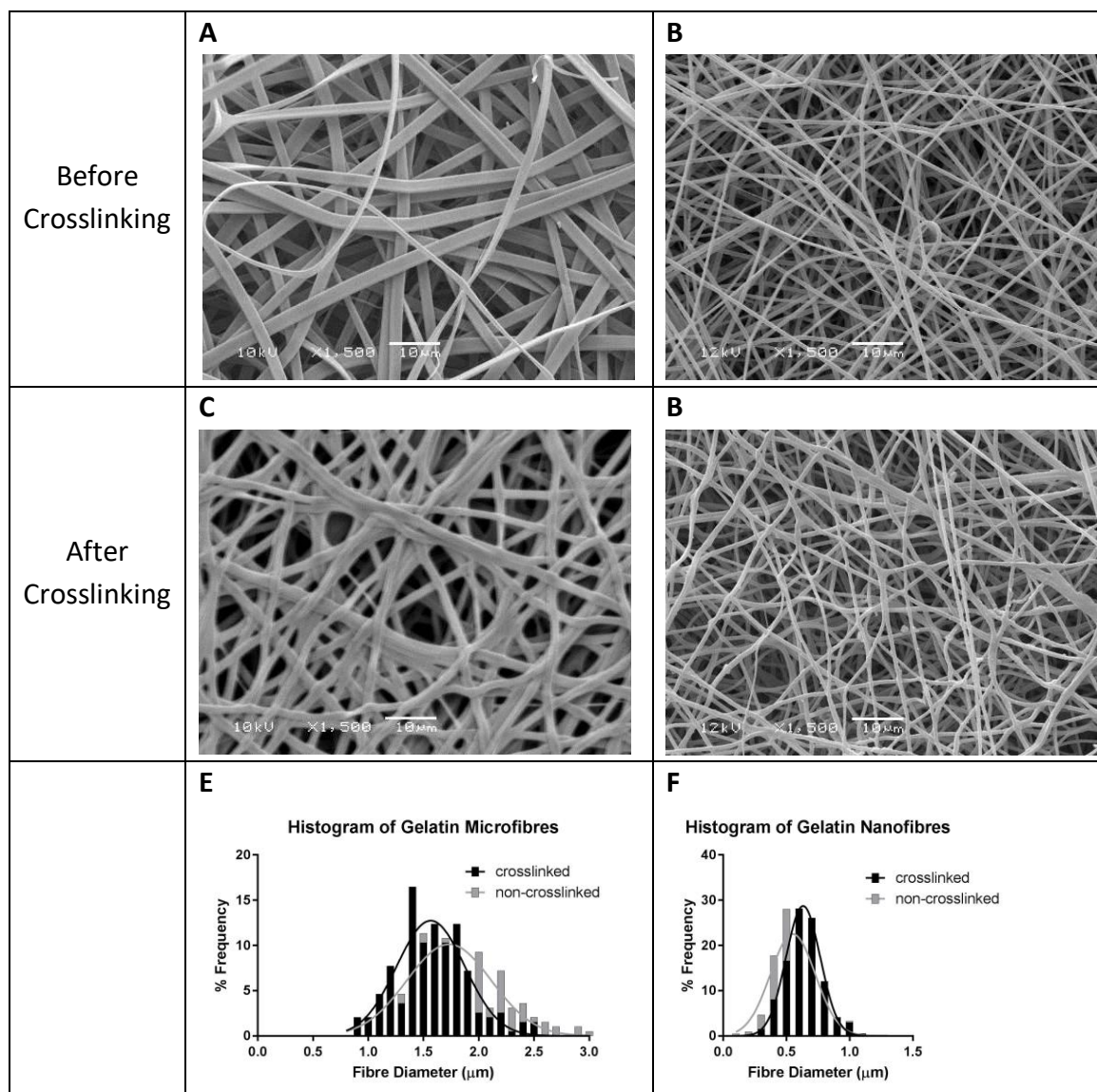


Figure 4.3.2: SEM images and fibre diameter distribution of gelatin microfibre and nanofibre electrospun scaffolds before and after crosslinking

Gelatin, 10% or 8% (w/v), was dissolved in HFIP and electrospun to produce scaffolds with fibre diameters either in the micro- or nano-metre range, respectively. Representative SEM images of the microfibre and nanofibre scaffolds before crosslinking are shown (A; B). The scaffolds were crosslinked in a solution of 25 mM EDC and 10 mM NHS at 4°C for 24 hours. SEM micrographs of crosslinked gelatin microfibrils and nanofibrils are shown (C; D). Fibre diameter distributions of scaffolds before and after crosslinking were obtained by measurement of at least 300 individual fibres from 3 batches of scaffolds (E; F). Scale bars = 10 µm.

The gelatin fibres, in particular the microfibres, appeared flat and ribbon-like, rather than circular. In order to investigate possible reasons for this, temperature and humidity were adjusted within the electrospinning chamber. By increasing the temperature and reducing humidity from ambient conditions, it was hypothesised that the solvent would evaporate more quickly thereby influencing fibre morphology. Increasing temperature had no effect on fibre shape and morphology as seen by representative SEM micrographs (Figure 4.3.3). When the temperature was set at 30°C, mean fibre diameter was 2.2 μm , which was a small but statistically significant increase compared with both lower and higher temperatures which generated mean fibre diameters of 1.9 μm . (Figure 4.3.5A). Reducing humidity caused the fibres to become less uniform with a larger range of fibre diameters, whilst remaining ribbon shaped (Figure 4.3.4). Alongside the increasing diameter range, decreasing relative humidity also resulted in an increase in the mean fibre diameter, 2 μm at 44% to 3 μm at 22% and almost 4 μm at 11% (Figure 4.3.5B). Overall, it is apparent that varying atmospheric conditions during electrospinning gelatin did not affect fibre shape, but did change fibre diameter.

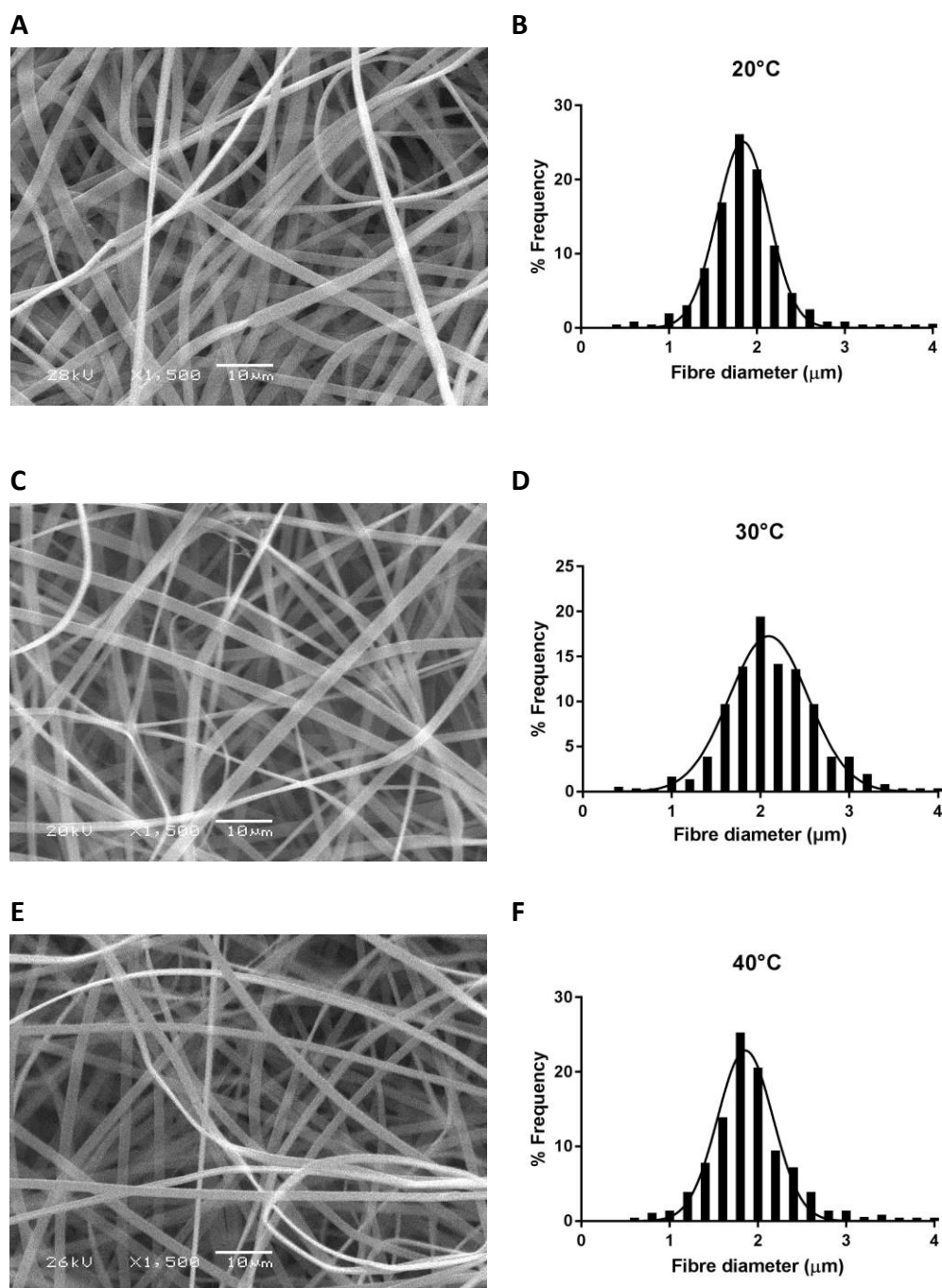


Figure 4.3.3: SEM images and fibre diameter distributions of gelatin microfibres electrospun at varying temperatures

Gelatin, 10% (w/v), was dissolved in HFIP and electrospun at varying temperatures (20°C, 30°C and 40°C) at a constant relative humidity of 44%. Representative SEM micrographs are shown (A, C, E) Fibre diameter distributions were obtained by measurement of 360 individual fibres from 3 batches of scaffolds (B, D, F)

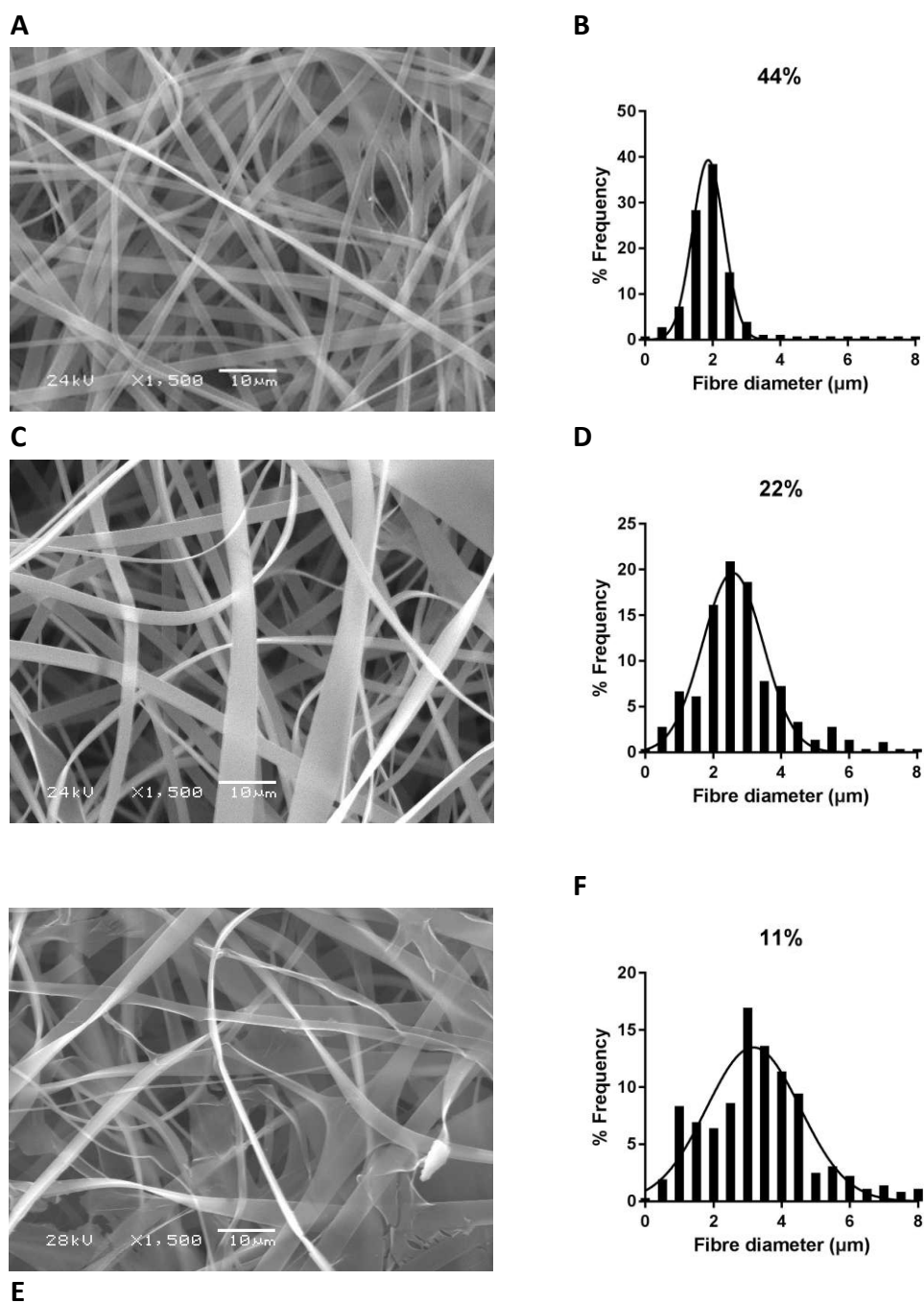
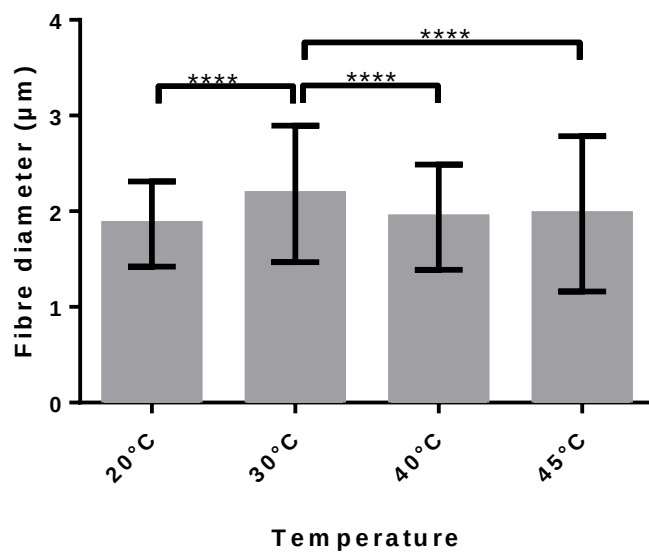


Figure 4.3.4: SEM images and fibre diameter distributions of gelatin microfibres electrospun at varying temperatures

Gelatin, 10% (w/v), was dissolved in HFIP and electrospun at varying relative humidities (44%, 22% and 11%) at a constant temperature of 45°C. Representative SEM micrographs are shown (A, C, E) Fibre diameter distributions were obtained by measurement of at 360 individual fibres from 3 batches of scaffolds (B, D, F)

A



B

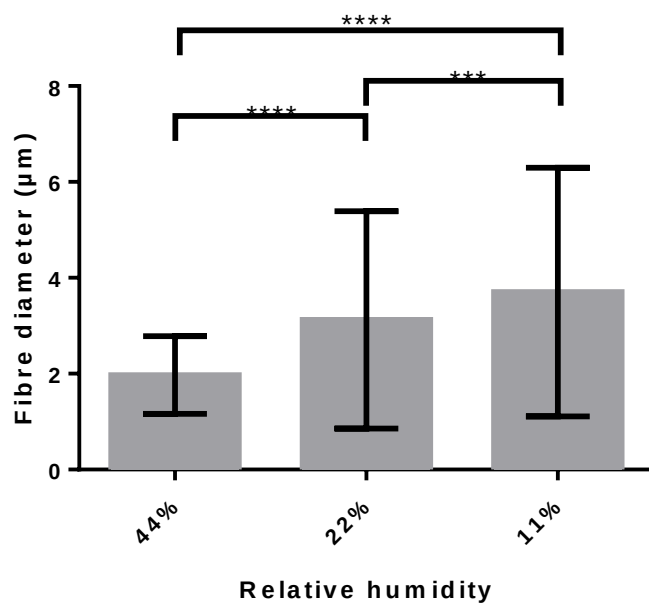


Figure 4.3.5: Fibre diameters of gelatin microfibres after varying temperature and relative humidity

Mean fibre diameters with varying temperature (A) and relative humidity (B). Data represents mean $\pm$ SD (n=360 from 3 batches of scaffolds). *** $p \leq 0.001$, **** $p \leq 0.0001$

Porosity was estimated using the gravimetric method in which scaffold density is calculated (Figure 4.3.6). The results show that all of the nanofibre and microfibre scaffolds were approximately 60% porous and there were no significant differences between the scaffold types.

Tensile strength testing revealed that PET electrospun scaffolds were more elastic and require more force to break than electrospun scaffolds made from gelatin (Figure 4.3.7). In addition to this difference between materials, it is also apparent fibre diameter influences scaffold tensile strength, as PET microfibres have a significantly higher Young's modulus and ultimate tensile strength compared with PET nanofibres. Gelatin scaffolds have only elastic tendencies as seen from the linear stress-strain graphs and instant breaks at the point of maximum stress. This is in contrast to the PET stress-strain graphs which are more bell-shaped indicating that after the initial elastic behaviour, the scaffolds undergo plastic, non-reversible deformation before a gradual breakage.

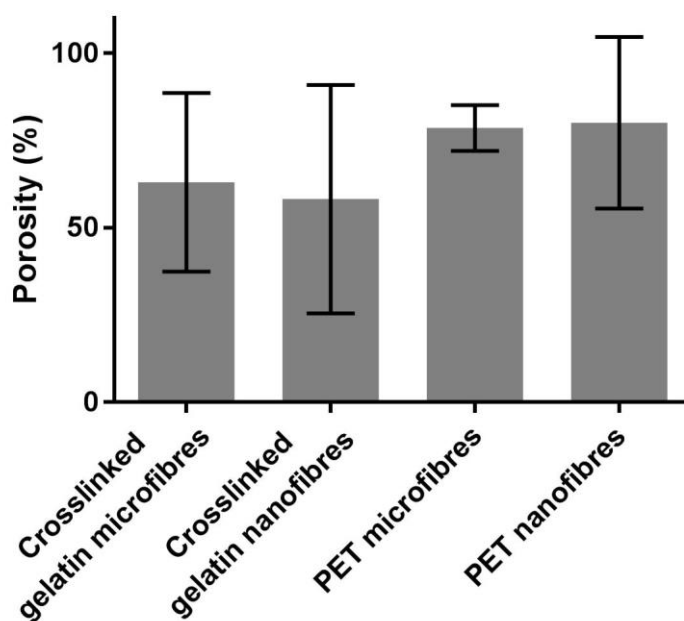


Figure 4.3.6: Porosity of electrospun fibre scaffolds

Total porosity was measured by gravimetric analysis. Graph shows mean $\pm$ SD (n=6 from 2 batches of scaffolds). Statistical significance was calculated using one-way ANOVA. Differences were found to be non-significant.

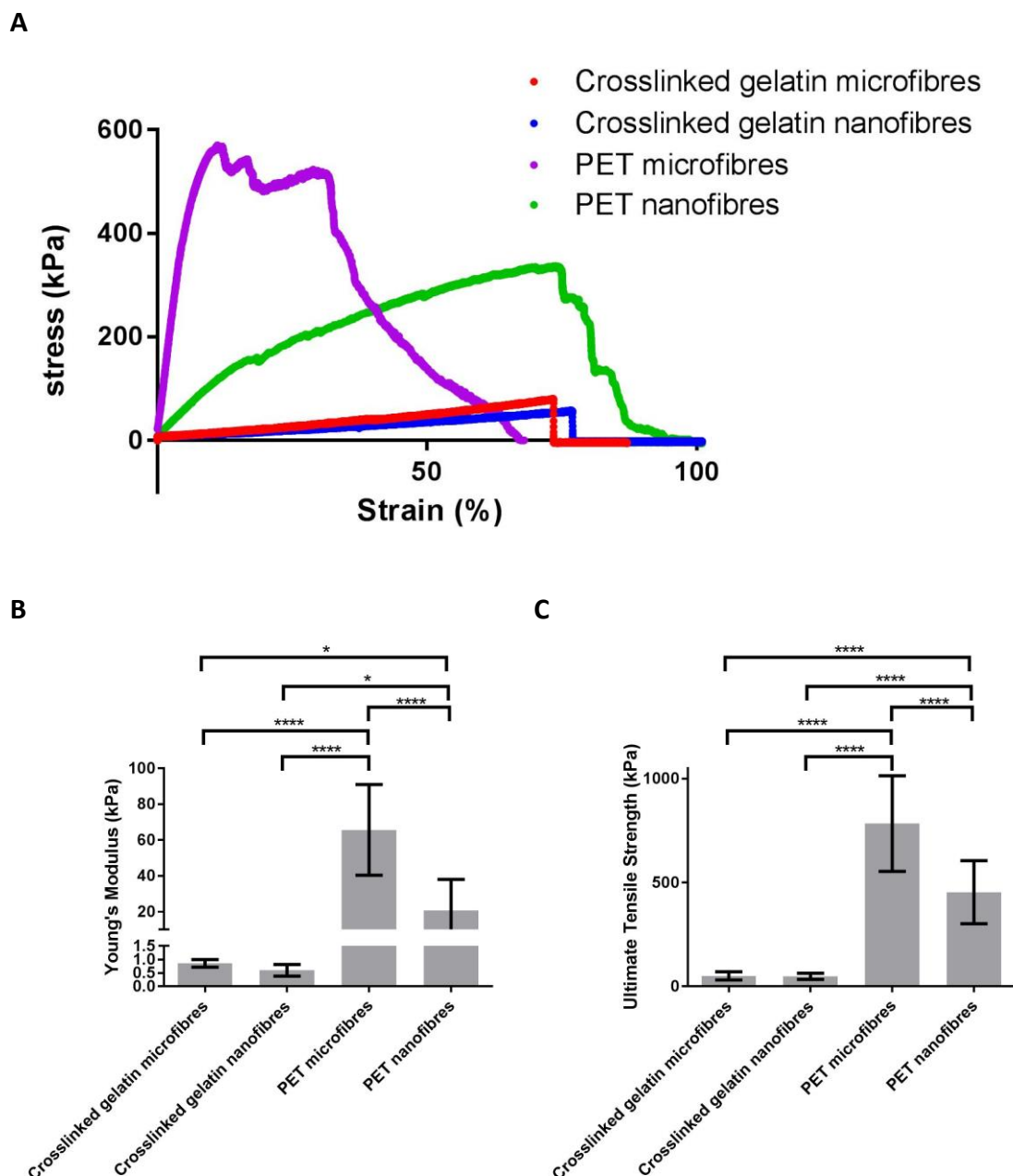


Figure 4.3.7: Tensile strength of the electrospun scaffolds

Typical stress-strain curves for each scaffold type are shown in A. Young's Moduli were calculated from the slopes of the initial parts of the stress-strain graphs (B). Ultimate tensile strength (C) is the maximum tensile strength before scaffold breakage. Graphs B and C show mean $\pm$ SD (n=9 from 3 batches of scaffolds). Statistical significance was calculated using one-way ANOVA. (* $p \leq 0.05$; **** $p \leq 0.0001$)

4.3.2. Cells proliferated on top of all the electrospun scaffold types

It is essential for cells to be able to adhere to and proliferate on scaffolds. A resazurin-based metabolic assay was conducted 6 hours after cell seeding to measure cell attachment, then every two-three days for a week to measure cell proliferation. All of the four scaffold types were able to support attachment and growth of the colonic epithelial cell lines SW480, Caco2 and HT29 (Figure 4.3.8). There were no significant differences in cell proliferation between the scaffold types and tissue culture plastic, although there did appear to be greater variation in proliferation on the scaffolds compared with tissue culture plastic. This could be caused by variability in cell attachment immediately after seeding due to cells not adhering to the scaffolds preferring the plastic underneath.

Morphology of cells on scaffolds after 7 days culture was assessed by SEM (Figure 4.3.9). Caco2 cells formed multicellular adherent layers in which individual cells could not be distinguished. HT29 cells grew in confluent layers on top of all the scaffolds, whereas SW480 cells adhered to fibres as individual, separate cells and remained spherical rather than flattened. There were no apparent differences in morphology of each cell line between the different scaffold types.

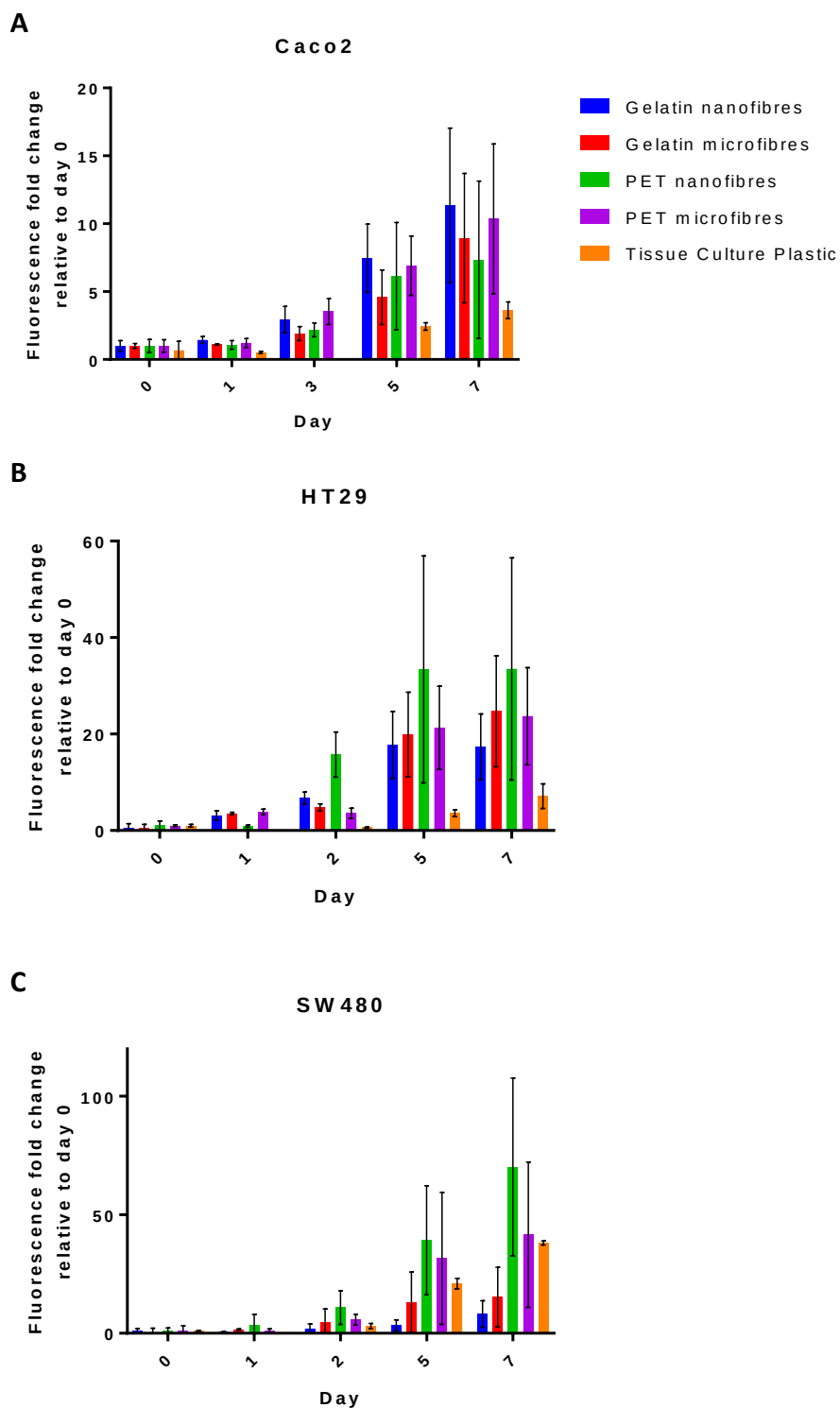


Figure 4.3.8: Cell proliferation on electrospun scaffolds

Caco2 (A), HT29 (B) and SW580 cells (C) were seeded onto scaffolds and cultured for 7 days. Presto Blue fluorescence data was normalised to day 0 show mean $\pm$ SD (n=9, 3 replicates each in 3 separate experiments). Results were analysed with repeated measures 2-way ANOVA. Differences were found to be non-significant.

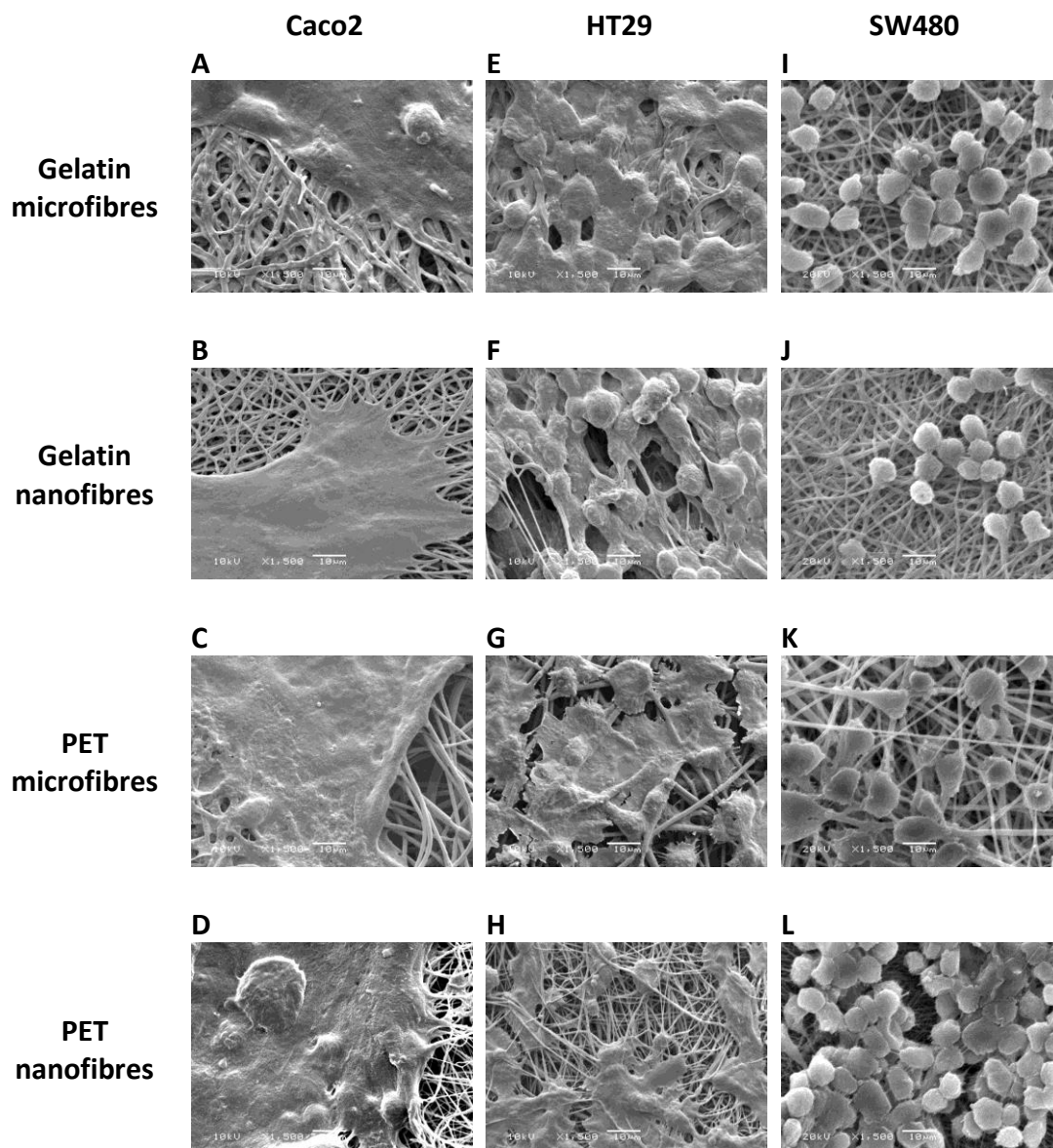


Figure 4.3.9: SEM micrographs of cells on electrospun scaffolds

Caco2 (A-D), HT29 (E-H) and SW480 cells (I-L) were grown on electrospun scaffolds for 7 days culture. The samples were fixed with glutaraldehyde and dehydrated with increasing concentrations of alcohol before imaging by SEM. Representative images of each sample are shown.

4.3.3 Cells differentiated to form cellular junctions on gelatin nanofibres and PET microfibres but no consistent changes in stem cell markers were observed

Zo-1 is a marker of tight junctions expressed on cell membranes giving a typical 'chicken-wire' pattern when identified using immunocytochemistry. Its expression is one indicator of the barrier function of intestinal epithelium as well as terminal differentiation. Immunocytochemistry was done to assess Zo-1 expression in Caco2 and HT29 cells after 7 days culture on scaffolds. SW480 was not used as this cell line does not express Zo-1. Caco2 cells displayed the characteristic 'chicken wire' expression associated with tight junctions (Figure 4.3.10), whereas zo-1 expression in HT29 cells was less distinct (Figure 4.3.11). As this is also the case on the glass controls, it is most likely an inherent feature of the cells rather than a response to the scaffold morphology or experimental artefact. Caco2 cells did not exhibit uniform zo-1 expression on any of the scaffolds, with many cells not appearing to be stained at all. From observations of immunostaining in both cell lines, it appears that cells expressed the least amount of zo-1 on gelatin microfibres. Gelatin nanofibres and PET microfibres appeared to support the strongest zo-1 expression.

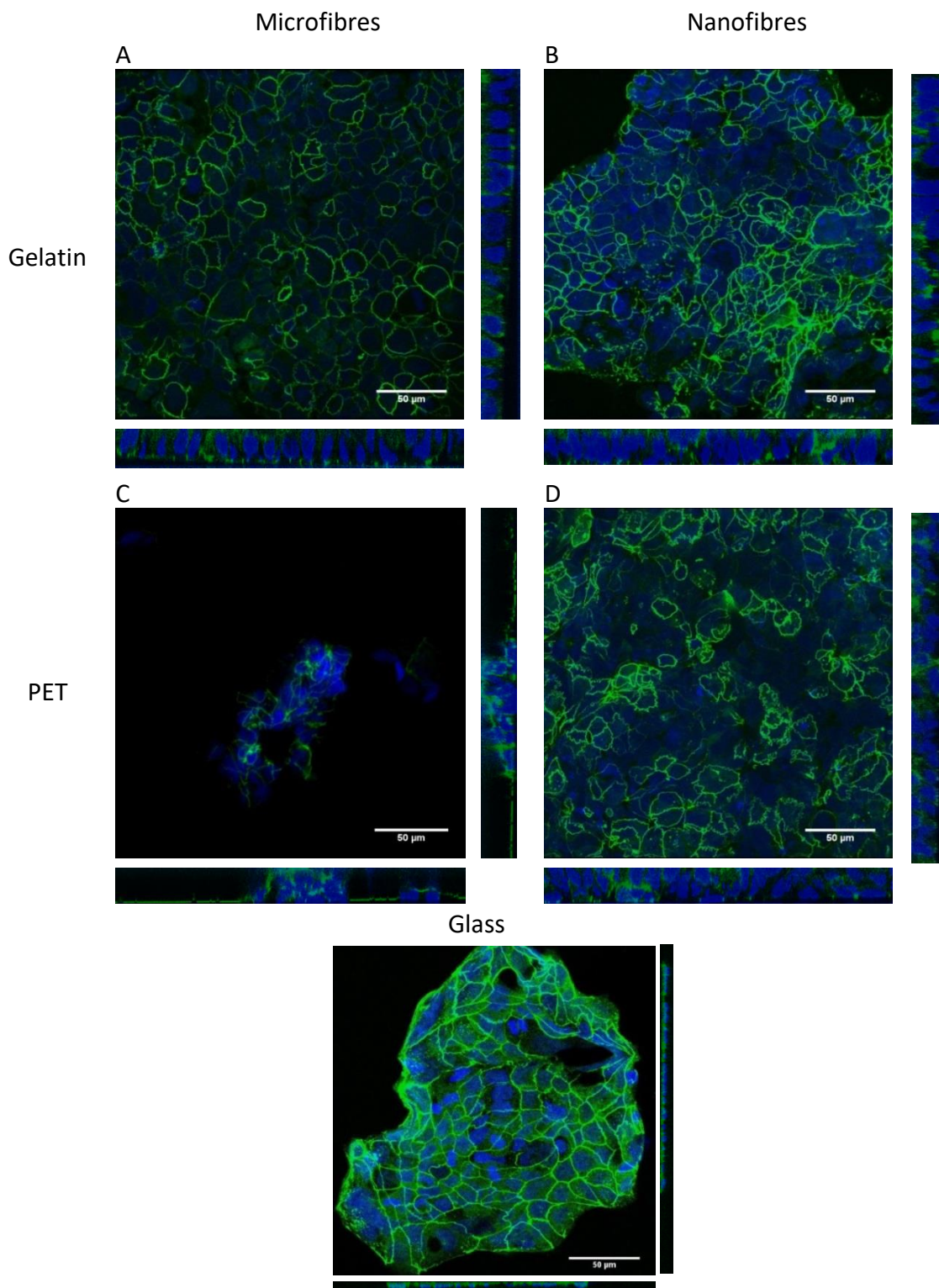


Figure 4.3.10: Caco2 zo-1 immunocytochemistry

Immunocytochemical staining for the tight junction marker Zo-1 in Caco2 cells after 7 days culture on scaffolds. Representative 3D projections of confocal z-stacks are shown as well as XZ and YZ orthogonal views. Zo-1 is in green, and Hoechst 33342 nuclear stain is in blue.

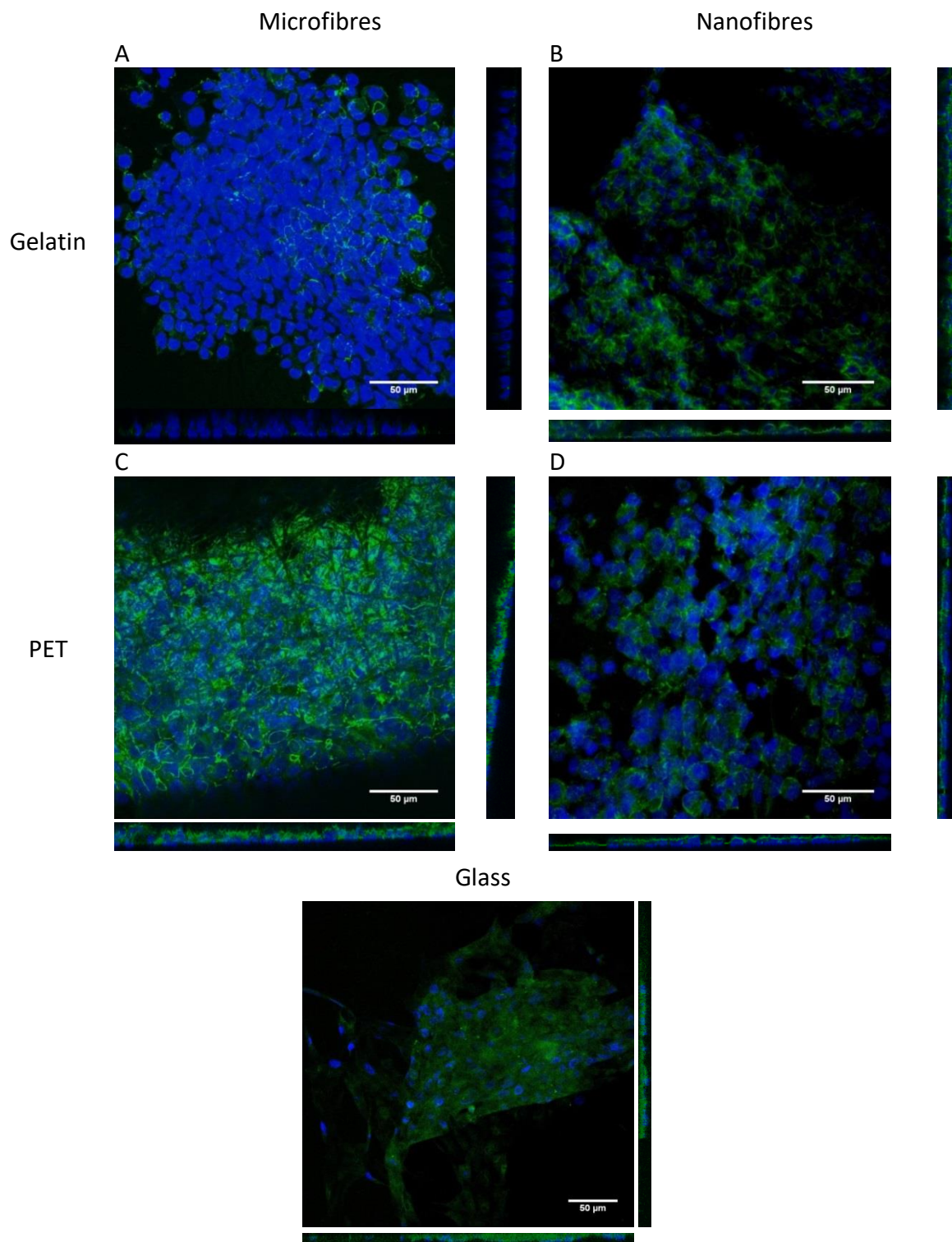
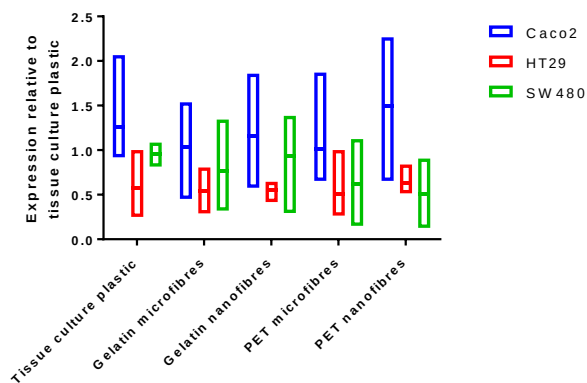


Figure 4.3.11: HT29 zo-1 immunocytochemistry

Immunocytochemical staining for the tight junction marker Zo-1 in HT29 cells after 7 days culture on scaffolds. Representative 3D projections of confocal z-stacks are shown as well as XZ and YZ orthogonal views. Zo-1 is in green, and Hoechst 33342 nuclear stain is in blue.

Changes in expression of stem cell markers *Lgr5* and *ABCG2* compared with tissue culture plastic were assessed using qPCR. *Lgr5* levels were unaffected with culture on the electrospun scaffolds. There were statistically significant increases in *ABCG2* expression of up to 6-fold in SW480 cells after culture on gelatin nanofibres, and up to 4-fold in Caco2 cells on PET microfibres. SW480 cultures also showed small but statistically non-significant increases in *ABCG2* on the other electrospun scaffolds (Figure 4.3.12).

A – *Lgr5*



B – *ABCG2*

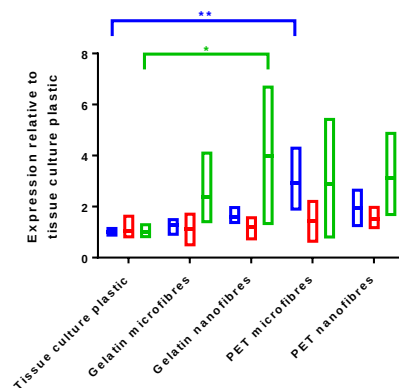


Figure 4.3.12: Expression of *Lgr5* and *ABCG2* after culture on electrospun scaffolds

The relative expression of *Lgr5* (A) and *ABCG2* (B) in colon epithelial cell lines after 7 days of culture on scaffolds compared with tissue culture plastic. Data represents mean, maximum and minimum expression relative to tissue culture plastic (n=6 from 2 separate experiments). Statistical significance compared to tissue culture plastic was assessed by one-way ANOVA. * $p \leq 0.05$, ** $p \leq 0.01$

Table 4.8: Summary of results

	Crosslinked gelatin microfibres	Crosslinked gelatin nanofibres	PET microfibres	PET nanofibres
Fibre diameter, mean $\pm$ SD (μm)	1.56 ± 0.33	0.64 ± 0.15	1.30 ± 0.37	0.38 ± 0.15
Ultimate Tensile Strength, mean $\pm$ SD (kPa)	50.53 ± 19.8	48.48 ± 14.59	783.7 ± 230.2	453.1 ± 151.9
Young's modulus, mean $\pm$ SD (kPa)	0.85 ± 0.14	0.60 ± 0.22	65.61 ± 25.28	20.70 ± 17.30
Porosity, mean $\pm$ SD (%)	63.01 ± 25.59	58.18 ± 32.75	80.08 ± 24.57	78.58 ± 6.554
Cell proliferation? (Y/N?)	Y	Y	Y	Y
Cell morphology	Multicellular aggregates, on top of fibres	Multicellular aggregates, on top of fibres	Multicellular aggregates, on top of fibres	Multicellular aggregates, on top of fibres
Tight junction formation	Low	High	High	Moderate
ABCG2 expression – min - max fold change compared to tissue culture plastic	1 - 4	1 - 6	1 - 5	1 - 4
Lgr5 expression – min - max fold change compared to tissue culture plastic	0.5 – 1.5	0.5 - 2	0.5 - 2	0.25 - 2

4.4 Discussion

The aims of this chapter were to fabricate electrospun scaffolds made from gelatin and PET and with fibres in the nano- and micro-range, compare their physical properties, and investigate their effect on cell growth using three different colonic carcinoma cells lines as exemplar cell types. The electrospinning parameters used had been previously optimised by other members of the research group. With these settings, it was possible to achieve uniform, smooth, bead-less fibres with a 2 to 4-fold difference between nanofibres and microfibres.

Despite the clear differences in fibre diameter, this was not translated into differences in porosity. There does not appear to be any difference in fibre density as has been found in previous research when microfibre and nanofibre scaffolds are compared [173,187]. It has been previously found that cells infiltrated the more porous microfibre scaffolds, whereas they grew in layers on top of less porous nanofibre scaffolds [166]. In the current study this was not found to be the case as cells grew on top of both the nanofibre and microfibre scaffolds. This supports the finding that there is no variation in porosity between the different electrospun scaffolds fabricated in this study.

Fibre diameter and pore size has also been found to influence the mechanical properties of electrospun scaffolds. Polycaprolactone (PCL) microfibre scaffolds were found to have higher tensile strength and Young's modulus than nanofibre scaffolds [187]. In the current study the same finding was observed with the PET scaffolds, but not the gelatin scaffolds. This could be due to the generally greater

mechanical strength of synthetic polymers compared with naturally derived materials.

In order to investigate the reason for gelatin microfibres appearing ribbon-shaped rather than circular, the effect of ambient conditions during electrospinning was investigated. The hypothesis was that by increasing temperature and reducing relative humidity in the electrospinning chamber, changes in solvent evaporation and solution viscosity would affect the shape of fibres [188]. It was found that ribbon-shaped fibres were formed at all the tested temperatures and humidities, but there were changes in fibre diameter and in the case of altering relative humidity there were changes in fibre uniformity.

Increasing temperature has two opposing influences on electrospinning. Firstly, it leads to faster solvent evaporation, and therefore increased polymer concentration and viscosity. This also causes faster polymer solidification, resulting in increased fibre diameter [189]. Conversely, at higher temperatures, electrical conductivity increases and solution viscosity and surface tension decreases, leading to thinner fibres [190]. This explains the finding that fibre diameter increases when the temperature is raised from 20°C to 30°C, as solvent evaporation dominates the process at this temperature. As the temperature increases further to above the melting temperature of gelatin [191], reducing viscosity takes over as the primary influence. This explains the reduced fibre diameters formed at 40°C and 45°C in this study.

Reducing relative humidity causes faster solvent evaporation, so that the polymer solidifies more quickly before it can be elongated in the jetting process, leading to the increased fibre diameters that were observed. As the solvent evaporates more quickly, the polymer solution becomes more viscous which may explain the entangled and disordered fibre morphology observed at lower humidities [192].

Ribbon-shaped gelatin fibres have also been observed in other studies. When high concentrations of gelatin (30% – 40%) in aqueous solutions were electrospun at temperatures elevated up to 50°C, ribbon shaped fibres in the nanometre range as well as the micrometre range were found [169]. When 8.3% gelatin was electrospun by Li et al ribbon-like fibres were formed, but round fibres were observed at lower gelatin concentrations [164]. They suggested this could be due to slow solvent evaporation and solidification after landing on the collector. The findings in this chapter do not support their hypothesis, as encouraging more rapid solvent evaporation by increasing temperature and reducing relative humidity did not alter fibre shape. However, their finding that round fibres are formed with a lower gelatin concentration is supported by the results in the current study in which nanofibres were round but microfibres were ribbon-shaped.

Due to its high water solubility of gelatin, it needs to be crosslinked prior to use. In the current study, gelatin was crosslinked using a carbodiimide coupling reaction which forms covalent bonds between amino acids, as previously described [169]. An advantage of this method of crosslinking is that all residues are water soluble and can be washed away so that no cytotoxic effects occur [193].

Cells were able to adhere and proliferate on all four scaffold types tested in this study. The influence of the different scaffold types on cell differentiation was also investigated. This was done in part by assessing the formation of intercellular tight junctions which are crucial for the barrier function of the intestinal epithelium. Tight junctions are found on the apical side of epithelial cells and are composed of transmembrane proteins such as occludins and claudins [194,195]. The tight-junction marker zonula occludens-1 (zo-1) displays a characteristic 'chicken-wire' staining when a tight barrier is formed. This staining pattern was observed in Caco-2 cells on all the scaffolds as well as the glass control, indicating that they are all capable of supporting cell differentiation and barrier formation. When Caco2 cells were grown on PCL microfibres with mean fibre diameter of 3.68 μm , they displayed poor epithelial morphology and zo-1 staining was found sporadically at only some cell-cell functions [196]. This was most likely due to the large fibre diameters and pore size resulting in cells penetrating into the scaffolds rather than forming a monolayer on top. As already mentioned, relatively low porosity was observed in the microfibre scaffolds in this study. This meant that all tested electrospun scaffolds were able to support cellular junction formation to some degree, with gelatin nanofibres and PET microfibres appearing to support zo-1 expression.

Scaffold material composition and form have been shown to play crucial roles in control of stem cell phenotype and determining cell behaviour [72,197–199]. In particular, differences between natural and synthetic materials have been investigated. Natural polymers tend to be weaker than synthetic polymers [200], a

finding that has been upheld in the current study. They can also mimic the *in vivo* niche as they have sites for binding cell surface receptors as well as providing physical and mechanical cues that regulate cell function [72]. Synthetic and natural polymers have been compared previously with limbal stem cells. It was found that cell proliferation and differentiation was higher on scaffolds made of natural materials, and a greater percentage of less-differentiated cells were found on the synthetic scaffolds [201]. In contrast, no clear differences in either proliferation or differentiation between synthetic PET and naturally derived gelatin were observed in this study.

Maintenance of the stem cell phenotype was evaluated by qPCR of the stem cell markers *Lgr5* and *ABCG2*. As described in chapter 3, *ABCG2* is a transporter that actively effluxes Hoechst dye, and is responsible for the side population phenotype. It was therefore of interest whether cell culture on alternative materials and topographies could alter *ABCG2* gene expression, as this would suggest that the scaffolds play a role in maintenance of stem cell phenotype. Nanofibre scaffolds have previously been shown to support stem cell self-renewal and pluripotency. Human embryonic stem cells have been cultured on electrospun nanofibres made of synthetic polymers [202]. In this study, the scaffolds were able to support stem cell self-renewal. Additionally, the cells retained pluripotency and expression of stem cell markers *OCT3/4* and *Nanog*. Furthermore, when mouse embryonic stem cells were grown on polyamide nanofibres, enhanced proliferation and self-renewal was observed, as well as increased expression of the stem cell marker *Nanog* [203].

In the current study no change in *Lgr5* expression was found, however there was a small increase in *ABCG2* expression in SW480 cells after culture on gelatin nanofibers and in Caco2 cells after culture on PET microfibrils. Due to the small changes in one cell line each, this warrants further investigation. However, this is the first report of a study investigating the effect of electrospun scaffolds on stem cell phenotype in colon cells and thus represents an exciting initial step into this area of research. In order to explore this further, different epithelial cell types could be investigated. Furthermore, changes in expression of a range of stem cell marker genes such as *Musashi-1*, *CD133*, *β -catenin*, *Ascl2* and *Sox9* may be assessed, as well as differentiation markers such as villin, mucin, lysozyme and chromogranin. This would provide more information regarding the effect of the scaffolds on differentiation and stem cell phenotype.

4.4.1 Conclusions

It was hypothesised that electrospun scaffolds manufactured with different biomaterials and fibre diameters would have varying influences on cell behaviour. This was investigated by assessing proliferation, differentiation and expression of stem cell markers in colon epithelial cell lines on the different scaffold types.

Cells proliferated and exhibited epithelial morphology on all four of the tested electrospun scaffold types. Expression of the *Lgr5* gene was unchanged compared with tissue culture plastic after culture on the scaffolds, whereas there was an increase in *ABCG2* expression after culture on gelatin nanofibres and PET microfibrils. As nanofibres are more commonly used for epithelial cell culture,

gelatin nanofibres were chosen as the scaffold to take forward and test with primary cells from human colon. This will be described in chapter 6. Additionally, gelatin has the property of being able to readily form hydrogels. As hydrogels are another scaffold form of interest with regards to intestinal epithelial cell culture, the next chapter will focus on the abilities of gelatin hydrogel scaffolds to support and control cell proliferation and differentiation. A comparison between hydrogel and fibrous morphologies (whilst maintaining the same biochemical composition) and their influence on primary cell culture will be investigated.

Chapter 5: Biomimetic gelatin hydrogel scaffolds made by 3D printing and their compatibility with human colon carcinoma cells

5.1 Introduction

Hydrogels are three-dimensional networks made of hydrophilic polymers that can be crosslinked to form highly hydrated polymer matrices [204]. They offer advantages such as providing bulk and structural integrity and the facility to incorporate drugs and growth factors within the gel [204]. One of the challenges of hydrogels in tissue engineering is poor mechanical strength, limiting their use to soft or non-load bearing tissues [205]. However, within this limitation it is possible to tailor mechanical properties to match the tissue type, as well as alter degradation rate according to the application.

A variety of naturally derived and synthetic hydrogels have been studied for tissue engineering applications. Poly(ethylene oxide) (PEO) and poly(ethylene glycol) (PEG) are two of the most commonly used synthetic hydrogels for tissue engineering applications [206]. They can be modified with the addition of acrylates or methacrylates, mixed with a photoinitiator and crosslinked by UV exposure [207,208]. Thermally reversible hydrogels have been formed from block copolymers of PEG and PLGA [209]. This gel was used as a scaffold for delivery of stem cells to diabetic wounds in order to increase cell engraftment. More recently, synthetic peptide hydrogels have been developed to create fibrous scaffolds that can mimic extracellular matrix (ECM) [210]. These gels are being investigated for use with a range of cells, including dermal fibroblasts [211] and osteoblasts [212].

They have been assessed both for 3D cell encapsulation as well as 2D cell culture of chondrocytes [213]. It was found that the peptide hydrogels could support cell viability in 2D as well as in 3D culture, and collagen was deposited resulting in increased scaffold stiffness.

The most frequently used naturally derived hydrogels for tissue engineering applications are either ECM components or have macromolecular properties which are similar to native ECM [206]. Collagen is the most abundant constituent of ECM, and is commonly used in hydrogel scaffolds [214,215]. Gelatin is derived by hydrolysis of collagen, and has advantages over unprocessed collagen such as increased aqueous solubility and reduced potential for antigenic and immunogenic responses [183]. Crosslinked gelatin hydrogels have been studied for tissue engineering of various tissues and organs. For example, in corneal repair applications, gelatin has been used as a cell sheet carrier for the delivery of corneal endothelial cells [216] and as cellularised scaffolds with keratocytes for corneal stroma regeneration [193]. Additionally, gelatin hydrogels have been investigated for cardiac tissue engineering [217] and mixed with chitosan and hyaluronic acid for the development of artificial skin [218].

Rapid prototyping, also known as scaffold freeform fabrication, is a technology that uses a computer model to rapidly produce scaffolds with complex macrostructures [90]. The term incorporates a range of methods, including laser-based techniques such as stereolithography and printing methods which use powder beds and deposition of a binder [219]. Droplet deposition is a method of rapid prototyping in

which electrical signals are used to control the ejection of individual molten droplets that join to droplets of the previous layer and solidify. This method is suitable for fabrication of scaffolds with a relatively regular structure. Thermoresponsive hydrogels such as gelatin are an attractive ink for printing. This is due to their temperature dependent gelation ability, as well as attractive properties for printing such as viscosity and shear thinning [220].

To date, gelatin has primarily been used for printing either after methacrylation to create the photo-crosslinkable material GelMA [221] or in combination with other polymers such as hyaluronic acid [222]. For example, methacrylated gelatin has been mixed with methacrylated hyaluronic acid to create a gel-like fluid that could be printed and photo-crosslinked [223]

Several studies have reported the creation of scaffolds with crypt-villus architecture for the culture of intestinal epithelium. Sung et al created a villi-shaped scaffold made of the natural hydrogel collagen or the synthetic hydrogel PEG-DA [115]. Their fabrication method involved making several moulds of various materials and techniques. A serial mould fabrication technique has been used to manufacture a PLGA scaffold with the crypt-villus topography that is typical of small intestinal epithelium [113]. Additionally, non-biodegradable materials have been used to create *in vitro* models for studying intestinal disease and differentiation, rather than for regenerative medicine. For example, a PDMS microwell substrate was manufactured with several moulding steps [117].

All of these reports require many elaborate steps in the fabrication process. Therefore, there is a need for a rapid, one-step method for manufacturing this structure using a commonly used, safe and biocompatible material.

5.1.1 Aims and hypotheses

In the previous chapter, electrospun scaffolds made from the synthetic polymer PET and the naturally derived material gelatin were investigated for their ability to support colon epithelial cell growth. Although the differences were small, the conclusion of that chapter was that gelatin nanofibres were best suited for the culture of colon epithelium. In this chapter, gelatin will be investigated in the form of a hydrogel as an alternative option to electrospun fibres.

It was hypothesised that as a biocompatible material, crosslinked gelatin hydrogel scaffolds would be able to support epithelial cell proliferation and influence cell differentiation. Furthermore, it was hypothesised that unmodified gelatin hydrogel could be used as a printing ink for rapid prototyping of biomimetic scaffolds. Therefore, the aims of this chapter were to use gelatin to fabricate planar hydrogel scaffolds as well as printed scaffolds with added topography that mimic the crypt-villus structure of the intestinal epithelium, and to investigate their effects of cell growth and differentiation.

These aims were achieved with the following objectives:

- Cast planar crosslinked gelatin hydrogel scaffolds.
- Evaluate the ability of the gelatin hydrogel to support colon epithelial cell proliferation, differentiation to a typical epithelial morphology and maintenance of a stem cell phenotype.
- Fabricate a 3D scaffold made from gelatin hydrogel, and characterise it with regards to its mechanical properties
- Evaluate the ability of printed gelatin scaffolds to support colon epithelial cell proliferation, differentiation to a typical epithelial morphology.
- Assess if printed gelatin scaffolds support of a stem cell phenotype.

5.2 Experimental

5.2.1 Optimisation of crosslinking concentrations

It was previously reported that an NHS to EDC molar ratio of 0.2 was optimal for gelatin crosslinking [224]. In the previous chapter, electrospun gelatin was crosslinked with an NHS to EDC ratio of 0.4. Additionally, it was reported that increasing the ratio between EDC and carboxylic acid groups (COOH) in the gelatin leads to a greater degree of crosslinking [224]. In order to assess whether reducing the NHS:EDC ratio and increasing the EDC:COOH ratio resulted in more extensive crosslinking and cytocompatibility, alternative EDC and NHS concentrations were tested (Table 5.9). Apart from the change in EDC and NHS concentrations, crosslinking was conducted as described in chapter 2, section 2.2.4.

Table 5.9: EDC and NHS concentrations that were tested for crosslinking optimisation experiments

EDC concentration (mM)	NHS concentration (mM)	NHS:EDC ratio	EDC:COOH ratio
25	10	0.4	0.313
50	10	0.2	0.625
25	5	0.2	0.313

5.2.1.i Assessment of crosslinking efficiency

A TNBS assay was performed to determine to amount of free, un-crosslinked amino groups. This can then be used to calculate crosslinking efficiency, as described in detail in chapter 2, section 2.3.5. Additionally, swelling is related to crosslinking efficiency. Amount of swelling has been shown to be inversely proportional to degree of crosslinking [127]. A detailed method for swelling measurements can be found in chapter 2, section 2.3.6.

For both the TNBS and swelling assays, 10% (w/v) gelatin hydrogel was cast as described in chapter 2, section 2.2.2. For each assay, a biopsy cutter was used to cut three circular scaffolds with diameters of 8 mm.

5.2.1.ii Cell proliferation on differently crosslinked gelatin scaffolds

In order to assess whether different crosslinking concentrations had an effect of cell attachment and proliferation, SW480 or Caco2 cells were seeded on scaffolds and a Presto Blue assay was performed over the course of a week. For each cell line, four circles with diameters of 15 mm were cut from each differently crosslinked gelatin hydrogel. The gels were placed in 12-well non tissue culture treated plates and

sterilised by soaking in 10x antibiotic-antimycotic solution (100 µg/mL streptomycin and 250 ng/mL amphotericin B) for at least 3 hours at 4°C. SW480 or Caco2 cells were cultured at 37 °C in complete medium as described in chapter 2, section 2.4.1. They were grown to confluence in T75 flasks, then removed by trypsin digestion and counted. Cells (2×10^4) in 50 µL complete medium were added to each scaffold in triplicate with one blank without cells, and incubated at 37°C for 3 hours to allow cells to adhere before 1 mL complete media was added to each well.

Cell adhesion and proliferation was assessed using the Presto Blue assay. This was conducted 24 hours after cell seeding (day 1) and repeated on days 3 and 7. At each time-point, samples were incubated with 10% (v/v) Presto Blue reagent in complete media at 37°C for 40 minutes, before fluorescence was measured at excitation wavelength 560 nm and emission wavelength 590 nm. Details of the Presto Blue assay can be found in chapter 2, section 2.5.1.

5.2.2 Ability of colon carcinoma cells to proliferate and differentiate on gelatin hydrogels

Gelatin hydrogels crosslinked with 25 mM EDC and 10 mM NHS were cut into circular scaffolds with 15 mm diameters. They were sterilised by soaking in 10x antibiotic-antimycotic solution for at least 3 hours at 4°C. The colon carcinoma cell lines Caco2 or HT29 were grown to confluence in T75 flasks at 37 °C in complete medium. Cells (2×10^4) in 50 µL complete medium were added to each scaffold, and incubated at 37°C for 3 hours to allow cells to adhere before 1 mL complete media was added to each well. Cultures on the gelatin hydrogel and tissue culture plastic

control were compared by proliferation, SEM, immunocytochemistry and qPCR as shown in the schematic in Figure 5.2.1.

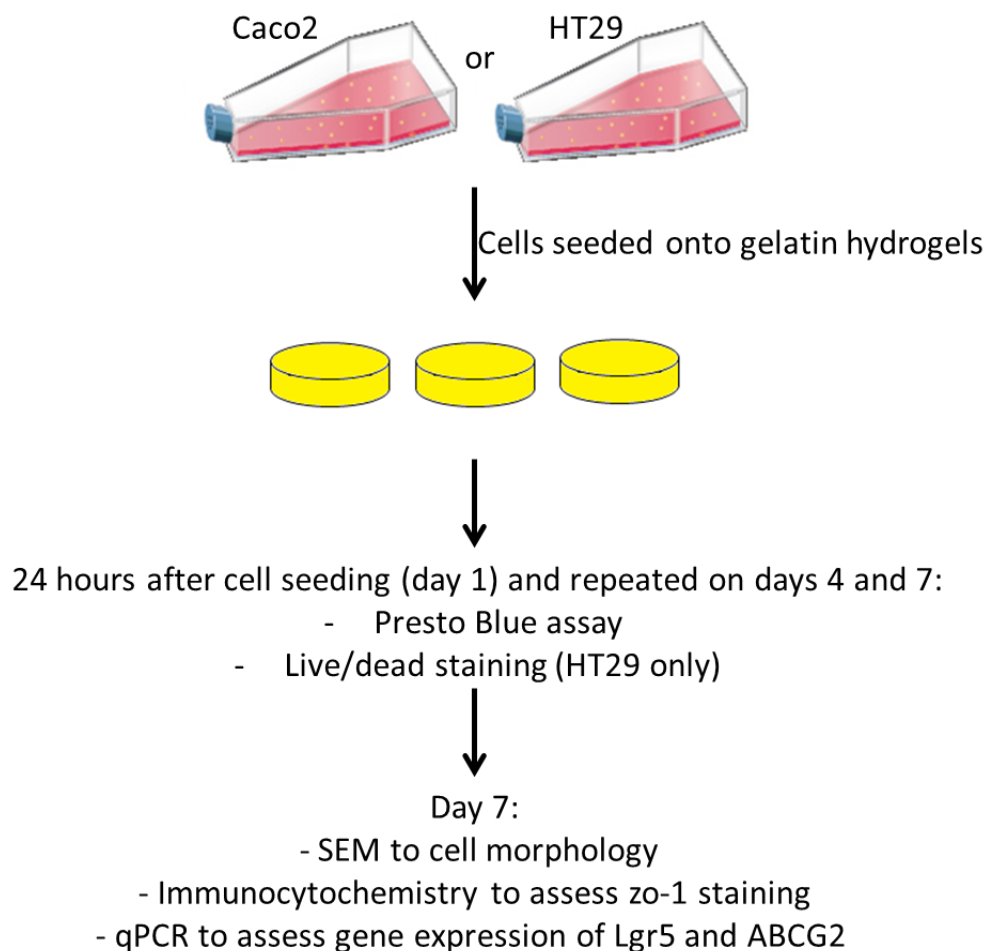


Figure 5.2.1: schematic showing experimental procedure for assessing ability of colon carcinoma cells to proliferate and differentiate on gelatin hydrogels

The purpose of this set of experiments was to assess colon epithelial cell proliferation, generation of epithelial cell phenotype and expression of stem cell markers on gelatin hydrogels

5.2.2.i Cell proliferation

Cell adhesion and proliferation was assessed using the Presto Blue assay and in the case of HT29 cells also by Live/Dead staining. Both assays were conducted 24 hours after cell seeding (day 1) and repeated on days 4 and 7. For details of the Presto Blue assay, see chapter 2, section 2.5.1, and details of the Live/Dead assay can be found in chapter 2, section 2.5.2.

5.2.2.ii SEM to assess cell morphology

Cellular morphology on the scaffolds was evaluated by SEM imaging. Scaffolds were fixed 7 days after cell seeding with 3% (w/v) glutaraldehyde then dehydrated with increasing concentrations of IMS. Details of the SEM method can be found in chapter 2, section 2.3.1.

5.2.2.iii Immunocytochemistry to assess tight junction formation

Immunocytochemical staining for the tight junction marker zonula occludens protein-1 (zo-1) was used to assess the ability of Caco2 and HT29 cells to differentiate and form an epithelial barrier. Scaffolds were fixed with 3.7% (w/v) paraformaldehyde 7 days after cell seeding. Cells were permeabilised, blocked to prevent non-specific binding and stained with anti-zo-1 antibody conjugated to Alexa Fluor 488. Samples were imaged by confocal microscopy. A detailed method can be found in chapter 2, section 2.6.

5.2.2.iv qPCR to assess gene expression of stem cell markers

Gene expression of the stem cell markers *Lgr5* and *ABCG2* was assessed after 7 days culture on the scaffolds. RNA was extracted, transcribed to cDNA and qPCR was conducted. All values were normalised to the housekeeping gene *GAPDH* and gene expression was reported in comparison to tissue culture plastic controls. Detailed methods can be found in chapter 2, section 2.1.

5.2.3 Manufacture and characterisation of printed gelatin scaffolds

5.2.3.i Imaging of printed villi

Printing and imaging of gelatin scaffolds was conducted by Hagit Gilon in the Additive Manufacturing and 3D Printing group at the University of Nottingham. Villi-shaped pillars were printed as described in chapter 2, section 2.2.3. Side on optical microscope images of the pillars were obtained by placing the glass slide with the scaffold perpendicular to the microscope stage. SEM images were obtained by freeze drying to dehydrate the scaffolds, and placing them on a tilted SEM stage.

5.2.3.ii Characterisation of printed villi

The gelatin in the scaffold base, and the gelatin in the printed villi were processed in different ways (i.e. base was set at room temperature whereas printed pillars were cooled very quickly on the cold stage). In order to investigate whether this difference had an impact on the mechanical properties, compression testing was carried out with gelatin that was prepared in each of these ways.

Gelatin was printed directly onto a glass slide on the cold stage. A 28x28 array was printed with a drop spacing of 300 μm , to generate a planar square of material. Circles with 8 mm diameters were cut from these printed squares to represent the printed pillars. To represent the base, circles were cut from gelatin hydrogel that was cast as before, as described in chapter 2, section 2.2.2. Compression testing was completed using a texture analyser to obtain Young's moduli. Details of this method can be found in chapter 2, section 2.3.4. For each scaffold type, 3 samples were tested.

5.2.4 Colon carcinoma cell proliferation and differentiation on printed gelatin scaffolds

Villi-shaped scaffolds were printed as described in chapter 2, section 2.2.3 and crosslinked with 25 mM EDC and 10 mM NHS. They were sterilised by soaking in 10x antibiotic-antimycotic solution for at least 3 hours at 4°C. The colon carcinoma cell lines Caco2 or HT29 were grown to confluence in T75 flasks at 37 °C in complete medium. Cells (2×10^4) in 50 μL complete medium were added to each scaffold, and incubated at 37°C for 3 hours to allow cells to adhere before 1 mL complete media was added to each well. Cultures on the gelatin hydrogel and tissue culture plastic control were compared by proliferation, SEM, immunocytochemistry and qPCR as shown in the schematic in Figure 5.2.2.

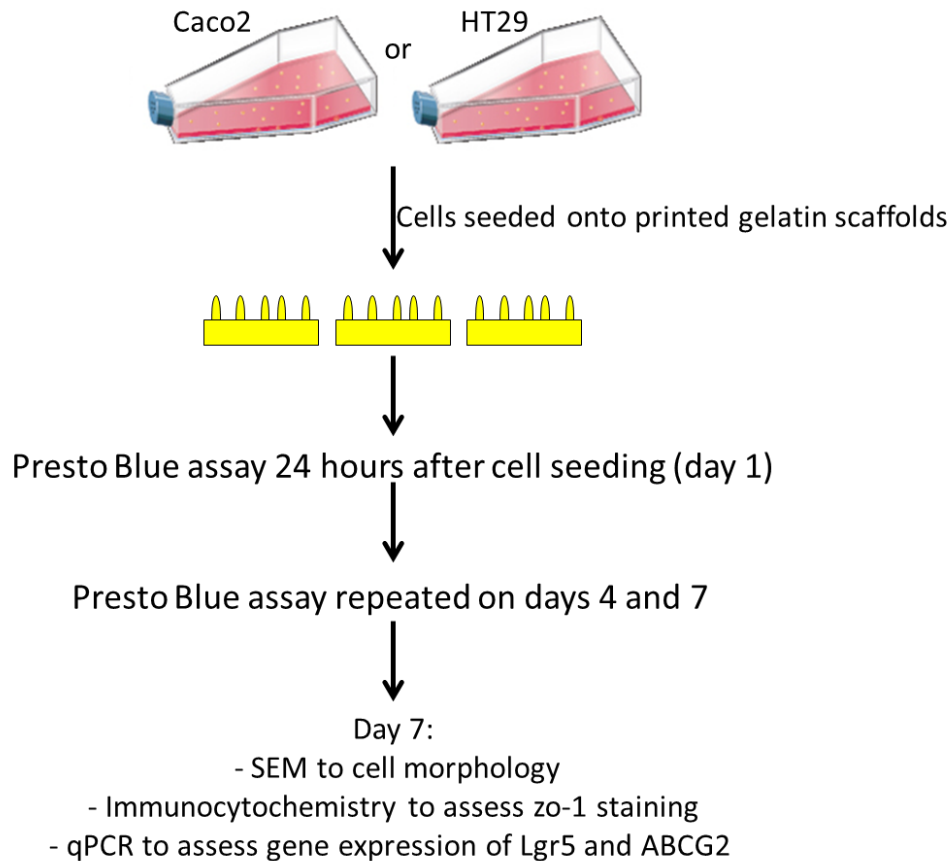


Figure 5.2.2: schematic showing experimental procedure for assessing ability of colon carcinoma cells to proliferate and differentiate on printed gelatin scaffolds

The aim of this set of experiments was to determine cell viability and behaviour on 3D printed gelatin scaffolds.

5.2.5 Statistical analysis

Data were expressed as mean $\pm$ standard deviation. GraphPad Prism 7 software was used to perform statistical analysis. One-way ANOVA was used to assess statistical differences, with the exception of Presto Blue data which was analysed with repeated measures ANOVA. Differences were deemed statistically significant if $p \leq 0.05$. Symbols of further levels of statistical significance are detailed in chapter 2, section 2.7.

5.3 Results

5.3.1 Different concentrations of EDC and NHS did not alter crosslinking efficiency

The aim of this set of experiments was to optimise crosslinking with EDC and NHS by altering the NHS:EDC and EDC:COOH ratios. Three different concentration combinations of EDC and NHS were investigated for their ability to crosslink gelatin and support cell proliferation (Table 5.9). Figure 5.3.1A shows the swelling ratio of each differently crosslinked gel, as an indirect measure of crosslinking. There were no differences in swelling between the gels, as they all had a swelling ratio of approximately 5, meaning that there was a 5-fold increase in mass between the freeze-dried and fully hydrated scaffold. Free amino groups were measured using a TNBS assay to directly measure crosslinking efficiency. Again, there were no differences between the gels (Figure 5.3.1B), as they were all found to be approximately 30% crosslinked. Proliferation of two different cell lines was also measured using the Presto Blue assay. SW480 cells were able to proliferate on all three gels over 7 days (Figure 5.3.1C), however the gels that were crosslinked with 50mM EDC and 10mM NHS led to significantly lower proliferation compared with tissue culture plastic control. Cell proliferation on the other two gels was not found to be different from tissue culture plastic. Caco2 cell proliferation plateaued on plastic by day 7 and cell number appeared to be lower on the gels crosslinked with 50 mM EDC / 10 mM NHS and 25 mM EDC / 5 mM NHS (Figure 5.3.1D). In contrast

the number of cells on the gels crosslinked with 25 mM EDC / 10 mM NHS continued to increase at day 7.

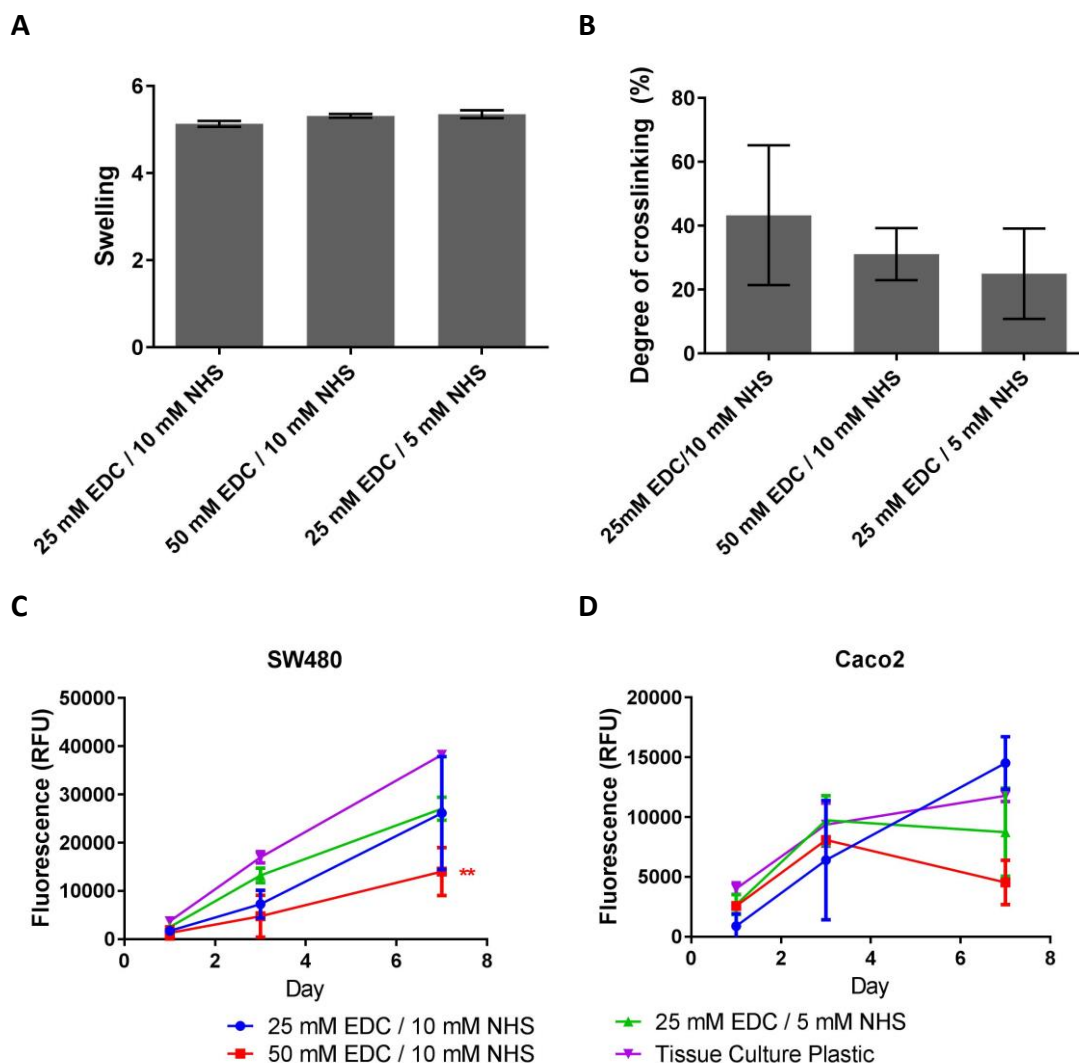


Figure 5.3.1: Crosslinking gelatin hydrogel with different EDC and NHS concentrations

After crosslinking with either 25 mM EDC/10 mM NHS 50 mM EDC/10 mM NHS, 25 mM EDC/5 mM NHS, swelling was measured (A) and degree of crosslinking was assessed with the TNBS assay (B); Proliferation of SW480 (C) and Caco2 cells (D) was determined using the Presto Blue assay. All results show mean $\pm$ SD (n=3). Statistical significance for swelling and degree of crosslinking was assessed using one-way ANOVA. Statistical significance compared to tissue culture plastic for cell proliferation was assessed using two-way ANOVA. ** $p \leq 0.01$, * $p \leq 0.05$

5.3.2 Cells are able to grow on crosslinked gelatin hydrogel

As it appeared that the gels crosslinked with 25 mM EDC and 10 mM NHS were best able to support cell proliferation, this was the concentration combination that was chosen for further experiments. Gels were crosslinked with 25 mM EDC and 10 mM NHS, and then Caco2 and HT29 cells were seeded on them to assess proliferation. Caco2 proliferation on the gel was found to be equal to tissue culture plastic (Figure 5.3.2A). HT29 proliferation when measured by Presto Blue was found to be significantly lower on the gel compared with plastic (Figure 5.3.2B). However, it is clear from live/dead staining that the cells are still viable and proliferating on the gel (Figure 5.3.2C).

Both Caco2 and HT29 cells were able to form adherent cell layers on top of the gels, as can be seen from scanning electron micrographs (Figure 5.3.3A-B). Zo-1 immunostaining was used to assess the level of tight junction formation and cell differentiation (Figure 5.3.3C-D). Caco2 cells displayed the typical 'chicken-wire' zo-1 expression very clearly. However, HT29 cells expressed very low amounts of zo-1 after 7 days of culture on the gels.

To investigate whether culture on these gels would influence the phenotype of the cell population, in particular the stem cell phenotype, after a week of culture on the gelatin, qPCR was carried out to assess expression of *Lgr5* and *ABCG2*. *Lgr5* expression was significantly increased in Caco2 cells, but remained the same in HT29 cells (Figure 5.3.4A). With regards to *ABCG2* expression, HT29 cells showed a significant increase in *ABCG2*, whereas a smaller increase was found in Caco2 cells

(Figure 5.3.4B). However, there was a large variation in *ABCG2* expression in both cell lines.

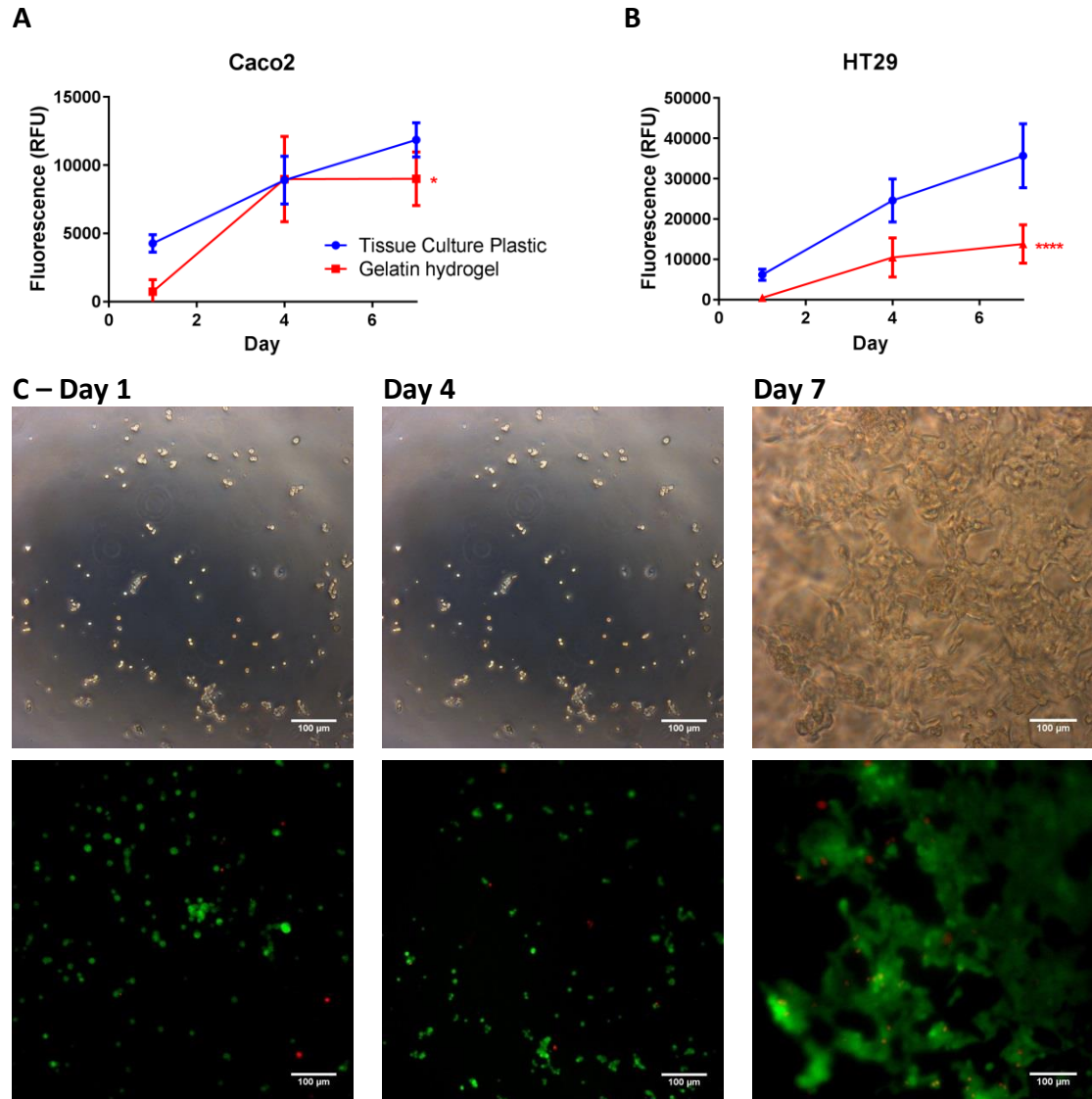


Figure 5.3.2: Cell proliferation on gelatin hydrogels

Caco2 and HT29 cells were seeded onto scaffolds and cultured for 7 days. Cell proliferation was quantified using Presto Blue assays (A-B). Live/dead stain was used to visualise HT29 cell growth. Representative live/dead images are shown (C), with brightfield images in the upper panel and fluorescent images in the lower panel. Live cells are in green and dead cells in red. Mean $\pm$ SD is shown ($n=6$, 3 replicates each in 2 separate experiments). Statistical significance compared to tissue culture plastic control was measured using 2-way ANOVA. * $p \leq 0.05$, *** $p \leq 0.001$. Scale bars = 100 μm .

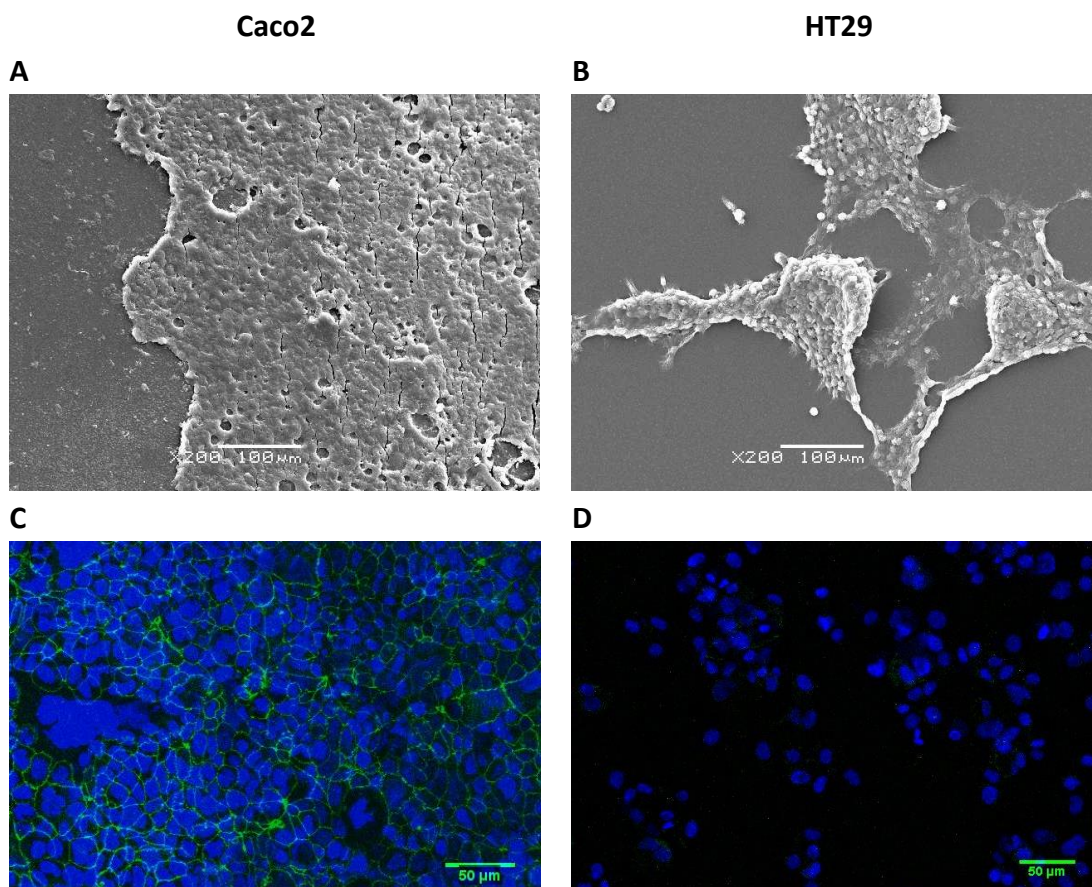
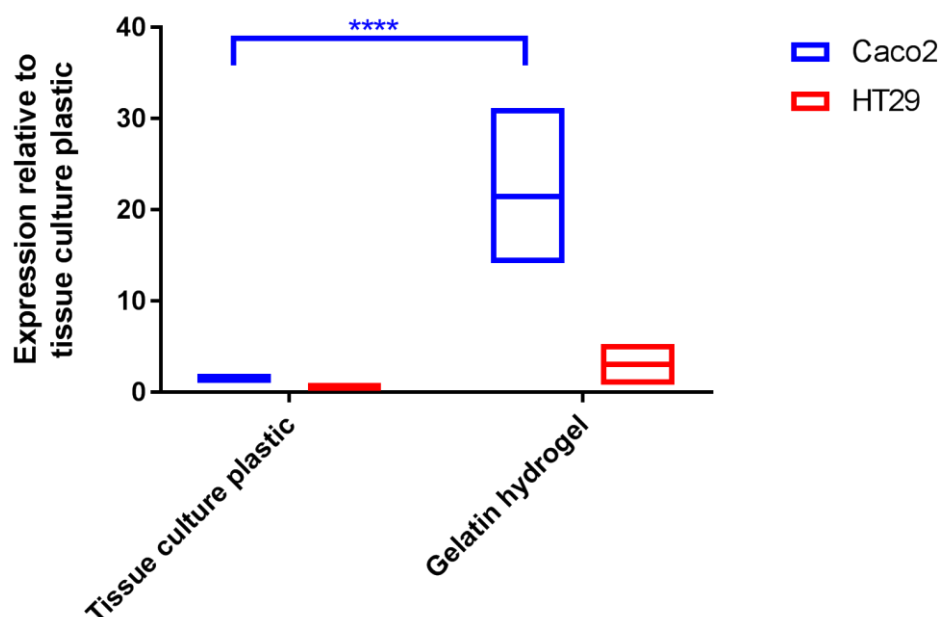


Figure 5.3.3: SEM and zo-1 immunocytochemistry images of cells on gelatin hydrogels

Caco2 and HT29 were grown on gelatin hydrogels for 7 days culture. Samples were fixed with glutaraldehyde and dehydrated with increasing concentrations of alcohol before imaging by SEM (A-B). For immunocytochemistry, samples were fixed, permeabilised and stained with anti zo-1 antibody conjugated to AlexaFluor 488. Zo-1 is in green, and Hoechst 33342 nuclear stain is in blue (C-D). Representative images of each sample are shown.

A – Lgr5



B – ABCG2

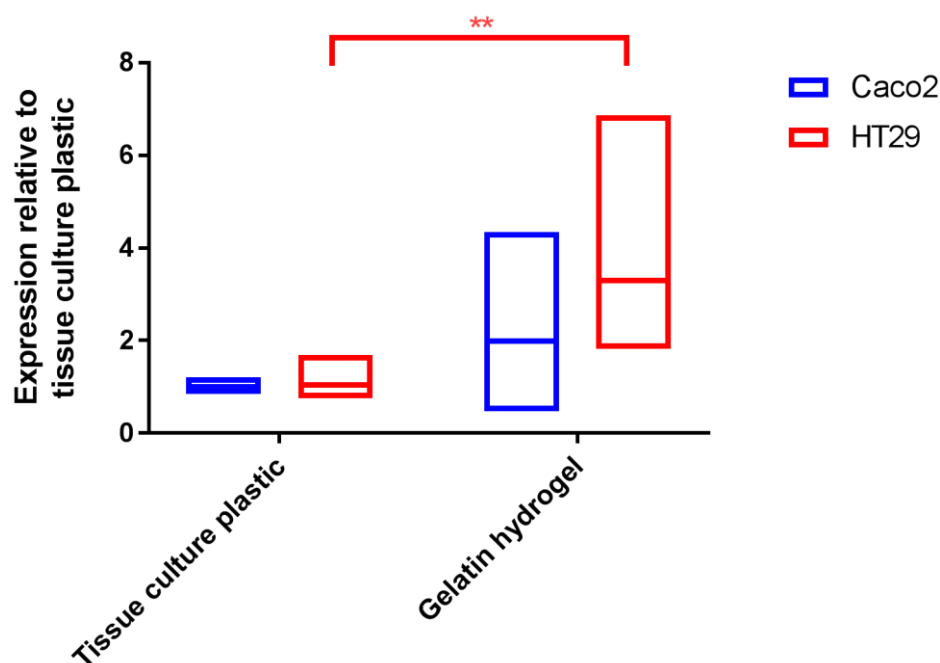


Figure 5.3.4: Expression of *Lgr5* and *ABCG2* after culture on gelatin hydrogel

The relative expression of *Lgr5* (A) and *ABCG2* (B) in colon epithelial cell lines after 7 days of culture on gelatin hydrogel compared with tissue culture plastic. Data represents mean, maximum and minimum expression relative to tissue culture plastic (n=6 from 2 separate experiments). Statistical significance compared to tissue culture plastic control was measured using a t-test. ** $p \leq 0.01$, *** $p \leq 0.001$

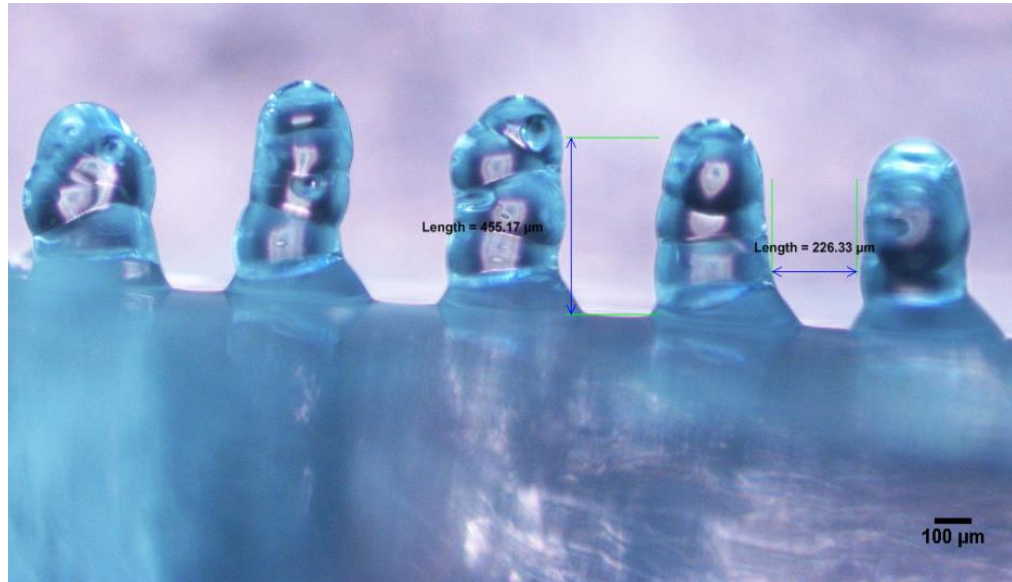
5.3.3. Gelatin ink can be used to print villi-like structures

Gelatin was used to print pillars that resemble villi. A representative side-on light microscope image (Figure 5.3.5A) shows that the pillars are approximately 400-500 μm high with spaces between villi of around 200 μm . Figure 5.3.5B shows a tilted SEM image on the pillars from above. From this image, it is apparent that the pillars are uniformly spaced and taper so that they are wider at the bottom and become narrower at the top. There are also many air bubbles present on the surface of the villi, which may be an artefact of the SEM preparation method.

The printed scaffolds are made up of two parts: the base which was cast in a mould and set at room temperature, and the printed villi that were chilled very quickly due to the printing process (Figure 5.3.6A). In order to investigate whether this difference in setting had any difference on the mechanical properties, printed planar gelatin scaffolds and gelatin that was poured in a petri dish at room temperature were used for compression testing. The differently set scaffolds were crosslinked in the same way, and compared with uncrosslinked gelatin. Figure 5.3.6B shows representative stress-strain graphs. Uncrosslinked gelatin has a non-linear curve in which elastic modulus increased with increasing stress, whereas crosslinked gelatin showed linear elastic behaviour. Young's moduli were calculated from the initial gradient of the stress-strain graphs. Both crosslinked gels had significantly lower Young's moduli compared with uncrosslinked gelatin (Figure 5.3.6C). Although there is no statistically significant difference between the

differently prepared crosslinked gels, there does appear to be a trend in which the printed gelatin has lower elastic modulus than the non-printed.

A



B

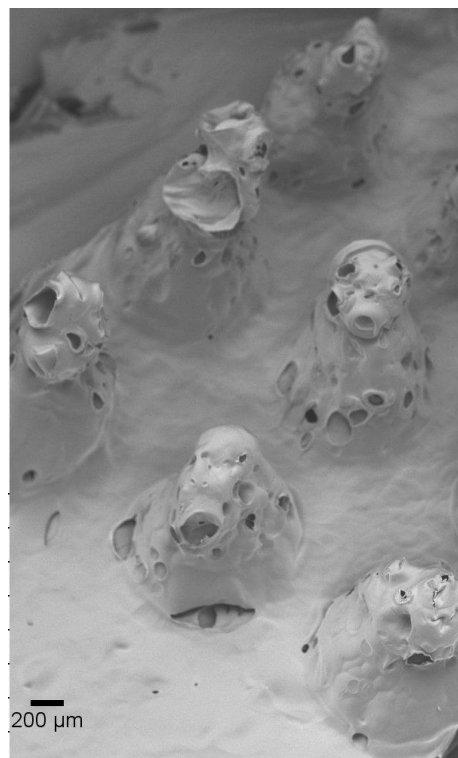


Figure 5.3.5: Gelatin printed villi

A representative light microscope image (A) and SEM image (B) of gelatin printed villi.

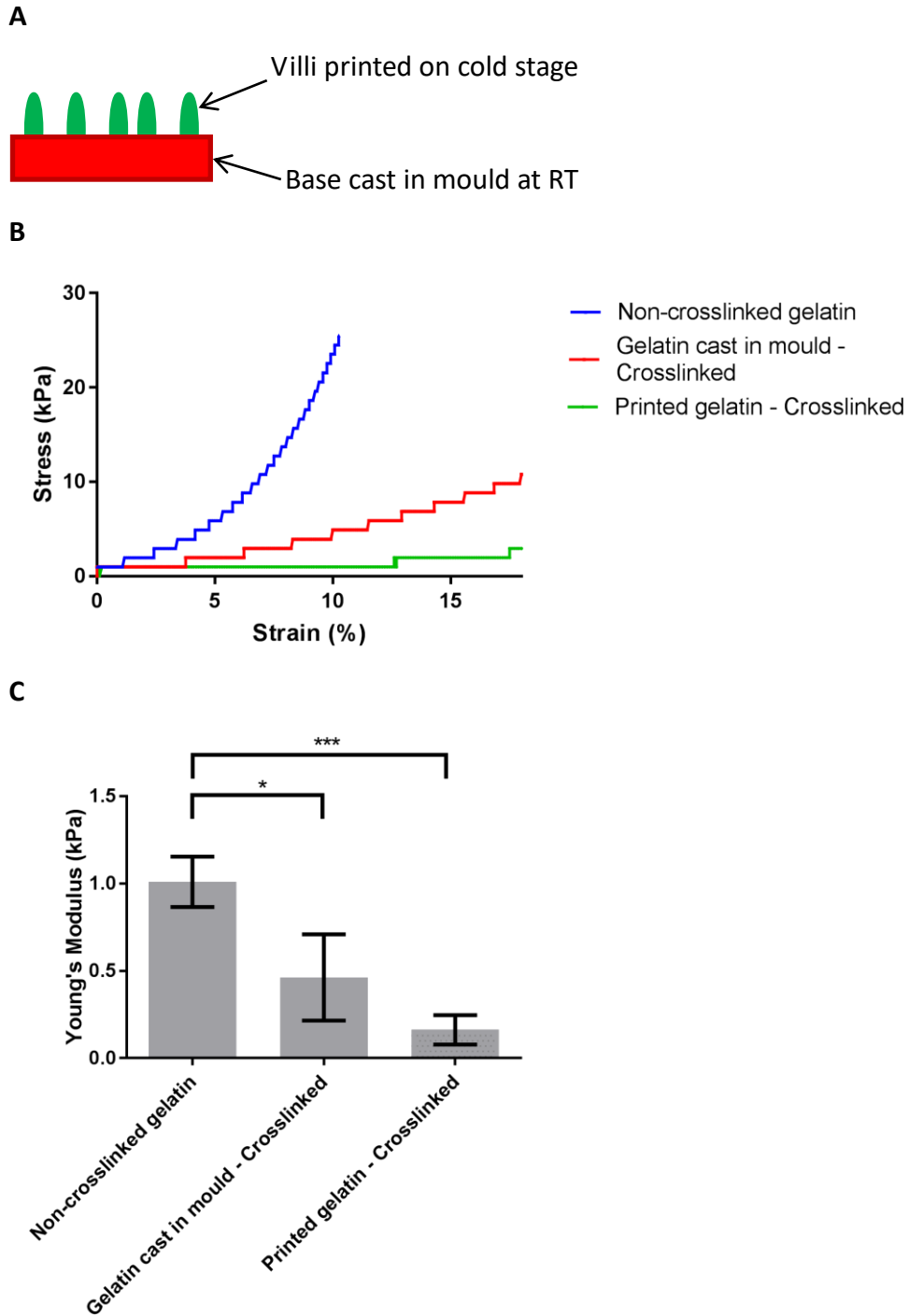


Figure 5.3.6: Mechanical properties of gelatin printed scaffolds

A schematic showing the different methods used to form base and villi parts of the scaffolds is shown in A. Compression testing using a texture analyser was performed on differently prepared gelatin. Representative stress-strain curves are shown in B. Young's modulus was calculated from slopes of stress-strain curves and shown in C. Data represent mean $\pm$ SD ($n = 3$) Statistical significance was assessed using one-way ANOVA, * $p \leq 0.05$, *** $p \leq 0.001$

5.3.4 Printed topography does not negatively impact cell growth and behaviour

To investigate the effect of printed topography on cell growth, Caco2 and HT29 cells were seeded onto the printed scaffolds. The results were very similar to the non-printed gelatin described earlier. Caco2 cell proliferation on printed scaffolds was equivalent to tissue culture plastic (Figure 5.3.7A). HT29 cell growth was slower than on tissue culture plastic, but the cells did still proliferate over the week (Figure 5.3.7B).

After a week on the scaffolds, the cells were fixed and either dehydrated and imaged by SEM or stained for zo-1 and imaged by confocal microscopy. SEM images show that the pillars have collapsed and crumpled rather than staying upright as they were prior to cell culture (Figure 5.3.8A-B). It is possible that this is artefact of the SEM preparation method in which the samples were dehydrated and imaged under vacuum. It appears that Caco2 cells have migrated partway up the villi, but not to the very top. HT29 cells are very sparse and are located primarily on the bottom of the scaffold. Confocal images support these observations (Figure 5.3.8C-D). Similar to the findings on non-printed gelatin scaffolds, HT29 cells do not express zo-1, whereas Caco2 cells have clear, strong zo-1 expression.

Expression of stem cell markers after a week on the printed scaffolds was assessed with qPCR in comparison to tissue culture plastic. *Lgr5* expression was significantly increased in both Caco2 and HT29 cells (Figure 5.3.9A). Compared to tissue culture plastic, there was a 20-fold increase in *Lgr5* expression in Caco2 cells. This is similar

to the *Lgr5* expression data of Caco2 cells on planar gelatin hydrogel. However, in HT29 cells there was a large variation between the 2 repeat experiments. In one experiment there was a greater than 80-fold increase, whereas in the other there was no change. This pattern was also found with *ABCG2* expression, with one experiment finding a 6-fold increase whereas no change was found in the other. Overall, *ABCG2* expression was significantly increased in HT29 cells on the printed scaffolds, whereas its expression was unchanged in Caco2 cells (Figure 5.3.9B).

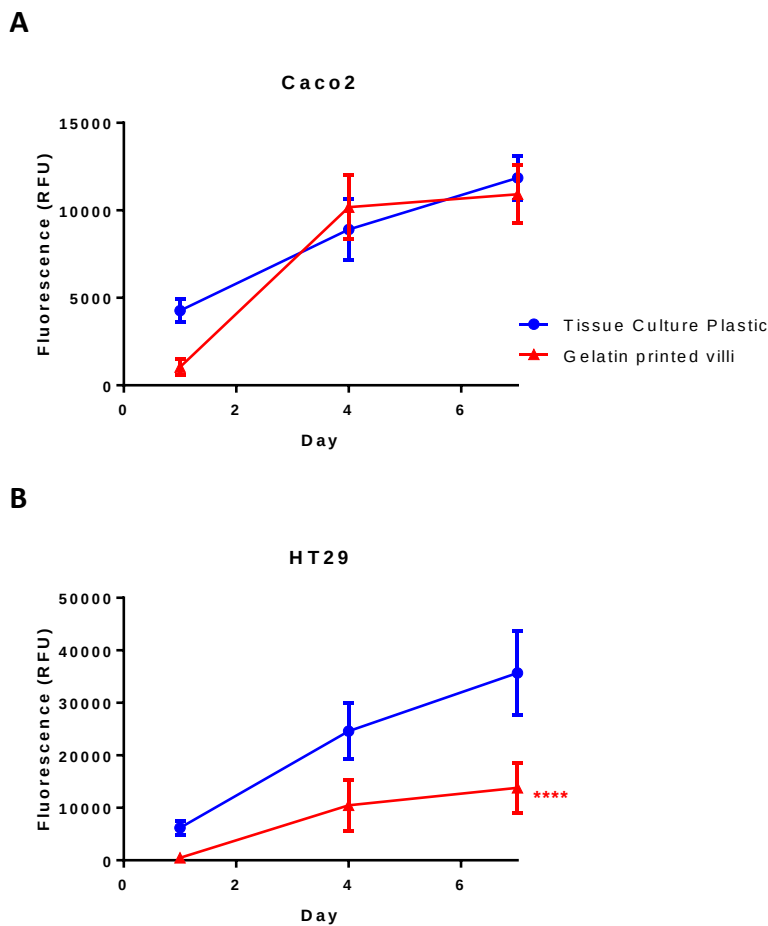


Figure 5.3.7: Cell proliferation on gelatin printed villi scaffolds

Caco2 (A) and HT29 cells (B) were seeded onto printed scaffolds and cultured for 7 days. Cell proliferation was quantified using Presto Blue assay. Mean $\pm$ SD is shown (n=6, 3 replicates each in 2 separate experiments). Statistical significance compared to tissue culture plastic control was measured using 2-way ANOVA. *** $p \leq 0.001$

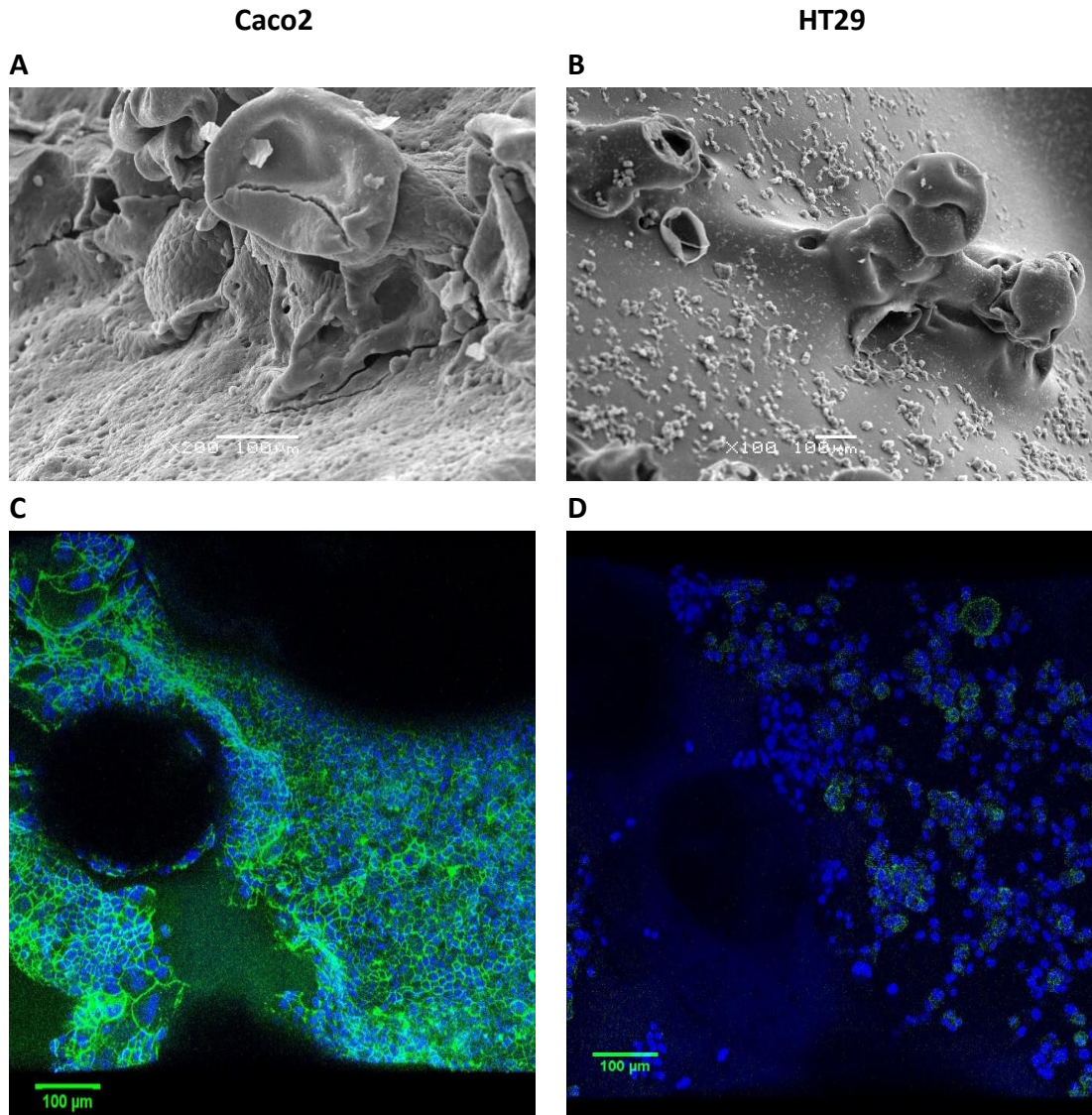
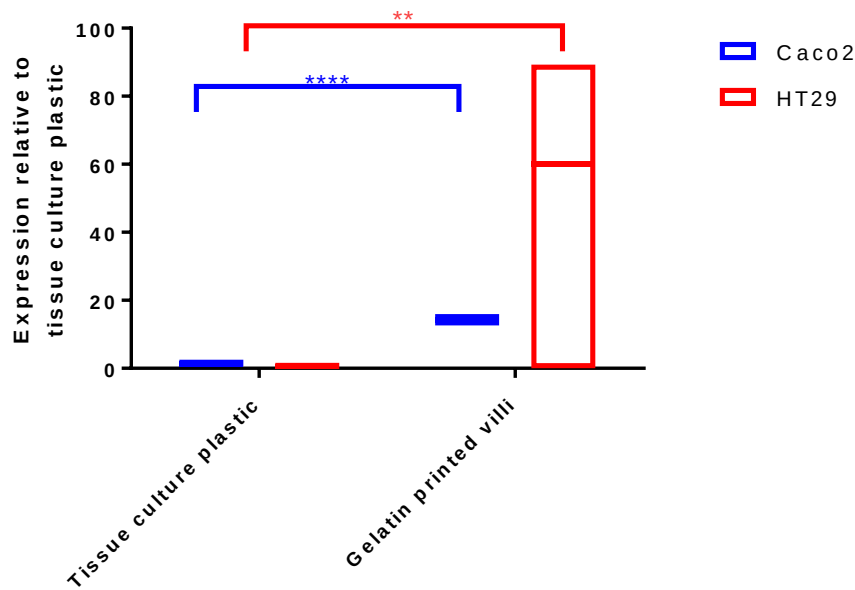


Figure 5.3.8: SEM and immunocytochemistry images of cells on gelatin printed villi

Caco2 and HT29 were grown on gelatin printed villi for 7 days culture. Samples were fixed with glutaraldehyde and dehydrated with increasing concentrations of alcohol before imaging by SEM (A-B). For immunocytochemistry, samples were fixed, permeabilised and stained with anti zo-1 antibody conjugated to AlexaFluor 488. Zo-1 is in green, and Hoechst 33342 nuclear stain is in blue (C-D). Representative images of each sample are shown.

A – Lgr5



B – ABCG2

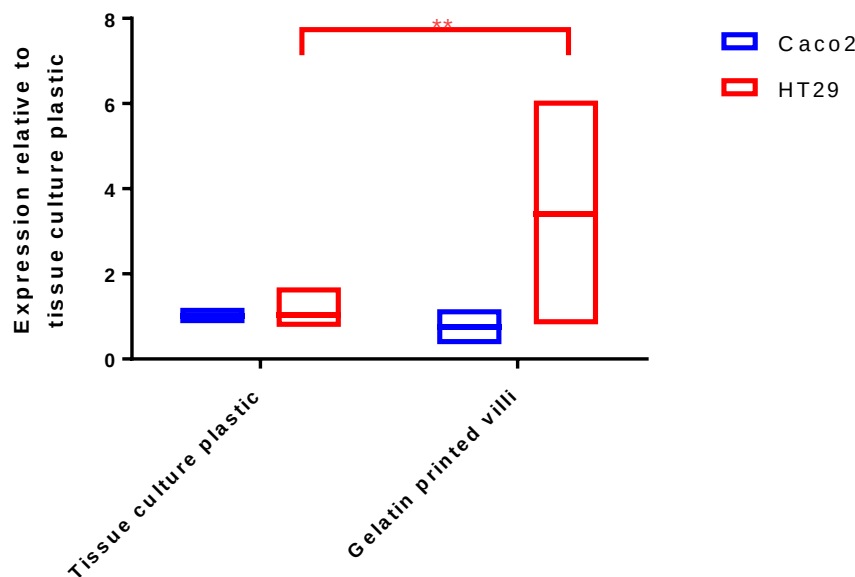


Figure 5.3.9: Expression of *Lgr5* and *ABCG2* after culture on gelatin printed villi

The relative expression of *Lgr5* (A) and *ABCG2* (B) in colon epithelial cell lines after 7 days of culture on gelatin printed villi compared with tissue culture plastic. Data represents mean, maximum and minimum expression relative to tissue culture plastic (n=6 from 2 separate experiments). Statistical significance compared to tissue culture plastic control was measured using a t-test. ** $p \leq 0.01$, **** $p \leq 0.0001$

5.3.5 Biomimetic crypt-villus structures can be printed

In order to assess the capabilities of the printing method, a crypt-villi structure was printed. Crypts with diameter of approximately 300 μm were formed and villi-like pillars were printed above the crypts (Figure 5.3.10). Although further optimisation is required to create dimensions that more closely resemble native epithelium, this demonstrates the potential of the printed method to fabricate biomimetic structures.

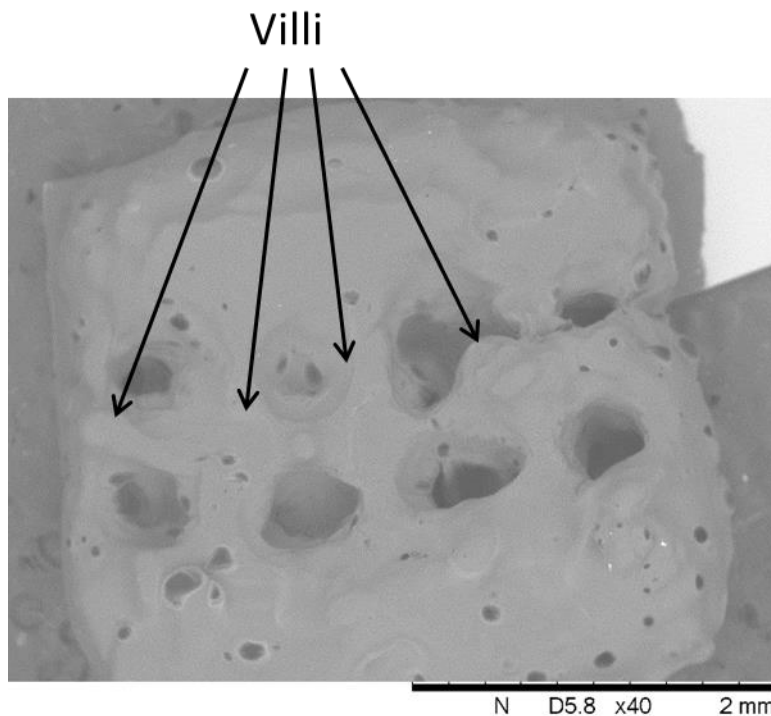


Figure 5.3.10: Gelatin printed crypts and villi

Scaffolds with crypt-villus topography were printed using gelatin ink. A representative SEM image is shown

5.4 Discussion

The aims of this chapter were to use gelatin to fabricate simple, planar hydrogel scaffolds as well as printed scaffolds with added topography and to investigate their effects of cell growth and differentiation.

Due to the high aqueous solubility of gelatin, it must be crosslinked prior to use in cell culture. The crosslinking of gelatin hydrogels with EDC and NHS has been previously optimised with an NHS to EDC molar ratio of 0.2 [224]. At higher ratios the reaction was found to be less efficient as NHS reacts with EDC, leading to less EDC being available for the crosslinking reaction. At lower ratios and in the absence of NHS, the reaction is less efficient as the EDC-activating carboxylic acid undergoes the hydrolytic side reaction more easily (see crosslinking reaction scheme in chapter 2, Figure 2.2.3). In the previous chapter, electrospun gelatin scaffolds were crosslinked with 25 mM EDC and 10 mM NHS as this had been reported previously [225], and was found to be sufficient to prevent dissolution in media. According to work by Kuijpers et al, this NHS to EDC ratio of 0.4 is too high for optimal crosslinking. Therefore, alternative concentrations with EDC:NHS ratios of 0.2 were tested.

The second ratio that has been reported to be important in crosslinking efficiency is the ratio of EDC to carboxylic acid groups in the gelatin. Kuijpers et al (2000) found that the higher the EDC:COOH ratio, the better the crosslinking efficiency. This was also observed by Coimbra and colleagues [226]. Therefore, as well as reducing the NHS:EDC ratio to 0.2, the EDC:COOH ratio was also doubled from 0.3 to 0.6.

Coimbra et al found that crosslinking efficiency increased from 20% to 40% when the EDC:COOH ratio was increased from 0.25 to 0.5. In contrast to these previous reports, it was found in the present study that there was no difference in crosslinking efficiency with the different EDC and NHS concentrations. A possible explanation for this is the thickness of the gels that were made. In order to make handling of the gels possible during cell culture experiments, the scaffolds were approximately 2 mm thick. As a result it is likely that many of the amino groups were deep within the gel and not available to the crosslinking reagents, as has been shown before [227].

When the differently crosslinked gels were tested with cells, the higher EDC:COOH ratio led to decreased cell proliferation. This could potentially be due to toxicity from the chemical residues if scaffolds were not washed well enough prior to cell seeding. As it appeared that the gels crosslinked with 25 mM EDC and 10 mM NHS could be handled easily and were able to support cell proliferation, this was the concentration combination that was chosen for further experiments.

Gelatin hydrogels have been previously shown to support adhesion and delivery of corneal endothelial cells for corneal regeneration [228,229]. It was found that the biocompatibility and biodegradation of gelatin made it a suitable material for this application. Additionally, EDC-crosslinked gelatin hydrogel membranes have been used to culture retinal pigment epithelial cells [230]. The cells were able to grow well and exhibited epithelial-like morphology. Interestingly, the cells were not able to grow on glutaraldehyde-crosslinked gelatin, indicating that cytotoxic

glutaraldehyde remained in the gels. The results from this chapter support the findings of these studies that gelatin hydrogel crosslinked with EDC is a suitable scaffold for the culture of epithelial cells.

As mentioned in chapter 4, scaffold material composition and form can have important roles in control of stem cell phenotype and determining cell behaviour [72,197–199]. For example, it has been found that the proportion of MCF7 cells with a stem cell phenotype is significantly increased following culture in collagen scaffolds [231]. The report indicated that there was a larger population of cancer stem cells following culture in the collagen scaffolds.

In this chapter, it was found that *Lgr5* expression increased in Caco2 cells after culture on both planar and printed gelatin hydrogels. Additionally, the cells were able to differentiate and form tight junctions. Conversely, HT29 cells did not form tight junctions and expression of *Lgr5* and *ABCG2* increased after culture on the gelatin substrates. This may be explained by the slower proliferation of HT29 cells on the gels. In future experiments, HT29 cells could be cultured for longer in order to reach confluence before expression of zo-1 and stem cell markers is evaluated.

The importance of using biomimetic matrices for obtaining appropriate differentiation has been highlighted in a previous study in which stem cells were seeded onto polyacrylamide gels coated with fibronectin or collagen [198]. It was shown that stem cells differentiated towards different cell types dependent on substrate stiffness and biochemical composition.

Matrigel is a hydrogel that is used for *in vitro* culture of intestinal crypts for the formation of organoids. The Young's modulus of Matrigel has been reported to be around 0.4 – 0.6 kPa [232,233], which is similar to both crosslinked gelatin measurements in this chapter (approx. 0.2 – 0.5 kPa). As this stiffness has been shown to support growth primary intestinal epithelial cells, it was hypothesised that the crosslinked gelatin could also be suitable for this purpose.

To manufacture scaffolds with printed topography, gelatin was printed onto a base made of gelatin that had been cast in a mould and set at room temperature. In contrast, the printed droplets were chilled immediately on the cold stage. It has been reported previously that properties of gelatin hydrogels are dependent on their drying conditions [226]. At low temperatures, gelatin chains re-form collagen-like triple helix structures at low temperatures forming crystalline structures. Whereas at room temperature, gelatin macromolecules retain their random coil formation and form semi-crystalline structures [234]. Mechanical properties of crosslinked gelatin that had been cast and set at room temperature or printed on a cold stage were assessed in order to investigate effects of the different preparation methods. The printed gelatin appeared to have a lower Young's modulus than the gelatin that was cast at room temperature. This could be due to the crystalline nature of the structure leading to a lower elasticity. To test this hypothesis in future work, differential scanning calorimetry could be used to measure percentage crystallinity following different solidification temperatures [235].

In a previous study, villi-shaped scaffolds were manufactured using a combination of various materials and techniques [115]. A plastic mould fabricated by laser ablation was used to create a reverse mould made of PDMS. This was then used to create a sacrificial alginate mould which was used to make the final villi-shaped scaffold made of either PEG or collagen. In another study, crypt-shaped scaffolds were manufactured by first fabricating a mould using the photoresist SU-8 [114]. Liquid PDMS was then poured onto this mould to create a crypt-like pattern. The printing method used in this chapter does not require the use of moulds or different materials. As both crypts and villi can be printed in a single step, it is faster and cheaper alternative to previously reported methods.

Gelatin is a biodegradable and biocompatible material suitable for clinical application. To date, nozzle-based rapid fabrication of scaffolds by 3D printing of gelatin alone has been reported only once. In this case, hepatocytes were encapsulated in gelatin which was crosslinked with glutaraldehyde after scaffold formation [222]. The glutaraldehyde did not appear to have any cytotoxic effects in that report, although toxicity has been reported in other studies [230]. Gelatin is most commonly used together with other materials such as hyaluronic acid [236], alginate, fibrinogen and chitosan [219,237]. It has been demonstrated in this chapter that the method can be used to print biomimetic crypt-villus structures in a simple, one-step method using unmodified gelatin.

5.4.1 Conclusions

The hypotheses of this chapter were that crosslinked gelatin hydrogel scaffolds would be able to support cell proliferation and influence cell differentiation, and that gelatin hydrogel could be used as a 3D printing ink for rapid prototyping of biomimetic scaffolds. The overall conclusions of the chapter are that gelatin hydrogel is a suitable substrate for cell proliferation and differentiation. Both planar and printed gelatin substrates were able to support tight junction formation in Caco2 cells. Furthermore, expression of stem cell marker *Lgr5* was enhanced after culture on the gelatin scaffolds, indicating that gelatin hydrogels are able to support maintenance of a stem cell phenotype. Additionally, gelatin can be used to rapidly print biomimetic topographies using a drop-on-demand jetting method. The addition of the printed topography did not have any negative effects on cell growth and behaviour. This is the first report of an investigation into colon epithelial cell culture differentiation and stem cell phenotype following culture on gelatin hydrogels. Additionally, the method for manufacturing crypt-villus architecture using gelatin with a one-step printing process is a novel outcome of this chapter. In the next chapter, gelatin hydrogel will be tested alongside gelatin nanofibres from the previous chapter to establish whether these substrates can be used to culture primary colon crypt cells.

Chapter 6: *In vitro* culture of primary human colon crypt cells

6.1 Introduction

The overall aim of this project is the development of a clinically applicable graft using primary colon epithelial cells that can be isolated from a patient biopsy and expanded *in vitro*.

As well as tissue specific progenitor cells, embryonic and induced pluripotent stem cells are alternative cell sources for regenerative therapies. Embryonic stem cells are derived from the inner cell mass of a blastocyst stage embryo. They can be expanded *in vitro*, and upon exposure to specific signals can differentiate to generate cells of any type [238,239]. However, their use is limited by ethical concerns, and by their allogenic origin which restricts their clinical application [240]. Induced pluripotent stem (iPS) cells are generated by de-differentiation of somatic cells by transduction of the transcription factors Oct3/4, Sox2, Klf4, and c-Myc [241,242]. Like embryonic stem cells, they can differentiate to form other cell types. However, iPS cells can overcome the ethical objections and clinical limitations of allogenic embryonic cells [243]. Intestinal organoids have been generated by the directed differentiation of pluripotent stem cells [244]. However, this is not a straightforward process and there is a risk that if pluripotent cells are not fully differentiated, they can cause teratomas [240]. Tissue specific adult stem cells have a restricted differentiation potential, and can usually only generate cells of the tissue from which they were isolated [245]. Invasive procedures may be required to obtain adult stem cells, and their widespread use is limited by challenges associated

with maintaining and expanding long term cultures [243]. However, they remain an attractive option as directed differentiation is not required.

Adult stem cells in intestinal epithelium reside at the bottoms of invaginations known as crypts of Lieberkühn [4]. These cells replicate and differentiate to generate cells of different types. As they differentiate, absorptive, goblet and enteroendocrine cells migrate upwards towards the lumen where they are eventually shed [4].

A non-enzymatic method of isolating individual crypts from colonic mucosa has been well-established [246]. The method uses the chelating agent ethylenediamine-tetraacetic acid (EDTA) which binds to calcium and causes separation of the epithelium from the underlying lamina propria. Dithiothreitol (DTT) is also included in the digestion mixture to remove mucus released by goblet cells in the epithelium. With this method, it is possible to yield intact crypts without fibroblasts or other contaminating cells [246]. Using this isolation method, short-term cultures of epithelial crypts have been maintained. Grossman et al seeded crypts onto tissue culture plates coated with collagen and used media supplemented with glial derived neurotrophic factor to keep cells viable for up to 72 hours [247].

Dame and colleagues reported a method for keeping crypts intact for up to five days [128]. This culture method involved embedding the crypts in Matrigel and overlaying with media containing epidermal growth factor (EGF), insulin, transferrin, hydrocortisone, epinephrine and bovine pituitary extract. When this method was used, crypt-like architecture was maintained with distinct base and apex.

Long term culture of human colon epithelium in the form of organoids was reported in 2011 [40]. When crypts suspended in Matrigel were overlaid with a media containing EGF, R-Spondin, Noggin, Wnt3a and nicotinamide, organoids could be cultured for at least one month.

Colon organoids have shown potential for mucosal regeneration when transplanted to damaged epithelium [59,58]. These studies found that Matrigel was required during transplantation in order to increase engraftment efficiency. However Matrigel is unsuitable for clinical use due to its batch-to-batch variability, undefined composition and patient incompatibility. In order to overcome this issue, Peng et al expanded organoids in collagen gel, then transferred to a collagen foam for delivery in the form of an enema [57].

There have been some previous reports of primary intestinal epithelial cell culture on biomimetic scaffolds. A serial mould fabrication technique has been used to manufacture a PLGA scaffold with the crypt-villus topography that is typical of intestinal epithelium [113]. The scaffolds were coated with Matrigel and seeded with murine intestinal progenitor cells. The cells were found to proliferate and differentiate on the scaffold, covering the entire villus structures. However the cells grew in enteroid structures along the villi, rather than a monolayer that is open to the lumen. In another study, a PDMS substrate with crypt-like topography was manufactured [117]. Primary murine colon crypts were suspended in Matrigel and placed inside the microwells. A combination of 3D and 2D culture resulted in the formation of organoids containing stem cells within the Matrigel at the bottom of the microwells, and a differentiated monolayer above the microwells in the absence

of Matrigel. With this method, they were able to replicate the polarised nature of the colon epithelium. However, as already mentioned, the inclusion of Matrigel in these studies limits their clinical applicability. Additionally, organoids do not fully reproduce the structure and function of native tissue, and may not lead to full integration into the host [1]. Therefore in order to make a regenerative graft it would be useful to be able to culture primary colon epithelial cells on biomimetic scaffolds without Matrigel.

6.1.1 Aims and hypotheses

In chapters 4 and 5, scaffolds were manufactured and colon carcinoma cell lines were used as a model for investigating scaffold effects on cells. The long term aim of this project is to use the scaffolds to create a regenerative tissue graft, which should be done with non-cancerous primary cells. It would also be ideal to use autologous cells taken from the patient to prevent immune reactions.

In chapter 3, the side population method for identifying and isolating stem cells was investigated. This was done with the aim of isolating a stem cell-enriched population from primary colon crypt cells in order to increase efficiency of primary cell culture. However, it was concluded that the side population method was too variable and unreliable and is therefore unsuitable for use with primary colon epithelial cells. Therefore in this chapter, the entire heterogeneous population of colon crypt cells was used.

It was hypothesised that primary human colon cells could be cultured on scaffolds that mimic the basement membrane to form a monolayer similar to that found in

native colon epithelium. The first aim of this chapter was to reproduce the published method for isolation of epithelial crypts from human colon mucosa and their *in vitro* culture to generate organoids [40]. The second aim was to investigate whether primary cells could grow on gelatin electrospun and hydrogel scaffolds described in the previous two chapters to form monolayers that resemble native colon epithelium.

These aims were achieved with the following objectives:

- Isolate epithelial crypts from human colon and culture them using the published organoid culture method in order to expand cell number
- Seed organoid cells on gelatin nanofibre and hydrogel scaffolds, and assess cell attachment and proliferation.

6.2 Experimental

6.2.1 Crypt isolation and culture with DMEM + 20% FBS, KGMG or Sato organoid media

Crypts were isolated from normal colonic mucosal tissue from surgical resection specimens and suspended in Matrigel and plated at a concentration of 50 crypts per 25 μ L, as described in chapter 2, section 2.4.2. After the Matrigel was solidified by incubating at 37°C for 15 minutes, it was overlaid with 250 μ L of either Dulbecco's modified eagle medium (DMEM) supplemented with 20% (v/v) foetal bovine serum (FBS) or Keratinocyte Growth Medium Gold (KGMG, Lonza).

6.2.1.i Keratinocyte Growth Medium Gold (KGMG)

KGMG was used by Dame et al [128] to maintain normal colonic crypts in culture for 3-5 days. It consists of a Keratinocyte Basal Medium Gold to which the following supplements were added from the KGMG Bullet Kit (Lonza): epidermal growth factor (EGF), insulin, transferrin, hydrocortisone, epinephrine, bovine pituitary extract.

6.2.1.ii Sato organoid media

A long-term culture method for normal human colon epithelium was described in 2011 [40]. When colon crypts in Matrigel were overlaid with media containing a combination of growth factors, self-renewing organoids were formed, which contain all differentiated cell types. In an attempt to replicate this method, organoid media was made which contained all the essential growth factors as described in the original article. Sato organoid media was made as detailed in Table 6.10. Media was changed every 2 days.

6.2.1.iii Cell viability

Viability of cells suspended within Matrigel was assessed using the CellTox Green cytotoxicity assay and CellTiter Glo 3D assay in multiplex. CellTiter Glo 3D is an endpoint assay therefore different samples are required to assess cell viability at different time-points. However it was chosen for use with cells embedded in Matrigel as the CellTiter Glo 3D reagent appeared to diffuse through the gel more effectively than Presto Blue reagent (see data in Appendix C). Both assays were

carried out on the same samples immediately after each other, so that data of cell toxicity as well as viability could be obtained.

For each timepoint, two wells containing cells, and one blank were plated. CellTox Green assay was performed as described in chapter 2, section 2.5.3. Immediately after this, the CellTiter Glo 3D assay was conducted as described in chapter 2, section 2.5.4. Blank measurements were deducted from sample measurements to account for background fluorescence and luminescence.

Table 6.10: Components of Sato organoid media as described by Sato et al [40]

<u>Reagent</u>	<u>Supplier</u>	<u>Solvent</u>	<u>Concentration</u>
Advanced DMEM/F12	Invitrogen	N/A	1x
Penicillin/streptomycin	Invitrogen	N/A	100 U/mL
HEPES	Invitrogen	N/A	10 mM
Glutamax	Invitrogen	N/A	2 mM
N2 supplement	Invitrogen	N/A	1x
B27 supplement	Invitrogen	N/A	1x
N-acetylcysteine	Sigma-Aldrich	dH ₂ O	1 mM
Nicotinamide	Sigma-Aldrich	dH ₂ O	10 mM
Human recombinant EGF	Invitrogen	1% BSA/PBS	50 ng/mL
Human recombinant R-Spondin	Peptotech	1% BSA/PBS	1 µg/mL
Human recombinant Noggin	Peptotech	1% BSA/PBS	100 ng/mL
Human recombinant Wnt3a	R&D Systems	PBS	100 ng/mL

6.2.2 Organoid media from Cambridge

An alternative organoid media protocol was obtained from Dr Bon-Kyoung Koo from the Cambridge Stem Cell Institute, University of Cambridge. This media was made as detailed in Table 6.11. In addition to the media which was ‘homemade’ using the protocol from Dr Koo’s group, complete organoid media was also received

as received as a kind gift from Dr Koo. Colon crypts were isolated as described in chapter 2, section 2.4.2, suspended in Matrigel and overlaid with media received from Cambridge or with media that was ‘homemade’ according to the same protocol. Images of organoids were obtained using a Nikon Eclipse TS100 microscope.

Table 6.11: Components of Cambridge organoid media

<u>Reagent</u>	<u>Source</u>	<u>Solvent</u>	<u>Concentration</u>
Advanced DMEM/F12 + 1x L-glutamine + 1xHEPES	Invitrogen	N/A	
Wnt3a conditioned medium	Kind gift from Dr Shams-Nateri, University of Nottingham	N/A	1x
Recombinant Human R- Spondin 1	R&D Systems	1% BSA/PBS	1 µg/mL
Recombinant Murine Noggin	Peprtech	1% BSA/PBS	100 ng/mL
B27 supplement	Invitrogen	N/A	1x
N-Acetylcysteine	Sigma-Aldrich	dH ₂ O	1.25 mM
Nicotinamide	Sigma-Aldrich	dH ₂ O	10 mM
Recombinant Murine EGF	Invitrogen	1% BSA/PBS	50 ng/mL
A 83-01 (Alk 4/5/7 inhibitor)	Tocris	DMSO	0.5 µM
SB 202190 (p38 inhibitor)	Sigma-Aldrich	DMSO	3 µM
Primocin	Invitrogen	N/A	1x
Y27632 (ROCK inhibitor)	Sigma-Aldrich	dH ₂ O	10 µM

6.2.3 Organoid culture with GSK3 β inhibitor CHIR99021 in place of Wnt3a

Due to difficulties in successfully growing organoids with recombinant Wnt3a and Wnt3a conditioned media, an alternative was tried in the form of GSK3 β inhibitor CHIR99021. This compound activates the Wnt cascade and has been used in place of Wnt3a in intestinal organoid cultures in previous studies [145,248].

Organoids were passaged as described in chapter 2, section 2.4.2.ii with the exception that dissociated cells were resuspended in Growth Factor Reduced Matrigel supplemented with components detailed in Table 6.12

Table 6.12: Matrigel supplements for CHIR99021 experiment

<u>Reagent</u>	<u>Source</u>	<u>Concentration</u>
Recombinant human Wnt3a	R&D Systems	40 ng/mL
Recombinant Murine EGF	Invitrogen	50 ng/mL
Recombinant Murine Noggin	Peptotech	100 ng/mL
Recombinant Human R-Spondin 1	R&D Systems	1 μ g/mL
B27 supplement	Invitrogen	1x
N-Acetylcysteine	Sigma-Aldrich	1.25 mM
Nicotinamide	Sigma-Aldrich	10 mM
A83-01	Tocris	0.5 μ M
SB202190	Sigma-Aldrich	3 μ M
Primocin	Invitrogen	1x
Y27632	Sigma-Aldrich	10 μ M

The Matrigel was solidified at 37°C for 15 minutes then overlaid with either organoid media from Cambridge, or with media containing CHIR99021 which was made as described in Table 6.13. Media was changed every 2 days and images of organoids were taken two days after plating with a Nikon Eclipse TS100 microscope.

Table 6.13: Components of organoid media in which CHIR99021 was used in place of Wnt3a

<u>Reagent</u>	<u>Source</u>	<u>Solvent</u>	<u>Concentration</u>
Advanced DMEM/F12 + 1x L-glutamine + 1xHEPES	Invitrogen	N/A	
Recombinant Human R-Spondin 1	R&D Systems	1% BSA/PBS	1 µg/mL
Recombinant Murine Noggin	Peprtech	1% BSA/PBS	100 ng/mL
B27 supplement	Invitrogen	N/A	1x
N-Acetyl- L -cysteine	Sigma-Aldrich	dH ₂ O	1.25 mM
Nicotinamide	Sigma-Aldrich	dH ₂ O	10 mM
Recombinant Murine EGF	Invitrogen	1% BSA/PBS	50 ng/mL
A 83-01	Tocris	DMSO	0.5 µM
SB 202190	Sigma-Aldrich	DMSO	3 µM
Primocin	Invitrogen	N/A	1x
Y27632	Sigma-Aldrich	dH ₂ O	10 µM
CHIR99021	Selleckchem	DMSA	3 µM, 6 µM, 9 µM or 12 µM

6.2.4 Primary cell seeding on scaffolds

Gelatin nanofibre scaffolds and gelatin hydrogel scaffolds were manufactured and sterilised as described in chapters 4 and 5.

A gift of organoid media was obtained from Dr Bon-Kyoung Koo from the Cambridge Stem Cell Institute. Crypts were isolated and cultured with this media, as described earlier in section 6.2.2. Organoids were grown for 6 days, then passaged as described in chapter 2, section 2.4.2.ii. Passaged organoids were seeded onto gelatin nanofibre scaffolds, gelatin hydrogel scaffolds or suspended in 50 µL Matrigel in 24-well plates. For each timepoint, two samples and one blank were

plated. Plates were incubated at 37°C for 1 hour to allow cell adhesion, then 500 µL organoid media was added to each well. After a further 5 hours, CellTox Green and CellTiter Glo 3D assays were conducted, as described in chapter 2, sections 2.5.3 and 2.5.4. These assays were repeated 2 days and 4 days after plating. Additionally, images of cells in Matrigel were obtained with a Nikon Eclipse TS100 microscope. The experimental procedure is summarised in Figure 6.2.1.

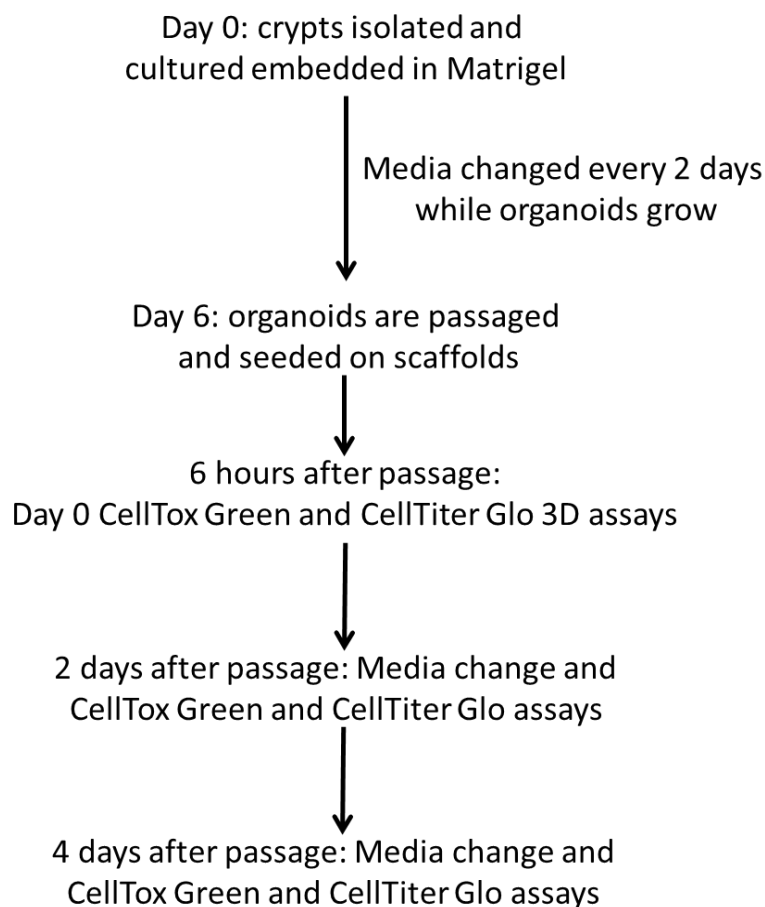


Figure 6.2.1: Schematic showing experimental procedure for assessing primary cell adhesion and viability on gelatin scaffolds

Crypts were isolated from human colon mucosa, expanded in Matrigel in the form of organoids, then dissociated and seeded onto scaffolds. Cell viability on scaffolds was assessed to using CellTox Green and CellTiter Glo 3D assay.

6.2.5 Statistical analysis

Data were expressed as mean $\pm$ standard deviation. GraphPad Prism 7 software was used to perform statistical analysis. Two-way ANOVA was used to assess statistical differences in cell viability data. Differences were deemed statistically significant if $p \leq 0.05$. Symbols of further levels of statistical significance are detailed in chapter 2, section 2.7.

6.3 Results

6.3.1 Crypts disintegrate when cultured in Matrigel and overlaid with DMEM or KGMG media

The first aim of this chapter was to culture epithelial crypts isolated from human colon so that they survive and grow. This was first attempted using DMEM supplemented with 20% (v/v) FBS and KGMG. When crypts were cultured with DMEM + 20% (v/v) FBS, they disintegrated within 2 days of plating. When KGMG was used, the crypts also degenerated but this happened more slowly than with DMEM. It took up to 6 days for the crypts to fully break up with KGMG (Figure 6.3.1). It is also apparent that crypt length reduced over time and the crypt bases stayed intact longer than the rest of the structure in both types of media.

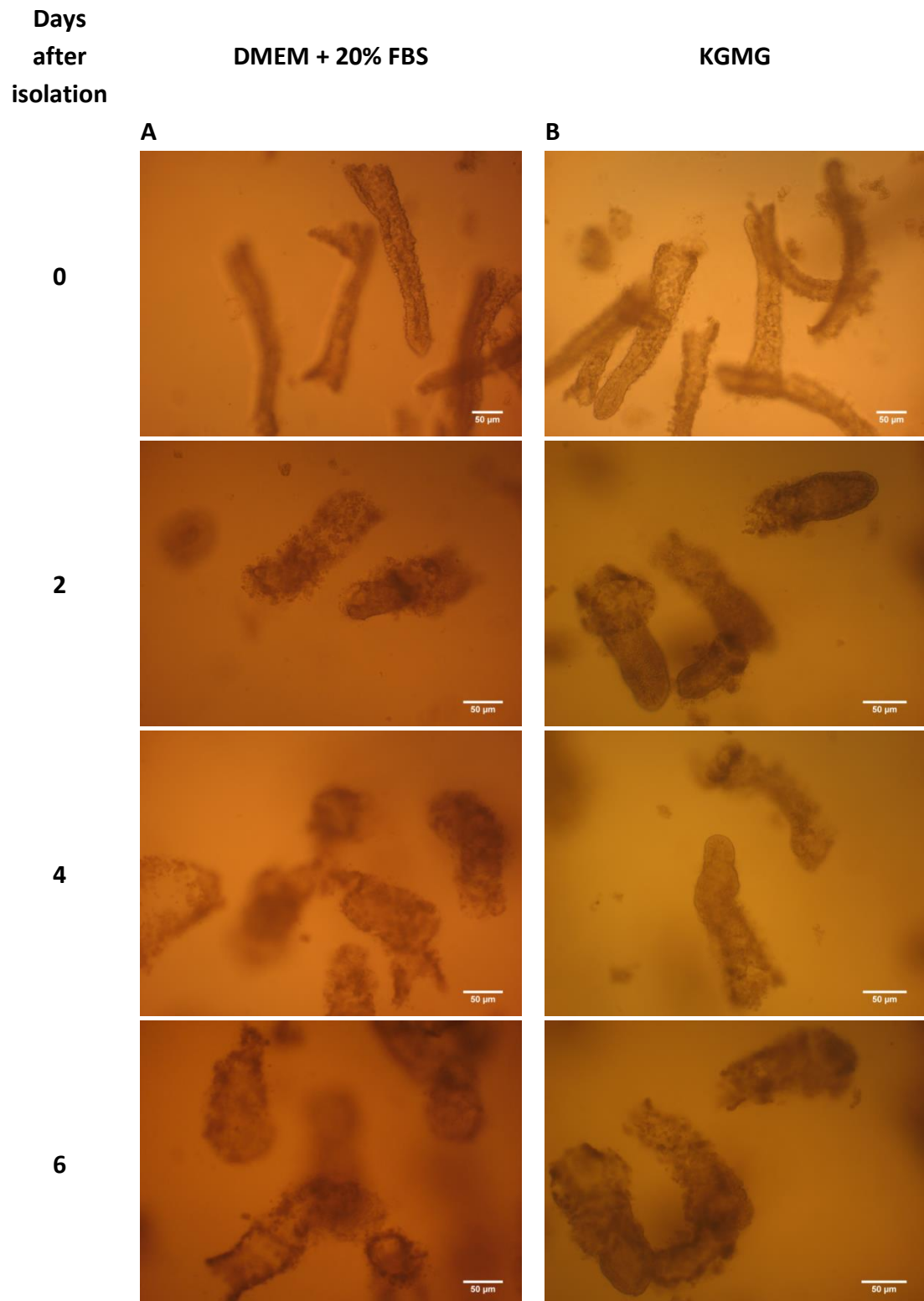


Figure 6.3.1: Human colon crypts cultured with either DMEM + 20 % FBS or KGMG

Individual crypts were isolated from human colon, embedded in Matrigel and overlaid with either DMEM supplemented with 20% FBS (A) or Keratinocyte Growth Medium Gold (KGMG; B). Media was changed every day. Representative images are shown

6.3.2 Organoids are formed with Sato media containing Wnt3a, R-Spondin, Noggin and EGF but they do not survive

Sato organoid media containing a combination of growth factors was used to culture isolated crypts. With this media, the crypts formed rounded structures similar to organoids described in other work. However, the organoids contained a lot of debris in the centre, which can be observed by the dark areas in the images. Additionally, the organoids did not grow, and began to deteriorate by day 2 after plating (Figure 6.3.2A).

In order to quantify what was observed under the microscope, CellTox Green and CellTiter Glo 3D assays were conducted (Figure 6.3.2B-C). After 6 days culture with DMEM + 20% FBS, both the CellTox Green assay as well as the CellTiter Glo 3D assay confirmed a loss of cell viability. When cultured with KGMG, it appeared that there were only dead cells remaining after 6 days. This is demonstrated by the increased CellTox Green signal and the reduction of CellTiter Glo signal to zero. When organoid media was used, the cells appeared to survive longer. After 6 days, CellTox Green signal remained the same as day 1, and CellTiter Glo signal reduced slightly. This indicates that there were fewer viable cells remaining but this reduction takes place at a slower rate when compared with the other tested media.

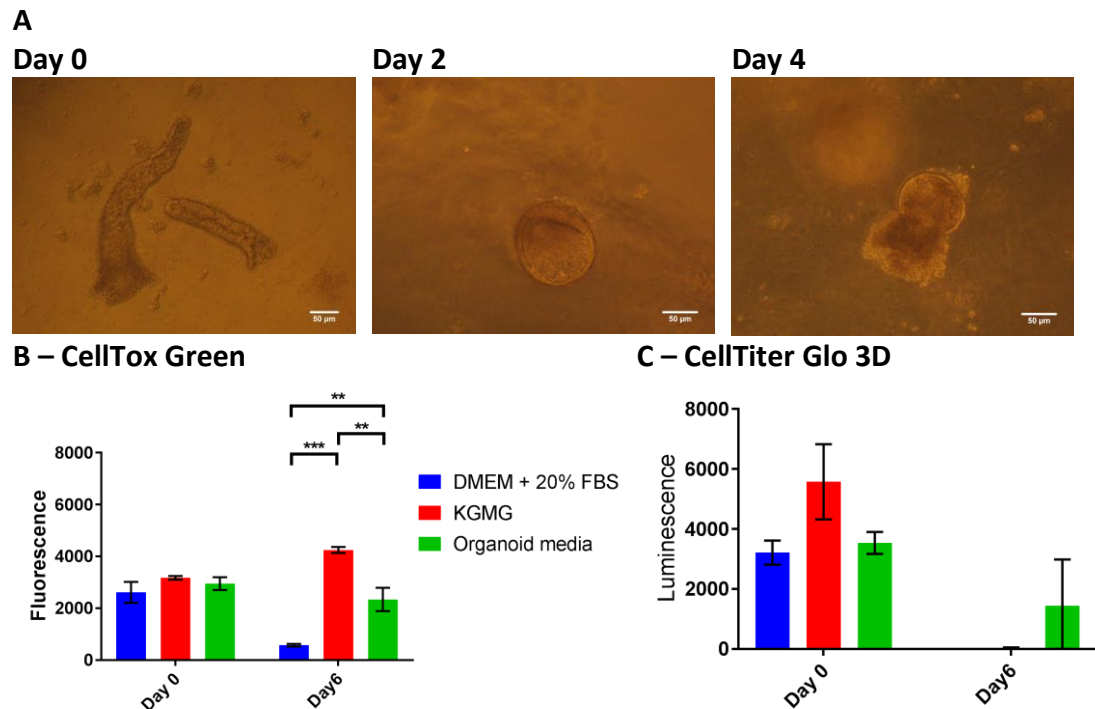


Figure 6.3.2: Colon crypts cultured with organoid media do not survive

Individual crypts were isolated from human colon, embedded in Matrigel and overlaid with organoid media containing Wnt3a, R-Spondin, Noggin and EGF. Representative images of isolated crypts with organoid media are shown in A. Cell viability with organoid media, KGMG and DMEM + 20% FBS was assessed with CellTox Green assay (B) and CellTiter Glo 3D assay (C). Data shows mean $\pm$ SD (n = 2). Statistical significance was assessed with 2-way ANOVA, ** $p \leq 0.01$, *** ≤ 0.001 . Scale bars = 50 μ m.

6.3.3 The source of organoid media constituents is crucial to their survival and growth

Following difficulties in achieving crypt and organoid survival, an alternative organoid media was obtained from Dr Koo at the Cambridge Stem Cell Institute. Gifted media was used alongside media that was 'homemade' according to the same protocol. Organoids were formed in both cases (Figure 6.3.3). However, it is apparent from the images that there is a lot of debris present within the centre of

the organoids when homemade media was used. The organoids grew well with the media received from Cambridge, but not with the homemade media.

A - Media from Cambridge



B - 'Homemade' media

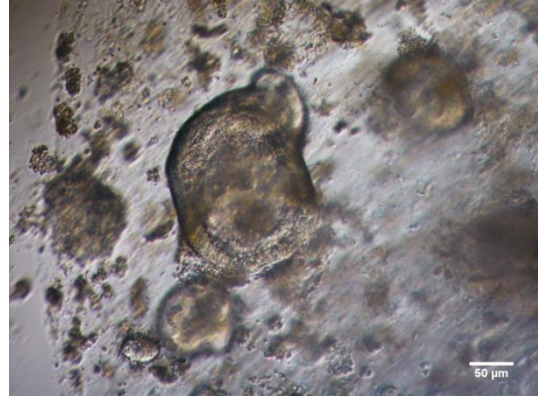


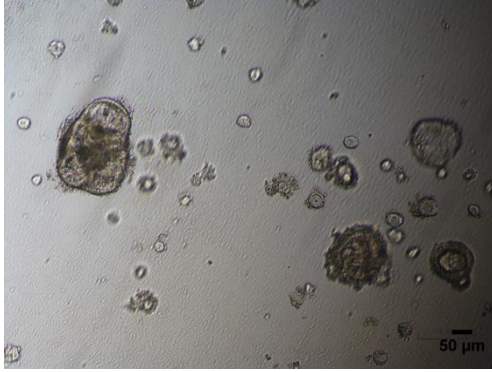
Figure 6.3.3: Organoids formed with Cambridge organoid media

Individual crypts were isolated from human colon, embedded in Matrigel and overlaid with organoid media that was gifted by Dr Koo from the Cambridge Stem Cell Institute (A) or with media that was 'homemade' using the same protocol (B). Images are representative of organoids formed after 4 days in culture. Scale bars = 50µm.

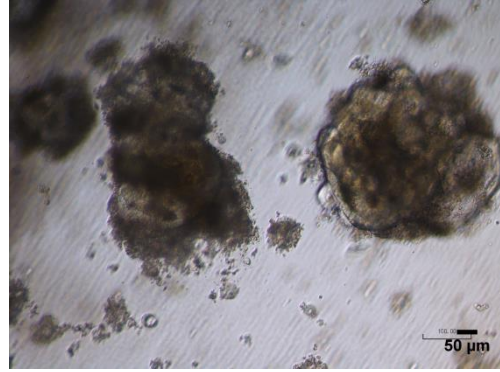
6.3.4 Small molecule inhibitor of GSK3 β may be used as a replacement for Wnt3a in organoid media

Images were taken of organoids 2 days after passage and plating with media containing 4 different concentrations of GSK3 β inhibitor CHIR99021 in place of Wnt3a or organoid media from Cambridge as a positive control (Figure 6.3.4). It is apparent that the lowest concentration of CHIR99021 resulted in slower cell growth as demonstrated by the small organoids present. The higher concentrations led to growth of larger organoids however there is a large amount of debris present within the organoids as seen by the dark area in the images. This is in contrast to the positive control in which there are large organoids with no debris present.

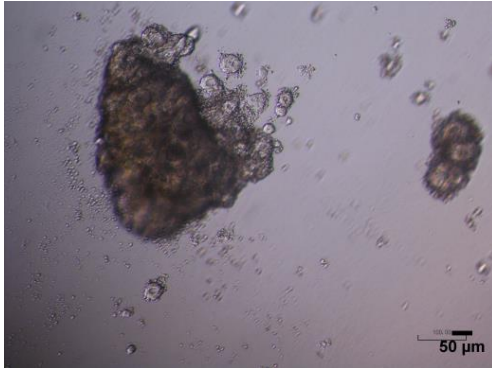
A – 3 μ M CHIR



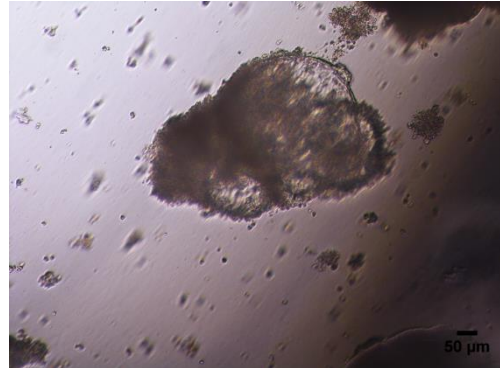
B – 6 μ M CHIR



C – 9 μ M CHIR



D – 12 μ M CHIR



E – Organoid media from Cambridge



Figure 6.3.4: P2 organoids cultured with media containing GSK3 β inhibitor CHIR99021

After two passages, colon organoids were suspended in Matrigel and overlaid with media containing varying concentrations of GSK3 β inhibitor CHIR99021 in place of Wnt3a conditioned media. Representative images are shown of organoids formed 2 days after passage with 3 μ M (A), 6 μ M (B), 9 μ M (C), 12 μ M (D) and organoid media gifted by Dr Koo from the Cambridge Stem Cell Institute (E). Scale bars = 50 μ m.

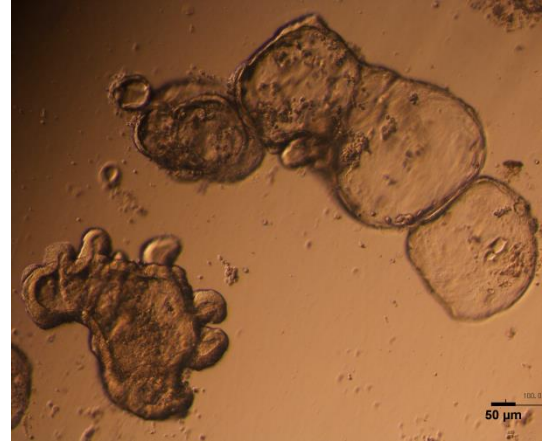
6.3.5 Organoid cells do not proliferate on electrospun or hydrogel scaffolds

As it was apparent that organoids could only be grown with media that was made in Cambridge, this was used to test scaffolds with primary cells. Within the first five days after crypt isolation, organoids grew well, forming large budding structures (Figure 6.3.5A-B). Six days after isolation, organoids were passaged and either seeded onto scaffolds or suspended in Matrigel as the positive control. The CellTox Green data remains the same throughout for both scaffolds and the Matrigel (Figure 6.3.5D), suggesting that non-viable cells were removed during media changes and washing. It is apparent from the images (Figure 6.3.5C) as well as the CellTiter Glo data (Figure 6.3.5E) that the cells in the Matrigel positive control proliferated well in the form of organoids. It is also apparent from the CellTiter Glo data that cells on electrospun gelatin nanofibres or gelatin hydrogels did not proliferate within the 4 days of the experiment.

A – Crypts immediately after isolation

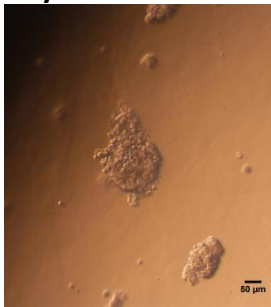


B – Organoids after 5 days in culture



C

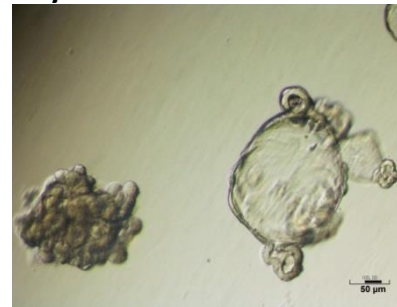
Day 0



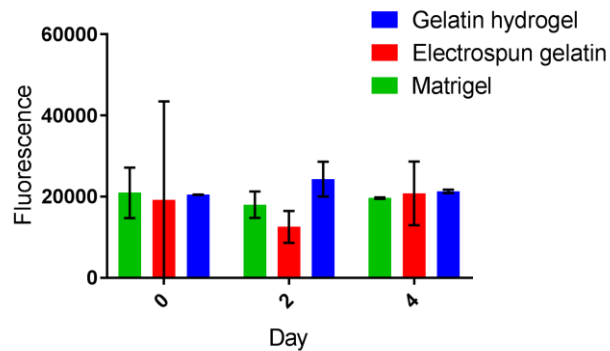
Day 2



Day 4



D – CellTox Green



E – CellTiter Glo 3D

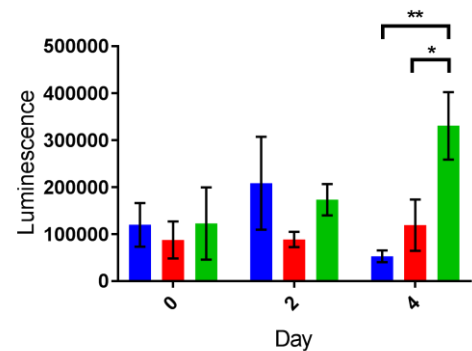


Figure 6.3.5: Organoids expanded, passaged and seeded on scaffolds

Crypts were isolated from human colon (A), embedded in Matrigel and overlaid with organoid media that was gifted by Dr Koo from the Cambridge Stem Cell Institute. Large organoids had grown after five days in culture (B). Six days after isolation, organoids were passaged and seeded on gelatin hydrogel, electrospun gelatin or in Matrigel. Images of positive control cells in Matrigel are shown in C. Cell viability on scaffolds was assessed with CellTox Green assay (D) and CellTiter Glo 3D assay (E). Data shows mean $\pm$ SD ($n = 2$). Statistical significance was assessed with 2-way ANOVA, * $p \leq 0.05$, ** $p \leq 0.01$. Scale bars = 50 μ m.

6.4 Discussion

The aims of this chapter were to reproduce the published organoid culture method and to investigate whether primary cells could grow on gelatin electrospun and hydrogel scaffolds from chapters 4 and 5.

Initial attempts to maintain colon crypt survival in culture used media containing a combination of growth factors and hormones. By culturing crypts with the commercially available Keratinocyte Growth Medium Gold, crypts were able to survive for up to 6 days. This had been demonstrated previously by Dame and colleagues [128]. Several early studies have reported that growth factors and hormones such as EGF, insulin, transferrin, hydrocortisone, epinephrine and bovine pituitary extract are required for maintaining intestinal epithelial cells *in vitro* [249,36,250,251]. Insulin has been shown to be involved in cell multiplication, whereas transferrin and EGF play a role in morphologic cell differentiation [249]. However, as this culture method did not result in cell expansion *in vitro* in the current study, media described by Sato et al was used to generate organoids [40]. This media did prolong cell survival in addition to generating spherical organoid structures. However, in this study, cell expansion was not achieved as has been reported in other studies [28,252].

Following communication with Dr Boo-Kyoung Koo from the Cambridge Stem Cell Institute, an alternative organoid media protocol was investigated. The primary difference with this media was the inclusion of conditioned media from HEK293 cells transfected with a Wnt3a expression construct instead of recombinant Wnt3a.

Organoid media was made according to their protocol using Wnt3a conditioned media that was a kind gift from Dr Shams-Nateri at the University of Nottingham. The Wnt activity of this media was tested by researchers in Dr Shams-Nateri's group using a TOPflash/FOPflash reporter assay and compared with a previous batch of conditioned media that was successful in growing mouse intestinal organoids. It was accepted for use if the Wnt activity was at least equivalent to the comparison batch of media. It is possible that the Wnt activity required for growing mouse organoids is less than what is necessary for human cells. Therefore, it may be possible that the conditioned media that was used did not provide sufficient Wnt activity for successful human organoid culture. This may explain the differences observed between media that was sent from Cambridge and media that was 'homemade' according to the same protocol.

GSK3 β is a serine/threonine kinase that phosphorylates β -catenin, leading to its degradation. Thereby GSK3 β is a negative regulator of the canonical Wnt signalling pathway [253]. CHIR99021 is a small molecule inhibitor of GSK3 β and can therefore activate the Wnt cascade. It has been used to differentiate definitive endoderm into CDX2⁺ midgut/hindgut cells that could generate intestinal organoids [254]. Additionally, it has been used successfully in place of Wnt3a in intestinal organoid media [145,248,27]. Due to difficulties in making organoid media using recombinant Wnt3a and Wnt3a conditioned media, CHIR99021 was investigated as a potential alternative. The approach was partly successful as organoids were grown indicating cell proliferation had occurred. However, when compared with the Cambridge organoid media it is apparent that the media containing CHIR99021 led to the

presence of more dead cells and debris. Further investigation of this is therefore required. Increasing CHIR99021 concentration to higher than 12 μM or using CHIR99021 in addition to Wnt3a may be tested in future work.

The only reported studies in which primary colon cells have been seeded onto biomimetic scaffolds have included Matrigel [113,117]. The current study is therefore novel for the attempt to grow primary colon epithelial cells on scaffolds that mimic either the topography of the basement membrane or the villus architecture of the intestinal epithelium, in the absence of Matrigel. However, the primary cells did not proliferate on the gelatin electrospun nanofibre scaffold or the gelatin hydrogel scaffold. As the cells were able to proliferate in Matrigel, it is possible that this was due to anoikis caused by inadequate extracellular matrix contact. It has been previously described that anoikis is induced by the loss of interaction of $\beta 1$ -integrin with ECM elements [255]. Therefore future work could involve using substrates that more closely resemble the chemistry of the ECM in addition to the topography, and allow for better cell interaction. A potential method for achieving this is the printing method discussed in chapter 5, which can be used to manufacture scaffolds with crypt-like topography.

6.4.1 Conclusions

The first aim of this chapter was to isolate epithelial crypts from human colon mucosa and expand them *in vitro* in the form of organoids. Whole crypts could be cultured using media obtained from Cambridge. It was found that the precise nature and source of organoid media components is critical for successful primary

cell culture. In particular, adequate Wnt activity appears to be crucial. Indirect activation of the Wnt signalling cascade by GSK3 β inhibition may be a potential alternative culture method. It was hypothesised that these cells could be grown on scaffolds in the absence of Matrigel to form a monolayer that resembles native epithelium. Therefore, the second aim was to investigate whether primary cells could grow on the electrospun gelatin nanofibres and gelatin hydrogel scaffolds that were manufactured and investigated in chapters 4 and 5. In the single preliminary experiment that was performed, it was found that primary cells did not proliferate on these scaffolds. Further work will include investigation of different substrates that more closely resemble the microenvironment of intestinal epithelial cells.

Chapter 7: General conclusions and future work

Inflammatory bowel disease (IBD), comprising Crohn's disease and ulcerative colitis, affects approximately 3 million Europeans [47]. This translates to a direct healthcare cost of 4.6–5.6 billion Euros/year [48]. Current therapeutic strategies focus on modulation and suppression of the immune system. However, key features of these diseases include continued epithelial damage and impaired epithelial healing [52,53]. Furthermore, some reports suggest that treating the persistent inflammation does not necessarily lead to complete mucosal healing [54,55]. Therefore strategies for aiding tissue regeneration are required in addition to conventional immune treatments.

Epithelial crypts isolated from normal colon mucosa have been cultured *in vitro* in the form of organoids. These organoids have been shown to have regenerative potential when implanted in mouse models of denuded colon mucosa [256,257]. However a major limitation of clinically translating these studies is the use of Matrigel which is clinically unsuitable due to its undefined composition and patient incompatibility. Additionally, enclosed organoids do not fully reproduce the structure and function of native tissue, and does not lead to full integration into the host [1].

Scaffolds can be used to mimic the native microenvironment, thereby allowing cells to adhere, proliferate and differentiate as they would *in vivo*. To date, the use of scaffolds to recapitulate the structure of intestinal basement membrane and underlying stroma has either involved the use of colon carcinoma cell lines such as

Caco2 to model cell response or Matrigel-coated scaffolds to culture enclosed organoids from primary cells. There are no reported studies in which primary cells from human colon have been cultured on scaffolds with the aim of reproducing the open epithelial structure for regenerative medicine applications.

Therefore, the overall aim of this project was the development of biomaterial scaffolds that are able to support growth of primary colon epithelial cells, ultimately targeted to the regeneration of the intestinal mucosa. This overarching aim was divided into two parts: the first was to isolate epithelial crypts from human colon, then identify and sort a population of stem cells to culture *in vitro*. The second aim was to manufacture and characterise scaffolds that mimic the extracellular matrix and stem cell niche found in intestinal mucosa, and investigate their influence on cell proliferation and differentiation using colon carcinoma cell lines to assess cell response. Scaffolds of interest were then investigated for their ability to support proliferation of primary human colon epithelial cells.

The first objective was to investigate the side population method for identifying and isolating stem cells based on their ability to efflux the Hoechst 33342 dye (chapter 3). In this study, the method was first validated with colon carcinoma cell lines with the intention of ultimately using the protocol to sort a population enriched with stem cells from dissociated primary colon epithelial crypt cells. However, consistent results could not be obtained with the cell lines. It was concluded that the side population method is too sensitive and variable and could not be used as a robust method for the identification of stem cells. This is supported by published findings in which differing results in the same cell lines were obtained by researchers in

different laboratories, and researchers in the same laboratory have found varying side populations by using a different dye or flow cytometer [145,147]. Therefore the method was not used with primary cells and instead, whole epithelial crypts were isolated and cultured.

In chapters 4 and 5, various scaffolds were manufactured, characterised and seeded with colon epithelial cell lines to investigate their influence on cell proliferation and differentiation. Electrospun nanofibre and microfibre scaffolds made from the synthetic material PET and the natural material gelatin were fabricated in chapter 4. All electrospun scaffolds supported cell proliferation. There were some small differences in their ability to maintain a stem cell phenotype while also supporting differentiation and formation of typical epithelial cell morphologies. Cells on gelatin nanofibre scaffolds differentiated to form tight junctions that are typical of epithelial cells, while also expressing increased levels of the stem cell marker *ABCG2*. Thereby, gelatin nanofibres demonstrated the ability to support appropriate cell differentiation while maintaining a pool of stem cells. Therefore, gelatin nanofibre scaffolds were chosen to test with primary cells from human colon in chapter 6.

In chapter 5, gelatin hydrogel scaffolds were investigated for their ability to support cell proliferation and influence cell differentiation. Additionally, a novel method of rapidly printing 3D scaffolds using unmodified gelatin was presented. It was concluded that gelatin hydrogels are a suitable substrate for cell proliferation and differentiation and that the addition of the printed topography did not have any negative effects on cell growth and behaviour. The printing parameters for forming

biomimetic villi-like topographies with dimensions that resemble native epithelium require further optimisation with the inclusion of crypt and villi structures. In the meantime, planar gelatin hydrogel scaffolds were investigated in chapter 6, to establish whether they can be used to culture primary colon crypt cells.

The final objective was to isolate and culture primary crypt cells from human colon and seed them onto chosen scaffolds to investigate cell proliferation (chapter 6). Media obtained from researchers at Cambridge University was successful in generating proliferating organoids from whole crypts. It was concluded that the precise nature and source of organoid media components is critical for successful primary cell culture. Specifically, adequate Wnt activity appears to be crucial. A previously reported method in which the Wnt signalling cascade is indirectly activated by GSK3 β inhibition [145,248] was found to be partially successful for growing organoids. This may be a potential alternative culture method in future work. Primary organoid cells were seeded onto gelatin nanofibre and hydrogel scaffolds; however, the cells did not proliferate. The seeding of primary human colon cells onto scaffolds in the absence of Matrigel has not previously been described. Therefore, the work presented in this thesis is the first report of this being attempted.

The main obstacle to achieving the aim of developing a tissue engineered construct using primary cells is the difficulties in maintaining survival and proliferation of human primary colon cells. This is evidenced by the difficulties faced when using the well-established protocol for organoid generation. It is apparent that the availability of growth factors, in particular Wnt3a is crucial to maintaining cells in culture.

Additionally, microenvironment and ECM contact is vital for cell survival. Both of these components may be addressed using tissue engineering strategies. A possible approach that may be explored in future work is the incorporation of growth factors within the scaffold so that they are in close contact with cells and can be released over a period of time. Furthermore, scaffolds can be designed to more closely replicate the biochemistry of the ECM and provide adequate interaction of β 1-integrin with ECM elements to prevent cells undergoing anoikis.

The architecture and composition of intestinal mucosa is far more complex than the scaffolds that were investigated in this project. However, the printing of scaffolds with crypt architecture as mentioned in chapter 5 should be investigated further. Results of previous studies have demonstrated the potential of replicating the *in vivo* topography in recapitulating the polarised layout of intestinal epithelium [115,117]. Moreover, bioprinting of cells within a hydrogel is another potential avenue of future research. The gelatin crosslinking method used in this project would not be suitable for inclusion of cells in the printing process due to reaction time and temperature. However, alternative materials and crosslinking methods have been previously described for this purpose. Hepatocytes have been printed in unmodified gelatin which was crosslinked with glutaraldehyde [222]. Additionally, methacrylated gelatin (GelMA) has been shown to be a suitable photopolymerisable hydrogel for bioprinting applications [258].

A further approach that has been reported previously is the addition of other cell types found within the intestinal stem cell niche, such as myofibroblasts, macrophages and bacteria [113]. The inclusion of the different cell types goes some

way to more closely replicating the complexities of the epithelial microenvironment. Bi-layered scaffolds in which the layers have different porosities have been used to co-culture cells of various tissues, including lung [166], bone [173] and tracheal epithelium [259]. Based on this idea, future investigation could involve the inclusion of a more porous scaffold containing fibroblasts that are able to secrete cytokine, growth factors and ECM to further support epithelial cell growth and differentiation.

Although the main goal of the project involved the use of primary cells for regenerative medicine applications, the results with colon carcinoma cell lines are also useful for alternative applications. Cell lines such as Caco2 and HT29 are commonly used as *in vitro* models for studying intestinal function and differentiation and for pharmacological testing [175,177]. It was shown in this study that gelatin nanofibres, PET microfibres and gelatin hydrogels can increase expression of stem cell markers as well as allowing differentiation to epithelial cell phenotypes in colon carcinoma cell lines. These are potentially useful properties for an *in vitro* model of intestinal epithelium, and provide a more accurate model of *in vivo* processes than culturing cells in 2D on rigid plastic surfaces [163]. As these scaffold types have not previously been used with colon epithelial cell lines, the results presented in this project provide an interesting and novel contribution to this area of research. However, for this application, further research is needed to ascertain whether cells differentiate into all of the various epithelial cell types. This can be done by assessing expression of markers for other cell types, such villin (absorptive cells), mucin (goblet cells), lysozyme (Paneth cells) and chromogranin

(enteroendocrine cells). Additionally, epithelial function would need to be assessed, with particular regard to the barrier function.

Overall, much has been learnt regarding the culture requirements of primary colon cells as well as how a tissue engineering scaffold can influence the phenotype of human primary colon epithelial cells. It is hoped that these lessons can be applied to future research in this area and contribute to the eventual goal of creating a clinically relevant tissue graft using primary intestinal epithelial cells for intestinal regeneration.

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Appendix A - PIPS Reflective Statement

Note to examiners:

This statement is included as an appendix to the thesis in order that the thesis accurately captures the PhD training experienced by the candidate as a BBSRC Doctoral Training Partnership student.

The Professional Internship for PhD Students (PIPS) is a compulsory 3-month placement which must be undertaken by DTP students. It is usually centred on a specific project and must not be related to the PhD project. This reflective statement is designed to capture the skills development which has taken place during the student's placement and the impact on their career plans it has had.

From July to October 2015, I worked at the non-profit 3Rs organisation, Fund for the Replacement of Animals in Medical Experiments (FRAME). The main project that I worked on during the placement was a survey of researchers working with animals at the University of Nottingham. The primary aim was to find out more about the justifications and motivations for using animals and the problems with current alternatives that make them unsuitable for use instead of animals. Other objectives were to investigate any variations between groups of researchers such as those using different species or with varying levels of experience, and to learn about sources of information that researchers use to look for alternatives. Lack of awareness of non-animal alternatives was given as an important reason why many responders undertook animal studies. The responses indicated that scientists believe that currently available alternatives do not provide enough information about whole-body processes and that animals are worthwhile when used in combination with other methods for furthering scientific and medical progress.

The key message from the survey was that many scientists have generally good intentions with regards to their animal work. If we can provide them with information on non-animal methods that yield the same type and quality of data to which they are accustomed, they would be open to using these alternatives. It is up

to organisations like FRAME to continue engaging with animal users and help them obtain this information on alternative options.

Alongside this main project, I also wrote an opinion piece, entitled “The 'Genomic Revolution' and its Impact on Medical Research”. The article was printed in FRAME’s journal ATLA (Alternatives to Laboratory Animals). In order to write this, I did a lot of reading around the Human Genome Project and genomics in general. I found it very enjoyable learning more about something I had only a passing knowledge of previously. It also inspired me to enter a science journalism competition run by *Naturejobs*. Winning this competition led me to consider this as a possible future career path. After the placement ended, I continued to write short pieces for FRAME’s blog PiLAS.

Overall the experience was very rewarding, particularly as I was able to work on projects that were so different to my previous experiences. It broadened my perspectives with regards to potential careers and highlighted the transferable skills that I have obtained over the course of the PhD, and how they may be applied to different roles.

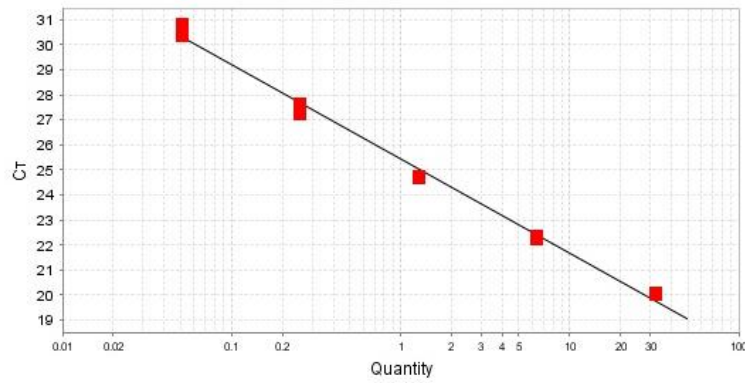
Appendix B – qPCR standard curves

For valid comparative C_T ($\Delta\Delta C_T$) calculations of qPCR results, amplification efficiency of target genes and endogenous controls (housekeeping genes) should be similar. Applied Biosystems 7500 Software v2.3 was used to generate standard curves using serially diluted cDNA undergoing qPCR reactions. Quantity of DNA is plotted on a logarithmic scale against cycle threshold (C_T) value. The slope of the resulting linear standard curve is used to calculate efficiency (E) of the PCR reaction using the following equation:

$$E = 10^{\left(\frac{-1}{\text{slope}}\right)}$$

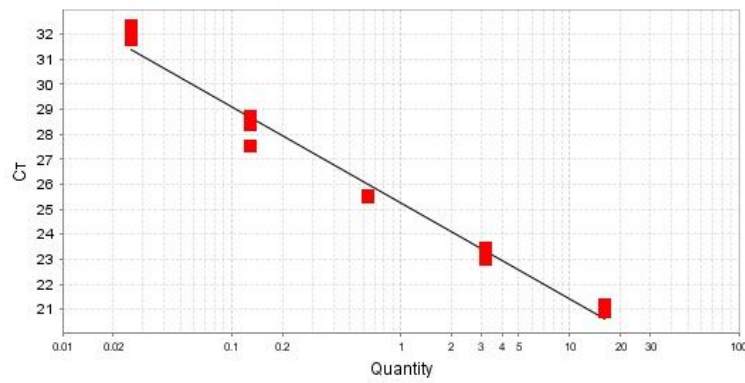
Standard curves for the housekeeping gene *HPRT* and the target genes *Lgr5* and *ABCG2* are shown in Figure B.1. As both the efficiencies of both target genes were similar to the housekeeping gene *HPRT*, relative quantification using the $\Delta\Delta C_T$ method can be considered valid.

A – HPRT



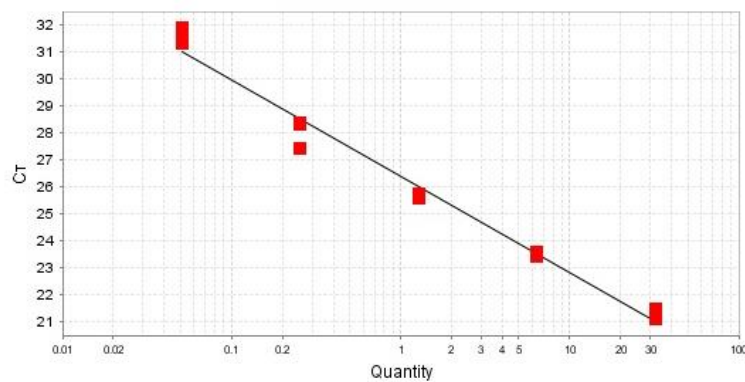
Slope = -3.759
Efficiency = 84.529%

B – Lgr5



Slope = -3.856
Efficiency = 81.706%

C – ABCG2



Slope = -3.582
Efficiency = 90.174%

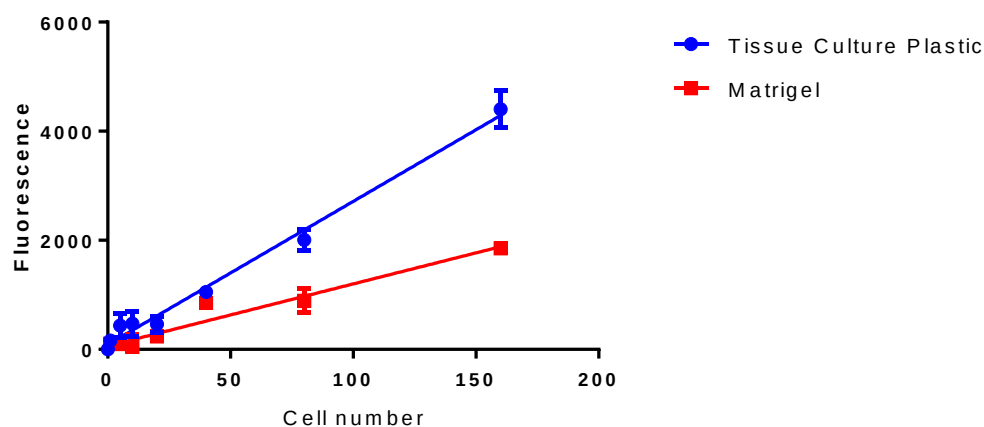
Figure B.1: qPCR standard curves and derived slopes and amplification efficiencies

qPCR reactions were conducted with serially diluted cDNA in order to assess amplification efficiencies of the housekeeping gene, HPRT (A), and the target genes Lgr5 (B) and ABCG2 (C). Each dilution was added to the reaction plate in triplicate and the C_T values of every reaction are plotted.

Appendix C - Comparison of Presto Blue and CellTiter Glo 3D assays for assessing cell viability in Matrigel

Experiments were conducted to compare the abilities of Presto Blue and Cell Titer Glo 3D assays to assess cell viability of SW620 cells suspended in Matrigel. Results are shown in Figure C.1. The data indicate that the CellTiter Glo 3D reagent was able to diffuse through the Matrigel to the cells more effectively than the Presto Blue reagent. As a result, CellTiter Glo assay was used to assess viability of primary colon crypts cells suspended in Matrigel.

A – Presto Blue



B – CellTiter Glo 3D

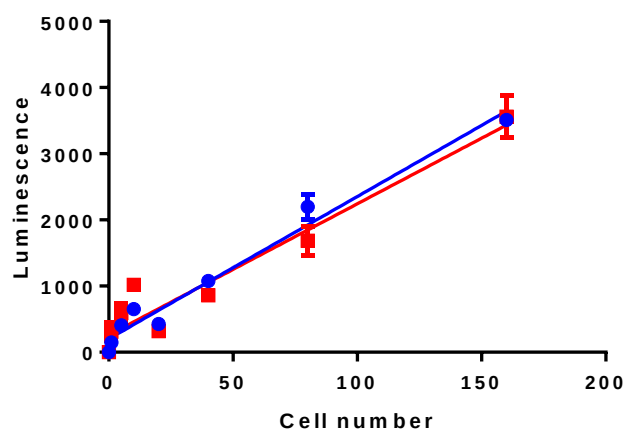


Figure C.1: Comparison of Presto Blue and CellTiter Glo 3D assays for assessing cell viability in Matrigel.

SW620 cells were counted and seeded either on tissue culture plastic or embedded within Matrigel. After 1 hour, either Presto Blue (A) or CellTiter Glo 3D (B) assays were conducted. Data points represent mean $\pm$ standard deviation ($n=2$) and were fit by linear regression.